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MEASUREMENT OF PLATELET INTRACELLULAR FREE CALCIUM ION CONCENTRATION BY RATIO FLUORESCENCE MICROSCOPY: A STUDY OF PLATELET ACTIVATION INDUCED BY CONTACT WITH BIOMATERIALS

by

Kip D. Hauch

A dissertation submitted in partial fulfillment of
the requirements for the degree of

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Chairperson of Supervisory Committee

Program Authorized

to Offer Degree: Department of Chemical Engineering

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Doctoral Dissertation

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Abstract

Measurement of Platelet Intracellular Free Calcium Ion Concentration by Ratio Fluorescence Microscopy: A Study of Platelet Activation Induced by Contact with Biomaterials

by Kip D. Hauch

Chairperson of the Supervisory Committee:
Professor Thomas A. Horbett
Department of Chemical Engineering

Ratio fluorescence microscopy was used to image and measure the intracellular concentration of free calcium ion, $[Ca^{++}]_i$, in single human platelets during attachment and spreading on several fibrinogen-coated biomaterial surfaces. The calcium-sensitive fluorophore Fura Red™ was characterized, loaded into platelets and a calibration of intracellular Fura Red™ was achieved using the imaging system.

Washed platelets settled to the surface from a static suspension. Initially, $[Ca^{++}]_i$ in quiescent cells was observed to be 50-100nM. Following a variable lag period of up to five minutes, responding cells exhibited a rise in $[Ca^{++}]_i$ of 100-300nM. Sporadic oscillations in $[Ca^{++}]_i$ were seen as the $[Ca^{++}]_i$ reached an elevated plateau. Other platelets showed little or no change in $[Ca^{++}]_i$ while in contact with the surface. Platelets that demonstrated larger transient rises in $[Ca^{++}]_i$, were found to have spread during the experiment, while the $[Ca^{++}]_i$ in non-spread cells remained low.

Platelet $[Ca^{++}]_i$ was also measured in single cells that attached to the surfaces from a flowing suspension of platelet and red blood cells under a shear rate of 300 inverse seconds. During the first five minutes after attachment, pseudopodial formation was observed without a large change in $[Ca^{++}]_i$.

This study demonstrates the visualization and quantification of a important signal transduction step in single platelets activated by contact with biomaterial surfaces. Imaging of platelet $[Ca^{++}]_i$ may be useful in relating the mobilization of intracellular calcium with other platelet activation events, such as the release of platelet granule contents, and the scrambling of membrane phospholipids that supports blood coagulation. The technique may also be useful in investigating the mechanisms of platelet activation at surfaces, and in evaluating the effects of surface modifcation on platelet activation.

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Dedication

This work is dedicated to my mother and to the memory of my father.

Chapter 1: Introduction

Advancements in the practice of modern medicine have led to a continual and growing need for medical devices; in particular, devices that are used to access, interrupt, reroute, repair and replace various elements of the circulatory system. Examples of such devices include: dialysis access ports, short and long term catheters, blood bags, prosthetic vascular grafts, prosthetic heart valves, cardiopulmonary bypass apparatus, and implanted circulatory assist devices. The contact of these devices with flowing blood is not without consequence, however. The circulatory system is protected by the processes of hemostasis which act quickly to prevent the traumatic loss of blood. This is accomplished partly by the response of blood platelets, which stick to the sites of vascular disruption or injury; and partly by the coagulation proteins in blood plasma, which polymerize to form a mesh, entrapping cells and restoring the integrity of the circulation. Hemostasis is critical to the body's response to injury or disease, but the adhesion of platelets and the onset of coagulation triggered by the contact of blood with biomaterial surfaces is an undesirable response. The thromboembolic complications that result have serious consequences for patients including: the clogging of catheters and shunts, damage to and consumption of platelets, reduced graft patency and the formation of emboli that eventually cause pulmonary, cardiac, cerebral and other organ damage. Thus, the thrombosis triggered by contact of blood with biomaterial surfaces is a serious problem in the use of many important medical devices [1].

Platelets plays a primary role in hemostasis and thrombosis. The smallest of the blood cells, platelets are specialized structures which respond to breaks in the vascular lining by sticking to the subendothelial components of the blood vessel and spreading. They stick to one another, forming aggregates; and also release agents that stimulate

other platelets and promote coagulation. Thus, the adhesion and activation of platelets is an important concern in the design of blood-contacting surfaces and devices.

Platelet adhesion and activation are complex and tightly controlled biologic processes. The interactions of platelet membrane receptors with adsorbed adhesive proteins are important, as are soluble agonists. Stimulatory signals that reach the platelet are transduced into physiologic outcomes such as changes in cell shape, cell spreading, granule release, and changes in membrane chemistry to promote coagulation. As in many other cells the concentration of free calcium ions in the platelet cytosol, $[Ca^{++}]_i$, functions as an important intermediary in this process of signal transduction. In the resting cell, $[Ca^{++}]_i$ is kept at nanomolar levels, while external concentrations of calcium in blood are typically millimolar. A rise in $[Ca^{++}]_i$ to micromolar levels is typically associated with platelet activation by most agonists. Thus, $[Ca^{++}]_i$ measurements have been made in stirred suspensions, and by flow cytometry, to study this aspect of platelet signal transduction in agonist stimulated platelets.

The use of calcium sensitive fluorophores and fluorescence microscopy has made it possible to image and measure $[Ca^{++}]_i$ in single adherent cells. Ratio fluorescence microscopy has been used on a variety of cells to image spatial and temporal changes in $[Ca^{++}]_i$. The goal of the work presented here was to make measurements of $[Ca^{++}]_i$ in platelets during their activation via contact with biomaterial surfaces using ratio fluorescence microscopy. As this work began, there were very few published accounts of the successful use of ratio fluorescence microscopy to image and measure $[Ca^{++}]_i$ in platelets.

Demonstrated in this dissertation is the successful imaging and measurement of $[Ca^{++}]_i$ in platelets during contact with and stimulation by biomaterial surfaces. The technique has been applied in both a settling, and a flowing regime. The results reveal

that aspects of the platelet $[Ca^{++}]_i$ response are, unsurprisingly, complex. The response is heterogeneous. There does appear to be an association between elevated $[Ca^{++}]_i$ response and cell spreading.

The technique developed provides a unique measure of the platelet activation induced by biomaterial surfaces. Other currently used measures of platelet activation, including granule content release, P-selectin exposure and integrin $\alpha_{IIb}\beta_3$ affinity modulation, measure the end result of the platelet activation. As a second messenger in the signal transduction proces, $[Ca^{++}]_i$ is believed to function to link the receipt of the stimulatory signals with effector pathways, and as such, $[Ca^{++}]_i$ measurement may provide a more direct measure of the stimulation of the platelet. The study of the $[Ca^{++}]_i$ response in platelets may add to the understanding of the different biochemical mechanisms involved in attachment and spreading. The design and improvement of blood contacting biomaterials relies on a better assessment of the platelet activation stimulated by material contact, and depends on a better understanding of the mechanisms involved. In the future, ratio fluorescence imaging of platelet $[Ca^{++}]_i$ may be used to correlate the $[Ca^{++}]_i$ response with other important platelet functions such as the release of granule contents and the promotion of blood coagulation. The technique represents an important advancement in it's ability to make measurement of an important aspect of signal transduction in cells at an interface. This may be of considerable utility in understanding how novel surface modifications are interpreted by a variety of cells in varied settings.

Chapter 2 provides general background and review of some of the topics related to this research. An overview of the hemostatic system is presented showing the intertwined manner in which the components of blood, elements of the vascular wall,

and the nature of blood flow, all act in concert to maintain the integrity of the circulation. The structure and function of the platelet is described, with emphasis on the mechanisms of platelet activation and control. This leads to a discussion of the role of $[Ca^{++}]_i$ and the various methodologies that have been applied to study this aspect of platelet signal transduction.

Chapter 3 reviews the development of ratio fluorescence microscopy and describes the methods used in this research.

Chapter 4 describes the *in situ* calibration of the fluorophore Fura Red using ratio fluorescence microscopy. Calibration is frequently lacking from many studies of $[Ca^{++}]_i$, especially those done using platelets. The results show that the affinity of the fluorophore for Ca^{++} may be altered in the intracellular environment.

Chapter 5 presents results of experiments performed with washed platelets that were observed while they settled onto biomaterial surfaces from static suspensions. Ratio fluorescence microscopy was used to image and measure $[Ca^{++}]_i$ in the cells after they had attached and while they spread and became activated on the surface. The results demonstrate an association between elevated $[Ca^{++}]_i$ and platelet spreading.

Chapter 6 presents the results of experiments performed with platelets flowing past biomaterials surfaces under defined shear conditions in a mixed cell suspension. Platelet $[Ca^{++}]_i$ was imaged as the cells attached to the surface, and it was seen that some cells demonstrated the initial formation of pseudopodia, while the $[Ca^{++}]_i$ values remained low, at least as compared to the values of $[Ca^{++}]_i$ seen in spreading cells in the static experiments.

Finally, Chapter 7 presents the conclusions of the research and suggests possible future directions.

Notes to Chapter 1

1. Edmunds Jr. L: Breaking the Blood-Biomaterials Barrier, *ASAIO J,* 41:824-830, 1995.

Chapter 2: Literature Review and Background

This chapter will introduce the field of hemostasis and thrombosis, and present an overview of the platelet; its physiology, its structure, and finally the biochemistry of its activation. This will lead to a discussion of $[Ca^{++}]_i$ (the concentration of intracellular free calcium ion) as a second messenger in the process of signal transduction in platelets, and an summary of current the studies of $[Ca^{++}]_i$ in single platelets.

The background material presented in this section rests mostly on other published reviews, which are cited in the text below near the conclusion of each topic.

2.1 Hemostasis, Thrombosis, and Virchow's Triad.

Wintrobe has documented the birth of hematologic science, including the discovery (and rediscovery) of the platelet [1]. Modern day hematology, and specifically hemostasis and thrombosis, are the topics of several clinical and scientific texts, e.g. [2-4]. More compact coverage of the material can be found [5]. A particularly concise yet thorough review can be found in reference [6].

Virchow's triad: the complexity of hemostasis: The mechanisms of hemostasis, are integral to blood itself, and as detailed in the reviews above, involve delicate balances, and complex and intricate pathways. But long before any of the details were even imagined, the very interdependent nature of hemostasis was appreciated. Although active in philosophy, politics and archeology, Berlin's Rudolf Virchow is known for his monumental contributions to medicine: as father of modern pathology and one of the first to describe leukemia during the mid-19th century [1]. Virchow recognized that the process of hemostasis involves a delicate balance between blood, the blood vessel, and the blood flow, a concept known as 'Virchow's triad'. In Figure 2.1, the triad has been

expanded to show six elements of hemostasis: blood cells, particularly platelets; coagulation, adhesive and other proteins in plasma; the living endothelium lining the blood vessels; the nature of the luminal surface; the effects of fluid shear; and the effects of transport. Table 1.1 lists the roles these elements play in hemostasis.

In the functioning of hemostasis, these six elements are all interdependent; each affecting the others. This complexity often has significant consequence for the isolated study of any one element of the hemostatic system. For example, in the study of isolated, i.e. washed, platelets the role of plasma protein ADPases and endothelial cell ecto-ADPases must be replaced with alternative enzyme systems to prevent platelet activation by ADP. Likewise, the role of red cells in enhancing the mass transport of platelets to surfaces must be considered in any studies using flowing platelet suspensions. Experimental study of any single, isolated aspect of blood (e.g. the platelets, or a protein or the surface) must at least address or acknowledge the possible impacts of all the other parts of this system.

The importance of hemostasis to human health, or conversely the tremendous impact of thrombosis on human morbidity and mortality, drive the enormous efforts to advance the understanding of this complex system protecting the circulation. Yet an additional impetus is the need of modern medicine to interrupt, to repair and to replace various parts of the circulatory system. When a medical device is used in contact with blood the complex balances between the various elements of hemostasis are likely to be altered. It is remarkable that it is in the face of this level of complexity that the success of modern day blood contacting devices has been achieved. It is also not surprising that unresolved problems of thrombosis associated with blood contacting materials often limit or preclude their use in a large number of circumstances.

The discussion above lays the framework for understanding hemostasis and thrombosis, and thus understanding many of the concerns and the different areas of study in the field of blood-biomaterial interactions. Next the focus shifts to the platelet, the primary cell involved in hemostasis.

2.2 The Platelet

2.2.1 Introduction

The platelet is the primary cell in the hemostatic system. The short life span of the cell is spent traveling through the blood stream in a quiescent or resting state. Only if and when the platelet encounters a break in the non-thrombogenic endothelial lining, does it adhere and begin a transformation that enables it to perform its hemostatic role. This transformation involves profound changes in shape and structure, directed and controlled changes in membrane receptors and in membrane phospholipids, and is orchestrated by complex and still poorly understood systems of signal transduction. The result is adhesion of the platelets to sites of vascular injury, the recruitment of additional platelets and other cells to the site, and the catalysis of coagulation and hence fibrin mesh formation.

General reviews describing the platelet can be found in textbooks on hematology or specifically hemostasis and thrombosis [2-6]. The platelet is one of the most intensely studied of all cells in modern human biology. This may be attributed to the platelet's importance to human life, or perhaps just due to the relative ease with which the platelets can be isolated for study. However, it is the complexity of platelets that has likely led to the now tens of thousands of research reports that appear yearly. The study of platelet adhesion receptors was (and is) paramount to our understanding of an entire class of adhesion receptors, the integrins; important to organisms ranging in complexity from

plants to insects to humans. Other platelet receptor systems have also been prototypical, e.g. adrenergic receptors, and the G-protein coupled receptors. Platelets have even served as models of neuronal tissue. Numerous mechanisms of signal transduction, important across the spectrum of cell biology, have received intensive study in platelets, e.g. membrane lipid metabolism, G-proteins, protein phosphorylation, and intracellular ion regulation.

2.2.2 Platelet Physiology: What Platelets Do

Platelets are formed in a unique way, by blebbing off of progenitor cells, megakaryocytes, found in the marrow sinuses. The cells are discoid, roughly 1 x 3um, and circulate for 8-10 days or so before removal from the circulation. Platelet have no nucleus, and have no capability for reproduction nor even synthesizing new protein. They are, in essence, membranous bags of biochemistry waiting for something to happen. Nonetheless they are extraordinarily complex. The platelet membrane is dense with specific glycoprotein receptors linked to numerous biochemical pathways. They contain at least three types of granules ready for secretion and contain contractile apparatus. Few cells exhibit such a combination of tremendous transformation with such speed. Once they have been triggered and perform their duty however, they eventually become debris. They are used once, if at all, and then discarded; their ranks renewed only by release of stored platelets from the spleen or by new platelet production.

Platelet responses can be very loosely split into two categories, primary events and secondary events. Quiescent platelets circulate in their discoid form until stimulated by contact with exposed subendothelium or until stimulated by local concentrations of soluble agonists from other nearby platelets. Upon activation, the microtubule bands responsible for holding the platelet in its discoid shape, are disassembled and the platelet

takes on a more spherical shape. Pseudopodia begin to appear giving rise to the name for this stage of platelet activation, the "spiny sphere stage." The platelets become sticky, that is they stick to one another and sometimes to other cells. The platelet attaches, extends pseudopodia and lammelapodia, and eventually spreads itself thinly across the broken endothelium or other stimulating surface. A fully spread platelet may achieve a diameter of 10um or more, three times the diameter of the unstimulated cell. The process of attachment and spreading is dynamic, not every contact with an activating surface results in adhesion and spreading.

The success of the platelet in providing hemostasis depends on the autocatalytic nature of the platelet response. A quiescent platelet can attach to an activating surface, but it is the ability of that activated platelet to recruit and activate other nearby platelets, which in turn activate even more platelets, and so on, ... etc. that is responsible for the eventual formation of a platelet plug at the site of vascular injury. When activated, platelets produce thromboxane A_2, a short lived and powerful platelet agonist. Platelet membrane receptors for fibrinogen (which are kept non-functional in the circulating platelet) are made active in stimulated platelets resulting in extensive platelet to platelet cohesion bridged by bivalent fibrinogen molecules. Platelets secrete the contents of their alpha and dense granules releasing ADP and serotonin, other platelet agonists. Closely related to secretion comes a loss of membrane asymmetry resulting in the capability of the platelet membrane to support the catalytic formation of thrombin; itself an extremely potent platelet activator as well as responsible for the final polymerization of fibrinogen to fibrin creating the meshwork of the platelet plug entrapping platelets, red cells and other blood cells.

So, the function of the platelet is multifaceted. By far, most platelets circulate during their entire lifetime unstimulated. When and if conditions of vascular injury are

encountered, platelets respond primarily by sticking to the damaged vessel wall, and spreading, and forming aggregates with other platelets. Secondarily, the platelets actively stimulate other nearby platelets and facilitate the local formation of thrombin, which in turn solidifies the platelet plug within a fibrin mesh. Platelets have other roles: in recruiting inflammatory cells to the sites of injury, in retracting and later dissolving clots after they have formed, and in facilitating the extravasation and metastasis of tumor cells, which are not discussed here.

There is one last note about the manner in which platelet physiology has been studied. In 1962, Born introduced the technique of optical aggregometry [7, 8]. In this technique a suspension of platelets in plasma is stirred in a small cylindrical cuvette. The transmission of light through the stirred suspension is monitored as platelets are stimulated by the addition of a suitable agonist. As the platelets are activated, the change in platelet shape and the clumping together of the cells into aggregates is readily apparent from the changes in light transmission. Platelet aggregometry has played an invaluable role in platelet research. It has become a mainstay to the study of platelet function in the laboratory and to the assay of platelet function in the clinic. In aggregometry the ability of platelets to clump together, which is paramount to their function *in vivo*, is directly measured. The technique is simple and quick; any soluble agonist or inhibitor can be used in the suspension (as well as some insoluble agonists, e.g. fibrillar collagen or polymeric beads). Over the years, the technique has been modified to measure aggregation of platelets in whole blood using impedance, to measure platelet secretion via released ADP, and measure platelet signal transduction via intracellular calcium. The shear stresses present in the stirred suspension are important to the platelet response, yet are complex, poorly understood and poorly controlled in the typical platelet aggregometer. The clumping measured in platelet aggregometry is a macroscopic result

of a myriad of platelet responses in conjunction with coagulation proteins in plasma, and emphasizes cell to cell aggregation over platelet adhesion.

2.2.3 Platelet Ultrastructure

The platelet plasma membrane is typical of most mammalian cells, a fluid lipid bilayer. It is supported by an extensive underlying cytoskeleton comprised of actin filaments [9, 10] and rich in a wide range of transmembrane glycoprotein receptors. In the resting platelet the membrane undergoes many tortuous and deep evaginations creating a system called the surface connected canalicular system. The discoid shape of the platelet is maintained by a circumferential band of microtubules (the microtubular band). The cytosolic space itself contains a dense mesh of short actin filaments.

Platelets contain three kinds of secretory granules. Alpha granules, released with moderate platelet stimulation, contain many large macromolecules including Factor V, fibrinogen, von Willebrand Factor, and a host of others. When the alpha granule is released, the granule membrane fuses with the plasma membrane, and receptors found in the granule membrane, including P-selectin and a portion of the platelet's integrin $\alpha_{IIb}\beta_3$ receptors, find their way to the platelet's surface. A detailed review of the platelet alpha granule can be found in reference [11]. The second type of granule, the dense granule, contains ADP, serotonin, and Ca^{++}. Dense granules are so named due to their characteristic dense appearance in transmission electron microscopy. Dense granules are released when platelets receive strong stimulation. Lysosomal granules contain various degradative enzymes and may play a role in clot dissolution. Upon activation and spreading, the contraction of cytosolic actin filaments brings the granules together in the center of the cell.

Another key structure in the platelet is the dense tubular system, believed to be a vestige of the endoplasmic reticulum in other cells. This organelle is where intracellular Ca^{++} is sequestered by a Ca^{++}-ATPase pump found in this organelle's membrane.

2.2.4 Platelet Membrane Receptors:

Many different receptors are found in the membrane of the platelet. Some like the integrin $\alpha_{IIb}ß_3$ are found in great number (80,000 copies per platelet) while others are represented by less than a hundred copies. Certain of the receptors which receive adhesive proteins as ligands, act to anchor the platelet to adhesive protein covered structures and surfaces. All of the receptors serve to link events or conditions external to the platelet, e.g. shear forces, or the presence of agonists, antagonists, or adhesive proteins, with the various biochemical pathways responsible for the platelet's physiologic responses. Discussed here are only a few of the more important platelet receptors.

2.2.4.1 Integrin $\alpha_{IIb}ß_3$

The integrin $\alpha_{IIb}ß_3$, also known as GPIIbIIIa, is a heterodimeric transmembrane glycoprotein receptor for fibrinogen, vWF, fibronectin, vitronectin and thrombospondin. It participates in both outside-in signaling (i.e. where binding of external ligand to the receptor results in signal transduction and the triggering of physiologic responses inside the platelet) and inside-out signaling (where as a consequence of other pathways of platelet activation the functional competency of the receptor is altered, in this case the non-functioning receptor in the resting platelet is made functional in the activated platelet). The major role of integrin $\alpha_{IIb}ß_3$ is in the binding of bivalent fibrinogen (and multivalent vWF) as part of the crosslinking responsible for platelet aggregation, and in binding to adsorbed fibrinogen and vWF in the process of platelet attachment and spreading. In spreading platelets, fibrinogen

bound receptors associate with the cytoskeleton and the receptors demonstrate movement towards the center of the spreading cell, a process which may have a role in thrombus formation and clot retraction [12] . Integrin $\alpha_{IIb}\beta_3$ is congenitally deficient in the disease Glanzmann's thrombosthenia.

As one of the prototypical integrins, the molecular level detail of how integrin $\alpha_{IIb}\beta_3$ works, and how its ligand affinity is controlled is a topic of great interest. The presence of external Ca^{++} is required for the proper formation of the heterodimer, although control of external Ca^{++} is not believed to represent a mechanism by which the function of the receptor is controlled. Integrin $\alpha_{IIb}\beta_3$ binds many of its ligands, including vWF and fibronectin, via an RGD sequence in the ligand, and fibrinogen has such sequences. However, it appears that $\alpha_{IIb}\beta_3$ primarily recognizes a sequence of 12 amino acids at the C-terminal region of the gamma chain of fibrinogen, at least during platelet aggregation. Resting or unstimulated platelets do not bind fibrinogen, which is important since fibrinogen is a major blood protein, and hence platelets circulate in an environment containing lots of available fibrinogen. Stimulation of the platelet by an agonist results in a conformational change in the receptor, apparently mediated via the cytoplasmic tail of β_3, that results in a high affinity for fibrinogen. The conformational change in the receptor can be detected by monoclonal antibodies. The particular signaling molecules that interact with $\alpha_{IIb}\beta_3$ and are responsible for this conformational change are unknown, but suspects in the process include: G-protein activated non-receptor protein tyrosine kinases such as pp60 ^{Src} (Src), activation of PI-3-kinase, protein kinase C, and small GTP binding proteins such as Rho and Rac, etc. In the early stages of stimulation only, this activation of $\alpha_{IIb}\beta_3$ can be blocked by increases in cAMP (e.g. treatment with PGI_2) or cGMP (NO). After several minutes of activation, the binding of fibrinogen to

the receptor becomes irreversible and $\alpha_{IIb}\beta_3$ is increasingly found linked to the reorganized cytoskeleton.

Outside -in signaling refers to the triggering of the variety of typical platelet responses (aggregation, cytoskeletal reorganization, procoagulant activity) by binding of ligand to the integrin $\alpha_{IIb}\beta_3$. While the resting platelet does not bind soluble fibrinogen with high affinty, the platelet does recognize and bind to fibrinogen adsorbed to a surface. This constitutive binding to adsorbed fibrinogen may play a role in platelet attachment, while subsequent modulation of integrin $\alpha_{IIb}\beta_3$ affinity may support platelet aggregation. Outside -in signaling also clearly involves the cytoplasmic tail of the integrin, and also involves a selection of protein kinases, phosphatases, small GTP-binding proteins, as well as the regulation of intracellular calcium. The clustering of receptors and the interaction of the receptors with the cytoskeleton via actinin and talin are also involved.

The molecular level details of the functioning of integrin $\alpha_{IIb}\beta_3$ in platelets has been and continues to be a major focus of interest. Its interaction with fibrinogen in platelet attachment and aggregation has already become a pharmacologic target of clinical utility. While in years past the integrin receptors have been considered more for their importance in adhesion than in transmembrane signaling, the later has received much more attention recently. The assembly of a signaling complex at the juncture of the cytoplasmic tails of the integrin and the cytoskeleton, involving $p125^{FAK}$ and various other kinases and phosphatases at the site of a focal contact like structure, is the focus of much current research. The following references ([10, 13-28, 29]) comprise a list of current reviews of integrin signaling specifically concentrating on platelets, and integrin $\alpha_{IIb}\beta_3$ and the platelet cytoskeleton. Caen and Rosa [30] have nicely summarized the

history of the major discoveries regarding the platelet adhesion receptors integrin $\alpha_{IIb}\beta_3$ and the GP Ib-IX-V complex discussed below.

2.2.4.2 Other Platelet Integrins and Integrin Signaling

Platelets contain other receptors of the integrin family. Integrin $\alpha_v\beta_3$, a receptor for vitronectin, is the only other β_3 family integrin found on platelets, however its importance is questionable since it appears with only a few hundred copies. It has been suggested that $\alpha_v\beta_3$ might play a role in platelet attachment to osteopontin found in disrupted atherosclerotic plaques found as a consequence of angioplasty [31]. There are three members of the β_1 family (previously the VLA or Very Late Antigen family) found on platelets: integrin $\alpha_2\beta_1$ (previously known as GP IaIIa, VLA-2, ECMR-II) binds collagen, integrin $\alpha_5\beta_1$, (previously known as GPIcIIa, VLA-5, ECMR V) recognizes fibronectin, and integrin $\alpha_6\beta_1$ (previously known as GPIc'IIa) which recognizes laminin. The integrin $\alpha_2\beta_1$ is believed to be an important primary receptor for attachment to collagen, while the *in vivo* importance (if any) of the other β_1 integrins is not generally known. However, the interaction of platelets with collagen also clearly involves GP Ib-IX-V and its interaction with vWF adsorbed to collagen fibrils in the subendothelium. GPVI may also have an important role in platelet collagen interactions. While it is generally accepted that integrin $\alpha_2\beta_1$ is important for attachment, several issues are still unresolved including: whether $\alpha_2\beta_1$ is responsible for outside-in signaling, whether $\alpha_2\beta_1$ is under inside-out affinity modulation similar to integrin $\alpha_{IIb}\beta_3$, and the importance of secondary mediators of platelet activation released during platelet attachment to collagen, e.g. ADP, TxA_2. Collagen induces a very strong secretory response in platelets, which can be synergistic with other platelet agonists such as thrombin. Nitric oxide/cGMP dependent pathways, and the later involvement

of integrin $\alpha_{IIb}\beta_3$ may influence adhesion and spreading on collagen [32, 33]. Good discussion of integrin $\alpha_2\beta_1$, in platelets can be found in references [18, 21, 24, 34-37]

The integrins as a whole represent an area of fundamental advancement in our understanding of modern cell biology, which started in the 1980's and continues to this day. They play important roles in cell attachment, cell orientation, motility and cell signaling. They are found in species as varied as plants to insects to humans. The role of the integrins as membrane receptors involved in signaling through their interactions with the cytoskeleton, and various other signaling molecules (e.g. G-proteins, intracellular kinases and phosphatases) is an area of burgeoning interest. Advancements in our understanding of integrin mediated signaling have implications not only for platelets and in vascular biology but in broader areas of cell biology including tumor metastasis [38, 39] and developmental biology. References ([23, 40-59]) comprise a list of current and thorough reviews that are important reading for those new to this field.

2.2.4.3 Glycoprotein Ib-IX-V Complex

The remaining important adhesive platelet receptor is the Glycoprotein Ib-IX-V complex. These membrane glycoproteins are not integrins, but members of the leucine-rich protein family. The glycoprotein Ib-IX-V complex is a receptor for vWF, found as part of the subendothelial matrix, and is critical for platelet attachment to subendothelium under high shear conditions, e.g. in arterial flows or in pathologic stenoses. The complex also serves as a receptor for α-thrombin. Glycoprotein Ib consists of two disulfide linked chains, which associate with GP IX. Two of the GP Ib IX pairs then associate with one GPV to complete the complex; there are roughly 12,500 of these complexes found on the platelet surface. The complete amino acid sequence and the

gene encoding for GPIb are known. The glycoprotein Ib-IX-V complex is deficient in the disease Bernard-Soulier syndrome.

The glycoprotein Ib-IX-V complex is believed to recognize residues 514-542 in the A1 domain of vWF. The A domains of vWF are also found in the structure of several $ß_1$ and $ß_2$ integrins (where they are referred to as I-domains) and have additional interactions with heparin, and collagen. Circulating platelets do not normally interact with circulating vWF, however unlike the affinity modulation of integrin $\alpha_{IIb}ß_3$, the glycoprotein Ib-IX-V complex is constitutively active; it is the conformation of vWF that is believed to be altered when found bound to collagen in the subendothelial matrix, revealing the otherwise cryptic binding site in the A1 domain. The glycoprotein Ib-IX-V complex is pinned to the underlying membrane cytoskeleton via an interaction with actin binding protein (ABP-280). This is believed to help maintain the resting discoid shape of the platelet, and is broken during platelet activation and cytoskeletal reorganization. Outside-in signaling is triggered by the binding of vWF to the glycoprotein Ib-IX-V complex resulting in further platelet activation steps including, significantly, the affinity modulation of integrin $\alpha_{IIb}ß_3$ and platelet spreading. The interaction with vWF is most important in platelet attachment under high shear flow, and is also involved in the shear induced aggregation of platelets in a sheared platelet suspension containing vWF. Many of the signaling mechanisms are still unclear, but it is known that in shear induced aggregation, a strong influx of Ca^{++} is regulated by the GP Ib-IX-V binding of vWF, and results in subsequent protein tyrosine phosphorylation [60-63]. Additional signaling molecules are found associated with the cytoplasmic tail of GP Ib, notably the 14-3-3 ζ protein. Finally, although controversial for many years, it is now accepted that the GP Ib-IX-V complex serves as a high affinity receptor for α-thrombin, in addition to the more widely recognized seven

transmembrane domain thrombin receptor. The following reviews [30, 34, 64-71] provide current and thorough discussion of the GP Ib-IX-V complex and its functioning as a platelet adhesive and signaling receptor.

2.2.4.4 The Thrombin, ADP and Other Receptors

Thrombin is a protease with many different functions at the site of vascular injury: it is involved in several steps in the coagulation cascade, notably the final polymerization of fibrinogen into fibrin; it is an extremely potent activator of platelets; and is chemotactic for monocytes and mitogenic for lymphocytes and fibroblasts. The platelet thrombin receptor is a member of the seven transmembrane domain, G-protein coupled receptor family. It has a unique method of activation in that the interaction of thrombin with the receptor is to cleave a portion of the receptor's flexible amino terminal exodomain. Several amino acid residues (SFFLRN) at the freshly cleaved tail of the exodomain, which is still attached, then serve as a ligand, binding in a pocket in the body of the thrombin receptor and causing activation. This is the so-called "tethered ligand" and the peptides themselves called thrombin agonist peptides (TRAPs). The thrombin receptor is linked to heterotrimeric G-proteins, and activation leads to rise in $[Ca^{++}]_i$, activation of phospholipase C, protein kinase C, etc. resulting in affinity modulation of integrin $\alpha_{IIb}\beta_3$, platelet aggregation and secretion. As discussed above, the GP Ib-IX-V complex also serves as a thrombin receptor, and there is still question as to whether other thrombin receptors are yet to be identified. The following reviews discuss the platelet seven transmembrane domain thrombin receptor [67, 72-79].

While ADP was one of the first recognized platelet aggregation agonists, the mechanism of its interaction with platelets remains remarkably unclear even today. Platelets respond to ADP which can be released by damaged red blood cells and endothelial cells, and is also released by the platelet itself via secretion of the platelet

dense granule. ADP stimulates platelet activation, shape change, rapid Ca^{++} influx, transient $[Ca^{++}]_i$ rise, and reversible aggregation via affinity modulation of integrin $\alpha_{IIb}\beta_3$. Prolonged exposure to low levels of ADP leads to desensitization (refractoriness). In the circulation, ADP is quickly catabolized by various endogenous APDases in plasma and on the surface of platelet and endothelial cells. In washed platelet suspensions, therefore, this function must be fulfilled by the addition of apyrase or creatine phosphate/creatine phosphokinase enzymes to the suspension buffer.

The purinoceptors responsible for mediating the effects of extracellular adenine nucleotides (P_2) are currently divided into two groups: the P_{2X} receptors, believed to be ligand gated ion channels, and the P_{2Y} receptors which are seven transmembrane domain, G protein coupled receptors. The platelet ADP receptor(s) (defined pharmacologically) has previously been designated P_{2T}, a unique classification since unlike other P_2 receptors, ATP functions as an antagonist to this receptor. Contributing to the confusion regarding the platelet ADP receptor is the observation that ADP evokes rapid (sub-second) Ca^{++} entry in platelets suggesting a P_{2X} type ion channel. A recent report by MacKenzie, et al, concludes that platelets posses the P_{2X1} receptor operated cation channel [80]. After many years of unsuccessful attempts at cloning, finally a group lead by Gachet very recently suggests that the P_{2T} receptor is indeed P_{2Y1}, which they have now cloned and stably expressed, and identified in platelets and megakaryocytes [81]. The P_{2Y1} receptor is a seven transmembrane domain G protein coupled receptor which likely interacts with adenylate cyclase. The particular signal transduction pathways that ADP stimulates are still unclear. Our understanding of ADP stimulation of platelets is clearly at a stage of rapid development. Like thrombin, ADP may play a very important role in the secondary amplification of platelet activation

during attachment and spreading. Current discussion of the platelet ADP receptor, or receptors can be found in references [82-85]

2.2.4.5 Other Platelet Receptors

There are a host of other important receptors on platelets. Many are G protein coupled receptors, the activating ones include receptors for (in addition to thrombin), serotonin (5-HT), platelet activating factor (PAF) [86], thromboxane A_2, and epinephrine. An important G protein coupled receptor that is inhibitory to platelets is the PGI_2/PGE_1 receptor which is linked to cAMP formation. The clustering of the FCγ-R II (recognizing the FC fragment of IgG) may play a role in platelet activation. Many of these platelet receptors are quickly reviewed in a very broad, yet brief review of platelet activation by Blockmans, et al. [87].

2.2.5 The Adhesive Proteins

The role of adhesive proteins in platelet adhesion and aggregation has been described above as the platelet receptors that bind these adhesive proteins were enumerated. They are reviewed again only briefly here. Fibrinogen is a major plasma protein which is polymerized by thrombin into a fibrin mesh during coagulation, and serves as a bifunctional crosslinker during platelet aggregation. Platelets also secrete a small amount of fibrinogen from their alpha granules. While unactivated platelets do not recognize fibrinogen in suspension, they do recognize fibrinogen adsorbed to surfaces, which strongly supports platelet attachment and spreading. The suggestion is that immobilized fibrinogen is presented in a conformation that is conducive to recognition by the integrin $\alpha_{IIb}ß_3$. The particular portion of fibrinogen responsible for this interaction has not been conclusively determined; and it is unclear if a multimeric ligand interaction is involved or required (although recent evidence suggests that the gamma chain dodecapeptide region of fibrinogen is sufficient to support some attachment to

fibrinogen on polystyrene, but multi-domain fibrinogen is required for firm attachment, and attachment under flow [88]). Von Willebrand factor is a multimeric protein found in plasma and throughout the subendothelium bound to collagen. It is released from the Weibel-Palade bodies of endothelial cells, and is also found in the platelet alpha granule. It may serve along with fibrinogen to crosslink platelets during aggregation, through an RGD mediated interaction with integrin $\alpha_{IIb}\beta_3$. The primary role of vWF appears to be its interaction with GP Ib-IX-V. VWF found bound to collagen in the subendothelium supports platelet attachment at high shear. Thrombospondin is another adhesive protein, also carried in the platelet alpha granule, which may be important in stabilizing platelet fibrin aggregates. Fibronectin, and vitronectin are important adhesive proteins for other cells in the extracellular matrix, their relative importance in platelet adhesion is unclear. Finally, on biomaterial surfaces, it appears that fibrinogen is the major adsorbed protein supporting platelet adhesion and spreading. The presentation of a platelet adhesive conformation is not permanent, however. Prolonged incubation of adsorbed fibrinogen in buffer leads to further conformational changes which result in a loss of platelet reactivity. These further changes can be prevented by the co-adsorption of albumin on the surface [89]. Many questions remain regarding the mechanism of platelet attachment and spreading on adhesive protein covered surfaces. The specific residues of fibrinogen responsible for supporting adhesion are undetermined, as is the conformation required. It may be that the surface density, or spacing of adhesive ligands may be critical in forming multi-ligand interactions with the platelet. The biochemistry of fibrinogen has been reviewed by Budzynski [90]. The structure and function of von Willebrand factor has received much attention and is reviewed in references [91-95]. Reviews of fibronectin and the thrombospondins can be found in references [96] and [97] respectively. The adsorption of adhesive proteins to surfaces,

the conformational changes which occur, and the interactions of cells with these ligands have been reviewed by Horbett [98, 99] and others [100].

2.2.6 The Biochemistry of Platelet Activation

Numerous biochemical pathways serve to transmit signals and accomplish the physiologic work of the platelet. As with receptor research, platelet biochemistry as a whole has received gret attention for many decades. Platelets have served as useful model cells in which several important signaling and activation pathways have been uncovered and studied. Some of the major topics in platelet biochemistry are: the G proteins and their role with the G protein coupled receptors; integrin mediated signaling via the cytoskeleton and interactions with various kinases; particularly phosphotyrosine kinases; the phosphoinositde pathway, the role of cAMP and adenylyl cyclase, the metabolism of arachidonic acid, the balance of phosphokinases and phosphotases, the 14-3-3 proteins, the disassembly and reassembly of actin filaments (cytoskeletal reorganization and dynamics), the role of calpain and calmodulin, the mechanisms of granule secretion and membrane vesiculation, and finally the function of lipid scramblase proteins to control membrane phospholipid asymmetry affecting the procoagulant activity of the platelet. By necessity, the review here must be very superficial, this section will try to suggest more comprehensive reviews on each of these topics.

First, several older and general reviews of platelet biochemistry make useful reading as long as one remains cognizant of where newer developments fit in. A reasonably current, broad review of platelet activation is available by Blockmans, et al [87]. There are other, somewhat current, broad reviews, including a particularly well referenced review by Rao [101], the first 50 references of which cover most of the major platelet biochemistry developments of the past several decades. Rao followed this a year later

with a review on the pharmacology of platelet activation [102]. A particularly compact, general review was written by Packham [103], and another which address various platelet disorders and their management in the clinic was written by Bennett [104]. Two other reviews which deserve special note are a thorough and compact review by Kroll [105] and an extremely thorough review on the subject (nearly 1100 refs.) from Siess [106], both of which have received, and still do receive, frequent citation. For many years the authoritative book on platelet biochemistry was that of Phillips and Shuman [107], and in many respects this is still a valuable compilation. Somewhat more recently, a quite comprehensive compilation stems from a 1992 Thrombosis Research Institute Symposium in London [108]. The contents of this volume are often cited as individual works under the series title, however the entire volume is a very useful reference. Finally the activation of flowing platelets under shear stress, resulting in both aggregation and adhesion is critical to the understanding of platelet function *in vivo*. Five established investigators in this field have recently authored a good review of the role of shear in platelet activation, including shear induced aggregation and platelet adhesion under shear [109]. Another good review of the role of biorheology in thrombosis is that of Hellums, [110].

2.2.6.1 G-Proteins

The heterotrimeric G proteins (GTP-binding proteins) represent an important signal transduction system coupling the events at certain membrane receptors with various intracellular effector systems. A general review of the heterotrimeric G proteins can be found in reference [111]. In platelets, G protein coupled responses include both stimulatory and inhibitory effects. The platelet G protein coupled receptors are of the typical seven transmembrane type, and include receptors for thrombin, thromboxane, prostacyclin, epinephrine, PAF, and others. The G proteins couple to effector systems

including adenylyl cyclase controlling cAMP formation and phospholipase C responsible for phosphoinositide hydrolysis. Brass has authored several authoritative reviews on the role of G proteins in platelets [75, 79, 112]. The role of monomeric G proteins (low molecular weight G proteins) has received more recent attention. Rap1B in platelets is a substrate for phosphorylation by protein kinase A, a step which results in its translocation from the membrane to the cytosol. After activation rap1 associates with $p120^{RasGAP}$ and phospholipase Cγ1. Lapetina, et al. have provided reviews of rap proteins in platelets [113, 114]. A recent report claims that an increase in $[Ca^{++}]_i$ is both necessary and sufficient for Rap1 activation in platelets [115].

2.2.6.2 Biochemistry of Integrin Signaling

The direct involvement of the integrin receptors in signal transduction is a topic of considerable interest, currently generating hundreds of papers per year. Some signaling molecules may interact directly with the cytoplasmic tail of the $ß_3$ integrin, while others are assembled in larger interactions with the receptor and the cytoskeleton. Components of integrin mediated signaling in platelets may include: kinases, particularly pp60c-src, $pp72^{syk}$, $pp125^{FAK}$; Src homology domain interactions, e.g. with PI-3-kinase and PLC; interactions with small G proteins. e.g. RhoA, ras; and other phospholipid mediators, e.g. PLA_2. As cited above (in the discussion of integrin $\alpha_{IIb}ß_3$ and other integrins) there are several good reviews discussing integrin signaling in platelets and other cells, particularly [13, 16, 23, 53]

2.2.6.3 Phosphoinositide Metabolism

Phosphoinositide metabolism has been recognized as an important signal transduction mechanism in platelets and other cells. The "classic" phosphoinositide pathway is the receptor ligand mediated, G protein triggered action of phospholipase C to hydrolyze PtdIns(4,5)P_2, located in the plane of the plasma membrane, liberating

diacylglycerol (DAG) and Ins(1,4,5)P_3 (IP_3). (Phospholipase C, is actually a family of phospholipase isoforms, PLCß is activated by thrombin stimulation, collagen stimulates PLC-γ2, ThxA_2 stimulates PLC-ß1, etc.). DAG then activates protein kinase C with various downstream effects, and the now cytosolic IP_3 diffuses to the membrane of the dense tubular system where it binds to the IP_3 receptor and triggers release of Ca^{++} from this calcium sequestering organelle into the cytosol. This pathway has been well established in platelets, and this description usually appears in most general reviews of platelet biochemistry, usually as part of the discussion of platelet $[Ca^{++}]_i$. Phospholipid signaling in general has been reviewed recently by Divecha and Irvine [116] and an older review of phosphoinositide metabolism in platelets is provided by Daniel [117]. More recently however, the attention has been focused on the D3 phosphoinositides and the role of phosphoinositide-3-kinase (PI-3-K) which has been identified in platelets; and, in a variety of cells participates in cytoskeletal dynamics, activation of the ras pathway, and secretion. A current general review of the phosphoinositide kinases is found in reference [118]. and their role in platelets is reviewed in reference [119]. Three research reports from Hartwig's group are indicative of the efforts in this area to understand the role of PI-3-kinase in actin assembly in platelets.[120-122]. Because PI-3-kinase is found associated with other signaling proteins in focal contacts in platelets, interest has focused on its cytoskeletal interaction, and possible regulation of, or regulation by the integrin $\alpha_{IIb}ß_3$ -ligand binding. References [123-127] are a sampling of current research in this area including a report of ADP-dependent PI-3-kinase activation in platelets adherent to a fibrinogen matrix.

2.2.6.4 Cyclic AMP and Cyclic GMP

Cyclic AMP and cGMP are both important second messengers of inhibitory signals in platelets. As is well known, cyclic AMP is formed from ATP by the enzyme adenylyl cyclase, and this enzyme is stimulated by G_s and inhibited by G_i. The receptor for prostacyclin (PGI_2) and PGE_1 is coupled to G_s and these antagonists result in increased cAMP levels, whereas some agonists (e.g. thrombin, ADP) act via receptor mediated G_i to reduce cAMP. Increased cAMP enhances Ca^{++} sequestration, and inhibits phosphoinositide hydrolysis presumably through an inhibition of phospholipase C. Increased cAMP also activates cAMP-dependent protein kinase, with numerous possible downstream effects. Increased cAMP inhibits platelet activation by reducing or preventing Ca^{++} mobilization, prevents the affinity modulation of integrin $\alpha_{IIb}\beta_3$, inhibits myosin light chain phosphorylation, actin polymerization, and cytoskeletal assembly. The actions of cAMP in platelet inhibition are clearly linked to Ca^{++} metabolism, but also include inhibition of other events or pathways downstream of Ca^{++} mobilization. The phosphorylation of rap1B by activated protein kinase A is one inhibitory event under investigation which is linked in increased cAMP. Similarly, guanyl cyclase produces cGMP and is stimulated by nitric oxide (NO, previously known as endothelial derived relaxing factor, EDRF) which is produced by the enzyme nitric oxide synthetase (NOS). Likewise increased cGMP is a strong inhibitor of platelet activation. There is likely crosstalk between the two pathways as increases in one cyclic nucleotide can affect the phosphodiesterases responsible for degradation of the other. Futhermore, both cGMP dependent protein kinase and cAMP dependent protein kinase act to phosphorylate a novel protein, VASP (vasodilator stimulated protein), which is associated with platelet microfilaments and focal contacts. Both prostacyclin and NO are released by endothelial cells and act locally as potent

vasodilators and platelet inhibitors. The stable prostacyclin analog ilprost has been suggested as a pharmacological platelet inhibitor, however its clinical use has been stymied by its potent vasodilatory effects. Walter, et al., have recently reviewed the important role of cyclic nucleotides in platelet biochemistry, as well as the interactions with endothelial and vascular smooth muscle cells [128, 129] It is becoming increasingly apparent that cAMP and cGMP interact with several other regulatory pathways in platelets, primarily the central mediator $[Ca^{++}]_i$ as well as aspects of integrin signaling and cytoskeletal dynamics. Other reviews that include discussion of this topic include references [79, 87, 101, 102, 130]. Examples of some current research on the involvement of these cyclic nucleotides in platelet activation include study of the role of PGI_2 and NO on phosphorylation of the platelet dense tubule IP_3 receptor [131] as well as their role in integrin $\alpha_2\beta_1$ mediated platelet adhesion to collagen [33, 132]. It has been suggested that NO and cGMP may play a role in calcium cycling [133]

2.2.6.5 The Arachidonate Pathway

Another well known pathway of biochemical activation in the platelet is the arachidonate pathway. Phospholipase A_2 acts on phosphatidylcholine and phosphatidylethanolamine in the inner surface of the platelet plasma membrane to liberate arachidonic acid. Phospholipase A_2 is regulated in part by $[Ca^{++}]_i$ but is also likely regulated by direct G protein interactions. The liberated fatty acid can diffuse from the platelet and bind to albumin. Albumin has a high affinity binding site for fatty acid, and as Roth points out in a review, albumin and fatty acid-free albumin affect arachidonate metabolism *in vitro* [134]. Inside the platelet, the arachidonate enters either the lipoxygenase pathway or the cyclo-oxygenase pathway. In the lipoxygenase pathway arachidonic acid is eventually converted to 12-HETE, which diffuses from the

platelet, and is chemotactic to PMNs and triggers the expression of tissue factor in monocytes (monocyte procoagulant activity). In the cyclo-oxygenase pathway, the arachidonic acid is converted by cyclo-oxygenase to cyclic endoperoxides which can diffuse to the endothelial cells and be converted to prostacyclin, a potent vasodilator and platelet inhibitor; or remain in the platelet and be converted by thromboxane synthetase to thromboxane A_2, a potent vasoconstrictor, platelet agonist, and stimulator of secondary platelet aggregation and secretion. Cyclo-oxygenase is irreversibly acylated by aspirin accounting for its therapeutic anti-platelet efficacy. These pathways are diagrammed nicely in the reviews by Rao [101, 102], summarized in the review by Blockmans, et al. [87] and reviewed in detail by Roth [134].

2.2.6.6 Protein Phosphorylation and Dephosphorylation

The phosphorylation of proteins by intracellular kinases represents a primary method to regulate protein function and cell physiology in many cells [135]. Platelets are no exception, in fact they contain unusually large amounts of various kinases and phosphatases. There are two classes; first those that phosphorylate on serine or threonine residues, and second those that phosphorylate on tyrosine. Several of the serine/threonine kinases have already been mentioned above. Diacylglycerol and Ca^{++} synergistically act to active protein kinase C, a ser/thr kinase whose major substrate is known as p47, pleckstrin and is phosphorylated in association with platelet secretion. Activated protein kinase C also leads to the activation of myosin light chain kinase, and the Ca^{++}/calmodulin dependent phosphorylation of the 20kDa light chain of myosin. The 14-3-3 proteins have been identified with several biologic roles including that of protein kinase C inhibitor. The finding of several isoforms of 14-3-3 in platelets and in particular the very recent finding that 14-3-3ζ interacts with the cytoplasmic domains of GPIb have focused the attention on this protein as a possible part of post receptor

occupancy signaling [136-139]. The cAMP and cGMP dependent kinases which act on VASP are also of the serine/threonine type. The action of the kinases is carefully balanced by the action of ser/thr phosphatases, and the recent use of okadaic acid compounds to block the these phosphatases is an important tool in their study.

The translocation of various kinases from the cytosol to the cytoskeleton associated with their activation is an important aspect of their functioning. This is particularly true for the tyrosine kinases, which have become the center of much attention in the past few years. Mentioned here are three of the more prominent tyrosine kinases in platelets: $pp60^{c\text{-}src}$ is one of four members of the src gene product family found in platelets. Platelets have an unsually large amount of this common and well known tyrosine kinase, which is found associated with the cytoskeleton upon activation, and whose activation appears to be dependent upon the binding of fibrinogen to integrin $\alpha_{IIb}\beta_3$. $pp72^{syk}$ is a recently described tyrosine kinase found only lymphocytes and platelets. Finally, $pp125^{FAK}$ (focal adhesion kinase) is found in many cells, including platelets, associated with the cytoskeleton, localized at areas of cell-matrix contact. The activation of $pp125^{FAK}$ also appears to depend upon integrin engagement and Ca^{++} in many cases. Where $pp60^{c\text{-}src}$ and $pp72^{syk}$ seem to be active early in the activation process, $pp125^{FAK}$ seems associated with later events, like full spreading and secretion. Several tyrosine phosphatases are found in platelets, including PTP1B (which can be cleaved by calpain), SHP-1 and SHP-2.

The role of the various kinases and phosphatases, particularly the role of the tyrosine kinases in the assembly of a signaling complex, is currently under tremendous investigation. The role of PI-3-kinase (inhibited by wartmannin) and its subunits which also demonstrate SH2 and SH3 domains, as well as possible involvement of the MAPK pathway in platelets are also closely linked. Many of these proteins have significant

homology with systems that are important in other cells. It is likely that this vein of research will shape a new landscape to our understanding of platelet activation, signaling, and interaction with adhesive proteins. The following thoughts should be kept in mind. Early in the study of these processes, a host of inhibitors for particular ser/thr or tyrosine kinases were developed and used. Later their specificity was brought into question. Furthermore the very interlinked nature of these pathways suggest that other kinases may compensate for inactivated ones in certain situations. So while inhibitors still are a very useful tool, their limitations must be considered as well. Likewise inhibitors of the phosphatases are also used in the study of these pathways: okadaic acid to inhibit ser/thr phosphatases, and pervanadate (vanadate and hydrogen peroxide) to inhibit the tyrosine phosphatases. Much remains to be discovered but it seems clear that protein phosphorylation and dephosphorylation is carefully controlled, and intricately linked to other platelet activation events including $[Ca^{++}]_i$, mobilization [140], and cytoskeletal reorganization.

Two reviews on this subject deserve special note. First, Levy-Toledano, et al. have recently reviewed the current situation regarding both ser/thr and tyr phosphorylation and dephosphorylation in platelets [141], and Jackson et al. have provided a review of the tyrosine kinases and phosphatases in platelets with discussion of structural homology [142]. Other key reviews are references [16, 40] as well as [23, 24, 53]. A small sampling of the primary research articles on kinase involvement, including platelet adhesion to immobilized fibrinogen, adhesion to collagen under flow, platelet spreading and other topics is found in references [143-151].

2.2.6.7 Cytoskeletal Dynamics

The dramatic shape change exhibited by the platelet during aggregation or attachment and spreading is a hallmark of platelet activation. Nearly 20% of the platelet's total

protein is actin. In the resting state, nearly 40% of the actin is in actin filaments, capped with gelsolin or capZ protein, the remaining actin bound to thymosin b4. The actin concentration in platelets is so high, that if not for the capped ends, the actin would be expected to immediately polymerize. Upon stimulation, the actin filaments are uncapped, the filaments are severed by gelsolin, activated by an increase in $[Ca^{++}]_i$ creating new ends, and the actin is polymerized again. While it is generally accepted that $[Ca^{++}]_i$ plays a role in cytoskeletal dynamics, platelets are still capable of spreading when $[Ca^{++}]_i$ is chelated. So, there are still unanswered questions regarding the mechanism of cytoskeletal reorganization in platelets. The interactions of the integrin $\alpha_{IIb}\beta_3$ and GP Ib-IX-V complex with the cytoskeleton via actin bind protein (ABP 280, filamin), along with the collection of proteins like talin and vinculin, and the assembly of signaling proteins, e.g. the tyrosine kinases discussed above are all important aspects of cytoskeletal dynamics and are discussed in several important reviews found in references [10, 19, 25, 152, 153].

2.2.6.8 Calpain

Calpain is an important Ca^{++}-dependent cysteine protease found in many cells including platelets, and has been linked to many aspects of platelet activation. Calpastatin has been identified as its endogenous inhibitor, and calpeptin is a useful cell permeant inhibitor. Calpain (calcium activated neutral protease, CANP) has been identified in two forms, m-calpain, and mu-calpain which exhibit their proteolytic activity (*in vitro*) at mM and micromolar concentrations of Ca^{++}, respectively. While this is orders of magnitude higher than calcium concentration in the platelet cytosol, it is believed that both forms of calpain are capable of proteolytic activity at typical $[Ca^{++}]_i$. Upon platelet activation and with increased platelet $[Ca^{++}]_i$, calpain is translocated from the cytosol to the membrane cytoskeleton. There it has been shown to be involved with

various signaling events. Calpain is known to cleave platelet filamin (ABP-280), p235 talin and other membrane skeleton proteins suggesting its role in cytoskeletal dynamics. Furthermore, Huang et al have noted the cleavage of tyrosine phosphorylated cortactin, an actin binding and cross linking protein that is tyrosine phosphorylated by $pp60^{src}$ [154]. Montsarrat, et al. have suggested that calpain is involved in PI-3-kinase signaling in manner dependent upon integrin $\alpha_{IIb}\beta_3$ [155]. Calpain has been shown to act on $pp60^{src}$, $pp125^{FAK}$ and related tyrosine kinases [156, 157]. Frangioni et al have shown that cleavage of phosphotyrosine phosphatase 1B (PTP1B) by calpain results in its translocation and activation, an event dependent upon integrin engagement and providing another mechanism to control tyrosine phosphorylation in platelet signaling [158]. Moreover, calpain has been shown to cleave the integrin β_3 subunit directly, possibly regulating affinity modulation of integrin $\alpha_{IIb}\beta_3$ and bi-directional signaling [159]. Several reports implicate calpain in procoagulant activity and microparticle formation [160]. Schoenwaelder et al report identifying three different isoforms of activated mu-calpain and attribute clot retraction to one isoform and procoagulant microvesiculation to another [161]. Pasquet et al, tie this process to increases in platelet $[Ca^{++}]_i$ [162]. Yuan et al have related the function of calpain to GP Ib-IX-V and integrin $\alpha_{IIb}\beta_3$ signaling in vWF stimulated platelets during adhesion and spreading [163] . Finally, Miyazaki et al have used shear induced aggregometry without agonists to show that vWF - GPIb binding, $[Ca^{++}]_i$ rises, and activated calpain are involved in microparticle formation in this system [164]. Several general reviews of the calpains are available [165-167]. Clearly calpain is the subject of much current research and will play a role in our understanding of signal transduction during platelet activation and adhesion.

2.2.6.9 Granule Secretion

Granule secretion is an important aspect of platelet physiology and, as described above, provides for local delivery of adhesive proteins and potent agents to stimulate and recruit additional platelets and other cells. As such this process must remain under tight control. The centralization of granules is a key consequence of the cytoskeletal dynamics discussed above. Recently, several proteins that regulate and target granule fusion have been identified in platelets. While there is significant similarity to the release of synaptic vesicles, platelet granules do not appear to be pre-docked with the membrane, which may explain in part the sizable (many seconds) lag between stimulus and secretion [168]. Also, the release of the alpha granule exposes P-selectin (previously known as GMP-140 or PADGEM or CD62) on the platelet surface and this has often been used as a marker of platelet activation. It now appears that in addition to cell -cell docking, this membrane protein also plays a role in cell signaling. In platelets, P-selectin undergoes rapid phosphorylation and dephosphorylation, although the significance of this is unknown. P-selectin binds to its counter receptor PSGL-1 on neutrophils and monocytes where it induces tissue factor (procoagulant activity) [169].

2.2.6.10 Membrane Phospholipd Scrambling

Platelet procoagulant activity is another important aspect of platelet activation. The distribution of membrane phospholipids in the resting platelets is asymmetric, in that phosphatidylserine and phosphatidylethanolamine are found exclusively in the inner leaflet of the plasma membrane. This asymmetry is maintained by an aminophospholipid translocase. Upon strong platelet activation and dependent upon $[Ca^{++}]_i$ and extracellular calcium, the aminophospholipid translocase is blocked and the action of a lipid scramblase protein facilitates the transbilayer movement of the aminophospholipids resulting in the loss of asymmetry and the exposure of

phosphatidylserine on the outer surface of the platelet membrane [170, 171]. This exposure facilitates the assembly, on the platelet membrane, of the tenase and prothrombinase complexes of the coagulation cascade, increasing the rate of thrombin formation by a factor of 10^6. Platelet procoagulant activity is the consequence of strong platelet activation, such as by the combination of collagen and thrombin agonists. It is a late event in the sequence of platelet activation, and is dependent upon a rise in $[Ca^{++}]_i$ and the presence of extracellular calcium. Platelet procoagulant activity can also be triggered by ionophores that artificially cause a rise in $[Ca^{++}]_i$ and by the membrane attack complex of complement, as reviewed in [172]. Recent works suggests that released ADP from the platelet dense granule may serve as a secondary stimulus to maintain the prolonged $[Ca^{++}]_i$ rise necessary for the phospholipid flip-flop to take place [173]. Membrane phospholipid asymmetry and platelet procoagulant activity have been recently reviewed [174-177].

Another event closely related to the expression of procoagulant activity is the vesiculation of the platelet membrane and formation and shedding of small platelet microparticles (platelet dust, platelet vesicles). These unilammelar structures are roughly 70-170nm in diameter, contain several platelet membrane glycoproteins, and also exhibit considerable procoagulant activity (25-30% of the total platelet procoagulant activity is associated with the released microparticles). They are formed under the same stimulatory conditions that trigger membrane phospholipid flip-flop (requiring increased $[Ca^{++}]_i$), as well as other conditions as a consequence of platelet damage, e.g. in cardiopulmonary bypass (under high shear), in prolonged platelet storage (blood bags) and during freeze and thaw cycles. Activated calpain likely plays an important role in microparticle shedding under all these conditions, except that blebbing caused by complement, which may caused by a different mechanism. The role of the released

procoagulant microparticles is unclear. It would seem a little antithetical to the idea that activated platelet membranes provide localized procoagulant activity limited to the site of vascular injury. Microparticles may augment the efficacy of plasma cryoprecipitate used to treat von Willebrand's Disease, and microparticles have been shown to augment platelet adhesion to fibrin in a perfusion study, where the microparticles align with the fibrin. Microparticles also bind to, activate and aggregate neutrophils. [178-180].

There are undoubtedly more signaling pathways yet to be uncovered in platelets. For example, recently CD38 was identified on human platelets where it functions in the synthesis of cyclic-ADP-ribose, a calcium-mobilizing compound [181]. Its possible role in platelet signal transduction and activation remains to be explored. There is certainly much to be clarified regarding the pathways that have been identified so far. Particularly unclear is how various pathways interact, to provide control and regulation, positive and negative feedback, during platelet activation. Under most circumstances *in vitro* and certainly *in vivo*, the distinction between the primary stimulus for activation and secondary platelet activation promoted by released agonists or coagulation factors is murky. The $[Ca^{++}]_i$ does appear to be related to many, if not most, of the platelet activation pathways, however, as a central mediator of signal transduction.

2.3 Platelet $[Ca^{++}]_i$

As Clapham has pointed out; in cells from bacteria to specialized neurons, intracellular ionized calcium is the most common signal transduction element. Unlike phosphorylated forms of nucleotides or proteins, Ca^{++} cannot be metabolized, so cells exert extraordinarily tight control over $[Ca^{++}]_i$, through calcium buffering proteins and specialized pumps to extrude and sequester Ca^{++}. Resting $[Ca^{++}]_i$, in most cells is

less than 100nM, compared with 2mM levels extracellularly [182]. Through its action on effector systems like calpain, the calcium-calmodulin kinase (CaM kinase), calreticulin, and a host of others, $[Ca^{++}]_i$ influences many cell process critical for life, e.g. regulation of parts of the cell cycle, muscle contraction and hormone release. Calcium transport across the plasma membrane is controlled by the plasma membrane calcium ATPase [183], the Na^+/Ca^{++} exchanger and direct calcium channels. Transport across the internal membranes of the organelles is controlled by the mitochondrial membrane and the sarcoplasmic/endoplasmic reticulum Ca^{++} ATPase (SERCA) pumps.

A key advancement in the understanding of calcium signaling came in the discovery of IP_3 as a second messenger, which when released as a consequence of the activation of phosphoinositide metabolism, acts on the IP_3 receptor in the sarcoplasmic and endoplasmic reticulum membranes to release Ca^{++} sequestered in these internal organelles. Clapham provides a brief and broad overview of calcium signaling [182, 184] and Carafoli provides a general review of calcium homeostasis [185]. A detailed and thorough review of the molecular and cellular physiology of intracellular calcium stores was written by Pozzan, et al [186]. The IP_3-Ca^{++} pathway and capacitative calcium entry are discussed in several reviews [187-192].

In platelets, $[Ca^{++}]_i$ is widely recognized as an important second messenger. A rise in $[Ca^{++}]_i$ is associated with platelet stimulation by nearly every soluble agonist. The list of platelet functions believed to be calcium dependent is impressive and includes: shape change, adhesion to surfaces, spreading, aggregation, secretion, clot retraction, binding of fibrinogen and a host of other intracellular biochemical events related to platelet activation. [193]. Resting platelet $[Ca^{++}]_i$ is controlled by both extrusion across the outer plasma membrane, and by sequestration of Ca^{++} into the dense tubule system. Sequestration is by a Ca^{++}-ATPase (a SERCA type 2b) which can be blocked by

thapsigargin and is believed to be stimulated by cAMP. The presence of the plasma membrane pump had been controversial, but a recent report shows evidence of a plasma membrane pump, similar to that found in erthyrocytes, in human platelet plasma membranes [194].

The rise in $[Ca^{++}]_i$ upon stimulation comes from both an influx of extracellular calcium, and from the release of sequestered calcium from the dense tubules. Influx of calcium could be due: to receptors that also function as calcium channels, to channels that are activated by receptor coupled G proteins, or to channels controlled by other second messengers. There do not appear to be any voltage-gated calcium channels in platelets. There have been numerous suggestions that the ADP receptor acts as either a direct calcium channel or is linked to a G protein activated channel. A popular model applied to calcium signaling is the capacitative model or store-regulated model put forth by Putney. In this model, the discharged state of the internal stores somehow triggers the influx of extracellular calcium across the plasma membrane. The messenger for such a signal is unclear; it could be signaled by IP_3 or by other protein phosphorylation events [195]. It has been suggested that such calcium entry might be responsible for the plateau phase of the transient $[Ca^{++}]_i$ response. Recently protein kinase Cß has been suggested as a negative or limiting factor in receptor mediated calcium entry [196]

Release of calcium from the internal stores appears to be mediated by IP_3 via the well characterized, tetrameric IP_3 receptor found in the dense tubule membrane; the IP_3 being generated as described earlier by the action of phospholipase C. It has been suggested that there are two types of intracellular pools in platelets, one that is sensitive to thapsigargin treatment and another that is sensitive to IP3 [197]. Oscillations in $[Ca^{++}]_i$ have also been observed in platelets in response to thrombin, serotonin and other agonists. The asynchronous nature of these responses meant that they were

obscured in population measurements. It was not until single cell imaging of platelets was achieved that this level of complexity was revealed. The importance of these oscillations is unknown.

As discussed above, $[Ca^{++}]_i$ is believed to affect several biochemical pathways of platelet activation including the function of phospholipase A2 and the arachidonate pathway, and the action of various kinases, e.g. protein kinase C, and myosin light chain kinase resulting in the phosphorylation of pleckstrin and myosin, respectively. While $[Ca^{++}]_i$ is clearly important in most platelet responses, there appear to be other, possibly redundant, routes of activation that do not require increased $[Ca^{++}]_i$. This has made it difficult to attribute or link specific platelet responses solely to increased $[Ca^{++}]_i$.

The role of platelet $[Ca^{++}]_i$ in platelet activation is usually discussed in any review of platelet physiology, e.g. references [87, 106]. Calcium signaling in platelets has been thoroughly reviewed by Sargeant and Sage [198] and also by Heemskerk and Sage in two complimentary reviews [199]. Likewise, the volume derived from a 1992 symposium, and edited by Authi, et al., contains three important reviews on the subject [200-202], as well as a review by Gear on the subsecond events in platelet calcium signaling [203]. Finally several older reviews explain the rationale for the interest given to this topic in the early 1990's, and in retrospect demonstrate the progress (and lack thereof) in understanding platelet calcium signaling [193, 204-207].

The study of platelet $[Ca^{++}]_i$ has involved several different methods. By far the most work has been done utilizing intracellular fluorescent calcium sensitive probes in platelets in stirred suspension in a spectrofluorimeter. To study the $[Ca^{++}]_i$ response to adsorbed proteins or materials, beads of various kinds have been added to a stirred platelet suspension in a spectrofluorimeter, or fluorophore loaded platelets have been

passed through bead columns, and the measurements made on the platelet suspension/effluent. Flow cytometry has been used to measure $[Ca^{++}]_i$ and reveal heterogeneity in responses in the platelet population and correlate $[Ca^{++}]_i$ with other markers of activation, e.g. P-selectin exposure. There has been considerable interest in shear induced aggregation, and this has been studied most readily in a cone and plate viscometer that has been adapted to measure platelet aggregation by light transmission and $[Ca^{++}]_i$ by spectrofluorimetry. Finally there have been about ten groups that have published reports of single platelet imaging of $[Ca^{++}]_i$, including only one report of imaging under flow conditions. Only a few of these have been interested in the platelet surface interaction, the remainder have been interested in other aspects of platelet pharmacology. The numerous studies of platelet $[Ca^{++}]_i$ during aggregation studied in stirred suspension by spectrofluorimetry are included in the reviews listed above and are not reviewed further in this section.

2.3.1 Beads and Bead Columns

Salzman et al were among the first to measure $[Ca^{++}]_i$ in stirred suspensions using the photoprotein aequorin or fluorescent probe Quin 2. Much of this group's early work measuring platelet $[Ca^{++}]_i$ in the effluent of a bead column is summarized in their discussion of the role of fibrinogen in the activation of platelets by artificial surfaces [208]. When fibrinogen was present in the suspension medium, they found a rise in $[Ca^{++}]_i$ of similar magnitude to that stimulated by low concentrations of thrombin. This result was taken to indicate that platelet activation in the bead column occurs via pathways common to that of soluble agonists. In separate experiments, fibrinogen crosslinked by antibodies to the E-domain also led to a rise in $[Ca^{++}]_i$ when added to stirred platelet suspensions without other agonists [209]. It is thought that high local concentrations of ordered fibrinogen, as found adsorbed to a biomaterial surface or

represented by the crosslinked fibrinogen in solution, might prompt activation via its interaction with integrin $\alpha_{IIb}\beta_3$. The relationship, if any, between the ordering of adhesive ligands on a surface, and the clustering of integrins during adhesion and signaling remains a subject of intriguing speculation. In the bead column technique, platelets are subjected not only to activation by the material surface of the beads, but are also subject to activation from shear stress, and released mediators of platelet aggregation, e.g. ADP, serotonin, and fibrinogen. Thus, it is difficult to discern the extent of purely biomaterial-induced mobilization of $[Ca^{++}]_i$.

Ware et al have published an extensive investigation of platelet activation by poly(methylmethacrylate) (PMMA) beads, in stirred washed platelet suspensions [210]. The small hydrophobic beads induced platelet aggregation, preceded by $[Ca^{++}]_i$ mobilization, in a dose-dependent manner. It appears that the strength of the stimuli (the polymer beads) is sufficient to induce the release of alpha-granule fibrinogen (as is thrombin to support the aggregation of the washed (external fibrinogen-free) platelets. The aggregation was found to be accompanied by protein phosphorylation (PKC substrate, p47), serotonin release, and accumulation of phosphatidic acid (a diacylglycerol metabolite). These responses were inhibited by both aspirin and apyrase (an ADP scavenger), however, only partially so. The authors utilized a variety of monoclonal antibodies and peptides to inhibit integrin $\alpha_{IIb}\beta_3$. This led to the complete inhibition of fibrinogen binding, and aggregation. However, the $[Ca^{++}]_i$ response was not significantly affected by the blockage of integrin $\alpha_{IIb}\beta_3$ function. This would appear to clearly indicate that biomaterial contact is capable of inducing platelet activation and response without the upregulation of $\alpha_{IIb}\beta_3$ and subsequent aggregation/adhesion. The authors treated the platelets with chymotrypsin, which cleaves many membrane receptors, most notably GPIb, but leaves $\alpha_{IIb}\beta_3$ intact and capable of binding

fibrinogen. Chymotrypsin treated platelets aggregated normally when stimulated with fibrinogen in agonist concentrations. However, PMMA-induced aggregation and $[Ca^{++}]_i$ response was abolished. The authors speculate on a role for GPIb in this biomaterial-induced platelet activation, and mention pilot data that both support and refute this speculation. GPIb is suspected of conferring shear sensitivity to platelet activation, and its role in shear induced calcium influx has more recently been studied (see below). It is not surprising that it may play an important role in the response of platelets to PMMA beads in a stirred suspension.

Another investigator, Yui, has made extensive use of coated beads in platelet $[Ca^{++}]_i$ measurement for biomaterial assessment. Yui et al [211] monitored $[Ca^{++}]_i$ in a stirred suspension of washed Fura 2 labelled rabbit platelets mixed with latex particles (beads). They found that exposure of the platelets to the particles resulted in an increase in $[Ca^{++}]_i$ of roughly 1500nM, similar to that induced by 0.1U/ml thrombin stimulation. The adsorption of albumin to the beads prior to their use diminished this platelet response in a dose dependent manner. These results are consistent with the observations that albumin adsorption reduces material-induced platelet adhesion and activation, e.g. references [212, 213]. The rise in $[Ca^{++}]_i$ was reduced but not abolished when Ca^{++} was removed from the extracellular medium. This indicates that both calcium influx and release from intracellular stores are involved, as is found to be the case with soluble platelet agonists. Subsequently, Yui applied the technique to a study of both polystyrene and poly(acrylamide-co-methacrylic acid) beads [214] and finds that polystyrene particles evoke a much larger increase in $[Ca^{++}]_i$ than the p(AAmMAc) beads. Studying poly(propylene oxide) segmented nylon, Yui finds that the pre-adsorption of plasma proteins reduces the platelet $[Ca^{++}]_i$ response, this time in the effluent of a bead column [215]. Yui also used the p(AAmMAc) beads to measure

membrane fluidity along with $[Ca^{++}]_i$ [216]. Yui then adapted the method to look at changes in $[Ca^{++}]_i$ in platelets in contact with the modified nylon surfaces in the form of small blood bags [217, 218]. Polyurethanes modified with PEG spacers and various endgroups (-OH, -NH2 and -SO3) have also been coated on beads and the -SO3 modified surface shows reduced $[Ca^{++}]_i$ response and platelet adhesion [219]. Finally, Yui used the bead column again to observe reduced platelet activation on certain novel phospholipid polymers [220].

Although Yui has reported differences in $[Ca^{++}]_i$ response between different materials or conditions, it is difficult to draw conclusions from any of this work. First, Yui has not given a detailed description of the calibration used in his system . Secondly, Yui exclusively uses rabbit platelets. One report on the Ca^{++} content of platelets of various species shows rabbit platelets to contain roughly half as much Ca^{++} as human platelets, and they demonstrate far less dense granule secretion; suggesting that there are species differences involving Ca^{++} homeostasis and biochemical process likely to be $[Ca^{++}]_i$ dependent [206]. Finally, as in all bead or bead column assays, it is difficult to attribute the platelet response solely to platelet surface contact. The platelets are sheared in the experiment, in an uncontrolled manner, and certainly are stimulated by various secondary aspects of platelet activation and aggregation, e.g. by agonists that are released from the platelets themselves. Those platelets that are not attached to the surface or left behind as aggregates (bead column and blood bag experiments) are then analyzed some time after the actual material contact takes place. As used by Yui these measurements may provide a rough estimate of platelet damage during the experiment, but it is difficult to draw any conclusions regarding the mechanisms of platelet activation by surfaces in these experiments.

2.3.2 Flow Cytometry

Flow cytometry has proven to be a useful tool in the study of various aspects of platelet activation (including that induced by biomaterials), primarily by measuring the expression of active integrin $\alpha_{IIb}\beta_3$ or the exposure of P-selectin as a consequence of alpha granule release [221]. The formation of platelet microparticles and platelet procoagulant activity have also been studied with flow cytometry [162, 222, 223]. Gemmell has used flow cytometry to assess both microparticle formation and complement involvement in platelet activation by biomaterials [224, 225].

Intracellular calcium can be measured in flow cytometry using the dual emission dye, Indo-1, and a UV laser system. Rabinovitch has reviewed the technical details of such measurements [226, 227]. Several groups have made measurements of platelet $[Ca^{++}]_i$ using flow cytometry. An early finding was that the response of the platelet population to submaximal stimulation with thrombin or ADP [228] showed a clearly heterogeneous response, i.e. subpopulations of responding partially responding and non-responding platelets [229]. These observations reveal what was previously impossible to detect in stirred suspension experiments; namely that, when stimulated with agonist, the platelet $[Ca^{++}]_i$ response is not the same in every platelet. Oda et al have used flow cytometry to correlate $[Ca^{++}]_i$ with alpha granule secretion and actin polymerization in platelets [230, 231] again stimulated by thrombin of ADP. Here again, heterogeneity was a clear feature of both resting and stimulated platelet populations. Finally, Haynes has recently reported flow cytometric measurement of platelet microparticles and P-selectin exposure in conjunction with spectrofluorimeter measurement of resting and stimulated platelet $[Ca^{++}]_i$ from a population of patients with acute coronary ischemias [232]. The results show increased resting $[Ca^{++}]_i$ and increased microparticle formation (but, interestingly, not P-selectin) in the patient group

with unstable angina or recent myocardial infarction. This report provides an excellent example of the clinical significance of platelet $[Ca^{++}]_i$ measurements.

2.3.3 Shear-Induced Platelet Aggregation and $[Ca^{++}]_i$

In vivo, platelets experience a range of shear stresses. As reviewed by Kroll et al [109], studies of platelet aggregation in stirred suspensions later gave way to devices with more controlled shear regimes, including parallel plate flow cells, and the cone and plate viscometer. The result was the description and investigation of shear induced aggregation.

The Ikeda laboratory and the Kroll laboratory have both modified cone and plate viscometers to measure the shear induced aggregation by light transmission and platelet $[Ca^{++}]_i$ via ratio fluorescence with the dual emission indicator Indo-1. The early reports were confusing, but did demonstrate a rise in $[Ca^{++}]_i$ concurrent with shear induced aggregation. The response was dependent upon extracellular calcium and was blocked by antibodies against either GPIb or vWF [233]. A more detailed report from this group [63] confirms that the multimeric interaction of vWF with GPIb results in a calcium influx, and subsequent activation leading to affinity modulation of integrin $\alpha_{IIb}\beta_3$ and the support of aggregation crosslinked by vWF. Interestingly, recombinant monomeric vWF fragments were unable to elicit the transmembrane calcium influx and failed to support aggregation. Also the affinity modulation of integrin $\alpha_{IIb}\beta_3$ could be blocked by increased cAMP, preventing aggregation. Separately, Chow et al report these same findings in a similar system [62]. They also report that extracellular or released ADP is required for the aggregation step, though not for the calcium influx. And similar findings have been reported by yet a third group, albeit using a less defined shearing system [60]. Further work by Ikeda shows increased tyrosine phosphorylation associated with shear induced aggregation, which can be blocked by

antibodies that interfere with either the vWF -GPIb or -integrin $\alpha_{IIb}\beta_3$ interaction [61], and more specifically the role of the Ca^{++}/calmodulin dependent myosin light chain kinase as well as protein tyrosine phosphatases 1 and 2A in this process [234].

2.3.4 Measurements of $[Ca^{++}]_i$ in Single Platelets

Since the introduction of Fura 2 in 1985, several groups have reported single platelet imaging of $[Ca^{++}]_i$ using ratio fluorescence microscopy. By far the majority have been interested in platelet response to soluble agonists, usually thrombin or ADP. More recently attention has been given to the $[Ca^{++}]_i$ response during attachment to substrata.

One of the first reports is that of Hallam et al [235] who report ed (in abstract form) the response of single adherent platelets (on glass coverslips) to stimulation by ADP and thrombin. In their studies ADP induced a rapid (1-2s) rise in $[Ca^{++}]_i$ from a resting 100-200nM to >1μM in more than 90% of the adherent platelets. By inference then, there was a subpopulation (10%) of adherent platelets that was less or unresponsive. Thrombin elicited a stronger response, giving rise to $[Ca^{++}]_i$ levels greater than 2μM, as is seen in stirred suspensions. Oscillatory behavior was also noted. Thus, both heterogeneity and delayed oscillating responses were noted in the stimulation of adherent platelet populations by soluble agonist. Next was a report from Tsunoda et al [236] who used both digital imaging and microspectrofluorimetry. They report resting $[Ca^{++}]_i$ values of 72nM and a rise to 300nM upon stimulation with 0.5U/ml thrombin.

In 1991-1992 Nishio et al published reports of the imaging of adherent single platelets used to study the response of rabbit platelets to serotonin [237], and concanavalin A [238], as well as thrombin and ADP [239]. They first reported the response of single adherent platelets to serotonin and other serotonin receptor agonists and antagonists [239]. In their studies they observed rises in $[Ca^{++}]_i$ upon stimulation

with ionomycin and the G-protein agonist $AlFl_4^-$. Serotonin induced receptor mediated oscillations in $[Ca^{++}]_i$. No response was observed in the absence of extracellular Ca^{++} indicating the response was primarily receptor mediated calcium entry. The protein kinase C activator, mezerein diminished or prevented the serotonin-induced response. This was reversed by the addition of a protein kinase C inhibitor, H-7, thus indicating a role for PKC in the serotonin-induced receptor mediated response. Concanavalin A also induced a $[Ca^{++}]_i$ rise with a delay of roughly 20sec and evidence of oscillations [238]. Thrombin stimulation elicited a $[Ca^{++}]_i$ transient, while ADP triggered oscillatory behavior [239].

Ozaki, et al observed oscillations in platelet $[Ca^{++}]_i$ in human platelets adherent to glass and stimulated with low dose (0.01U/ml) thrombin [240]. Halim et al demonstrate a platelet $[Ca^{++}]_i$ response to endothelin-1, a vasoconstrictive peptide derived from endothelial cells, in single platelets on glass coverslips. [241]. Finally, Majima et al demonstrate that the heterogeneity in platelet responses (at least in rabbits) might be related to platelet density [242]. They separate washed platelets by density over Percoll and show that platelets from the lower density subpopulation show much reduced responses to thrombin stimulation, higher density platelets were more sensitive to aggregating agents, had higher reactivity during granule release and stronger $[Ca^{++}]_i$ responses.

A unique use of $[Ca^{++}]_i$ imaging was that of Pelletier et al [243]. In an investigation of integrin signaling, Pelletier et al stably transfected a 293 cell line with the integrin $\alpha_{IIb}ß_3$, conferring upon this cell line the ability to adhere and spread on a fibrinogen coated surface, a phenotype not expressed by the parent cell line. Stimulation of the cells by adhesion to fibrinogen resulted in $[Ca^{++}]_i$ oscillations followed by the tyrosine phosphorylation of $pp125^{FAK}$. When the intracellular calcium

was chelated with BAPTA-AM, the cells adhered but did not spread, and phosphorylation of pp125^{FAK} was blocked.

Heemskerk et al, have demonstrated spiking of $[Ca^{++}]_i$ in individual adherent human platelets [244]. In contrast to the reports discussed so far, the platelets in this study were allowed to settle onto a fibrinogen coated glass substrate. Heemskerk rationalized this choice of substrate as a "physiologically appropriate means of immobilization for study (of agonist stimullated platelets) by ... calcium imaging." as opposed to the bare glass used in much of the previous work. They found however that some cells showed spiking in $[Ca^{++}]_i$ upon attachment to the substrate (without agonists), while others did not. The addition of the agonist ADP increased the frequency of the spiking. Furthermore, in contrast to earlier work reported in rabbit platelets, the spiking continued in the absence of external Ca^{++}. When treated with prostacyclin or RGDS peptide, the cells were not very firmly attached, and the observed $[Ca^{++}]_i$ remained low and constant. This study also took advantage of newer technology, using a low-light CCD camera in video mode, and dedicated hardware to provide ratio images at impressive 0.8sec intervals. Also of note in this report is the fact that the authors were unable to calibrate the ratio fluorescence signal to actual $[Ca^{++}]_i$ values due to the low fluorescence signal and small size of the cells, a situation which, for many groups, continues to this day. In a subsequent study published the following year [245], Heemskerk et al report that the spiking of otherwise unstimulated platelets attached to fibrinogen substrata could be eliminated by the use of apyrase to catabolize adventitious ADP. They further demonstrate spiking in response to low doses of thrombin, and sustained $[Ca^{++}]_i$ elevation with higher doses. In general the platelet responses followed in these studies were during the first three to five minutes after attachment.

In a brief report, Rao et al describe the use of the ACAS laser cytometer/confocal microscope, and the use of the dual emission dye INDO-1 to measure $[Ca^{++}]_i$ in single adherent platelets from a patient with giant platelet syndrome [246]. Elevation of $[Ca^{++}]_i$ was seen upon attachment of the platelets to the glass substrate.

In the early studies of $[Ca^{++}]_i$ in single platelets, the investigators were mainly interested in the $[Ca^{++}]_i$ reponse of the single platelets to soluble agonists, i.e. the platelets were allowed to settle, and non-adherent cells rinsed away, then the $[Ca^{++}]_i$ observations were conducted, and the response to the addition of soluble agonist evaluated. In more recent work, Poole and Watson use ratio fluorescence microscopy to study the $[Ca^{++}]_i$ response during platelet attachment to collagen [247], i.e. the attachment process itself is viewed as the "agonist" in these studies. There has been controversy as to whether collagen is able to directly elect a $[Ca^{++}]_i$ rise in platelets without the involvement of secondary mediators, e.g. thromboxane and ADP. This study shows that, after a variable lag phase, apyrase and indomethacin treated (cyclo-oxygenase inhibited), platelets adherent to collagen coated polyethylene exhibited a rapid rise in $[Ca^{++}]_i$ of nearly 400nM. The $[Ca^{++}]_i$ elevation was sustained throughout the observation. As for many adhesion receptors, it is suspected that the adhesion event is signaled via tyrosine phosphorylation resulting in the activation of phospholipase C-γ2 and subsequent IP_3 mediated Ca^{++} release from stores. Indeed in this experiment, a tyrosine kinase inhibitor reduced the number of cells exhibiting a $[Ca^{++}]_i$ response, and resulted in reduced oscillating responses in the remaining responders, without affecting (the authors claim) the adhesion event. This suggests that the $[Ca^{++}]_i$ rise triggered by collagen involves a tyrosine kinase mediated step or pathway. The authors note that the sustained nature of the calcium response is unusual in comparison to the other soluble agonists studied in single platelet work, such as thrombin, ADP and serotonin, which

all triggered oscillations. Platelets attached readily to a laminin coated substrate in these studies but did not show a $[Ca^{++}]_i$ rise. Observations were made for ten minutes or so in these experiments, and the lag periods were described as variable, from 15sec to two minutes. A lag phase before activation has long been known as a hallmark of collagen stimulation in platelet aggregometry.

Ariyoshi and Salzman have recently published two studies of platelet $[Ca^{++}]_i$ using ratio fluorescence imaging. In the first, they have combined the use of Fura 2 with the pH indicator SNARF-1 for the first reported simultaneous measurement and imaging of $[Ca^{++}]_i$ and pH_i in single platelets [248]. Use of the monovalent ionophore monensin to swamp the Na^+/H^+ antiporter resulted in a rise in pH_i but not in $[Ca^{++}]_i$. Platelets spreading on glass, or stimulated by thrombin or ADP all showed rises in $[Ca^{++}]_i$ and in pH_i, but the two responses appeared to be independent, the alkanization occurring later than the rise in $[Ca^{++}]_i$. In their second report [249], they report localized gradients in $[Ca^{++}]_i$ within single adhering platelets, and correlate these gradients with the distribution of f-actin, gelsolin and integrin $\alpha_{IIb}\beta_3$ in cells that were fixed immediately following the $[Ca^{++}]_i$ imaging. The authors report that the $[Ca^{++}]_i$ rise was greatest during pseudopodia formation, and spatially the $[Ca^{++}]_i$ was found in the pseudopodia and at the core of spread platelets. The authors show that regions of increased $[Ca^{++}]_i$ within single cells correlated with the distribution of the cytoskeletal elements f-actin, gelsolin and integrin $\alpha_{IIb}\beta_3$. Treatment with cytochalasin D, an inhibitor of actin polymerization, prevented the cytoskeletal organization but did not inhibit the rise in $[Ca^{++}]_i$. The methods attempted in this study were ambitious and the images are tantalizing but often lacking in resolution due to the investigator's use of a intensified SIT camera of relatively low sensitivity and resolution. It is difficult for the reader to independently reach all of the authors conclusions from the images presented,

but one might respect the authors greater familiarity with the data set, and take this report as an intriguing preview of future work to be done with improved equipment.

A unique report has appeared recently by Tao, Rose and Haynes [250]. In this study the authors have used a siliconized glass coverslip sandwich (with spacers) to create a small reaction chamber (a siliconized glass coverslip sandwich) for the study of individual platelet $[Ca^{++}]_i$ responses induced by soluble agonists in a population of non-attached cells. The overall conclusion of this study is that while resting $[Ca^{++}]_i$ is relatively uniform in the platelet population, there is significant heterogeneity with respect to the $[Ca^{++}]_i$ response to thrombin and ADP stimulation. This study seemed fraught with numerous technical complexities, and it appears as though the goal of this study would be better addressed by other techniques, e.g. flow cytometry, or imaging in a well defined flow cell or stopped flow system.

Only one group has published studies employing single platelet imaging of $[Ca^{++}]_i$ in the study of the platelet-biomaterial interactions. Waples, Goodman and Albrecht, et al, first reported their work in 1992 [251]. In this first study, conducted relatively early in the development of ratio fluorescence work, they constructed a specialized apparatus for the simultaneous observation of the platelets by ratio fluorescence (Ca^{++}) and transmitted light (asymmetric illumination contrast, for morphology). They observed platelets spreading upon glass and formvar surfaces. In this study the images are understandably noisy due to the limitation of the ISIT camera used. Nonetheless, the authors demonstrate $[Ca^{++}]_i$ increases with activation induced by contact and report that this $[Ca^{++}]_i$ rise corresponds to platelet spreading. They report that platelet $[Ca^{++}]_i$ falls to a steady value after the initial response. Subsequently, this group reported the extension of this work to other biomaterials surfaces: polystyrene, polyurethane (Pellethane), and a $-SO_3$ modified polyurethane as well as formvar [252, 253]. The

platelets again demonstrated an initial $[Ca^{++}]_i$ rise upon activation by Pellethane and formvar, followed by a slow decrease to near resting values in fully spread platelets. In contrast, platelets contacting the $-SO_3$ modified polyurethane demonstrated much higher elevations $[Ca^{++}]_i$ and these were sustained throughout the experiment. High quality scanning electron microscopy and high voltage TEM, confirmed the fully spread results on the Pellethane and formvar, and showed that the platelets on the $-SO_3$ modified polyurethane appeared as though they had suffered damage during contact with the surface, showing a very dendritic and non- viable shape, and never reaching a fully spread morphology. These strange morphologic responses on the $-SO_3$ modified polyurethane had been noted before in previous work from this laboratory. The $[Ca^{++}]_i$ values reported for the platelets on $-SO_3$ modified polyurethane are orders of magnitude higher than those ever reported in other studies for platelets activated by any surface or soluble agonist.

Finally, two relatively recent published studies highlight the current use of single platelet $[Ca^{++}]_i$ imaging in the study of platelet adhesion to surfaces. The first, by Jen is the only published report of platelet $[Ca^{++}]_i$ imaging using flowing conditions and the second by Heemskerk, is the first to correlate measurements of platelet procoagulant activity, secretion and $[Ca^{++}]_i$ in single adherent platelets.

In 1996, Jen and co-workers, who have previously made numerous studies of platelet adhesion in parallel plate flow cells, published their first report of $[Ca^{++}]_i$ measurements in single platelets [254] during attachment and spreading upon fibrinogen coated glass coverslips in a flow cell. In their work they find that there is no immediate rise in $[Ca^{++}]_i$, rather there was a $[Ca^{++}]_i$ elevation in most cells following a highly variable lag period which ranged anywhere from 10 to 200 seconds. They note, as many do who have studied individual platelet responses, that the $[Ca^{++}]_i$ changes

varied tremendously among individual cells, i.e. there was significant heterogeneity in the platelet $[Ca^{++}]_i$ response. Despite this, they do note that majority of platelets that were observed to have spread, also had demonstrated a $[Ca^{++}]_i$ elevation. Interestingly, they report that platelets treated with a cell permeant calcium chelator to prevent any $[Ca^{++}]_i$ increase still showed normal shape change, but if actin polymerization was inhibited with cytochalasin D, both the $[Ca^{++}]_i$ response and shape change were inhibited. Surprisingly, neither colchicine (microtubule dissociating reagent) or the membrane permeable calpain inhibitor, calpeptin, had any effect on $[Ca^{++}]_i$ or shape change. One observation that is mentioned in passing in this report by Jen, and also previously by Waples, is that when a platelet arrives at the surface from the suspension, and lands on a platelet that has previously spread onto the surface, it frequently triggers a $[Ca^{++}]_i$ response in both cells. These observations of what might be cell-cell signaling have remained wholly anecdotal. The imaging equipment used in Jen's study is similar to that used by Heemskerk, and the images are of moderate resolution, but collected at frequent, 4sec intervals. Like Heemskerk, Jen claims the reported ratios could not be accurately calibrated due to poor fluorescence signal and the small size of the cells.

Recently Heemskerk et al have reported using a combination of phase contrast, fluorescence and ratio-fluorescence microscopy to observe platelet morphology, granule secretion, procoagulant activity and $[Ca^{++}]_i$ in single platelets [255]. In this study they report that most platelets spread onto fibrinogen coated surfaces with no detectable $[Ca^{++}]_i$ response, while platelet interactions with sprayed collagen fibrils were associated with large and prolonged increases in $[Ca^{++}]_i$, the exposure of P-selectin and the exposure of phosphatidylserine (as measured by the accumulation of fluorescently labelled antibodies to P-selectin or fluorescently labeled annexin V binding at the

surfaces of the cells). Previously they have suggested that adventitious ADP has been responsible for the $[Ca^{++}]_i$ response seen during activation and spreading on fibrinogen, and have used apyrase to remove ADP. (Heemskerk has conceded though,[Heemskerk, personal communication, Oct. 1997], that even with high doses of apyrase they have been unable to eliminate all spiking events in platelets spreading on fibrinogen.) They note that the $[Ca^{++}]_i$ response to collagen is not immediate, but is preceded by a lag of almost three minutes. They also observe that platelets stimulated by collagen contact show evidence of blebbing, which may be the vesiculation or formation of microparticles that has been the subject of interest in many platelet studies done in suspension, and in flow cytometry. The procoagulant activity triggered by contact with collagen was strongly inhibited by PGE_1 or chelation of $[Ca^{++}]_i$ with BAPTA. Futhermore, inhibition of protein tyrosine phosphorylation also resulted in reduction of bleb formation and PS exposure. These observations suggest that both tyrosine kinases and $[Ca^{++}]_i$ signaling are involved in the procoagulant response of platelets on collagen. One interesting finding in their work is that of the platelets spread on fibrinogen, and then further stimulated by thrombin, almost 90% showed a $[Ca^{++}]_i$ increase, but less than 3% showed any evidence of PS exposure, as shown by annexin V binding. However, of the platelets spread on collagen, and then further stimulated by thrombin, again most (75%) showed $[Ca^{++}]_i$ increases, but here, 80% showed binding of annexin V. The authors state that the binding of annexin V correlates with the ability of the collagen adherent platelets to support thrombin generation using *in situ* thrombin generation assays, but the data was not included in this publication. The combination of thrombin and collagen has repeatedly been shown to be the most potent in triggering platelet procoagulant response. With this and other studies there is evidence that the activation of platelets during adhesion to fibrinogen and

collagen coated surfaces result in very different platelet outcomes. Similarly, the question can be posed: are these differences evident in the platelet response to attachment and spreading on various protein covered biomaterial surfaces used in cardiovascular applications?

2.5 Platelet - Material Interactions

The importance of platelet functioning to human health through its role in hemostasis and thrombosis was explained at the opening of this section. The prevalence of thrombosis and its contribution to the morbidity of the American population has meant that the platelet and its interactions with adhesive protein covered structures of the vasculature have received continued strong research attention. Platelet interactions with artificial materials have also been crucial, however; and perhaps this is best appreciated in an observation by Missirlis [256]. He notes that although platelets have been known for over 150 years, it was the advent of siliconized glassware (aggregometry cuvettes, pipets, tubes) and the introduction of laboratory plasticware (largely preventing platelet activation by material contact during handling) that ushered in the modern era of platelet physiologic study in the 1950s and 60s.

Today, modern medicine has a continual need for devices involved in interrupting, rerouting, repairing or replacing parts of the human circulatory system. Platelet interactions with the materials in these devices are associated with complications that adversely affect the patients that depend upon them. The success of devices as simple as blood bags and short term catheters, or as complicated as the machinery in cardiopulmonary bypass or the artificial heart, depends on the platelet-biomaterial interaction. The adhesion, activation, and aggregation of platelets on foreign surfaces, followed in some cases by embolization of platelet thrombi from the foreign surface,

describe the complications that arise from the interactions of platelets with biomaterial surfaces [257]. A recent detailed, examination of pyrolitic carbon heart valves implanted in sheep showed evidence of platelets and platelet aggregates leading to the recommendation of the use of antiplatelet agents [258]. Measurements showing enhanced platelet destruction rates [259, 260], the occurrence of free-flowing platelet emboli [261], the entrapment of these emboli in distal organs [262] or downstream filters [263], losses in platelet fibrinogen receptors caused by recirculation in extracorporeal oxygenator systems [264], the clogging of plasmapheresis units with platelet rich thrombi [265], the inhibition of thrombi deposition in oxygenators [266] or vascular grafts [267] upon the addition of naturally occurring peptides that bind to platelet receptors, and the thromboembolic events causing thrombus deposition in valves in left ventricular assist devices [268] clearly show the nature and magnitude of the platelet's involvement in blood-materials interactions under conditions of high blood shear rates. Thus, the destruction of blood platelets, the clogging of catheters and grafts, and serious thromboembolic events, such as strokes are examples of the consequences that improved biomaterials attempt to address. But a rational approach to the improvement of blood contacting biomaterials requires an understanding of the underlying mechanisms by which platelets interact with foreign materials.

Expanded discussion of the consequences of platelet material interactions can be found as part of any clinical textbook on surgery or vascular surgery, e.g. references [269-274]. Packham has authored a short review that summarizes the issues involved in platelet-biomaterial interactions [257], and a useful and more recent review that focuses on platelet-surface interactions is that of Goodman and Albrecht [275]. Another recent, short review that nicely summarizes the state of affairs with regard to blood-material interactions is that of Edmunds [276]. Hanson and Harker have authored a

brief tutorial on platelet function and the coagulation cascades as they relate to biomaterials contact [277]. A research symposium held at the end of 1987 yielded a remarkable and comprehensive compilation outlining the research directions in blood-material interactions [278]. The chapters of this volume mirror the complexities of hemostasis and thrombosis discussed at the beginning of this section. This volume also provides an interesting benchmark against which the progress in understanding platelet-biomaterial interactions, or lack thereof, can be measured. Another somewhat more recent compilation resulting from a symposia is reference [256], which is useful not only because of the international representation of its participants, and its focus on clinical devices, but because of the discussion, faithfully reproduced in this volume, provides a snapshot of the thinking that has shaped some research efforts over the past five years.

The unique surface properties of the materials used, and key surface analysis techniques are discussed in reference [279]. A review of the blood compatibility of polyurethanes in particular has been published by Ito and Imanshi [280] Ratio fluorescence microscopy will be reviewed in the next chapter.

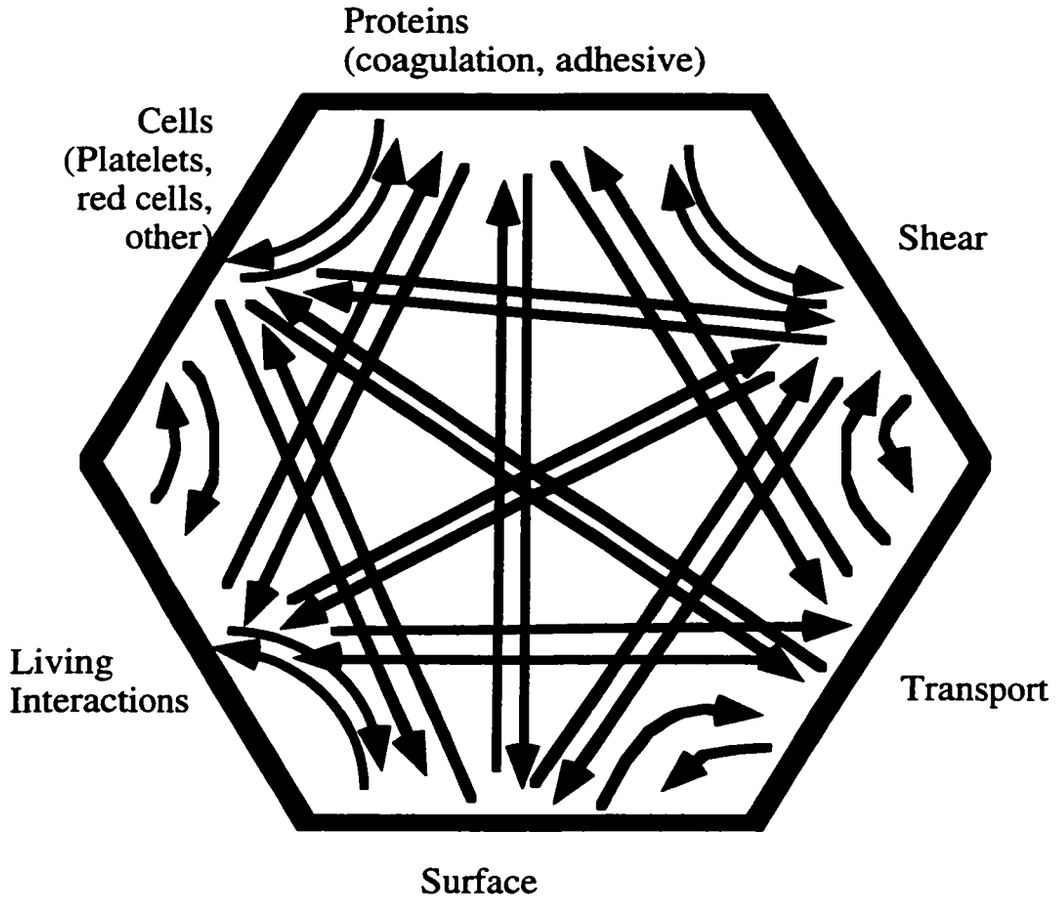

Figure 2.1: The Complexity of Hemostasis and Thrombosis as exemplified by Virchow's Triad, expanded to show six elements of the hemostatic system. Arrows show the action or importance of one aspect of the system on another.

Table 1.1 Elements of Hemostasis and their roles

Plasma: Plasma proteins that are critical to hemostasis include the proteins of the coagulation cascade and the adhesive proteins, e.g. von Willebrand Factor (vWF), fibrinogen, fibronectin, thrombospondin. The coagulation cascade, when triggered results in a fibrin mesh entrapping red cells and platelets at the site of vascular injury. The adhesive proteins provide for attachment of platelets to subendothelium (vWF) and other surfaces (fibrinogen/fibrin) as well as to each other.

Blood Cells: The platelet is the primary hemostatic formed element in blood. The platelet attaches to exposed sub-endothelium at sites of vascular disruption, changes shape, becomes sticky, aggregates with other platelets, excretes factors that stimulate other platelets and other cells, and provides a catalytic surface for enzymes of the coagulation cascade. ADP released from red blood cells can be important in activating platelets. Other cells, e.g. monocytes and PMNs also have interactions with platelets.

Blood vessel surface: The surface lining of the conduit through which blood passes is critical to the hemostatic process. The native surface is the lipid membrane of intact healthy endothelium, rich in glycoproteins. The surface can be modified by changes in membrane receptors presented by the endothelium, or by destructive removal of the endothelium by disease or injury resulting in the exposure of subendothelium, or atherosclerotic plaques. Of course any medical device in contact with blood presents a entirely different surface which is often modified or tailored to prevent or elicit a particular biological response.

Blood vessel 'Living interactions': Intact healthy endothelium secretes powerful agents which act locally to suppress platelet activation, and control vascular tone. Endothelium delivers adhesive proteins (vWF), secretes growth factors, metabolizes adenine nucleotides, locally provides anti-platelet compounds, and secretes factors of the fibrinolytic system. Thus the conduit through which blood normally flows is not an inert structure, but rather a living organ, capable of organized and varied responses which are a key part of the hemostatic system. It is usually assumed that when blood vessels are replaced with medical devices that this function is irreparably lost. Yet the design of medical devices that are coated with or release anticoagulants or anti-platelet agents, as well as the efforts of device designers to support living endothelium as part of implanted devices, are testament to the respect given to this important aspect of hemostasis.

Blood flow, shear: The flow of viscous blood through its conduits results in fluid shear stresses that act on the blood cells suspended in the flow, on the proteins in the flow and on the walls of the conduit.

Blood Flow, transport: Mass transport considerations are crucial in several aspects of hemostasis and thrombosis, affecting the local concentrations of cells, proteins and enzymes, as well as small molecules.

Table 1.2 Effects of Other Elements of Hemostasis on Platelets

The effect of coagulation and adhesive proteins on platelets: Thrombin is a potent platelet activator. Fibrinogen (and sometimes vWF) is the crosslinker that binds platelets together into aggregates. The binding of adhesive proteins to platelet integrin receptors triggers mechanisms of platelet activation, e.g. protein phosphorylation.

The effect of shear on platelets: Platelets respond directly to shear stress in suspension, and this response is believed to be mediated by the GP Ib-IX-V receptor system. The mechanisms of platelet adhesion differ under high and low shear conditions.

The effect of transport on platelets: In flowing blood, platelets benefit from augmented mass transport to the surface due to their margination in the blood stream by red cells.

The effect of surfaces on platelets: The glycoprotein rich surface of the endothelial cell is non-adhesive to platelets, yet exposed subendothelial collagen results in quick platelet attachment and activation. The response of platelets at the surface of any material is usually associated with the presence of an adhesive protein adsorbed to the surface.

The effect of living interactions on platelets: The activation of platelets is strongly suppressed by prostacyclin and nitric oxide (NO) released from living endothelium. Another platelet agonist, ADP is metabolized by ectoenzymes of the endothelial cells.

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Chapter 3: Methods for Ratio Fluorescence Microscopy, the Preparation of Washed Platelets and the Preparation of Polymeric Surfaces

3.1 Review

Calcium ions have long been known to play an important role in cell physiology. In platelets, the role of calcium as a signal coupling agent was suggested as early as 1962 in work with thrombin stimulated platelets (reviewed in [1]). Later, the extraordinary stimulatory effects of the divalent cation ionophore A23187 (which artificially raises $[Ca^{++}]_i$), as well as studies with calcium antagonists, solidified the interest in platelet calcium throughout the 1970s, but efforts to make measurements of $[Ca^{++}]_i$, e.g. by chlortetracycline fluorescence, were hampered with many serious technical difficulties. The first direct measurements of $[Ca^{++}]_i$ came in the early 1980s with the advent of the calcium indicator Quin 2 and its acetoxymethylester derivative which enabled its loading into the cell. The photoprotein aequorin was also popularized for making measurements of $[Ca^{++}]_i$ during this same time, and studies with the two indicators often reulted in very different results for similar experiments [2, 3]. Such studies were generally carried out on platelets in stirred suspension, and in fact modified agregometers were made available commercially to make such measurements. The fluorescence yield of Quin 2 is quite low and its tendency to buffer $[Ca^{++}]_i$ transients is also problematic. Aequorin required harsh loading conditions and suffered from other limitations as well [2, 3].

The report of a new family of fluorescent calcium indicators in 1985 by Tsien and co-workers marked the introduction of what would become the most commonly used Ca^{++} indicators to date, Fura-2 and Indo-1 [4]. The new indicators were not only 30-

fold brighter than Quin 2, but exhibited changes in their spectra upon binding calcium, specifically the excitation spectra for Fura-2 and the emission spectra for Indo-1. This meant that quantitative fluorescence measurements could be made at two different excitation or emission wavelengths and the result expressed as a ratio, with the advantage that many confounding factors affecting the quantitation of the fluorescence, e.g. uneven dye concentrations or illumination, photobleaching, variations in the sensitivity of the detector or pathlength, could be eliminated from the measurement. Using these dyes, investigators soon began to report calcium gradients in single living cells using ratio fluorescence microscopy [5].

Ratio imaging avoids several artifacts associated with the use of single wavelength indicators including: variations in signal arising from cell thickness (critical in experiments of spreading platelets), uneven or unequal dye loading or illumination, photobleaching and dye leakage. Since the introduction of these dyes tremendous improvements in the sensitivity and speed of electronic imagers and the exponential advances in computing power have fostered a large effort in ratio fluorescence imaging in many fields of biology. Perhaps because of their small size, the imaging of platelet $[Ca^{++}]_i$ has lagged years behind that of other cells, and even today the total number of published studies of single platelet $[Ca^{++}]_i$ imaging is suprisingly small (reviewed in the previous chapter).

Ratio fluorescence microscopy, and the Ca^{++} indicators used, have been the benficiary of numerous, thorough technical reviews and tutorials. The rapid advances in electronic detectors (cameras) and computing mean that most reviews older than three or four years are outdated with regard to these technical aspects. Taylor and Wang edited an important volume on various aspects of quantitative fluorescence microscopy [6]. Likewise, Foskett and Grinstein have edited a volume describing various important

optical techniques in cell biology [7]. Tsien address the newer fluorescent probes for cell signaling, and the practical aspects of ratio fluorescence microscopy in two shorter reviews, [8] and [9]. The journal Cell Calcium devoted a combined issue (vol. 11, issue 2/3, 1990) specifically to the practical aspects of ratio fluorescence imaging. The following year they did the same for the topic of oscillations in $[Ca^{++}]_i$ (Cell Calcium, vol.12, issue 2/3, 1991). More recently, the volume edited by Shotton is an excellent resource for digital microscopy [10], and in their contribution to this volume, Bolsover et al. have clearly derived the calibration equation described first by Grynkiewicz et al., and applied routinely in the vast majority of ratio fluorescence work [11]. A volume edited by Mason contains several chapters applicable to real-time quantitative imaging [12]. While it doubles as the product catalog for Molecular Probes, Inc., a handbook authored by Haugland is an invaluable source of technical information [13]. Finally, Nuccitelli has edited a volume specifically on the methods employed in the study of calcium in living cells [14].

3.2 Ratio Fluorescence Microscopy

The divalent cation fluorophores are typically constructed with the tetracarboxylate binding region of EGTA on one end of the molecule and complex fluorophores on the other end. The binding of Ca^{++} in this binding pocket is favored over Mg^{++} or other divalent cations by several orders of magnitude. Calcium binding introduces bends and changes in the electronic structure of the molecule leading to changes in its fluorescent properties. To measure $[Ca^{++}]_i$ the dye must be loaded into living cells. The hydrophillic tetracarboxylate groups are masked with acetoxymethylester groups leaving the whole molecule decidedly hydrophobic and capable of diffusing through the plasma membrane. The cells are incubated with the dye and once inside the cells, endogenous

esterases cleave the masking groups, releasing the functional form of the dye trapped in the cell (Figure 3.1). This use of -AM ester loading is common practice for many ion indicators in a variety of cells. Fura Red and Fura-2 exhibit changes in their excitation spectrum upon binding Ca^{++}. This means that when exicted at a particular wavelength, e.g. 490nm for Fura Red, the intensity of the fluorescence emmision of the dye is dependent upon the presence of Ca^{++}, whereas at another wavelength of exictation, 425nm, the fluoresence is independent of Ca^{++}. Unlike Fura 2, the use of Fura Red™ has not yet become wide spread. It has been selected for use here since, unlike Fura-2 which is excited by UV wavelengths, Fura Red's visible wavelength excitation allows for its use with glass and polymeric substrata.

In practice, the fluorescently labeled cells attach to a polymer coated coverslip mounted in the epifluorescent microscope. Two fluorescent images are acquired, one using the Ca^{++}-sensitive, and the other using the Ca^{++}-insensitive excitation wavelength for illumination. A senstive, high resolution, cooled-CCD imager, acquires the fluorescence images and a computer controlled acquisition system coordinates the filter wheels (selecting the illumination wavelength) and shutters, as well as the image acquisition from the digital camera. In this system the aquisition of an image pair is complete within about one second, and the image pairs are collected at 10-30 second intervals for the duration of the experiment. After subtraction for background, a ratio image is mathematically constructed (METAFLUOR, Universal Imaging Corp., Brandywine, PA) by dividing the fluorescence intensity at each pixel in the 425nm image by the intensity of the corresponding pixel in the 490nm. But before the ratio image is calculated, a threshold algorithm is applied which ensures that, at each point, the intensity in BOTH of the corresponding pixels is sufficiently above noise to result in

a meaningful ratio value for that pixel in the image. The ratio values are scaled and the image is displayed in pseudocolor.

In off-line analysis, individual cells in the ratio images are identified, the average ratio value of the pixels comprising a single cell is determined, and each cell's ratio value is tracked throughout the image series.

The fluorescence contributions of Ca^{++}-free and Ca^{++}-bound forms of the indicator are related by the dissociation constant of the dye and it can be shown [11] that the ratio of the fluorescence at the two excitation wavelengths is related to $[Ca^{++}]$ by equation (3.1):

$$[Ca^{++}] = K_d \beta \frac{(R - R_{min})}{(R_{max} - R)} \tag{3.1}$$

where R is the ratio of fluorescence, R_{max} is the value of the ratio when the dye is saturated with Ca^{++}, R_{min} is the value of the ratio of the free dye and ß is an instrumental and scaling parameter. K_d is the dissociation constant for the Ca^{++}- dye complex. This is a non-linear relationship. Calibration is discussed in chapter 4.

3.3 Ratio Fluorescence for Measuring Platelet Activation by Biomaterials

To a degree, ratio fluorescence provides a unique advantage in the measurement of platelet activation by biomaterial surfaces. In other assays, the assesment of platelet activation is made on a population of cells that have been subject to: 1) possible pre-stimulation, as is the case in any prepared or manipulated platelet suspension, 2) stimulation by contact with the material of interest, and 3) adventitious activation by contact with other materials in the experimental system or by cell-cell interactions. The exact same can be said for the conditions in ratio fluorescence experiments. However, a

key advantage is realized by the fact that a measure of platelet activation is available immediately as the platelet makes contact with the material of interest. Platelet $[Ca^{++}]_i$ is then monitored throughout the duration that the platelet is in direct contact with the material of interest. Thus the change in $[Ca^{++}]_i$ during the period of observation is a more direct reflection of the response induced by the material contact during that period, a measurement that is less influenced by the other confounding factors in the experiment.

3.4 Preliminary Experiments

Preliminary studies were conducted at the Image Analysis Facility at the Fred Hutchinson Cancer Research Center in Seattle. These first attempts at ratio fluorescence microscopy of platelets used Fura-2. During this time Fura Red™ became available and replaced Fura-2 as the calcium indicator in most of the work in this dissertation. In these first experiments, washed labeled platelets were allowed to settle on glass and plasma coated glass coverslips in a static settling chamber or in a few experiments to attach, from flowing suspension, to coverslips mounted in a parallel plate flow cell. The apparatus used in these early experiments is described below.

Observations were made with a Zeiss Axiovert inverted microscope equipped for epi-fluorescence with a 550nm dichroic and 610nm barrier filter and a Zeiss 100x 1.3NA oil or Nikon 100x 1.4NA objective. Illumination from the Xe light source was limited to 450nm or 490nm by narrow (±10nm) band pass filters and neutral density filters held in a computer controlled filter wheel (Ludl Electronic Products). A low light level, silicon intensified target camera provided video output (Model 66 SIT, Dage-MTI). The video signal was digitized and frame-averaged (Data Cube Products, Inc.). The system was controlled by a Unix workstation (Sun) running RATIOTOOL software

(Inovision Corp.) which controlled the filter wheels, interfaced with the video digitizing hardware and performed the image analysis and display and storage. To achieve temperature control, a portion of the microscope was enclosed in foamboard, and heated with a hairdryer controlled by a thermocouple (extremely fine tip, Omega), thermometer (BAT10) and temperature feedback controller.

The settling chamber assembly consisted of a stainless steel plate, a silicon rubber gasket and a teflon block, each piece with a square well cut from the center. Coverslip samples were sandwiched between the plate and silicon rubber gasket, creating a small open chamber with a volume of roughly two milliliters. The bottom of the coverslip/sample is then accessible to the objective. One milliliter of platelet suspension was placed in the chamber and allowed to settle onto the sample. Pairs of images (one image at each excitation wavelength) were recorded once every five seconds for the first 10-15 minutes of settling and every 30sec thereafter. Acquisition of a pair was complete within one second, and each image was the average of 8 (digitized) video frames. A ratio image was formed by digitally subtracting the appropriate background, dividing the image acquired under 450nm illumination by the image under 490nm illumination, and presented in 8-bit psuedocolor.

These preliminary experiments demonstrated that Fura Red™ could be sucessfully loaded into platelets and used for ratio fluorescence imaging of $[Ca^{++}]_i$. Observation of $[Ca^{++}]_i$ in platelets landing on glass coated with dilute (0.1%) blood plasma showed that the platelet did respond with a $[Ca^{++}]_i$ rise, that there was a variable lag before response, and there was heterogeniety in the response. The encouraging data from these experiments prompted the purchase and assembly of a ratio fluorescence microscopy system for our own laboratories. That system is described below.

3.5 Ratio Fluorescence Microscopy System

The ratio fluorescence microscopy system that used in the work reported in this dissertation is shown schematically in Figure 3.2 and Figure 3.3. Microscopy was performed with a Nikon Diaphot (100% transmittance) inverted research grade microscope equipped for epifluorescence. A Xe arc lamp (XBO/75) was chosen for illumination due to its more uniform spectral output as compared to Hg, and a quartz collector lens was used in the illuminator housing. Excitation filters and neutral density filters are housed in a dual filter wheel assembly controlled under a Mac2000 controller subsystem (Ludl Electronic Products, Hawthorne, NY). The dichroic mirror (580nm) and barrier filter (610 nm) for use with Fura Red™ were purchased from Nikon mounted in OEM filter cubes for use in a dual filter slider, for quick interchange between filter sets. The objectives used are the Nikon UVFluor 40x1.4 and 100x 1.3 NA oil immersion objectives which were selected for their excellent light collection and transmittance, and compatiblity with Fura-2 and other near UV fluorophores.

The camera subsystem (Photometrics) includes a Photometrics CH250 thermoelectrically cooled CCD camera head, containing a grade 2 Kodak KAF1400 imager. Cooling water to the camera head is provided by gravity feed. This slow scan, full frame, front illuminated imager was chosen for its low noise (improved sensitivity), very small pixel size (good spatial resolution), and general flexibility for use in a variety of fluorescence imaging applications. The camera head is paired with a Photometrics CE200 camera electronics unit providing camera control, and an AT200 A/D interface unit which is mounted in the host computer on a PC-ISA bus.

The original host computer system was a 486/33MHz Intel system running under the Windows for Workgroups 3.11/MS-DOS operating system. This unit was outfitted

with 16Mb of RAM, 200Mb of hard disk storage, a tape archival system, and a 14 inch color display. Originally, Photometrics Imaging System (PMIS) software was used to interface with the camera and acquire images. Macro code was written to interface with the filter wheel controller and the camera controller to coordinate and schedule the acquisition of images during an experiment and perform the ratioing calculation and display and store the images. Later a dedicated ratio fluorescence software package (METAFLUOR v.2.5, Universal Imaging Corp.) was employed for image acquisition and analysis. Also, the host computer was replaced with a Pentium 100MHz Intel system with 64Mb of RAM, 2Gb of hard disk storage, a 1.3Gb mageneto-optical archival storage unit, and an accelerated dual 21 and 17 inch dual color display system. The computer was connected to the University's network and configured to serve as a fileserver for remote access by multiple users.

A thermoelectrically controlled microscope stage microincubator and bidirectional temperature control unit was also purchased. The microincubator holds a circular teflon coverslip dish, a so-called "Leiden" dish, which comprises a teflon main body and an open stainless steel base. A 25mm round glass coverslip (#1 thickness) is sandwiched between the two parts to form the bottom of the assembled dish, which can contain roughly 3ml of fluid (cell suspension) and keep it at 37°C. Recently, a small insert was designed and machined from stainless steel to replace the Leiden dish with a chamber of much smaller volume (0.2ml). This unit also uses 25mm glass coverslips for the chamber bottom and uses tubing for the sidewalls of the chamber.

Parallel plate flow cells have been used previously to study the adhesion of platelets to surfaces from flowing blood or mixed cell suspensions. Such a flow cell has been modifed previously for use in epifluorescent video imaging of platelet adhesion events.

This flow cell was used to image platelet $[Ca^{++}]_i$ in cells attaching to polymer surfaces. This apparatus is fully described in chapter 6.

3.6 Materials

Fura Red™-AM, Fura Red™ tetraammonium salt , Pluronic® and Calcium Calibration Kit II with Mg^{++} were purchased from Molecular Probes (Eugene, OR). The calibration buffers contain 10mM MOPS buffer, 100mM KCl, a 10mM Ca-EGTA buffer adjusted such that the free calcium concentration ranges from 0 - 40μM, and free $[Mg^{++}]$ is constant at 1mM, pH7.2. Ionomycin, monensin, and thapsigargin were purchased from Calbiochem (LaJolla, CA) and stored as concentrated stock solutions in DMSO. Human fibrinogen, Sepharose 2B and apyrase (A-9149) were purchased from Sigma Chemical Co. (St. Louis, MO). Buffers salts were of reagent grade. The platelet suspension buffer with albumin (PSB-albumin) contains (in mM): NaCl, 145.5; KCl, 2.7; $MgCl_2$, 1; $CaCl_2$, 2; NaH_2PO_4, 0.4; HEPES, 4.7; dextrose, 5.55; along with: bovine serum albumin, 4mg/ml and apyrase, 0.03mg/ml.

3.7 Preparation of Washed Labeled Platelets

Washed platelet suspensions provide for the study of platelet adhesion and activation events in a controlled environment. Specifically, the studies can be conducted on surfaces of known protein composition. Interactions of platelets with the coagulation system can be removed. Washed platelet suspensions are commonplace in the study of platelet biochemistry and physiology. Properly prepared, washed platelet suspensions supplemented with fibrinogen aggregate normally when stimulated by agonists in aggregometry and adhere to and spread on surfaces. Techniques for the preparation of washed platelets are fairly well established, but do vary slightly among various

laboratories. Predominately, there are two methods used: the separation and washing of platelets by centrifugation, sometimes over an albumin cushion, and the separation of platelets from platelet rich plasma by size exclusion chromatography over Sepharose gel. Various claims are often made about the superiority of one method over another, usually woithout firm supporting data.

Two particular points concerning the platelet suspension buffers are worth noting. The first is that buffers that utilize carbonate to control pH, as is often the case in both older and modern platelet studies in the literature, require that the cell suspension be kept in a CO_2 controlled environment (usually 5% CO_2). This is often overlooked.

Second, adventitious ADP released from red cells and platelets can easily stimulate platelets. Plasma (and endothelial cells) contains endogenous ADPases to catabolize this leaked ADP *in vivo*, however these systems are obviously removed during platelet isolation. Left unchecked, this low-level ADP concentration can build over time, with the result that the platelets will become unintentionaly stimulated. More likely, if the platelets are faced with prolonged low level ADP stimulation they will become refractory, unresponsive to ADP and remain in a rounded non-functioning state. For this reason, ADPase systems are included in platelet buffers; most commonly the potato enzyme apyrase or the creatine phosphate / creatine phosphokinase enzymes are used. The use of PGI_2 to increase cAMP or the use of aspirin or indomethacin to destroy cyclo-oxygenase, represent pharmacologic treatments that are sometimes used to block certain platelet activation pathways (as discussed in the previous chapter) in washed platelet suspensions. The various platelet isolation methods are reviewed in an important volume edited by Hawiger, which serves as a tutorial on a wide range of techniques used in the study of platelet biochemistry and physiology [15]. The preparation of washed platelets in our laboratory has been previously described [16].

Healthy volunteers were recruited to be blood donors and remunerated in accordance with procedures approved by the University of Washington Human Subjects Institutional Review Board. A copy of the informed consent form is included in Appendix A. Donors denied taking any medications during the preceding two weeks. Venipuncture was performed at the antecubital or suitable peripheral vein using a larger bore 19G Butterfly infusion set (Becton-Dickenson, Rutherford, NJ) coupled to a syringe (BD PlastiPak, Becton Dickenson, Rutherford, NJ). The use of a tourniquet was minimized, and as much as 60ml of blood was drawn slowly into the syringe containing citrate anticoagulant (1:9 vol, acid-citrate-dextrose, ACD, NIH formula A). Phlebotomy was performed by trained and licensed personnel at the University of Washington Medical Center and the blood was transported at room temperature to the laboratory. The blood was transferred to 15ml polystyrene or 30ml polyethylene conical centrifuge tubes and platelet rich plasma (PRP) was prepared from the blood by centrifugation at 180g for 20min at room temperature. The PRP was harvested carefully using polyethylene pipets and labeled with 10μM Fura Red™. The platelets were incubated with the dye mixture (50μg Fura Red™AM mixed dissolved in 50μl DMSO and 5μl of 25%w/v Pluronic®) for 45-60min at 37°C. To separate the cells from the unincorporated dye and other plasma proteins, the cells were loaded onto a Sepharose 2B column and eluted into PSB-albumin without Ca^{++}. The column was fashioned from a 10ml polyethylene syringe barrel, using 30μm nylon mesh as a column support. The column was pre-equilibrated with many column volumes of PSB-albumin without Ca^{++}. The platelet-containing fractions were pooled and the platelet count was determined using a Coulter counter (model ZBI, Hialeah, FL) and adjusted to the desired concentration in calcium-free PSB-albumin buffer. The calcium concentration in the final suspension was brought to 2mM by addition of 1M $CaCl_2$ and the platelets

were incubated at 37°C for at least 30min before the experiments were conducted. The preparation of a mixed cell suspension containing washed platelets and washed red blood cells, both prepared by centrifugation, is described in chapter 6 . Washed fluorophore labeled platelets were shown to aggregate normally in response to 20μM ADP.

3.8 Surface Preparation

Detailed description of the methods used to prepare polymeric thin film coatings on glass coverslips can be found in the sections reporting the results of experiments using those surfaces. In general, 25mm diameter round glass coverslips, (#1) were cleaned thoroughly and coated first with a silane copolymer to enhance interfacial adhesion and then with the polymer of interest. Polymer coatings were centrifugally cast onto the surfaces from dilute solutions in solvent using a photoresist spinner (Headway Research, Garland, TX). The surfaces were dried and stored in polystyrene petri dishes, sealed in sample bags under dry N_2. Some surfaces were submitted to the National ESCA and Surface Analysis Center for Biomedical Problems for surface compositional analysis.

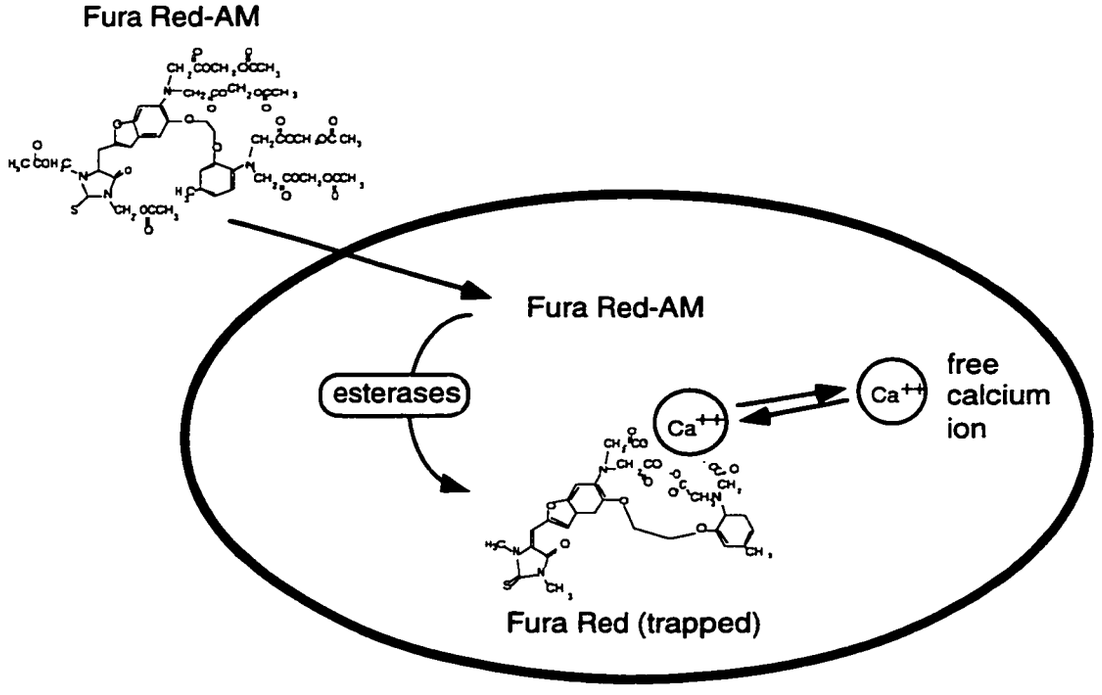

Figure 3.1. Loading of the intracellular calcium ion indicator Fura Red™. The indicator is provided in the acetoxymethylester form, in which the hydrophillic tetracarboxylate groups of the calcium ion binding pocket are masked. The dye is incubated with the cells, and once inside, intracellular esterases clease the masking groups leaving the fluorophore trapped in the cell.

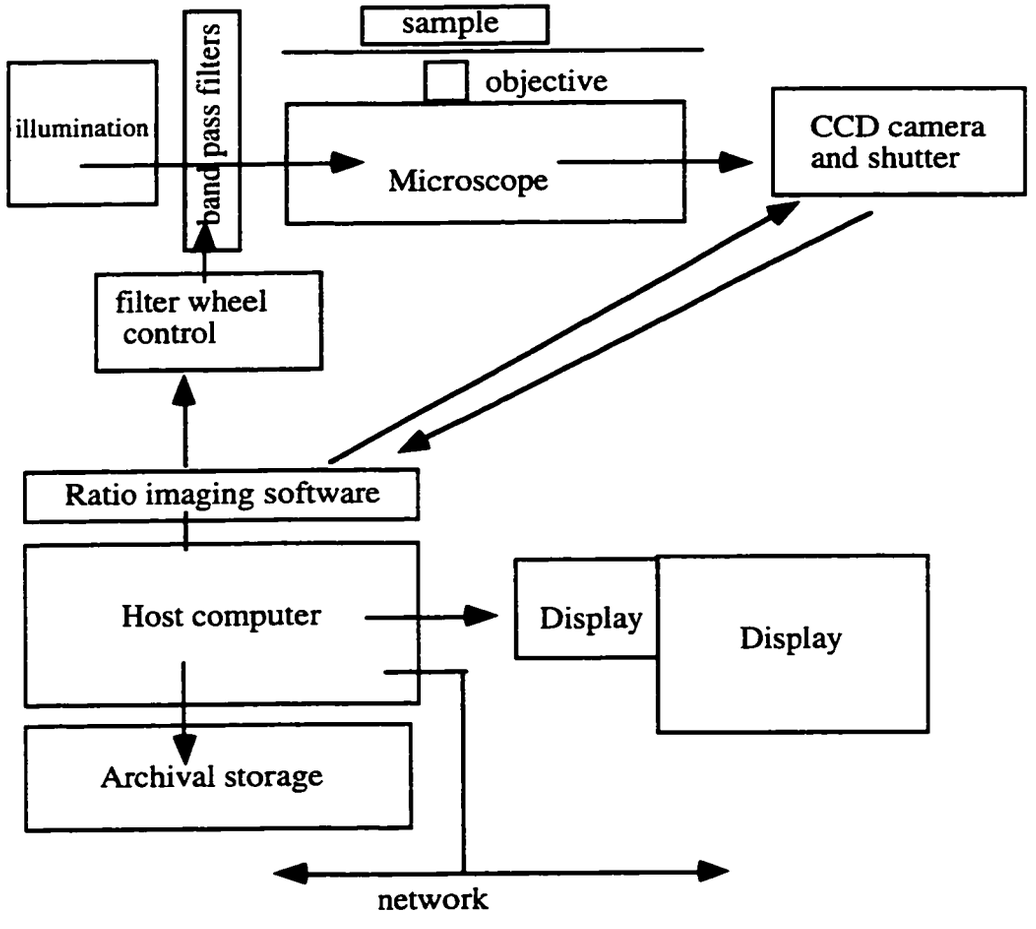

Figure 3.2. Schematic of the components of the ratio fluorescence microscopy system. The host computer runs the image acquisition and analysis software, which coordinates and controls the selection of the excitation filters and the exposure and acquisition of the images by the cooled-CCD camera subsystem.

Epi-fluorescent Ratio Microscopy:

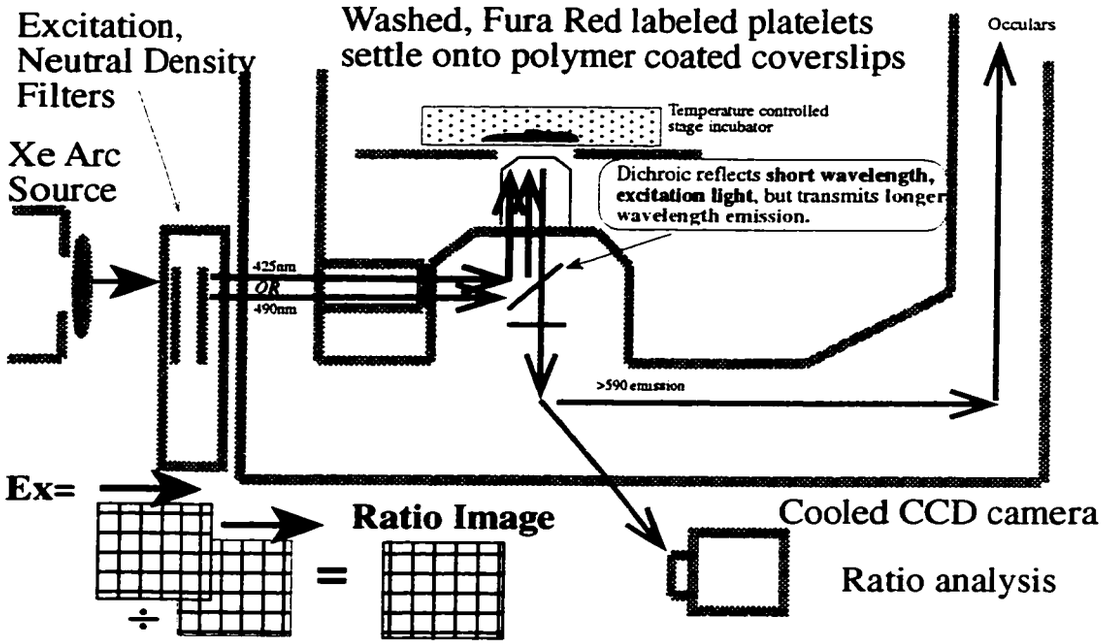

Figure 3.3 A schematic of the light path in the epifluorescent ratio microscope. Light from the Xenon arc lamp is passed through bandpass filters (selected under computer control) to the dichroic mirror. The shorter wavelength excitation light is reflected upwards through the objective and to the sample. The excited dye molecule fluoresces at a longer wavelength (a Stokes shift) . This fluorescence in the cells is imaged by the objective, passes through the dichroic mirror and emission band pass filter and is imaged by the cooled CCD camera.

Notes to Chapter 3

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Chapter 4: *In Situ* Calibration of the Calcium Indicator Fura Red™ in Human Platelets

4.1 Summary of Chapter 4

Fura Red™ is a dual excitation, ratiometric dye used in the study of intracellular calcium, $[Ca^{++}]_i$. While the properties of most ion indicators in buffered solution are readily available, the dissociation constant and other fluorescence properties affecting the calibration of these dyes are often altered in the intracellular environment. We have therefore performed an *in situ* calibration of Fura Red™ inside adherent human platelets at 37°C. We report a $K_{1/2}$ in the range of 550-650nM with an R_{min} and R_{max} of 0.77 and 5.9 respectively. The $K_{1/2}$ and R_{max} for intracellular Fura Red™ are considerably higher than those measured for Fura Red™ in buffered solution.

4.2 Introduction to Chapter 4

The advent of Ca^{++} -sensitive fluorescent dyes has made possible a variety of useful techniques for studying the role of this ion as an important second messenger in living cells. Spectrofluorimetry, ratio fluorescence imaging, flow cytometry and laser scanning confocal microscopy are all techniques that utilize these important fluorescent probes to report the intracellular concentration of calcium ion ($[Ca^{++}]_i$) in living cells.

Ratio fluorescence imaging of $[Ca^{++}]_i$ has proven particularly useful in measuring spatial and temporal changes of $[Ca^{++}]_i$ in single or small numbers of cells. The technique is less expensive than flow cytometry or confocal microscopy, and the use of an inverted microscope provides for flexibility in experimental design. Because imaging collects data from individual cells, this technique can reveal heterogeneity in

cell populations hidden in spectrofluorimetry, can provide details of morphology and structure not possible with flow cytometry, and depending upon configuration, can provide greater temporal resolution than confocal microscopy. In our studies of biomaterial-induced activation of platelets, imaging of individual cells has the additional advantage of allowing one to exclude bulk phase platelets whose activation may not be due to contact with the material.

The use of a ratiometric dye avoids several artifacts associated with single wavelength dyes including: variations in signal arising from cell thickness, unequal or uneven dye loading, photobleaching and dye leakage. By far the most commonly used ratiometric dyes sensitive to Ca^{++} are Fura-2 and Indo-1 which require excitation in the near UV. As an alternative, Fura Red was developed with excitation wavelengths in the visible [1, 2]. Visible wavelength excitation obviates the need for expensive UV optics and UV laser illumination, avoids most cellular autofluorescence, permits use in conjunction with caged compounds, and allows the use of cells on polystyrene and other polymeric substrata. Nonetheless Fura Red™ has yet to achieve widespread use as a stand alone ratiometric dye for Ca^{++}, perhaps in part due to its low quantum yield. It has been used alone to study $[Ca^{++}]_i$ transients in skeletal muscle [3] and we have reported on its use in human platelets and monocytes [4, 5]. More commonly it has been co-loaded into cells with Fluo-3 to act as a partner in a dual emission Ca^{++}-sensitive dye pair, that is conveniently excited by the argon laser in confocal microscopy [6].

Calibration of ratiometric indicators requires the determination of the parameters K_d, R_{min}, R_{max} and ß (see Theory). The parameters R_{min}, R_{max} and ß are comparatively easy to determine experimentally since they only require single point determinations under Ca^{++}- free and saturating Ca^{++} conditions. However

determination of K_d requires measurements over a range of [Ca^{++}]. For this reason K_d is often taken from the literature, and applied despite disparate experimental conditions. Construction of a complete calibration curve, and thereby determination of $K_{1/2}$ (= K_d ß), R_{min} and R_{max}, can be performed for the dye in suitable calcium buffered solutions in a spectrofluorimeter. However several investigators have noted that the behavior of the dye in buffer solution can be dramatically different than that observed by an imaging system when the dye is loaded in the cytosol of a particular cell and subject to possibly quite different environs; particularly of viscosity, ionic composition, pH and the presence of high concentrations of intracellular protein [7]. These changes can affect both the dissociation constant and the other optical calibration parameters. Reports have also described changes in indicator behavior between various cellular compartments, e.g. nucleus and cytosol [8, 9]. From these observations, the importance of full *in situ* calibration for intracellular ion indicators has become increasingly clear [10]. For these reasons we have performed an *in situ* determination of the calibration parameters for the dye Fura Red™ in adherent human platelets imaged with ratio fluorescence microscopy.

4.3 Theory

The calibration of ratiometric dyes has been discussed in several reviews [11-14], and uses the analysis of Grynkiewicz et al. [15], as summarized by Bolsolver et al. [16]. First, the affinity of the dye for its ligand is described:

$$\left[Ca^{++}\right] = K_d \frac{C_b}{C_f} \tag{4.1}$$

where K_d is the dissociation constant, C_b and C_f are concentrations of the Ca^{++}-bound and Ca^{++}-free forms of the dye. The measured fluorescence intensity is the

sum of the fluorescence contributions of the two species, namely the bound and free forms of the dye:

$$F_1 = S_{f,\lambda 1} C_f + S_{b,\lambda 1} C_b \tag{4.2}$$

where $S_{f,\lambda 1}$ and $S_{b,\lambda 1}$ represent the specific fluorescence of the respective species at the specified wavelength of excitation or emission ($\lambda 1$). Repeating the measurement at a second wavelength ($\lambda 2$) adds two additional parameters, $S_{f,\lambda 2}$ and $S_{b,\lambda 2}$.

$$F_2 = S_{f,\lambda 2} C_f + S_{b,\lambda 2} C_b \tag{4.3}$$

When the ratio is defined as $R = F_1/F_2$ and equations 4.1-4.3 are combined, the result is the commonly used equation 4.4:

$$[Ca^{++}] = K_d \beta \frac{(R - R_{min})}{(R_{max} - R)} \tag{4.4}$$

where:

$R_{min} = S_{f,\lambda 1} / S_{f,\lambda 2}$, the limiting value of R at zero $[Ca^{++}]$,

$R_{max} = S_{b,\lambda 1} / S_{b,\lambda 2}$,the limiting value of R at saturating $[Ca^{++}]$, and

$\beta = S_{f,\lambda 2} / S_{b,\lambda 2}$. Note that the subscripts max and min refer to the calcium-free and calcium-saturated forms of the dye, they do not refer to the magnitudes of the ratio values themselves.

Alternatively, K_d ß can be lumped as $K_{1/2}$, a parameter combining the affinity of the dye for its ligand and the sensitivity of the fluorescence response using the denominator, i.e. second, wavelength:

$$[Ca^{++}] = K_{1/2} \frac{(R - R_{min})}{(R_{max} - R)} \tag{4.5}$$

where $K_{1/2} = K_d$ ß = $[Ca^{++}]$ for which $R = (R_{min} + R_{max}) / 2$.

Thus, to relate a measured R to $[Ca^{++}]$ one must determine K_d and ß, or alternatively $K_{1/2}$, as well as R_{min} and R_{max} for the system used.

The equations reveal several practical issues. The sensitivity of the dye in reporting changes in [Ca^{++}] as changes in fluorescence rests in the values of S_f, and S_b , at the chosen wavelengths. The values of S_f and S_b, while primarily dependent upon the spectra of the dye, are nevertheless unique to each imaging system as they are also influenced by the light source, optics and detectors. In equations (4.4) and (4.5), the optical parameters, S_f, and S_b, are contained in R_{min}, R_{max} and ß. If the change in measured fluorescence upon binding Ca^{++} is small, than the values of R_{min} and R_{max} will be small and the range of measured ratios narrow. In practical terms, this means that the ratio, and hence the individual fluorescence intensities, will have to be measured with much greater accuracy to make meaningful distinctions in [Ca^{++}]. On the other hand, if the changes in fluorescence upon binding are large, then the values of R_{min} and R_{max} will also be large. In practice, the large range in ratio values necessitates the simultaneous measurement of both exceedingly dim and exceedingly bright individual fluorescence intensities, which may exceed the dynamic range of many detectors. Furthermore, the balance between free and bound species of the dye is most sensitive to changes in [Ca^{++}] when the [Ca^{++}] is the range of the dissociation constant K_d. Yet, the measured ratio changes most rapidly with respect to [Ca^{++}] when the concentration is near $K_{1/2} = K_d$ß.

This discussion illustrates the connections between the underlying variables and suggests that optimal ratio fluorescence imaging will require careful decisions. These include: the selection of an appropriate indicator with a useful K_d ; the judicious selection and assignment of numerator and denominator wavelengths, and finally confirmation of the optical parameters, R_{min}, R_{max} and ß, under the actual experimental conditions. The result of this process, hopefully, is an indicator system capable of accurate measurements of [Ca^{++}] in a region centered around $K_{1/2}$.

4.4 Materials

Fura Red™-AM, Fura Red™ tetraammonium salt , Pluronic® and Calcium Calibration Kit II with Mg^{++} were purchased from Molecular Probes (Eugene, OR). The calibration buffers contain 10mM MOPS buffer, 100mM KCl, a 10mM Ca-EGTA buffer adjusted such that the free calcium ranges from 0 - 40μM, and free [Mg^{++}] is constant at 1mM, pH7.2. Ionomycin, monensin, and thapsigargin were purchased from Calbiochem (LaJolla, CA) and stored as concentrated stock solutions in DMSO. Sepharose 2B and Apyrase (A-9149) were purchased from Sigma Chemical Co. (St. Louis, MO). Buffers salts were of reagent grade. The platelet suspension buffer with albumin (PSB-albumin) contains (in mM): NaCl, 145.5; KCl, 2.7; $MgCl_2$, 1; $CaCl_2$, 2; NaH_2PO_4, 0.4; HEPES, 4.7; dextrose, 5.55; along with: bovine serum albumin, 4mg/ml and apyrase, 0.03mg/ml.

4.5 Methods

4.5.1 *In vitro* Calibration Using Spectrofluorimetry

Fura Red™ salt was diluted to 10μM in each of the members of the calibration buffer kit. The free [Ca^{++}] value used for each buffer was that given by the manufacturer, but multiplied by 0.695 to account for the change in K_d of Ca^{++}-EGTA at 37°C. Fluorescence spectra were measured in a Hitachi F4500 spectrofluorimeter using 2ml of sample in a temperature controlled (37°C) cuvette. For each sample, the Ca^{++}-dependent fluorescence emission spectra was measured in the range of 550 - 750nm using an excitation of 490nm. The fluorescence excitation spectra was also measured as a function of excitation wavelength in the range 380 - 550nm, measuring emission at 650nm. Using the excitation spectra, the fluorescence intensity when

excited at 425nm (F_{425}) was divided by the fluorescence intensity when excited at 490nm (F_{490}) to yield the measured ratio at each [Ca^{++}].

4.5.2 *In situ* Calibration Using Epifluorescence Microscopy

In order to calibrate the dye under more relevant conditions, namely the environment of the dye as loaded and used in the cells in our studies, platelets were loaded with Fura Red™, and allowed to attach and spread onto clean glass coverslips. The cells were then bathed in a buffer of controlled external [Ca^{++}] containing a Ca^{++} ionophore to bring the cytosolic [Ca^{++}] to the external level. Thapsigargin was used to inhibit the sequestration of Ca^{++}, one of the normal mechanisms of $[Ca^{++}]_i$ control used by platelets.

Platelet rich plasma was prepared from the citrated whole blood of healthy volunteers by a single centrifugation, and labelled with 10μM Fura Red™. The platelets were incubated with the dye mixture (50μg Fura Red™AM mixed dissolved in 50μl DMSO and 5μl of 25%w/v Pluronic®) for 45-60min at 37°C. To separate the cells from the unincorporated dye and other plasma proteins, the cells were loaded onto a Sepharose 2B column and eluted into PSB-albumin without Ca^{++}. The platelet containing fractions were pooled and the platelet count was determined using a Coulter counter and adjusted to $2x10^7$/ml in the calcium-free PSB-albumin buffer. The calcium was brought to 2mM by addition of a small volume of 1M $CaCl_2$ solution.

Glass coverslips (25mm diameter, #1) were cleaned by sonication in 100% ethanol and rinsed with water. A 0.4ml aliquot of platelet suspension was placed on each of the surfaces and the platelets were allowed to settle for two hours at 37°C during which time some of the platelets attached and spread. The surfaces were then gently

dip rinsed in PSB with albumin to remove unattached cells. Then, each surface was incubated in a small polystyrene dish for 2hrs or more at 37°C with one ml of one of the various members of the calibration buffer set to which had been added: the Ca^{++} ionophore, ionomycin (5μM); the Na^{+} ionophore, monensin (2μM) to defeat the actions of the platelet Na^{+}/H^{+} antiporter which could possibly result in pH_i changes; and thapsigargin (2μM), which inhibits sequestration of cytosolic Ca^{++} in platelets [17]. These additions were of small volumes of concentrated stock solutions and resulted in neglible dilution. It has been noted [11] , and we also found, that the action of ionomycin can be slow and pH dependent. The ionophore A23187 or its non-fluorescent analog, Br-A23187 may be a suitable alternative to ionomycin.

The coverslip with attached platelets was then installed in a Leiden™ PTFE (Teflon) coverslip dish, covered with 3ml of the same incubation medium, and kept at 37°C in a microincubator mounted on the microscope stage (Medical Systems Corp., Greenvale, NY). Ratio fluorescence microscopy was performed using a Nikon Diaphot microscope equipped for epifluorescence, with a 40x, 1.4 NA objective; and 425, 490nm excitation and 580nm dichroic filters. Image pairs were acquired with a high resolution Photometrics CH250 cooled-CCD imager and acquisition of each image pair was complete within one second. Several randomly chosen fields were imaged on each sample.

4.5.3 Data Analysis

A representative background intensity was determined and this intensity value was then subtracted from each the raw images, and a minimal threshold (equal to about 5% of the intensity of the brightest pixels in the platelets) was applied to ensure that adequate signal existed in corresponding pixels of the two images. Using

METAFLUOR software, (Universal Imaging Corp., Brandywine, PA) ratio images were constructed and displayed in pseudocolor. Individual platelets were identified. The ratio values of all the pixels comprising each platelet were averaged. The average ratio values of the platelets from 12-18 distinct fields were averaged to determine an overall average ratio value for the platelets at that [Ca^{++}].

Ratio values were fit to the calibration equation (Equation 4.5) with three adjustable parameters ($K_{1/2}$, R_{min}, R_{max}) (Kaleidagraph 3.0 for Macintosh, Abelbeck Software)

4.6 Results

4.6.1 *In vitro* Calibration with a Spectrofluorimeter

The emission spectra for Fura Red™ excited at 490nm shows a single broad peak which shifts only slightly from 655nm to 645nm as a function of [Ca^{++}] (not shown). The excitation spectra of Fura Red™ in calibration buffer at 37°C is shown in Figure 4.1. The isosbestic point is fairly broad and appears to be between 420 and 430nm. The fluorescence of the dye when excited at 490nm decreases as the [Ca^{++}] increases. These spectra differ from those provided by the dye supplier, who report an isosbestic between 440-450nm, possibly because of differences in pH and temperature between our studies and those used by the supplier [2].

In Figure 4.2 the ratio (F_{425}/F_{490}), obtained from the spectra in Figure 4.1, is shown as a function of [Ca^{++}]. Also shown is the fit of Equation 4.5 to the data, which yields the values of the calibration parameters as shown in Table 4.1. Fura Red™ is unusual in that its Ca^{++}-sensitive fluorescence (F_{490}) decreases with increased binding of calcium. For this reason, we placed the F_{490} term in the

denominator of the ratio. As a consequence, the values of ß are considerably greater than unity, ranging from roughly two for buffered solutions of Fura Red™ measured using spectrofluorimetry, to an estimated five or greater for intracellular Fura Red™ measured using microscopy.

4.6.2 *In situ* Calibration

The ratio values of between approximately 60 and a few hundred individual platelets were averaged to determine an overall ratio value for each $[Ca^{++}]$. The average ratio value is shown as a function of $[Ca^{++}]$ in Figure 4.3. Equation 4.5 has been fit to the data in Fig. 5, yielding the calibration parameters for adherent platelets as shown in Table 4.1. The range of observed ratio values in the *in situ* measurements is much extended as compared with the results *in vitro*, and R_{max} was much higher for Fura Red ™ in adherent platelets than for bulk phase Fura Red™.

In some cases, at higher $[Ca^{++}]$, there appeared to be a subpopulation of platelets (as much as 25%) which exhibited a distinctly lower ratio value, far lower than majority of the population. For example, Figure 4.4 shows a histogram of the ratio values for the platelets pooled at 940nM $[Ca^{++}]$, demonstrating a subpopulation of the platelets with a ratio value of around two compared with the large majority at nearly four. Omission of these data resulted in slightly higher average ratio values for the given Ca^{++} concentrations, and if these values were used in the fit, the $K_{1/2}$ was reduced to 500nM.

4.7 Discussion

The widespread importance of Ca^{++} as a second messenger of signal transduction has led to intense interest in the study of $[Ca^{++}]_i$. As a result the combination of

ratiometric dyes and epifluorescent microscopy used to measure $[Ca^{++}]_i$ in single cells has found application in a variety of cell types, including neuronal and muscle cells, blood and tissue cells, and plant cells. Qualitative changes in $[Ca^{++}]_i$ are easily detected using these indicators. However the technical challenges involved in the quantitative measurement of $[Ca^{++}]_i$ are substantial, and hence often overlooked. *In situ* calibrations can be difficult since most cells utilize a variety of mechanisms to maintain $[Ca^{++}]_i$ at low resting levels. These mechanisms must be overcome by ionophores, poration or other means to bring the intracellular ion concentration to fixed values for calibration without destroying the integrity of the cell or causing the loss of intracellular indicator. *In situ* determination of ß can be particularly difficult since it requires the adjustment of $[Ca^{++}]_i$ to both the Ca^{++}-free and Ca^{++}-saturated levels in the *same* cell or group of cells. *In situ* calibrations are important since the fluorescence behavior of many ion indicators have been found to differ dramatically in the intracellular environment. In addition to temperature and pH, it appears that the binding of indicator to intracellular proteins can, in some cases, alter the fluorescence spectra, and the dissociation constant of the dye for its ligand resulting in altered values all of the calibration parameters. The relationship between the measured ratio and the actual $[Ca^{++}]$ that it reports (Equation 4.5) means that the usefulness of these indicators is greatest in a range centered at K_dß hence the importance of determining the $K_{1/2}$ under the assay conditions.

The spectral results for Fura Red™ in buffered solution shown in Figure 4.1 prompted us to use 425nm as the Ca^{++}-insensitive excitation in our imaging studies, as opposed to 450nm that is suggested by the supplier. Others have found 420nm and 480nm as useful Ca^{++}-insensitive and Ca^{++}-sensitive excitation wavelengths, respectively for Fura Red™[3]. The K_d of Fura Red™ in buffered 37°C solution of

138nM obtained in this study is surprisingly close to the K_d reported by the manufacturer for Fura Red™ at 20°C, 140nM. Kurebayashi et al. have reported a K_d of 360nM measured in a different buffer [3].

The $K_{1/2}$ *in situ* in platelets obtained in this study was around 600nM. This means that the dissociation constant in our system is considerably less than the K_d of 1100 - 1600nM determined *in situ* in the myoplasm of frog skeletal muscle fibers by Kurebayashi, et al.[3]. This difference may be due to the higher temperature used in our studies and the different protein milieu found in the cytosol of platelets. Furthermore, the current study relied on acetoxymethyl ester loading, while Kurebayashi, et al. used solely the salt form of Fura Red™, loaded by microinjection. For platelets, their small size precludes confident use of microinjection techniques or poration, leaving acetoxymethyl ester loading and ionophores as the primary methods used to load indicator and adjust $[Ca^{++}]_i$.

Although the fit of the calibration equation to the *in situ* data is quite good, there are other factors which impact the accuracy of the $K_{1/2}$ determination. As noted, the exclusion of the lower ratio sub-population found at some calcium concentrations lowers the estimate of $K_{1/2}$ to 500nM. We note that the *in situ* value of R_{max} measured for Fura Red™ in the imaging system was almost five fold higher than that measured by spectrofluorimetry for the indicator in solution. We have no definitive explanation for this, but speculate that in addition to the differences in the optical systems the binding of Fura Red™ to intracellular proteins may have affected the fluorescence spectra. Such high ratios, e.g. those in excess of five, begin to cause some problems in our imaging studies. For example, when the measured signal in the denominator wavelength falls closer to threshold, it is more susceptible to noise, and the ratio measured becomes less certain. Furthermore, the value of $K_{1/2}$ from the fit is

quite sensitive to the ratio values measured at saturating calcium concentrations. For these reasons the estimates of $K_{1/2}$ obtained in the curent study are believed to have an uncertainty of roughly 50-75nM.

The influx (or outflow) of large amounts of Ca^{++} ion across the membrane itself is a strong stimulant to platelet activation. Living cells would be expected to respond, and take action to modulate their $[Ca^{++}]_i$ accordingly, which would be at odds with the goal of acheiving a known, specific $[Ca^{++}]_i$ for calibration purposes. Several factors mitigate this problem. First, in this study the platelets have presumably already undergone significant stimulation associated with their attachment and spreading on the glass coverslip (a strongly activating surface). Second, in addition to the high concentrations of ionophore used to swamp endogenous platelet membrane Ca^{++} pumps, thapsigargin was used in an attempt to prevent sequestration of intracellular calcium. At the extremes (zero and very high Ca^{++}) these efforts appeared to be uniformly effective, as evidenced by the homogeniety of the measured ratios in the adherent population. However, in the range of $[Ca^{++}]$ closer to those that the cell would have during its normal physiological resting state, there was a subpopulation in which the $[Ca^{++}]_i$ was found to be closer to nominal resting values. These bimodal results seen at some calcium concentrations may simply mean that, for some platelets, the chosen mixture of ionophores and thapsigargin was unable to defeat the platelet's normal calcium homeostasis. While it is not an area of investigation in our laboratory, the differential sensitivity of individual platelets in the platelet population to these and other such perturbations may be useful in answering questions in regard to platelet heterogeneity and the maintenance of platelet $[Ca^{++}]_i$ in general.

This study is believed to provide a fairly accurate *in situ* calibration of Fura Red™ in platelets. Further improvements of the calibration might include the use of A23187 or its non-fluorescent brominated analog as the ionophore. It might useful to evaluate the ratio values as a function of time after the addition of ionophore to gauge the process of bringing the $[Ca^{++}]_i$ to extracellular levels. Finally a detailed *in vitro* study (via spectrofluorimetry) of the effects of cytoplasmic protein concentration and composition on the fluorescence properties of the indicator might lead to a better prediction of these properties in the intracellular environment.

4.8 Conclusions

An *in situ* calibration curve for the Ca^{++}-sensitive indicator Fura Red™ as loaded in human platelets was constructed. The calibration parameters $K_{1/2}$, and the ratio at saturating Ca^{++}, R_{max}, were much higher in the intracellular environment, measured by microscopy compared with similar measurements in buffer. While it is beyond the scope of this study to address why the dissociation constants and optical parameters of Fura Red™ and other carboxylate ion indicators vary in the intracellular environment, the fact that they do necessitates caution when interpreting $[Ca^{++}]_i$ results and careful attention to the details of calibration in the system at hand. It would seem prudent that similar efforts at *in situ* calibration should be attempted for each new cell type investigated, or when conditions such as temperature, protein concentration or pH are varied.

Despite its drawbacks, Fura Red™, can be used successfully, on its own, as a dual excitation dye in widefield fluorescence microscopy, especially with the aid of a cooled-CCD detector. With image pair acquisition times of approximately one second or less, moderate temporal resolution is achieved. Currently, there is growing interest

in the combination of standard epifluorescence microscopy with computational deconvolution techniques as a lower cost alternative to conventional laser scanning confocal microscopy. As such, the use of dual excitation ion indicators such as Fura Red™ may be expected to grow as well.

Table 4.1. Calibration Parameters for Fura Red™ in Buffered Solution and in Platelets

	In Vitro (*buffered solution*)	*In Situ* (*in platelets*)
$K_{1/2}$ (nM)	266±17	611 ± 45
R_{max}	1.19±0.01	5.86 ± 0.1
R_{min}	0.6±0.007	0.77 ± 0.05
ß	1.93	
K_d (nM)	138	

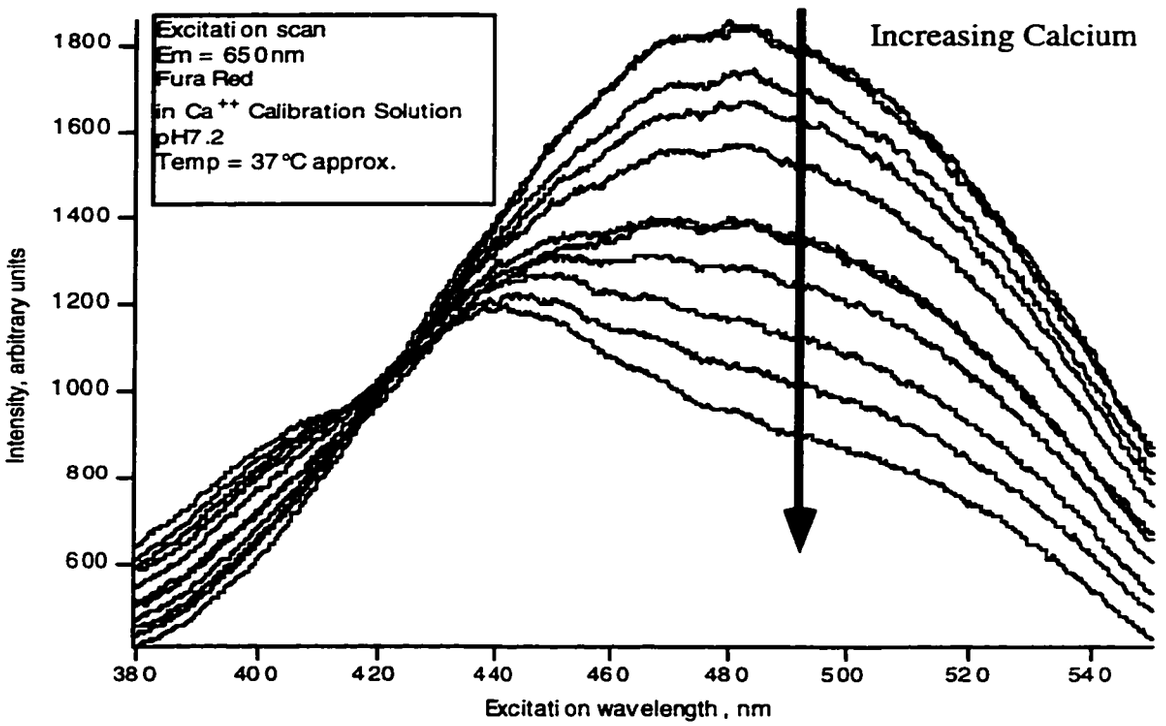

Figure 4.1. Excitation spectra of Fura Red™ salt in Ca^{++}-calibration buffer, pH 7.2, 37°C, measured spectrophotometrically. The isosbestic point is fairly broad, occurring between 420-430nm.

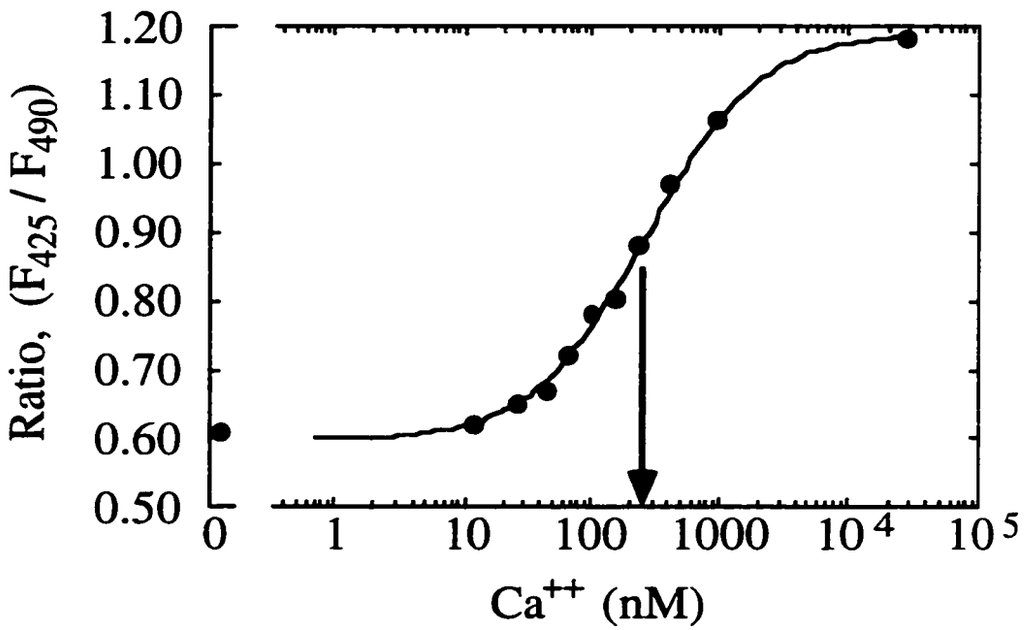

Figure 4.2. *In vitro* calibration of Fura Red™. The fluorescence intensity at 425nm excitation (F_{425}) was divided by F_{490} for each of the calibration solutions (data from Figure 4.1) and plotted as a function of [Ca^{++}]. The solid line shows a fit of Equation 4.5 to the data, yielding values of $K_{1/2}$ = 266nM (arrow), R_{max} = 1.19 and R_{min} = 0.60 for the calibration parameters. ß = 1.93 resulting in a K_d of 138nM.

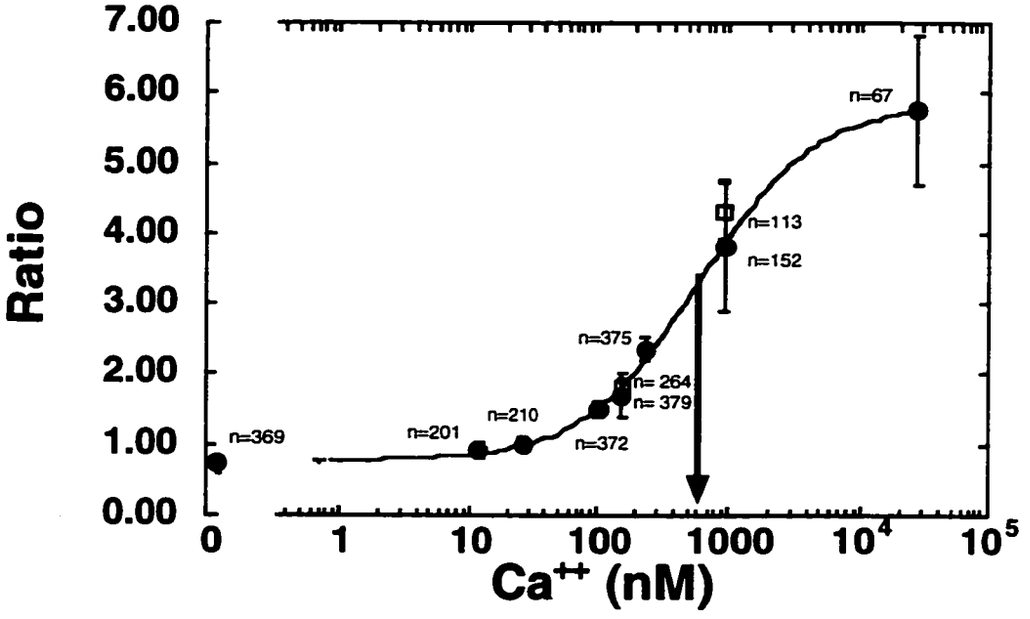

Figure 4.3. *In situ* calibration of Fura Red™. The ratio values of adherent platelets from several fields were averaged to one overall ratio value for each [Ca^{++}] (solid circles). The solid line shows the fit of Equation 4.5 to the data yielding values for the three calibration parameters. Omission of the lower ratio subpopulation results in a higher ratio average (open squares seen at [Ca^{++}] = 160 and 940nM, data not used in fit, discussed in text).

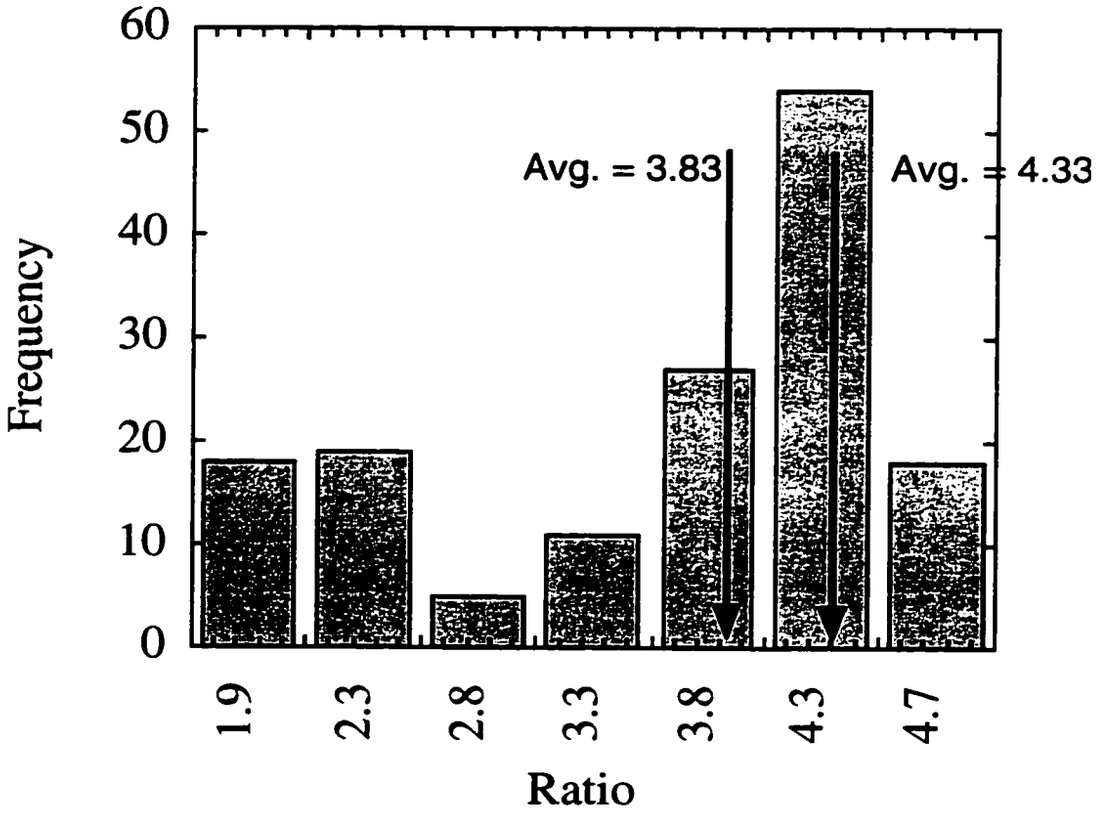

Figure 4.4. Histogram of ratio values from platelets at 940nM [Ca^{++}]. A sub-population is seen with lower ratio values around 2.0. If these data are omitted, the overall average is increased from 3.83 to 4.33.

Notes to Chapter 4

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Chapter 5: Measurement of $[Ca^{++}]_i$ in Platelets Adhering to Biomaterial Surfaces from Static Suspensions

5.1 Introduction

The previous chapters have described the functioning of the platelet, and its role in hemostasis and thrombosis, including thrombosis induced by biomaterial contact. The design of blood contacting devices depends on the availability of suitable blood contacting surfaces. The rational design of improved or novel biomaterial surfaces requires an understanding of the mechanisms involved in platelet adhesion and activation.

The activation of platelets by biomaterial surfaces involves a variety of different biohemical processes. Many methods have been used to study the adhesion and activation of platelets by biomaterial surfaces. Methods to quantify platelet adhesion include the use of radiolabeled platelets under both static and flowing regimes, as well as the use of colorometric assays (e.g. LDH) to quantify the amount of platelet material adhered to a surface at some endpoint. Dynamic measurement of platelet adhesion (attachment and detachment) can be made using epi-fluorescence video microscopy. To assess the state of platelet activation, granule release is most commonly measured. This is done by quantifying the release of granule contents, e.g. serotonin or ß-thromboglobulin, or by quantifying the exposure of the granule membrane protein, P-selectin, using immunochemical techniques. The affinity modulation of integrin $\alpha_{IIb}ß_3$ is also an important marker of platelet activation that can also be measured immunochemically (PAC-1 antibody).

The measurement of $[Ca^{++}]_i$ as a marker of platelet activation provides different information on the state of platelet activation than these other methods. Since $[Ca^{++}]_i$ is a central mediator of signal transduction, its response is linked to many different activation pathways. Measurement of $[Ca^{++}]_i$ might be a more sensitive marker of platelet activation than granule release, since the act of granule release is an endpoint, and the mobilization of $[Ca^{++}]_i$ is believed to act earlier in the signal transduction process. However, while $[Ca^{++}]_i$ as been implicated to play a role in many of the biochemical steps of platelet activation, the complexity of the system has made interpretation of $[Ca^{++}]_i$ results difficult.

As discussed in the previous chapters, $[Ca^{++}]_i$ can be measured in stirred suspensions. $[Ca^{++}]_i$ has been measured in individual cells in larger cell types to study signal transduciton in both excitable and non-excitable cells. Platelet $[Ca^{++}]_i$ shows a response during stimulation by soluble agonists such as thrombin, ADP, and others. The study of $[Ca^{++}]_i$ has received comparatively little attention in single platelets. The few published reports do confirm that platet $[Ca^{++}]_i$ in single platelets shows a transient in response to soluble agonists.

This section focusses on the measurement of $[Ca^{++}]_i$ in single platelets during attachment and spreading upon several biomaterial surfaces. $[Ca^{++}]_i$ was montored with ratio fluorescence microscopy as the platelets settled to the surface from static suspensions, attached to the surfaces, became activated and spread. Washed, fluorophore labeled platelets were used.

Several surfaces were tested. Early experiments used glass, and glass with dilute plasma. Other surfaces tested are: Biospan, a biomedical polyetherurethane; Octadecyl chain extended polyetherurethane (ODCE-PEU), polystyrene, and

hydroxyethylmethacrylate - ethylmethacrylate copolymers. In many experiments the surfaces were pre-adsorbed with fibrinogen.

5.2 Methods

The preparation of gel filtered, washed and labeled platelets has been described in Chapter 3. Briefly, human whole blood was used, with ACD as the anticoagulant. Platelet rich plasma was prepared by a single centrifugation and the platelets were labelled by incubation with Fura Red™-AM. The platelets were then separated from the plasma proteins and the unincorporated dye by gel filtration. The platelets were eluted from the column into a platelet suspending buffer - a HEPES/Tyrodes buffer with 4mg/ml albumin and 0.1U/ml apyrase, ph7.4. Ca^{++} was not included in the buffer during washing. The platelet fraction was collected and the platelet count adjusted. The Ca^{++} was replenished to 2mM, and the platelets were warmed to 37°C before use. Platelet suspension was added to a volume of suspension buffer in the coverslip dish to yield approximately 1.5×10^7platelets/ml in the final suspension.

5.2.1 Surfaces

A variety of polymeric biomaterial surfaces were used in these studies. Polyurethanes are frequently used in blood contacting situations. Their mechanical properties make them attractive for use in many devices, however the blood-compatibility of the polyurethanes has often been questioned. Numerous efforts continue regarding the surface modification of polyurethanes to improve blood compatibility by modifying the surface chemistry of the interfaces, while retaining the desirable bulk properties of this polymer family.

All surfaces were cast on circular glass coverslips (25mm diameter,#1, VWR Scientific) which had been cleaned by ultrasonication in Isopanasol (4%w/v in water, C.R. Callen, Seattle, WA) and three times in deionized water. The coverslips were blown dry with dry N_2 and dried in an oven for several hours.

All polymer films were formed by centrifugally casting the films from dilute solutions in solvent, using a photo-resist spinner. The surfaces were first coated with an appropriate silane copolymer; styrene-silane for polystyrene surfaces, and ethylmethacrylate-silane (EMA-silane) for all HEMA/EMA, Biospan™ and ODCE-PEU surfaces. This coating is described in detail in Chapter 6.

Biospan™ surfaces were prepared as follows: Biospan™ PTMO-based polyurethaneurea Primary Reference Material, (lot #071785) was provided by Prof. B. Ratner as a stock solution (25%w/v in DMAC, Polymer Technologies Group, Emeryville, CA). The Biospan™ was diluted to 3%w/v in dry DMAC, and then filtered using a solvent resistant 0.5µm filter. The Biospan™ film was centrifugally cast onto the EMA-silane coated coverslips using 100µl aliquots, a rotational speed of 4000rpm and a spin time of 30sec. A gentle stream of dry N_2 was blown over the surface during the spin. Coatings appeared uniform to the eye. Samples so prepared were submitted to the National ESCA and Surface Analysis Center for Biomedical Problems, where ESCA survey and high resolution C_{1s} spectra were generated and analyzed. The results of their analysis is as follows: The spectra are consistent with that seen for other samples of Biospan™ polyurethane. Spectra were collected from two spots on the sample, one near the center and the other near the edge. The spectra were similar suggesting the coating was intact and uniform across the coverslip. The survey scan did reveal some small silicon signal, which was attributed to the thinness of the Biospan™ coating. An additional spectra taken at glancing angle (more shallow sampling depth)

showed half the silicon signal as the other spectra, suggesting that this silcon signal was due to thinness of the Biospan™ coating over the EMA-silane copolymer layer. Results of the survey spectra are shown in Table 5.1. The ESCA spectra are shown in figure 5.1.

An octadecyl-chain extended polyetherurethane urea (ODCE-PEU) was provided by Prof. Ratner. This polyurethane modification is believed to result in a surface rich in hydrocarbon groups, and the C-18 pendant group may be responsible for enhanced adsorption of albumin. Previous results suggest that the fibrinogen adsorbed to the ODCE-PEU does not support adhesion as well as that bound to Biospan, resulting in reduced platlet adhesion in both static and flowing regimes, as compared to Biospan. [1]. Casting of the ODCE-PEU is detailed in Chapter 6.

Polystrene films were cast from dilute solutions of polystyrene secondary standard and analyzed as described in Chapter 6.

The hydroxyethylmethacrylate-ethylmethacrylate (HEMA-EMA) copolymers comprise a series of polymers of varying hydrophilicity, from very hydrophillic (100% HEMA) to hydrophobic (100% EMA). As part of this work, four members of the series were chosen. HEMA-EMA A is hydroxyethylmethacrylate and is hydrophillic. HEMA-EMA C is a copolymer of 80% hydroxyethylmethacrylate and 20% ethylmethacrylate. HEMA-EMA F is a copolymer of 50% hydroxyethylmethacrylate and 50% ethylmethacrylate. HEMA-EMA J is a copolymer of 10% hydroxyethylmethacrylate and 90% ethylmethacrylate. These polymers have been the subject of other studies of protein adsorption and platelet adhesion in this laboratory. The polymers were cast from 5% solutions in dimethylformamide onto coverslips previously coated with the EMA-silane coupling agent described above.

5.2.2 Fibrinogen Adsorption

In some experiments the surfaces were adsorbed with fibrinogen before use. Fibrinogen is a major plasma protein, and is known to adsorb to many biomaterial surfaces where it supports the attachment, adhesion and spreading of platelets. It was used in this work to potentiate platelet adhesion. Human Fibrinogen (lyophilized, with buffer salts, Sigma Cat.#F-3879) was dissolved in distilled deionized water to 20mg fibrinogen/ml. This solution was stored in aliquots at -80°C. This solution was then diluted 100fold in CPBS to yield 0.2mg/ml fibrinogen solution which was allowed to adsorb to the sample surfaces at 37°C for at least 1hr. The samples were rinsed in PSB and then used immediately in the experiments.

5.2.3 Ratio Fluorescence Microscopy

The ratio fluorescence microscope system was described in the previous chapter. Briefly, the system comprises a Nikon Diaphot`™ inverted microscope fitted for epifluorescent illumination (model TMD-EF). Illumination is from a Xenon arc lamp (XBO-100W, Nikon) and filtered through band pass filters (425 and 490 ± 10nm, Omega Optical, Brattleboro, VT) held in a filter wheel which is controlled by in conjunction with the image acquisition computer. A 580nm dichroic mirror and 610nm long pass barrier filter were used. The objective used was a Nikon 40X UV Fluor 1.3NA oil immersion objective.

The ratio fluorescence imaging software METAFLUOR (version 2.5, Universal Imaging Corp., Brandywine, PA) was used for both data collection and analysis. Images were acquired using a Photometrics CH250 cooled CCD imager with a Kodak KAF1400 chip. The chip was used with 2x2 binning, a 300ms exposure time for each individual image, with image pairs taken at 30sec intervals. Acquisition of each image

pair was complete within one second. To subtract for background, a fixed value of 230 gray scale units was subtracted from the 425nm wavelength image (image acquired using 425nm excitation) and 210 units from the 490nm wavelength image. The 12-bit, individual wavelength images were stored directly to computer hard drive and later moved to magneto-optical drives for storage.

A coverslip with a spin cast thin film was adsorbed with fibrinogen as above. The coverslip was removed from the fibrinogen solution, dip rinsed briefly in PSB-albumin, and installed in the telfon coverslip dish assembly as described in the previous chapter. Using a diamond scribe, a small mark was made on the surface to aid in establishing the focal plane. The sample dish assembly was immediately placed in the microincubator mounted on the microscope stage and kept at 37°C. Platelet suspending buffer (37°C, with albumin, apyrase and replenished Ca^{++}) was added to the dish. Focus was established by transmitted light microscopy using the scribe mark, and then the field of view was moved away from that mark. An aliquot of the washed labeled platelet suspension was added directly to the dish, diluting it ten fold and resulting in 1.5×10^7 platelets/ml in the final suspension. The image acquisition sequence was started immediately following the addition of the platelets to the dish. Image paris were acquired at 30sec intervals for 45 minutes. Infrequent adjustments to the focus were made as needed throughout the experiment.

Ratio images were formed from the individual wavelength images after background subtraction by dividing the intensity of each pixel in the 425nm image by the intensity of the corresponding pixel in the 490nm image. A threshold mask was applied to exclude any pixels in the ratio image for which the intensity in either of the individual images was below a small and arbitrarily set threshold, namely 75 greyscale units above background. This value was chosen to exclude artifacts found usually at the fringes of

cells where the intensity drops near background, causing this noise to be unduly magnified in the ratio. Pixels that were excluded by this threshold were displayed with a ratio value of 0 (as is all of the background), but this value was not included in the determination of an average ratio for a particular region during analysis, i.e. these pixels were excluded from the analysis. The ratio images were scaled for 8-bit display as described in Appendix B .

In off-line analysis, the image series was analyzed using METAFLUOR (version 2.5). Individual platelets were identified in the ratio images and each assigned a unique analysis region. In these static platelet experiements, cells settled to the surface and often drifted across the surface for a short time, due to Brownian motion, and thermal convection. Platelets that are in motion during the interval between individual wavelength acquisitions are easily noted by a characteristic artifactual ratio pattern, and are not included for analysis. Platelets were selected for analysis if they remained stationary for more than two or three consecutive frames. The average ratio value of the pixels in each analysis region (corresponding to each individual platelet) was determined and this value was logged to a computer file. Likewise, for each image in the series: the ratio image was viewed, small adjustments were made if necessary to the placement of the analysis region around adherent platelets, new analysis regions were assigned to new arrivals, and the data was logged. Also for each image in the series, each platelet was viewed and, in the event that there was interference from other non-attached cells, or in the event that the signal became so poor, such that the image was no longer recognizable as a cell, this was noted manually in the log and these data were omitted. The ratio values for each platelet, or the $[Ca^{++}]_i$ calculated from the ratio value and the calibration equation as described in Chapter 4, were displayed as a function of the time after platelet addition. Also in off-line analysis, a crude qualitative assesment of platelet

spreading was made for each platelet based on changes in platelet size, shape and thrickness, as best as could be determined from the thresholded ratio images.

5.3 Results

5.3.1 Preliminary Work at FHCRC

Experiments were first conducted on a ratio fluorescence imaging system at the Image Analysis Facility at the Fred Hutchinson Cancer Research Center. This system is described in Chapter 3. Figure 5.2 provides an example of these early results. In this experiment a cleaned glass coverslip was pre-adsorbed with dilute (0.1%v/v in CPBS) plasma and washed, labeled platelets were allowed to settle onto the surface. Fifteen platelets were analyzed in this experiment and the ratio time course of a select five of these platelets is shown in figure 5.2. First, these results, which were acquired using a video-rate camera (DAGE 66, silicon intensified target video rate camera) demonstrated the feasibility of imaging platelet $[Ca^{++}]_i$ and making such measurements. Several features of the platelet $[Ca^{++}]_i$ response are evident. There is a lag period in some platelets before the $[Ca^{++}]_i$ response. This is variable, and was as long as six minutes in some cells. The $[Ca^{++}]_i$ response itself was heterogenous. Some cells demonstrated a rapid rise in ratio to a sustained plateau value, some cells showed a weaker, slower and more transient response in ratio. Other cells remained quiescent on the surface throughout the experiment. Of the 15 cells analyzed, five showed $[Ca^{++}]_i$ responses, while ten did not.

Washed platelets that settled on Biomer, pHEMA and EMA-Silane copolymer surfaces without pre-adsorption of adhesive proteins from dilute plasma, rarely attached and did not show changes in $[Ca^{++}]_i$ on these surfaces.

The remaining studies were conducted on the ratio fluorescence imaging system that I assembled at our laboratory at the University of Washington, and is described above and in detail in Chapter 3.

5.3.2 BioSpan

Figure 5.3 shows an example of a pair of fluorescence images and the resulting ratio image of four adherent platelets. Few if any platelets attached to surfaces that were not coated with fibrinogen. The response of platelets to fibrinogen coated Biospan™ is heterogenous. Most platelets arrive at the surface with a resting $[Ca^{++}]_i$ of 50 - 100nM. Some cells remain attached to the surface but are otherwise non-responding, i.e. these cells do not show evidence of a large or rapid $[Ca^{++}]_i$ response. The $[Ca^{++}]_i$ in these cells stays below 200nM. In cells that do demonstrate a $[Ca^{++}]_i$ response several features are noted. First, in most cells there is a lag period, after attachment but before $[Ca^{++}]_i$ rise. This lag period is variable ranging from no lag to as long five minutes. Second, a rise in $[Ca^{++}]_i$ of 100 - 200nM is seen, and this is often accompanied by oscillations in $[Ca^{++}]_i$. Finally, these responding cells demonstrate an elevated plateau value. Some oscillations in $[Ca^{++}]_i$ are also evident at this stage, and the $[Ca^{++}]_i$ shows a trend of decreasing again, slowly towards the resting value. Figure 5.4 shows the time courses of two platelets that are representative of repsonding and non-responding cells. Figure 5.5 shows a series of eight ratio images, at 30sec intervals, that show platelets attaching adhering to Biospan. Newly adherent cells are seen in the images at 3.5min, and 5.5min timepoints. One platelet shows a lag of 3min beofre showing a sharp rise in $[Ca^{++}]_i$. Figure 5.6 shows ratio images taken at different fields of view at the end of the experiment. Several platelets with spread morphology and varying $[Ca^{++}]_i$ values are seen.

In one experiment on fibrinogen-coated Biospan, 37 platelets were observed to attach at various times and remain stationary. As best as could be determined from the ratio images, roughly half (19/37) of these platelets subsequently spread. Examination of the $[Ca^{++}]_i$ time courses of these spread platelets revealed, in most cases (15/19), several peak values of $[Ca^{++}]_i$ greater than 350nM. When the $[Ca^{++}]_i$ time courses of the non-spread platelets were examined, it was found that the $[Ca^{++}]_i$ in most of these cells remained below 250nM at all times (12/18). These data suggest that platelet spreading may be associated with $[Ca^{++}]_i$ values in excess of some threshold value between 250 and 350nM. Put another way; spread platelets are associated with $[Ca^{++}]_i$ levels in excess of 350nM, and quiescent platelets that are not spread, are associated with $[Ca^{++}]_i$ values less than 200nM. This hypothesis was tested using these data expressed as categorical results. The Yates-corrected Chi-square test for 2x2 contingency tables was applied and confirm that the data reveal differences between the peak $[Ca^{++}]_i$ values in spread and non-spread platelets which are significant at the $p<0.025$ level [2]. These results are shown in Table 5.2.

5.3.3 Polystyrene

As on the Biospan™ surface, few platelets attached at all to the polystyrene surfaces without fibrinogen coating. The characteristics of the $[Ca^{++}]_i$ response to attachment on the polystyrene surface are as for the Biospan™ surface: namely, the response is heterogeneous, there is a variable lag time before response, responding cells show a sharp rise in $[Ca^{++}]_i$ with oscillations and reach a plateau. The platelet $[Ca^{++}]_i$ in non-responding cells remains low. Figure 5.7 shows the time courses of two platelets that are representative of responding and non-responding cells. In one experiment on fibrinogen coated polystyrene, 40 attached platelets were analyzed. Slightly fewer of

the platelets subsequently spread than on the Biospan™ surface (16/40). Examination of the $[Ca^{++}]_i$ time courses revealed that most of the spread platelets had shown peak $[Ca^{++}]_i$ values above 210nM, while the $[Ca^{++}]_i$ values in most of the platelets showing no morphologic change remained below 210nM. These results are also shown in table 5.2. While the differences in $[Ca^{++}]_i$ values between spread and non-spread platelets were not as clear on the polystrene as on the Biospan; these differences were, nonetheless, significant at the $p<0.025$ level.

5.3.4 OCDE-PEU

On fibrinogen coated ODCE polyurethane, only 21 attached platelets were available for analysis, and none showed marked changes in shape. Five platelets did show weak rises in $[Ca^{++}]_i$ to values just over 200nM, and lag periods from 0 to 20 minutes, while the majority remained below 170nM. Previous results from others in this laboratory suggest that the fibrinogen adsorbed to ODCE-PEU does not support platelet adhesion as well as that bound to Biospan, resulting in reduced platelet adheison in both static and flowing regimes as compared to Biospan™ [1].

5.3.5 Other Surfaces

Several experiments using the HEMA-EMA surfaces had to be aborted due to technical reasons. Nonetheless, in separate and duplicate experiments for each, it was found that an insufficient number of platelets attached for analysis to HEMA-EMA A, C, and J surfaces, both with or without fibrinogen.

Six experimental runs were devoted to surfaces prepared by radio frequency glow discharge using hexafluoroethane monomer, and a downstream "afterglow" coating technique. These surfaces were coated directly onto cleaned glass coverslips without a coupling agent, and were prepared and provided by Prof. B. Ratner's laboratory.

Unfortunately subsequent analysis of these surfaces by ESCA conducted by the National ESCA and Surface Analysis Center revealed that these surfaces were poorly coated and the polymer coverage mostly incomplete. Since these surfaces were ill-defined, likely irreproducible, and inhomogeneous, the results of these experiments are not presented.

5.4 Discussion:

As described in Chapter 2, there was (and still is) very little precedent for the measurements made in this study, namely the measurement of $[Ca^{++}]_i$ in single platelets during attachment and spreading upon biomaterial substrates. The result of these studies confirms that there is a $[Ca^{++}]_i$ response in many (but not all) platelets during contact with biomaterials. The response is characterized by a lag period, a rise in $[Ca^{++}]_i$ of 100-200nM or more, and oscillations. There is considerable heterogeneity in the $[Ca^{++}]_i$ response among the population of cells that attach.

5.4.1 Lag Period

First, the finding that there is any lag period at all in the platelet $[Ca^{++}]_i$ response to biomaterial contact is an important result. Previously the platelet $[Ca^{++}]_i$ response had only been characterized in response to soluble agonists like thrombin or ADP. When studied in stirred suspension, or in stopped flow appratus, the platelet $[Ca^{++}]_i$ response to these agonists is rapid; on the order of a few seconds and in some cases, less than one second. The finding in this work is that most platelets do not immediately generate a $[Ca^{++}]_i$ response upon attachement to a protein coated biomaterial surface. Rather, most platelets attach to the surface and remain at a low $[Ca^{++}]_i$, typically 50-100nM for

several minutes before demonstrating a more rapid rise of 100nM or more during an observation interval.

These results suggest that there might be a fundamental difference in the nature of the platelet activation induced by agonists from the platelet activation induced by biomaterial contact. It is easy to speculate as to why this might be the case. For example, the delayed $[Ca^{++}]_i$ response may due to a requirement for the integration of several stimulatory signals before the moblization of $[Ca^{++}]_i$ is triggered, thereby signaling other downstream activation processes. It may be that the stimulatory signals from many copies of a membrane receptor engaged with the surface must be integrated before further steps of platelet activation are triggered; or that different signals from distinct types of receptors must be intgrated before $[Ca^{++}]_i$ is increased. Alternatively, the delayed response could be due to the assembly of a so-called signaling complex, comprising several signal transduction elements and assembled at the cytoplasmic tails of integrin receptors where they are anchored to the cytoskeleton. In any case, platelets stimulated with very low doses of the soluble agonists ADP or thrombin, can exhibit reversible aggregation; but full aggregation or spreading, and granule release are irreversible, and represent a committed and irrevocable functional response on the part of the platelet. This may be one reason why signal integration might be required before a $[Ca^{++}]_i$ response is triggered. It is interesting to note that the aggregation of platelets in stirred suspension by collagen fibrils is also characterized by a lag of roughly one to two minutes, and recently, single platelet imaging of $[Ca^{++}]_i$ in platelets attaching to collagen fibrils was reported with a variable lag of 15-120 seconds [3].

Unfortunately, the experiments presented here are not optimal for the definitive study of such a lag period. With image pairs acquired at 30 second intervals, and with

the sometimes transient nature of the inital contact, it is difficult to identify the moment of attachment with precision. Likewise, the definition of the onset of the response is somewhat arbitrary; many cells demonstrate a jump of 100nM or more over a single 30 sec interval, while other show more gradual increases in $[Ca^{++}]_i$. As a consequence the estimation of a lag period in this system is associated with an uncertainity of as much as one minute or more. Increased time resolution, and perhaps a video based system could provide better assesment of the lag between platelet atachment and $[Ca^{++}]_i$ rise.

While the lag period observed indeed suggests a difference in the activation of platelets by biomaterial contact from the activation by agonists, there are alternative explanations that are also plausible. For example, it may be that platelet attachment and spreading is entirely independent of $[Ca^{++}]_i$ and that the $[Ca^{++}]_i$ rise is part of other biochemical processes in the platelet, e.g. granule release or procoagulant activity, that might be triggered solely and randomly by secondary activating agents; i.e. ADP released by other nearby stimulated platelets, or locally formed or released thrombin. This remains the realm of speculation, of course, as all of these questions will require further study.

5.4.2 Heterogeneity

The heterogeneity observed in the $[Ca^{++}]_i$ response is at once a novel observation and a confounding aspect of data. Platelet heterogeneity in size, in age and in composition have all been known for some time. But heterogeneiety in functional response, particularly in $[Ca^{++}]_i$ response is completely obscured in stirred suspension measurements; it was not until the first measurements of single platelet responses in flow cytometery that this became apparent. The heterogeneity becomes very evident in single platelet imaging experiments, and is mentioned (though often not emphasized) in

nearly all of the published reports of single platelet $[Ca^{++}]_i$ imaging. There could be biologic significance to platelet functional heterogeneity; as this would result in a subpopulation of platelets that might respond rapidly with the slightest provocation to the hemostatic system; while other platelets might be held in reserve, only to respond to the most catastrophic hemostatic challenges. Platelet heterogeneity is a subject of current interest and it study may be aided by the use of single platelet $[Ca^{++}]_i$ imaging. For example, platelet $[Ca^{++}]_i$ responses might be correlated with platelet size or initial granule content.

The fact that not every platelet responds to a particular surface in a like manner makes the evaluation of the platelet response to different surfaces difficult. By comparison, in the standard two hour static platelet adhesion assay, the outcome for each cell is binary; it either sticks or was washed away, it is either counted as adherent or not. The platelet $[Ca^{++}]_i$ response is more complex, however. Some remain quiescent or show gradual rises in $[Ca^{++}]_i$. Others show a sharp rise to various values with oscillations, and yet others are in between. An arbitrary metric must be imposed, as was done above by separating those that demonstrated peak $[Ca^{++}]_i$ values above a chosen point, to binarize the data collected. Furthermore, the variability in platelet response in radiolabeled static platelet adhesion assays can be offset by many replicates, and long adhesion times; both of which are difficult aspects to translate to these dynamic microscopy experiments.

The heterogeneity in platelet functional response that others have repeatedly observed, and was observed here is likely an accurate reflection of the functioning of the platelet population *in vivo*. However, since some manipulation of the platelets was required for their use in these experiments, specifically the preparation of washed and

labeled platelets, the possiblity cannot be excluded that the heterogeneity observed might be the result of perturbations to some of the platelets during preparation.

5.4.3 Spreading

The data presented in Table 5.2 for two different fibrinogen coated surfaces, suggest that the spreading of platelets upon the substrate is correlated with platelet $[Ca^{++}]_i$ time courses where the peak $[Ca^{++}]_i$ values exceeded a certain level one or more times during the experiment. As discussed in Chapter 2, a rise in $[Ca^{++}]_i$ is associated with many different biochemical pathways of activation in the platelet; nearly every platelet agonist induces a $[Ca^{++}]_i$ response. Alone, the action of ionophores to artificially cause a rise in $[Ca^{++}]_i$ leads to platelet activation, and aggregation. Yet, increased $[Ca^{++}]_i$ is not always required for platelet activation to take place. Thus, platelets treated with a cell-permeant calcium chelator (BAPTA-AM) are seen to attach and spread on fibrinogen coated surfaces without any increase in $[Ca^{++}]_i$ [4], (and Hauch, unpublished observations). Data presented in Chapter 6 suggest that the beginnings of psuedopod formation can be seen in platelets prior to a rise in $[Ca^{++}]_i$. The determinations of platelet morphology in Table 5.1 were made at the end of the experiment, and as such are not temporally correlated with the dynamic $[Ca^{++}]_i$ responses.

While the data do not definitively show that a rise in $[Ca^{++}]_i$ precedes or causes platelet spreading; they do show that the processes of platelet spreading and $[Ca^{++}]_i$ control are somehow linked. Recent work by Heemskerk suggests that the rise $[Ca^{++}]_i$ is necessary for the later steps in platelet activation on surfaces, namely granule release and procoagulant activity [5], but not platelet spreading on fibrinogen.

The determination of morphologic change in these experiments was necessarily made by a crude qualitative assessment. The raw fluorescence images of these very

small cells are fairly blurry. When the ratio image is formed, a thresholding algorithm is used to prevent noise in the dim signal found near the edges of objects from being magnified in the ratio image. The effect of this algorithm is to exclude a small number of the pixels nearest the very edge of the cells, especially in thin spreading cells. The value of the threshold in the algorithm is chosen carefully so that the platelets in the ratio images are as close as possible in size and shape to the platelets as they appear in the fluorescence images, and under transmitted light microscopy. However, the necessary application of the thresholding algorithm precludes a quantitative morphology assessment of platelet spreading from these ratio images. So a qualitative, subjective determination of "spread" or "no change" was made by viewing the platelet throughout the experiment, taking into consideration the initial platelet size and change in size, platelet shape, and platelet thickness (as indicated by the intensity of the Ca^{++}-insensitive fluorescence).

This aspect of the experiment would clearly benefit by more robust technologies to better assess the shape of attached and spreading platelets. Such end point determinations might beter be accomplished by scanning electron microscopy. This would require a rapid fixation step at the conclusion of the experiment, and some efforts spent on the logistics of reconciling the scanning electron micrographs with the imaging experiment, e.g. by the use of reference marks on the substrates. Alternatively, Waples, et al. have reported simultaneous ratio fluorescence and transmitted light microscopy in a specially adapted microscope. This provides for the direct temporal correlation of changes in platelet shape with platelet $[Ca^{++}]_i$ [6].

5.4.4 Oscillations

Oscillations are another aspect of the platelet $[Ca^{++}]_i$ response that are obscured in measurements of $[Ca^{++}]_i$ in stirred suspensions. In fact, oscillations in $[Ca^{++}]_i$ in many cell types have been revealed with the advent of ratio fluorescence microscopy. Oscillations or spikes in platelet $[Ca^{++}]_i$ have been noted in the study $[Ca^{++}]_i$ in single platelets in response to the agonist ADP and thrombin. Neither the mechanism of platelet $[Ca^{++}]_i$ oscillations, nor its physiologic importance are understood.

Oscillations are seen as a prominent feature of the $[Ca^{++}]_i$ response in responding platelets during attachment and spreading (platelets not stimulated by additional agonists). Unfortunately, the phenomenon appears sporadic; the amplitudes and frequency are highly variable even within a single platelet time course and also vary greatly from platelet to platelet.

It is possible and indeed likely that the frequency of the oscillations might be more rapid than can be appreciated in the work reported here using 30 second acquisition intervals. Indeed, using near video rate $[Ca^{++}]_i$ imaging, Heemskerk has reported spiking in platelet $[Ca^{++}]_i$ in single platelets stimulated with ADP, at frequencies of about 5/min. Heemskerk has also observed oscillations during the attachment of platelets to fibrinogen coated surfaces, but feels that this phenomenon may be due to released adventitious ADP from other nearby adherent platelets. However, high does of apyrase, used to catabolize adventious ADP, were unable to completely eliminate platelet spiking on fibrinogen coated surfaces (Heemskerk, personal communication, Oct. 97).

5.5 Summary

The control of $[Ca^{++}]_i$ is a key aspect of signal transduction in a variety of cells. In platelets, $[Ca^{++}]_i$ has been implicated as being important in several biochemical

pathways of activation. Studies of $[Ca^{++}]_i$ in stirred platelet suspensions have shown that $[Ca^{++}]_i$ rises during the stimulation of platelets with agonists, and indeed, the use of ionophore to artifically increase $[Ca^{++}]_i$ leads to platelet activation and aggregation. Platelets are stimulated by contact with protein covered surfaces where they often attach and spread; becoming activated, releasing secondary mediators of platelet activation and promoting blood coagulation. The goal of these studies was to use newly available techniques of ratio fluorescence microscopy to image the platelet $[Ca^{++}]_i$ response during attachment and activation on biomaterial surfaces.

The observations presented in this chapter are part of the first handful of observations ever made of $[Ca^{++}]_i$ as imaged in single adherent platelets. This work sucessfully demonstrates that the techniques of ratio fluorescence microscopy can be adapted and refined to image $[Ca^{++}]_i$ in these, the smallest of the blood cells. The experiments show that the platelet $[Ca^{++}]_i$ response during attachment and spreading on protein coated biomaterial surfaces is heterogenous. Resting $[Ca^{++}]_i$ was observed to be 50-100nM, and cells that attached to the surfaces demonstrated a resting $[Ca^{++}]_i$. It does not appear, therefore, that increased $[Ca^{++}]_i$ is involved in platelet attachment. Platelets that showed a $[Ca^{++}]_i$ response often did so after a variable lag period typically lasting up to five minutes. Thus, the response of platelet $[Ca^{++}]_i$ to stimulation by surface contact is unlike that in response to soluble agonists, e.g. ADP or thrombin, which evoke an immediate rise in $[Ca^{++}]_i$. Some platelets remained attached to the surface without showing a $[Ca^{++}]_i$ response. Other platelets demonstrated a rise in $[Ca^{++}]_i$ of 100-200nM or more. Oscillations were found to be a prominent feature of the $[Ca^{++}]_i$ response, acompanying the $[Ca^{++}]_i$ rise. The $[Ca^{++}]_i$ reached an elevated

plateau and in some cases fell slowly towards the resting values over the remainder of the experiment.

The morphology of the platelets was correlated with the $[Ca^{++}]_i$ response. On Biospan™ it was seen that spread cells were associated with $[Ca^{++}]_i$ time courses that showed peak values greater than 350nM, while platelets that did not spread were associated with $[Ca^{++}]_i$ that remained below 250nM throughout the experiment. Likewise, on polystyrene a similar demarcation was found with regard to $[Ca^{++}]_i$ values above and below 210nM. The findings suggest that platelet spreading and $[Ca^{++}]_i$ response are indeed linked.

These studies provide for the direct observation of a key signal transduction event in platelets during the activation triggered by contact with a biomaterial surface. This technique may be useful in studyiung the mechanisms involved in platelet adhesion and activation, as well as in understanding how changes in activating substrate are perceived by the platelet. The $[Ca^{++}]_i$ response triggered during attachment and spreading may be directly related to other aspects of platelet activation, e.g. the release of granules and their stimulatory agents, or the support of procoagulant activity, which have further impacts on the activation of platelets at surfaces.

Table 5.1 Surface Analysis of Biospan™ by ESCA

	atom %			
Sample	**O(1s)**	**N(1s)**	**C(1s)**	**Si(2p)**
edge	21.02	1.82	75.59	1.57
center	20.48	2.22	76.15	1.14
glancing angle(20Å)	20.85	1.46	76.92	0.77

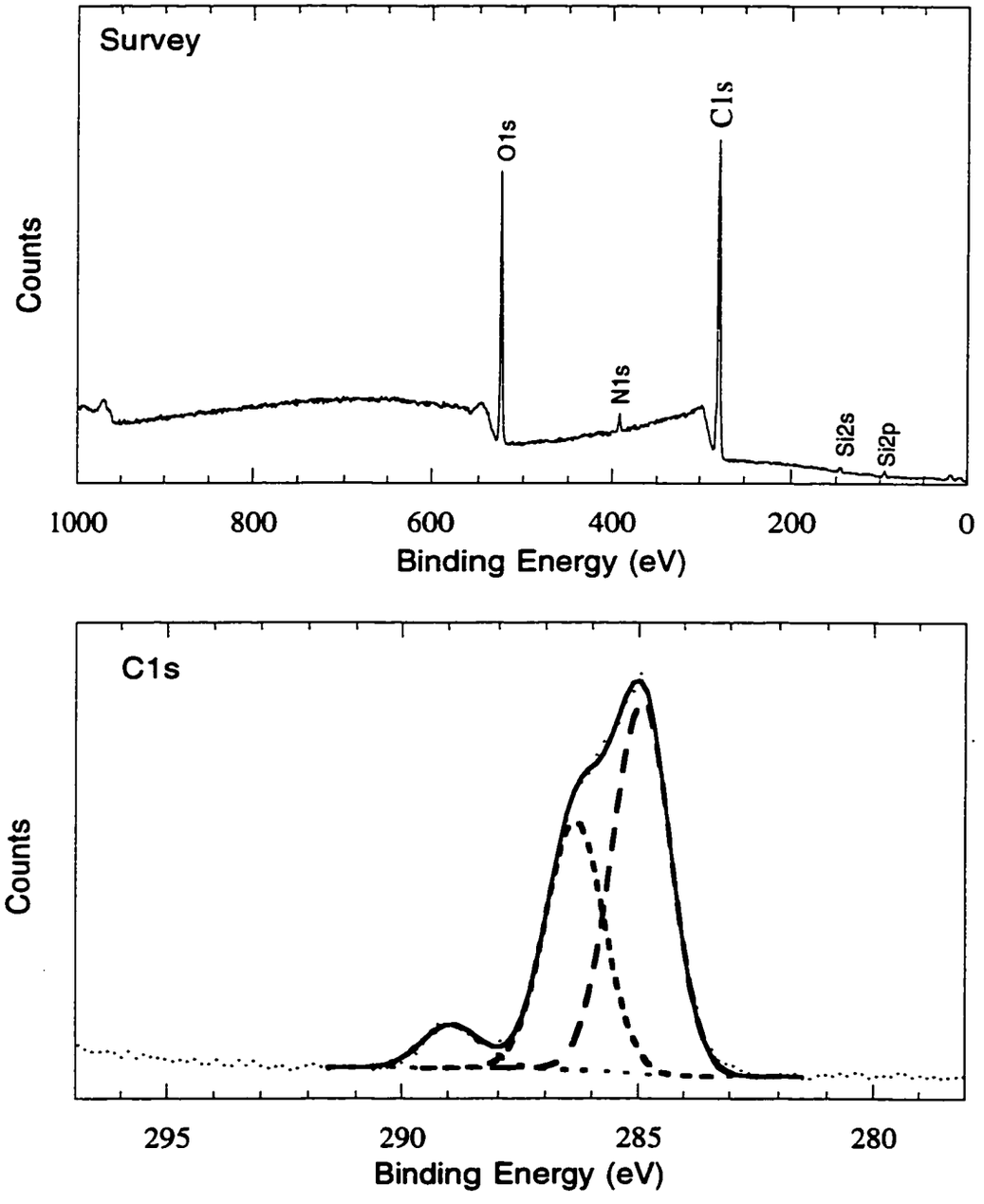

Figure 5.1 Survey and C1s ESCA spectra of Biospan™ coated on EMA-silane coated glass coverslips as used in the experiments.

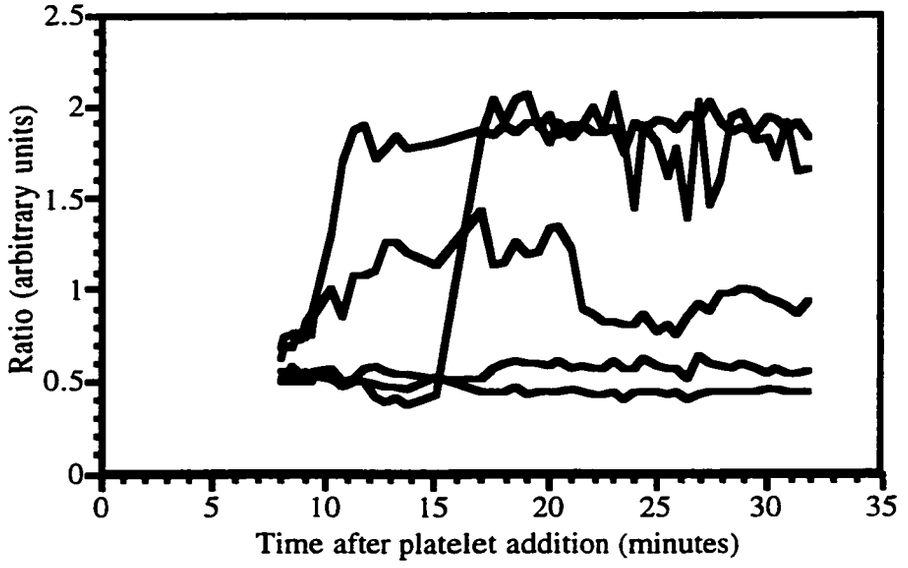

Figure 5.2 The average ratio value of five selected, individual platelets was determined in a series of ratio images as a function of time. Washed platelets were allowed to settle onto a cleaned glass coverslip that had been adsorbed with dilute (0.1%v/v in CPBS) plasma. Data was collected at the Fred Hutchinson Cancer Research Center as described in the text.

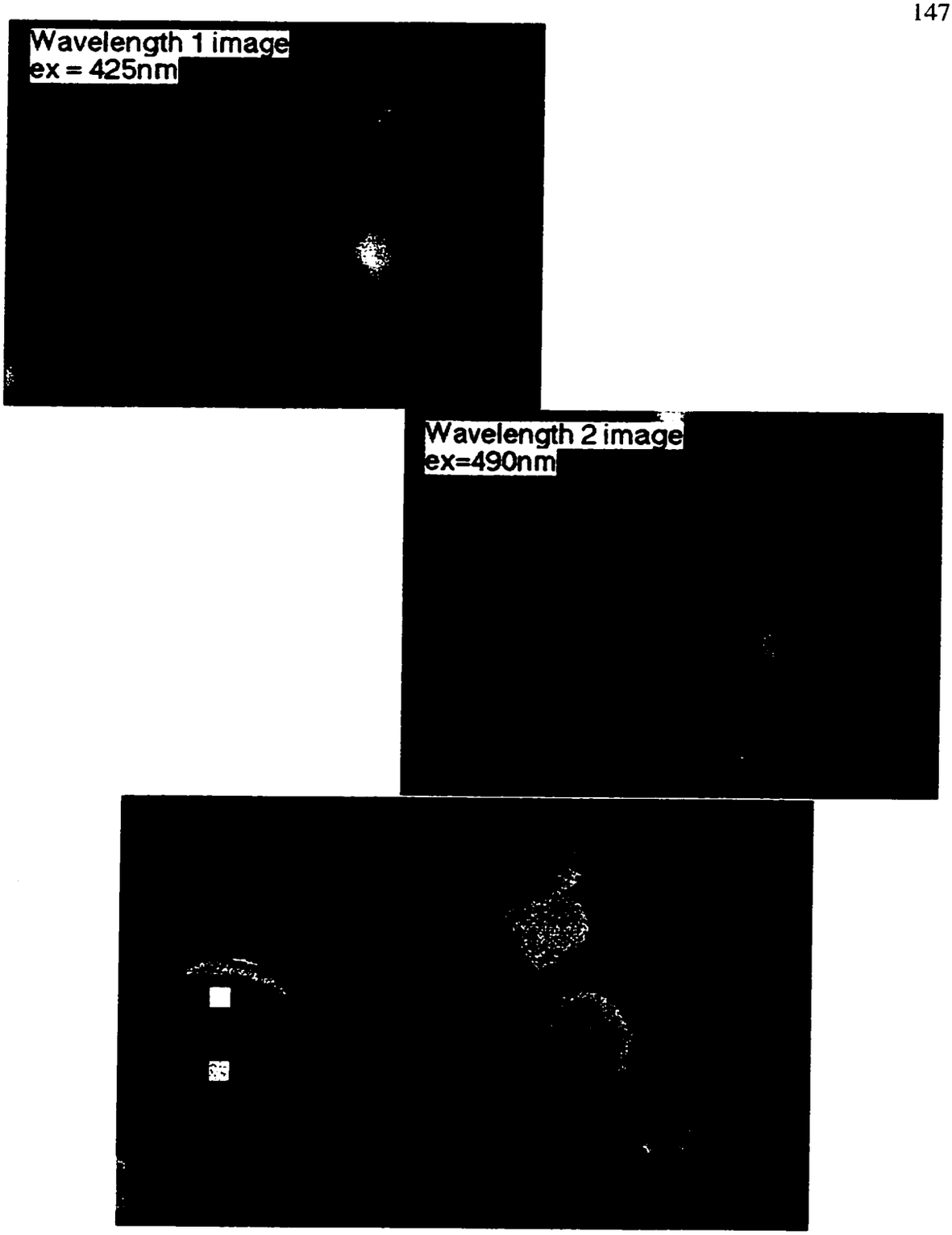

Figure 5.3 An example ratio image

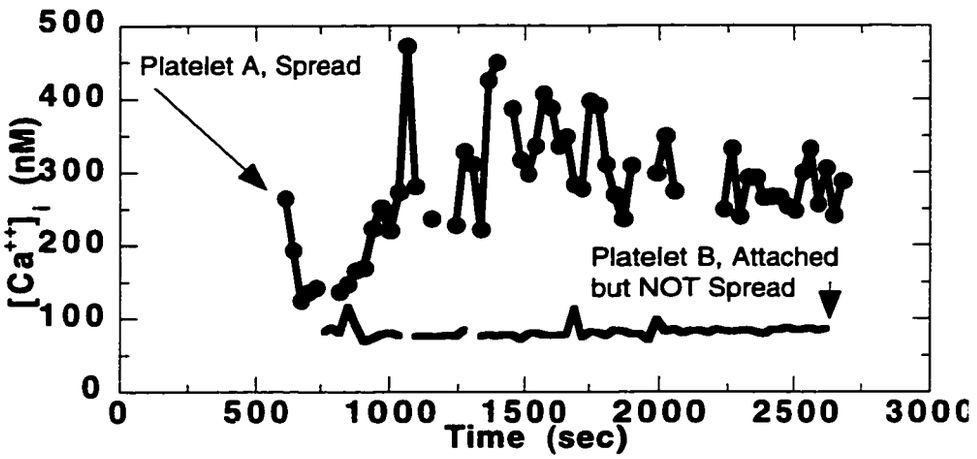

Figure 5.4 Time courses of $[Ca^{++}]_i$ of two selected platelets adherent on fibrinogen coated Biospan™. Washed, fluorophore labeled platelets settled to the Biospan™ surface which had been pre-adsorbed with 0.2mg/ml human fibrinogen. Platelet $[Ca^{++}]_i$ in individual cells was determined by ratio fluorescence microscopy at 30sec intervals. Gaps in the data indicate time points where measurements could not be made due to interference from other cells. As indicated, Platelet A was observed to have spread during the experiment, while platelet B was attached to the surface but did not spread.

Table 5.2, Platelet Adhesion, Morphology and $[Ca^{++}]_i$

Platelet Morphology	Platelet [Ca↔]i
Biospan™ (n = 37) *	
Spread 19/37	15/19 > 350nM (peaks)
No morph. change 18/37	12/18 < 250nM
Polystyrene (n=40) *	
Spread 16/40	12/16> 210nM (peaks)
No morph. change 24/40	16/24 < 210nM
ODCEpolyurethane (n=21)	
No platelets showed morphologic change	5/21 > 200nM

* The correlation between the time courses with elevated $[Ca^{++}]_i$ (transients > 350nM) and time courses where the $[Ca^{++}]_i$ remained low, with the observed platelet morphology were significant at the $p<0.025$ level for both Biospan™ and polystyrene. For Biospan™ $\chi^2 = 6.09$ and for polystyrene $\chi^2 = 5.10$.

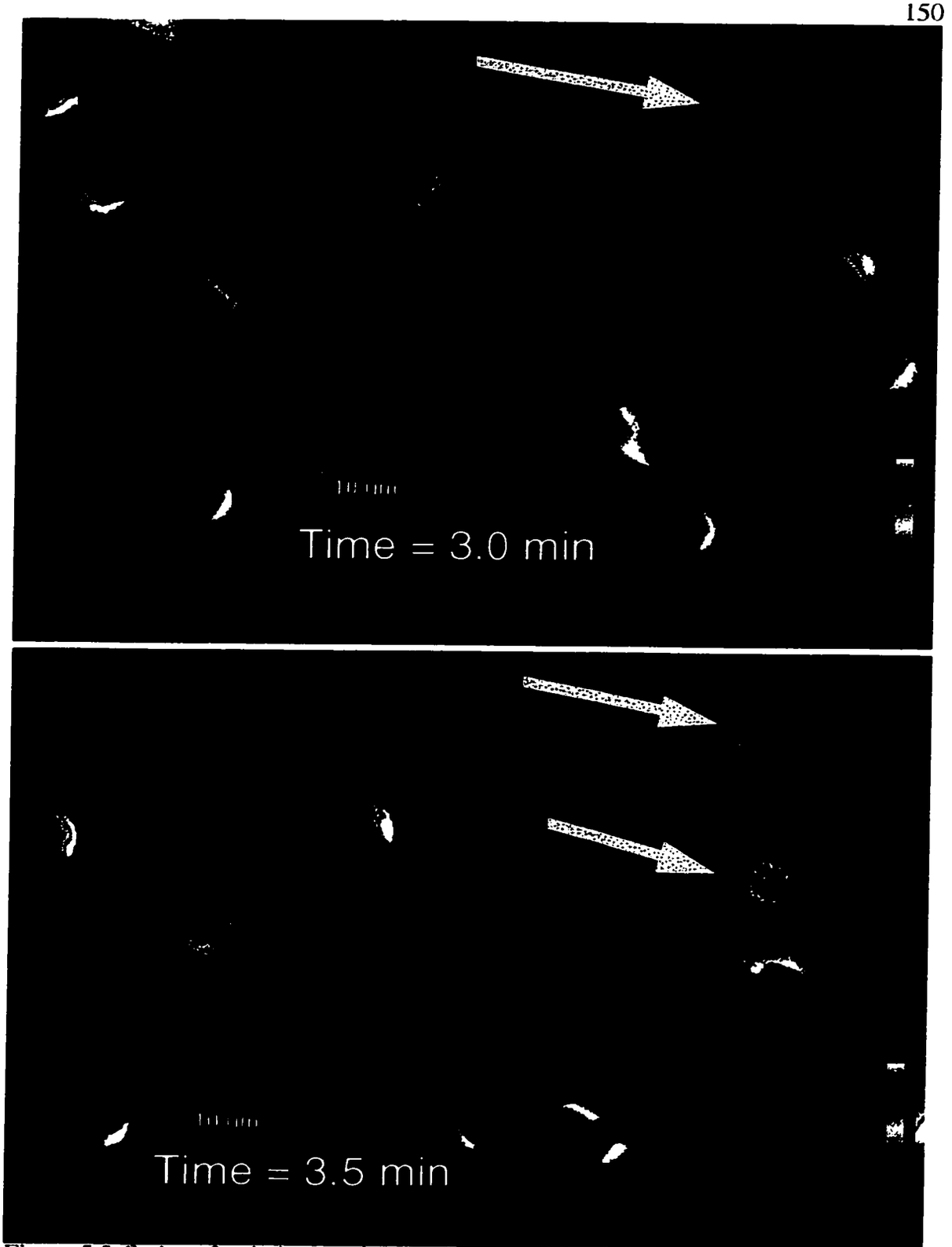

Figure 5.5 Series of ratio images for platelets on Biospan™

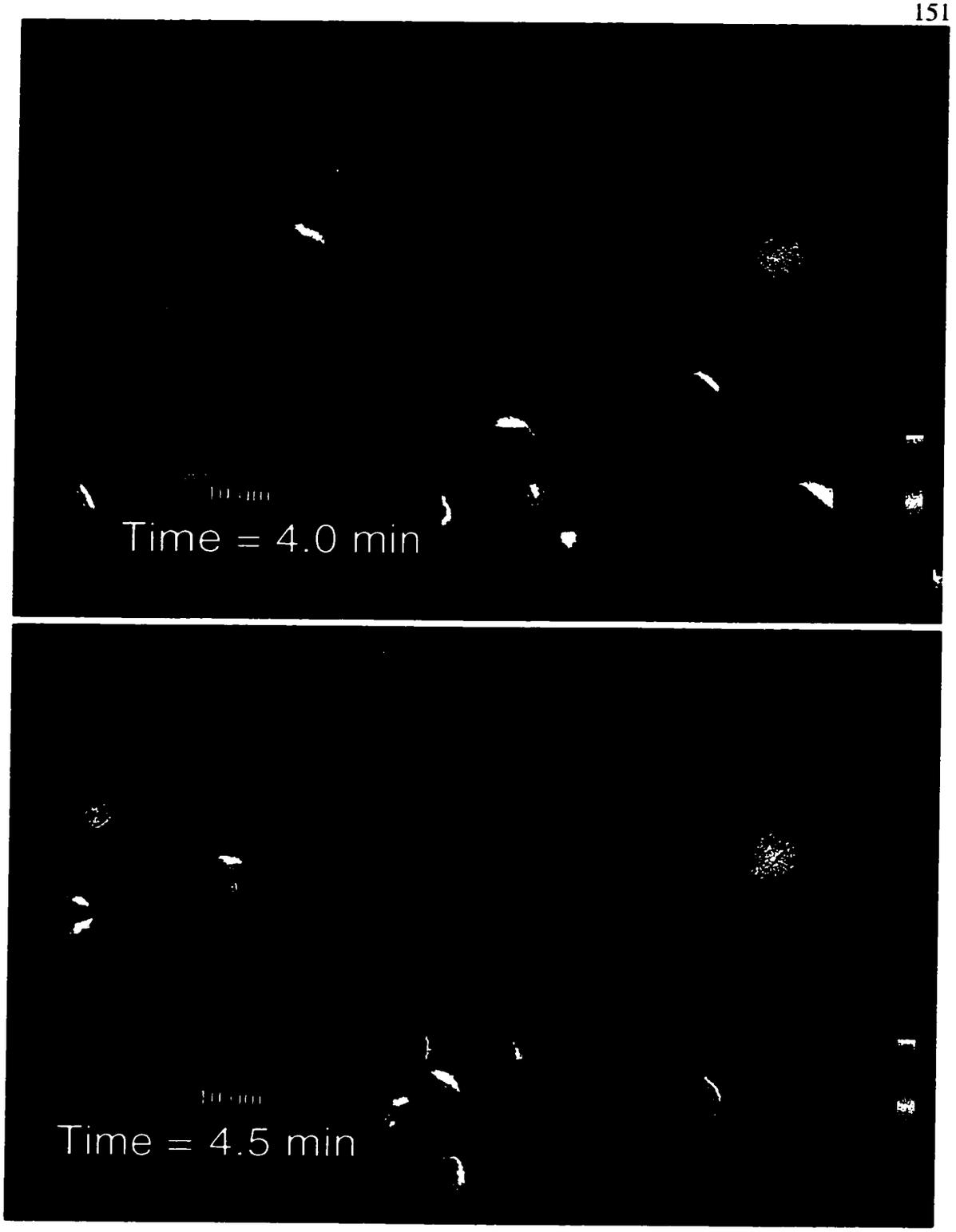

Figure 5.5 (continued)

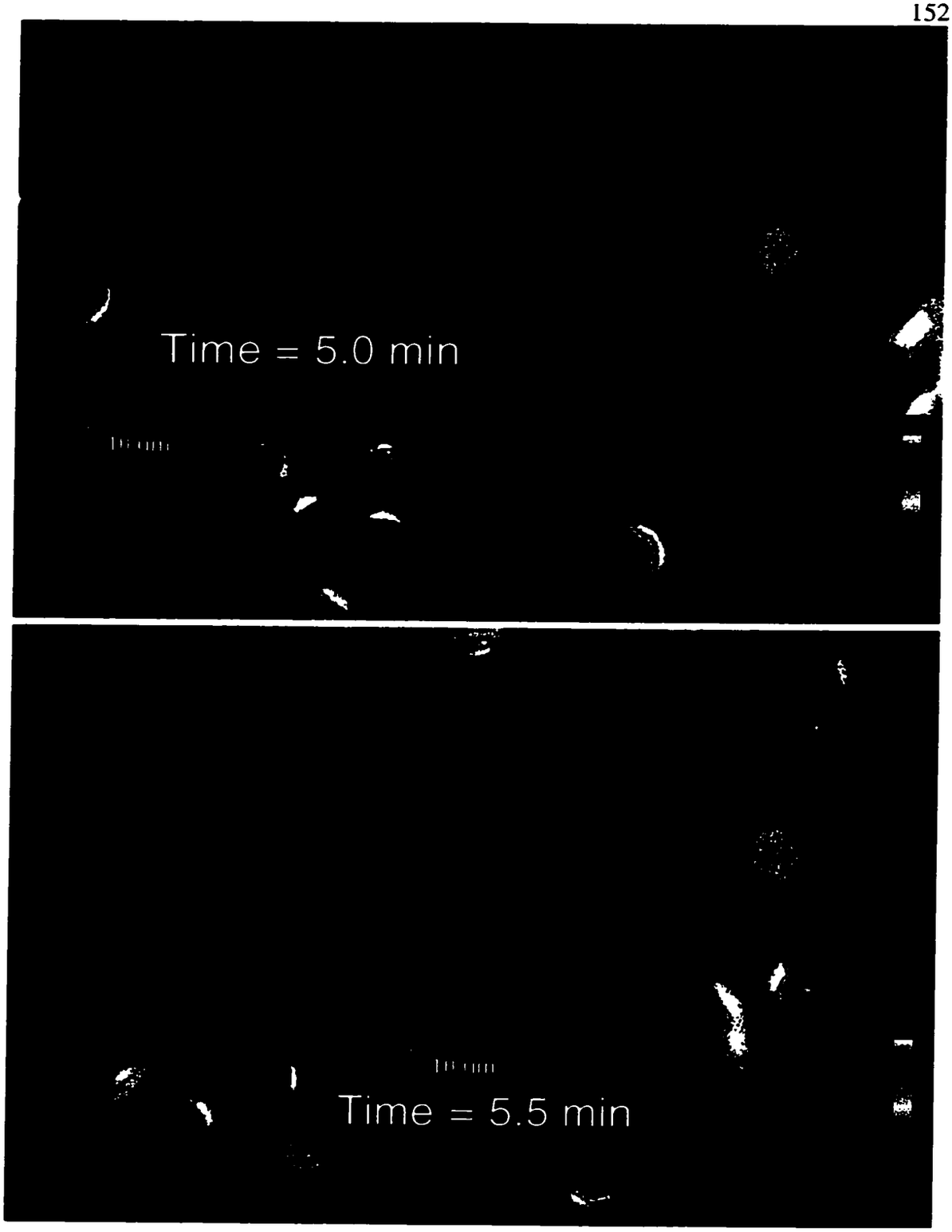

Figure 5.5 (continued)

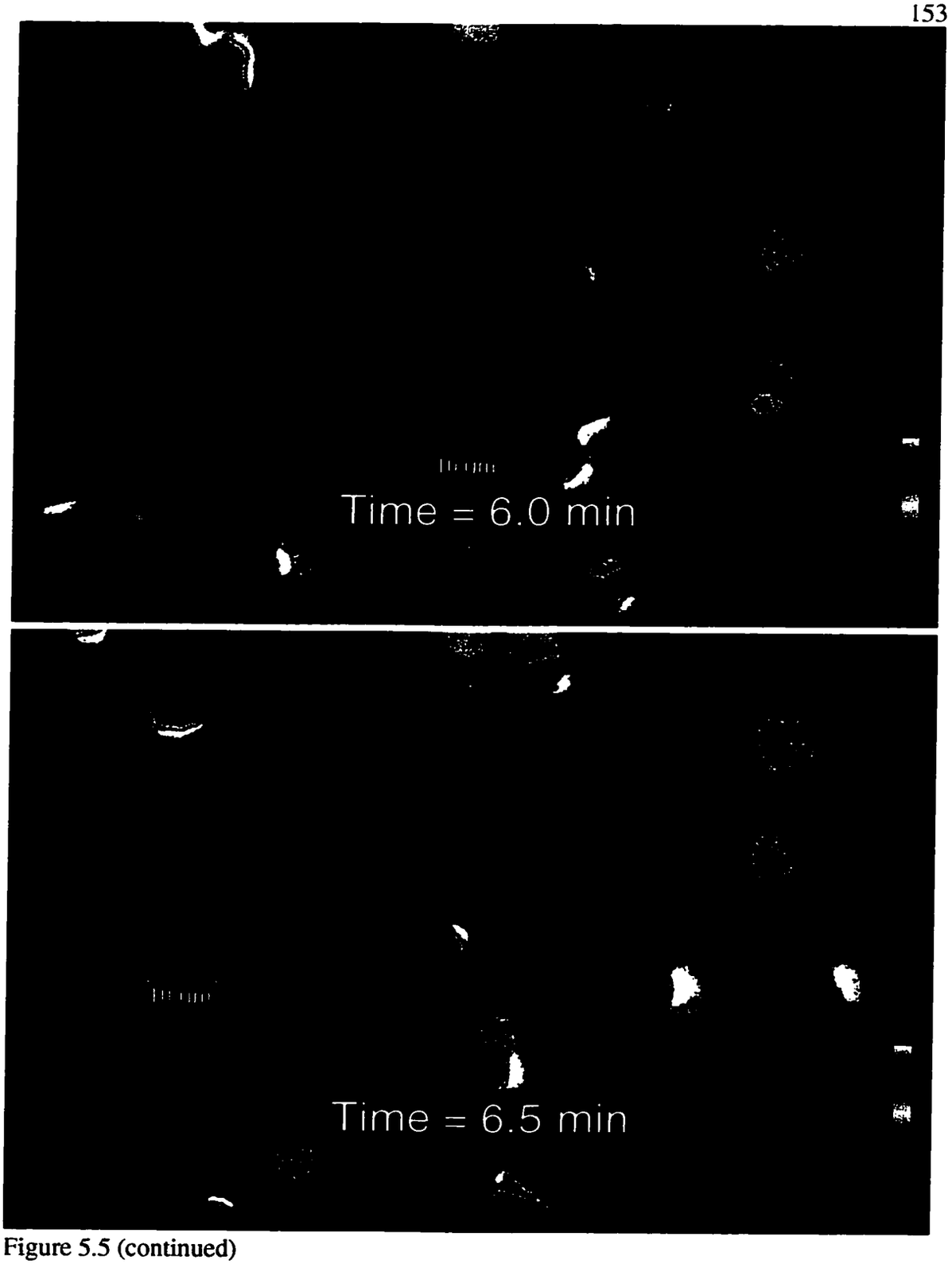

Figure 5.5 (continued)

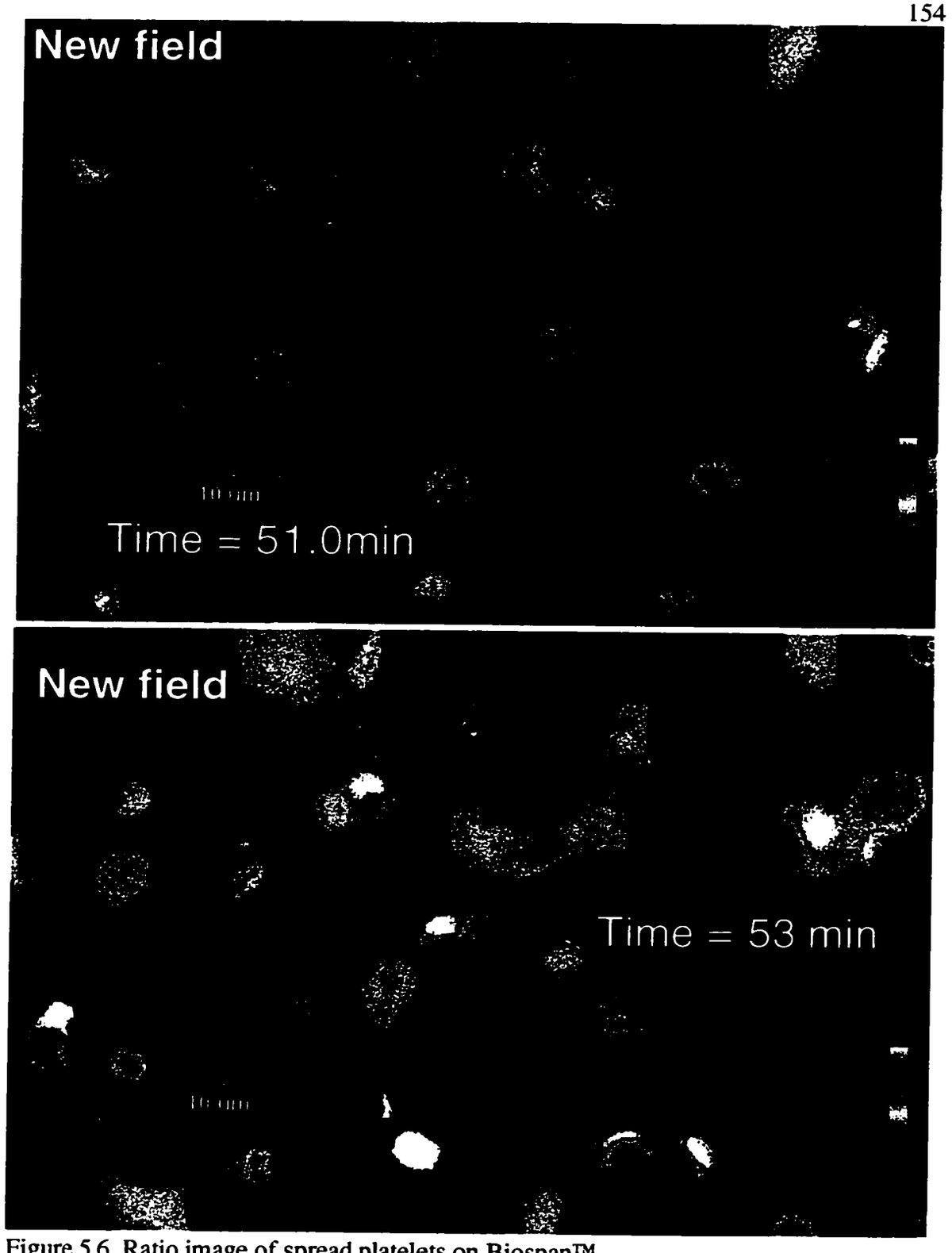

Figure 5.6 Ratio image of spread platelets on Biospan™

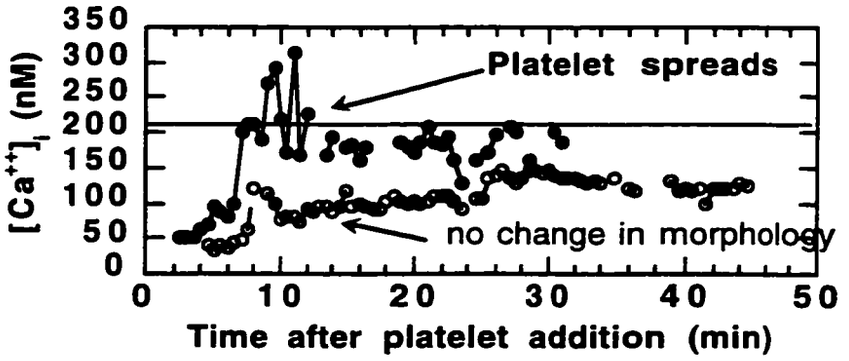

Figure 5.7 Time courses of $[Ca^{++}]_i$ of two selected platelets adherent on fibrinogen coated polystyrene. Washed fluorophore labeled platelets settled to the polystyrene surface which had been pre-adsorbed with 0.2mg/ml human fibrinogen. Platelet $[Ca^{++}]_i$ in individual cells was determined by ratio fluorescence microscopy at 30sec intervals. Gaps in the data indicate time points where measurements could not be made due to interference from other cells. As indicated, one of the platelets was observed to have spread during the experiment, while the other was attached to the surface but did not spread.

Notes to Chapter 5

1. Haycox C, Ratner B: In Vitro Evaluations of Platelet Activation in Whole Blood Exposed to Polyethylene Tubing Coated with a Range of Biomaterials using a Modifed Chandler Loop Assay., *J Biomed Mater Res,* 27:1181-1193, 1993.

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Chapter 6: Measurement of Platelet $[Ca^{++}]_i$ During Attachment and Adhesion to Surfaces From Flowing Mixed Cell Suspensions

6.1 Introduction to Chapter 6

The process of hemostasis, has evolved as a effective, and exquisitely sensitive system of human physiology. The platelet plays the primary cellular role in responding to sites of vascular injury, where circulatory flow is disturbed. Thus platelets spend nearly the entirety of their existence under flowing conditions. The parameters of that flow, e.g., the shear stresses imparted on the cell, changes in flow pattern, regions of stasis, and forces acting upon adherent platelets, are all critical to the response of the platelet. Thus, flow plays an important part in determining the outcome of the platelet response [1].

Recognition of the importance of flow has led to a large number of studies of the activation and adhesion of platelets under flowing conditions. This work has greatly added to our understanding of the processes involved in platelet activation and the interaction of platelets with the components of the vessel wall in normal hemostasis and pathologic thrombosis [2, 3]. These studies and a host of others like them, have provided the observations key to our understanding of the interactions between platelets, proteins and the vessel wall, including: the characterization of important platelet disorders, e.g. Bernard-Soulier Syndrome; the roles of key platelet glycoproteins, e.g. GPIb in attachment and adhesion; the roles of plasma proteins, e.g. von Willebrand factor, fibronectin, fibrin(ogen) as well as subendothelial collagen; the influence of red cells on platelet distribution across the flowing blood stream; and the influence of flow patterns, stasis and shear on platelet-platelet cohesion and thrombus growth.

As part of artificial replacements for blood vessels, heart valves and other organs, biomaterials are frequently used in contact with flowing blood. From its earliest days, therefore, the study of the blood compatibility of materials has often concentrated on flowing systems. Measurement of the patency of implanted vascular grafts or downstream infarction, as in the case of Gott's vena cava ring test, provides direct assessment of the blood compatibility of a material in a natural, albeit very complicated, flowing, whole blood system [4]. The simplistic Chandler loop test recirculates whole blood through tubing in an *in vitro* setting [5]. *Ex vivo* arterio-venous shunts also have proven popular in animal models to measure the platelet adhesion and consumption related to various biomaterials [6, 7].

Perfusion chambers, or flow cells, have also played an important role in the study of platelet biomaterial interactions. Such chambers provide several advantages. Virtually any polymeric material or material surface modification that can be provided as a thin film can be used. The roles of particular adhesive proteins can be determined using washed cell suspensions and specified adsorbed protein layers. Finally, flow geometry and shear rate can be varied easily and carefully controlled [8].

More recently microscopy has been combined with the use of the flow cell to provide a more visual perspective on the dynamic process of platelet contact, attachment and adhesion to biomaterial surfaces. Epifluorescent video microscopy has been used to examine platelet-biomaterial interactions [9, 10], including the effects of surface modifications [11, 12], the transient nature of platelet contacts [13, 14], and the dynamics and morphology of platelet adhesion and thrombus formation [15]. The technique has also been used in detailed studies demonstrating the role of specific adsorbed adhesive proteins and specific platelet receptors in the attachment process under various shear regimes [16, 17].

In a variety of cells including platelets, intracellular calcium ions play an important role as a second messenger in the signal transduction system. The $[Ca^{++}]_i$ presumably links activation signals triggered by receptor-ligand binding events and by other agonists, with the physiologic responses of the cell [18]. The use of fluorescent indicators to measure and monitor intracellular calcium ion concentration, $[Ca^{++}]_i$, has proved to be a useful method to assess platelet activation. A few investigators have applied this technique to study the signal transduction process during platelet activation under well-defined shear conditions. Ikeda and coworkers [19, 20], have modified a cone-and-plate viscometer for the study of shear-induced platelet aggregation and simultaneously, the study of calcium ion mobilization and/or influx. Their findings suggest that, during shear-induced platelet aggregation, the interaction of multimeric von Willebrand Factor (vWF) with the platelet's GPIb receptor is associated with a dramatic influx of Ca^{++} across the membrane of the cell. Subsequently, modulation of the integrin $\alpha_{IIb}ß_3$ to its competent form, provides for interplatelet bridging mediated by vWF multimers (aggregation). The multimeric nature of the vWF - GPIb interaction as well as the presence of extracellular Ca^{++} is essential to the aggregatory response. In very similar work, Chow et al. [21], find essentially the same results. Furthermore, their work shows that, while released ADP is essential for the shear-induced aggregatory response, the Ca^{++} influx is independent of exogenous ADP.

While the cone-and-plate viscometer is useful for studying this process of vWF-mediated, bulk phase, shear induced aggregation, the authors can only speculate as to how these mechanisms might be comparable in the case of platelet adhesion and spreading upon a vWF-coated substrate. Furthermore the temporal correlation of the platelet functional response (aggregation) and the signaling response ($[Ca^{++}]_i$) can only be made for an averaged population.

This chapter presents preliminary studies showing the feasibility of using ratio fluorescence microscopy to measure platelet $[Ca^{++}]_i$ in cells as they attach to surfaces from red cell suspensions flowing through a parallel plate flow chamber. This approach provides several advantages. First, the use of a well-characterized flow cell provides for laminar flow, and control of shear rate. The cell utilizes standard glass coverslips as the substratum, allowing for the investigation of platelet contact with a variety of coated thin films (of novel polymers, specified proteins or even monolayers of cells). Anticoagulated whole blood (representative of *in vivo* conditions) or washed cell suspensions (useful for the isolated study of particular adhesive proteins) can be used in either single-pass or recirculating configurations. Finally, this technique provides for the direct measurement of $[Ca^{++}]_i$ in individual cells concurrent with their attachment and spreading upon the substrate. Furthermore, only those activated platelets in contact with the surface are analyzed, which represents a significant advantage over other techniques, where it is more difficult to separate platelet activation events in the bulk phase from those events on the surface.

While a few reports have appeared describing the use of ratio fluorescence microscopy to observe $[Ca^{++}]_i$ in individual platelets during adhesion under static conditions, there has been but one report describing these measurements under flow. Work by Jen et al. [22] ,describes the changes in platelet $[Ca^{++}]_i$ found during adhesion to fibrinogen-coated glass coverslips from a flowing suspension of washed red cells and platelets. Many of our preliminary results are similar to their observations including: a variable lag time between the time of adhesion and the $[Ca^{++}]_i$ response, significant heterogeneity amongst the individual platelet responses, and general correlation between the strength of the $[Ca^{++}]_i$ response and morphologic change.

6.2 Experimental

The approach used in these preliminary experiments was to prepare washed platelets by differential centrifugation and label them with the Ca^{++}-sensitive fluorophore. The red cells were also washed, and the platelets and red cells were then combined into a mixed cell suspension. The cells were combined and resuspended in a modified HEPES-Tyrodes buffer with albumin and apyrase to a volume roughly equal to that of the original blood volume, so the concentration and proportion of red cells and platelets were roughly equal to that found in the original whole blood sample. This cell suspension was pulled through the flow cell and across the sample using a syringe pump. Fluorescence image pairs were acquired at six second intervals as the cells attached to the surface. The same cell suspension was used for up to three repeated passes through the cell.

6.2.1 Materials and Buffers

Fura Red™-AM, and Pluronic® were purchased from Molecular Probes (Eugene, OR). Sepharose 2B, lyophilized human fibrinogen, (F-3879) and apyrase (A-9149) were purchased from Sigma Chemical Co. (St. Louis, MO). Fibrinogen (with salts) was reconstituted with water to 20mg/ml and stored in 0.1ml aliquots at -80°C. Apyrase was diluted to 3mg/ml in sterile saline and stored in 2ml aliquots at -80°C. Buffer salts and solvents were of reagent grade. Bovine serum albumin was purchased from ICN (cat.# 103700, Cleveland, OH). The platelet suspension buffer with albumin (PSB-albumin) contains (in mM): NaCl, 145.5; KCl, 2.7; $MgCl_2$, 1; $CaCl_2$, 2; NaH_2PO_4, 0.4; HEPES, 4.7; dextrose, 5.55; along with: bovine serum albumin, 4mg/ml and apyrase, 0.03mg/ml (0.09U/ml). Citrated phosphate buffered saline (CPBS) contains (in mM): NaCl, 120; Citrate, 10; monobasic phosphate, 10, and the pH was adjusted to

7.4 with NaOH. Ringer's-citrate-dextrose (RCD) buffer for cell washing was prepared by adding citrate and dextrose to prepackaged sterile Ringer's buffer (Cat.#2132303, Baxter, Deerfield, IL), yielding a buffer containing (in mM): NaCl, 147.2; $CaCl_2$, 3; KCl,4, with 6.23g/l sodium citrate and 5g/l dextrose. The pH was adjusted to 6.5 and apyrase was added from stock to yield 0.03mg/ml (0.09U/ml). Buffers were sterile filtered before use.

6.2.2 Preparation of Platelets and Mixed Cell Suspension

Blood donors were recruited and selected in accordance with protocols approved by the University of Washington Human Subjects Institutional Review Board. Donors were healthy and denied taking any medications, including aspirin, during the preceding two weeks. Phlebotomy was performed by staff of the Outpatient Blood Draw facility, (Department of Laboratory Medicine, University of Washington Medical Center), and the blood was transported at room temperature to our laboratories. Whole blood was drawn slowly from the antecubital or suitable peripheral vein via a 19G Butterfly infusion set directly into a 60ml syringe containing ACD anticoagulant (1:9 v/v, NIH Formula A: citric acid, dihydrate, 7.3g/l; sodium citrate, 22g/l; dextrose monohydrate 24.5g/l). The hematocrit was measured using a capillary tube microcentrifuge and was found to be between 40-45 for all donors.

The blood was transferred to 15ml polystyrene or 30ml polyethylene conical centrifuge tubes and centrifuged at 180g for 20 minutes at room temperature. The platelet rich plasma (PRP) layer was carefully harvested. The PRP was centrifuged at 1500g for 15 minutes. The platelet poor plasma was drawn off and the platelet pellet gently resuspended in 10ml of PSB with albumin and apyrase. To label with fluorophore, 100µg of Fura Red™-AM was dissolved in 100µl of dry dimethyl sulfoxide (DMSO) with 10µl of Pluronic surfactant (25%w/v in DMSO). This

fluorophore mixture was added to the washed platelet suspension and the platelets were incubated at 37°C for an hour. After labelling, the unincorporated label was removed by again centrifuging the cells at 1500g for 15 minutes. The pellet was resuspended to 10ml in PSB with albumin and apyrase, and calcium was brought back to 2mM by addition of 1M $CaCl_2$ stock. The functionality of the washed platelets so prepared was tested and verified by aggregometry. Aliquots of washed platelets supplemented with fibrinogen responded normally to ADP stimulation (20μM) in the aggregometer.

After the harvesting of the PRP, the buffy coat containing white cells was removed from atop the packed red cells. The red cells were resuspended to 60ml in RCD with apyrase and centrifuged for 15min at 1500g. This was repeated for a total of three washings, with the final resuspension of the red cells to 50ml in PSB with albumin and apyrase. Calcium was returned to 2mM from 1M $CaCl_2$ stock.

The washed platelets were combined with the washed red cells resulting in 60ml of mixed cell suspension, in PSB with albumin and apyrase. The suspension contained 2mM calcium and the concentration and proportion of the platelets and red cells were roughly that of the original whole blood sample less small losses during processing.

6.2.3 Surfaces

Platelet adhesion was studied on two surfaces in these preliminary experiments: a polystyrene surface and an octadecyl chain extended (ODCE-PEU) polyetherurethane surface. Both surfaces were cast on circular glass coverslips (25mm diameter,#1, VWR Scientific) which had been cleaned by ultrasonication twice in Isopanasol (4%w/v in water, C.R. Callen, Seattle, WA) and three times in deionized water. The coverslips were blown dry with dry N_2 and dried in an oven for several hours.

Prior to coating the final polymeric film, the glass surface was coated with an appropriate silane copolymer: styrene-silane copolymer for polystyrene surfaces and

ethylmethacrylate-silane copolymer for the ODCE surfaces. These copolymers were prepared and provided by Prof. Ratner's group. The copolymer was centrifugally cast onto the clean glass coverslips using a photoresist spinner (Model EC101, Headway Research, Garland, TX). These films were cast using 100µl aliquots of dilute copolymer solution (2.5%w/v in ethyl acetate), a rotational speed of 2000-4000 rpm and a spin time of 40sec. (The lower rotational speeds are believed to lead to thicker coatings.) In the case of the styrene-silane, the casting technique was to dispense the 100µl aliquot rapidly while the surface was spinning. Coatings were made on one side only and appeared uniform to the eye. The coated coverslips were placed in a glassware oven overnight (60°C) and then extracted twice with ethylacetate and once with methanol (45-60min. each). The samples were again dried in an oven and stored in polystyrene petri dishes, which were kept inside sealed polyethylene sample bags under dry N_2 for later use.

Polystyrene was provided by Prof. Ratner's group as follows. Polystyrene Secondary Standard (catalog #039C, Lot 04, Scientific Polymer Products, Ontario, NY) was dissolved and precipitated once in methanol and stored for future use. The polymer was dissolved at 2% w/v in reagent grade toluene and filtered (0.5µm Millex-SR, cat.#SLSR025NB, Bedford, MA). The polystyrene film was centrifugally cast onto the styrene-silane coated coverslips using 100µl aliquots, a rotational speed of 4000rpm and a spin time of 30sec. The coatings appeared uniform to the eye. The coverslips were dried in an oven for an hour and stored as above for further use. Samples so prepared were submitted to the National ESCA and Surface Analysis Center for Biomedical Problems. The results of their analysis of survey and high resolution C_{1s} spectra were consistent with a uniform coating of polystyrene showing no evidence of contamination nor any evidence of the underlying copolymer or glass substrate.

Octaldecyl chain extended polyetherurethane was provided by Prof. Ratner's group. The material was provided as rubbery translucent chunks (labelled 6:5:1 MDI:Batyl Alcohol:PTMO-2000, lot 9-15-88 Pete Edelman). The polymer was dissolved in "Chem Stores" grade tetrahydrofuran (THF, 2%w/v) with shaking overnight, and then filtered (0.5 μm Millex-SR, Millipore, Bedford, MA). Because tetrahydrofuran dissolves the glue used to hold the liners to the inside of the caps of many of our glass vials (20ml scintillation vials #986568, Wheaton Glass), special liners were cut from thin sheets polytetrafluoroethylene (7/8" diameter, #15076-P065005, Berghof/America, Concord, CA), rinsed in methylene chloride and then in methanol, and used to line the caps of all vials used to hold THF. The ODCE film was centrifugally cast onto the ethylmethacrylate-silane coated coverslips using 80μl aliquots, a rotational speed of 4000rpm and a spin time of 40sec. The coatings appeared uniform to the eye. The coverslips were dried in an oven for an hour and stored as above for further use.

Fibrinogen was adsorbed to both types of surfaces by incubating the sample in fibrinogen solution (0.2mg/ml in CPBS) for more than an hour at 37°C. The sample was then dip rinsed in PSB-albumin and installed in the flow cell as described below. A small scribe mark was made on the surface to aid in establishing the appropriate focal plane prior to the start of flow.

6.2.4 Flow Cell Description and Operation

The parallel plate flow cell used in this work was designed by Prof. Sakariassen and purchased from Profs. Sakariassen and Sixma. The flow channel cross section is a rectangular slit with a slit height of 0.6cm, slit width of 1cm and the chamber has an entrance length of 10cm. The flow characteristics of the chamber have been studied previously and found to be uniform and laminar under the conditions used [23, 24]. The chamber was originally designed to hold samples for off-line analysis of platelet

adhesion by radioisotope labelling or electron microscopy. The chamber was later modified by Haycox [11], to provide a microscope objective with access to the backside of the coverslip. The top side of the flow cell, including the portion holding the coverslip in place, was redesigned and the replacement machined from polycarbonate. The flow geometries remain unchanged from the original design. This top piece was designed for use on an inverted microscope, with a Zeiss 63X oil immersion objective (plan apo, 1.3NA). We have used it with a Nikon 100X oil immersion objective (UV fluor, 1.3NA). The design is incompatible with the Nikon 40X oil objective. The flow chamber is depicted schematically in Figure 6.1.

The flow cell with its sample was mounted on the stage of the microscope. A small 37°C water bath was placed next to the microscope and a holder fashioned for several 50ml centrifuge tubes to be used as reservoirs. Approximately 40cm of Silastic tubing (medical grade silicone rubber tubing, cat.# 601-335, 0.132ID x 0.183 OD, Dow Corning, Midland, MI) was used from the reservoir to the entrance of the flow cell. At the exit, a length of Silastic tubing (approximately 15cm) connected the flow cell to a 60cc disposable syringe (BD Plastipak, Becton-Dickenson, Rutherford, NJ) mounted in a syringe pump with a fixed speed motor (Model AD, Razel Scientific Instruments, Stamford CT). When operated, the syringe pump withdrew the syringe plunger and pulled fluid from the reservoir, into the flow cell, across the sample and into the syringe. The flow rate was 11ml/min and the corresponding shear rate was calculated to be 300 sec^{-1}. This shear rate, used in previous flow studies in this laboratory, corresponds to the lower shear rates found in the large arteries [25]. While the temperature of the fluid in the reservoir was held at 37°C, the tubing was uninsulated, and there was no temperature control nor insulation surrounding the flow cell or the syringe. While not measured, the temperature of the fluid probably dropped a degree or

two in transit from the reservoir to the sample, and several more degrees during collection in the syringe.

The cell was first flushed by passing 60ml of PSB-albumin through. During this time the microscope was focused on the scribe mark, and then the field of view shifted slightly to exclude the scribe mark. Flow was stopped, the inlet tube was clamped and transferred to the reservoir of mixed cell suspension, and the flow resumed. As the mixed cell suspension entered the flow cell, image acquisition was begun.

6.2.5 Image Acquisition and Data Analysis

The ratio fluorescence microscope system comprises a Nikon Diaphot inverted microscope fitted for epifluorescent illumination (model TMD-EF). Illumination is from a Xenon arc lamp (XBO-100W, Nikon) and filtered through band pass filters (425 and 490 ± 10nm, Omega Optical, Brattleboro, VT) held in a filter wheel and alternated under computer control. A 580nm dichroic mirror and 610nm long pass barrier filter were used. The objective used was a Nikon 100X UV Fluor 1.3NA oil immersion objective.

The ratio fluorescence imaging software METAFLUOR (version 2.5, Universal Imaging Corp., Brandywine, PA) was used for both data collection and analysis. Images were acquired using a Photometrics CH250 cooled CCD imager with a Kodak KAF1400 chip. The chip was used with 3x3 binning, a 250ms exposure time for each individual image with image pairs taken at 6sec intervals. Acquisition of each image pair was complete within one second. To subtract for background, a fixed value of 230 gray scale units was subtracted from the 425nm wavelength image (image acquired using 425nm excitation) and 210 units from the 490nm wavelength image. These values were based on previous images acquired under the experimental conditions but with no fluorescently labelled cells present. Typically, it took anywhere from 5-20 frames to reestablish the focus due to the flexing of the thin coverslip under flow. The

12-bit, individual wavelength images were stored directly to computer hard drive and moved to magneto-optical drives for storage.

Ratio images were formed from the individual wavelength images after background subtraction by dividing the intensity of each pixel in the 425nm image by the intensity of the corresponding pixel in the 490nm image. A threshold mask was applied to exclude any pixels in the ratio image for which the intensity in either of the individual images was below a small and arbitrarily set threshold, namely, 75 greyscale units above background. This value was chosen to exclude artifacts found usually at the fringes of cells where the intensity drops near background, causing this noise to be unduly magnified in the ratio. The ratio images were scaled for 8-bit display as described in Appendix B, and viewed as an image series.

Upon review, individual platelets were identified and each assigned to an analysis region. The average ratio value of the pixels comprising each platelet was measured in each of the ratio images of the sequence. The ratio values were converted to values of $[Ca^{++}]_i$ using the calibration equation and parameters determined previously (see Chapter 4).

6.3 Results

Several general observations can be garnered from the image series. First, because of problems with loss of focus during the early frames, a few platelets were already attached when the measurements began. Despite this problem, however, the response of many new arrivals could be captured in their entirety. Second, some small morphologic changes can be seen in the platelets during the course of the experiment. Third, small but distinct changes in $[Ca^{++}]_i$ can be seen in the platelets during the experiment.

6.3.1 Polystyrene with Fibrinogen

Figure 6.2 presents the time course of $[Ca^{++}]_i$ of several platelets as they attached to a polystyrene surface coated with the adhesive protein fibrinogen. The time index is referenced to the time that the mixed cell suspension first entered the flow chamber. Individual curves begin where the platelet was first found to be stationary in consecutive frames. Breaks in the curve represent frames where the $[Ca^{++}]_i$ could not be determined, most often due to a loss of focus in these experiments.

In general, the initial value of $[Ca^{++}]_i$ is quite low, around 50nM. Most cells show a slow gentle rise in $[Ca^{++}]_i$ during the experiment but most remain at less than 150nM during the 5.5 minute run of the experiment. One platelet shows a more striking $[Ca^{++}]_i$ response as, after a gradual rise in $[Ca^{++}]_i$ over 3min, the $[Ca^{++}]_i$ response becomes more erratic, showing sign of fluctuation and a sharper rise to a value of nearly 200nM near the end of the observations.

6.3.2 ODCE-PEU with Fibrinogen

Figure 3 presents the time course of $[Ca^{++}]_i$ of several platelets as they attached to an ODCE-PEU surface. In this experiment as well, the early, if not initial, value of $[Ca^{++}]_i$ in the platelets is seen to be quite low, generally below 50nM. There is a slow gradual rise in $[Ca^{++}]_i$ during the course of the experiment, but the platelet $[Ca^{++}]_i$ remains at less than 150nM during the 5.5 minute run of the experiment.

6.4 Discussion

As discussed above, the study of platelet attachment, activation and spreading upon surfaces from flowing suspensions plays an important role in dissecting the mechanisms of these processes both *in vitro* and *in vivo*. It is generally accepted that a rise in platelet $[Ca^{++}]_i$ is an important step in the process of signal transduction associated with platelet

activation. However most of the details regarding the platelet $[Ca^{++}]_i$ response and its relationship to attachment and spreading are still unknown.

These preliminary experiments show that it is possible to image and measure platelet $[Ca^{++}]_i$ in platelets attaching to a biomaterial surface from a flowing mixed cell suspension. In theory, the measurements should also be possible using labelled platelets in flowing, anticoagulated whole blood.

The initial or resting value of platelet $[Ca^{++}]_i$ seen in these experiments is low, between 0-50nM. This agrees with previous studies under static conditions as well as the work of others in the literature where the resting value of platelet $[Ca^{++}]_i$ is taken as being something less than 100nM [26].

6.4.1 Lag Time

Previous experiments of platelets settling and attaching to biomaterial surfaces from static suspensions, showed that platelets often exhibit a lag time of 3-5 min after attachment before demonstrating a marked rise in $[Ca^{++}]_i$. The experiments here seem to confirm that there is no immediate rise in platelet $[Ca^{++}]_i$ associated with attachment to the biomaterial surface, at least that can be discerned at the time resolution used in these experiments (six seconds). The meaning of the lag time is unclear. A lag period between stimulus and the aggregatory response is well established for stirred suspensions of platelets responding to the stimulus of collagen fibers in the low shear of aggregometry. As well, Poole and Watson have reported a lag in the $[Ca^{++}]_i$ response during attachment of platelets to collagen coated surfaces from static suspensions [26]. In contrast, the platelet $[Ca^{++}]_i$ responses to agonists such as thrombin or ADP, in suspension or in single attached cells, are nearly immediate. The lag suggests that perhaps different signal transduction pathways are involved during the $[Ca^{++}]_i$

response to attachment. However, the details of the signal transduction pathways in platelets are still very unclear, and the subject of much speculation and study.

6.4.2 Heterogeneity

As seen in the time courses, there is considerable variability in the platelet $[Ca^{++}]_i$ response among the population that was observed. While all the cells seem to show a gradual rise in $[Ca^{++}]_i$ during the experiment, the initial values differ considerably. After a few minutes of attachment, the $[Ca^{++}]_i$ response is somewhat more erratic, although still generally rising. Only a few cells show signs of a marked $[Ca^{++}]_i$ rise of the kind seen during longer observations of cells during static experiments, and this unfortunately comes near the end of the observations.

6.4.3 Pseudopodia Formation Without Ca^{++} Rise

It was previously observed that a platelet $[Ca^{++}]_i$ rise to values of 200-350nM was associated with cells that spread on fibrinogen coated biomaterials over 45 minutes in static adhesion experiments. In these experiments using the same surfaces, platelets were observed that attached to the surfaces from flowing suspensions and the observations made over the initial six minute period. The images were made at a higher magnification than the earlier work, using a 100X objective instead of a 40X objective. In these experiments the initial changes in shape, what appear to be the extension of pseudopodia, are seen in several of the cells. There does not appear to be a sharp rise in platelet $[Ca^{++}]_i$ immediately associated with these morphologic changes. This is an important observation because it implies that the platelets are capable of attaching to a fibrinogen coated surface, presumably through an integrin mediated interaction, and then subsequently transducing a signal resulting in the initiation of cytoskeletal reorganization and the formation of actin fibers in extending filipodia; all independent of

or at least prior to the wholesale influx of Ca^{++} or release of Ca^{++} from stores. This is not to diminish the importance of platelet $[Ca^{++}]_i$ in response to activation. It may simply be that the $[Ca^{++}]_i$ rise is associated with later steps in activation, e.g. more extensive cytoplasmic spreading, or granule release or the expression of procoagulant activity by a change in membrane phospholipid asymmetry.

6.5 Comparisons with the Published Work of Others

Recent work by Jen and coworkers,[22], describe the platelet $[Ca^{++}]_i$ response during platelet adhesion to a fibrinogen coated glass coverslip in a parallel plate flow chamber. While their study is more detailed and involved, several of their observations are mirrored in the work presented here.

Before making comparisons however, there are several experimental differences to note. Jen et al. report conducting the experiments at room temperature, while in this work the mixed cell suspension was kept at 37°C and then flowed directly through a room temperature flow cell. Jen et al. utilized a silicon intensified target (SIT), video rate camera for imaging. The cooled CCD detector used in the present work is more sensitive to light, has a greater dynamic range (i.e., range of intensities between the lower detection limit and the saturating level of light), and has better spatial resolution. Jen et al. performed morphology from scanning electron micrographs taken of fixed samples prepared at the end of the experiment. The samples in the present study were not analyzed by SEM. The shape of the attached platelets is generally evident in the ratio fluorescence images, and the extension of some small pseudopodia can be seen. However, because the ratio images are thresholded, it is possible that some finer details of the platelet structure, especially at the periphery, may be lost. Finally, Jen et al. report that due to the weakness of the fluorescence signal from their Fura-2 loaded

platelets, and subsequent quenching, their attempts at calibrating the ratio fluorescence failed. For this reason they report the platelet $[Ca^{++}]_i$ response as a change in ratio. Fura Red™ has been calibrated as a Ca++-sensitive dye used in platelets (this dissertation) and the ratio measured in these studies has been converted to a $[Ca^{++}]_i$ value using that calibration.

Notwithstanding these differences, the studies reported in this chapter agree with the published work of Jen et al. in several respects. First, there appears to be no immediate rise in platelet $[Ca^{++}]_i$ upon adhesion to a fibrinogen coated surface. Jen et al. observe a relatively stable baseline value immediately after attachment to the surface. The data presented here indicates a gradual and small rise in platelet $[Ca^{++}]_i$, but that is consistent with the observations of Jen et al. given the limited sensitivity used in their study. Second, Jen et al. report a lag period of 10-200 seconds before they observe a sharp rise in platelet $[Ca^{++}]_i$. In studies of platelets adhering to fibrinogen coated surfaces from static suspension, described earlier (Chapter 5), a lag was also seen before a sharp rise in platelet $[Ca^{++}]_i$, usually 3-5 minutes, a little longer than the lag observed by Jen et al. In the flow studies reported here, most of the cells remain generally quiescent, demonstrating only a small gradual rise in $[Ca^{++}]_i$ throughout the 4-6 minutes of observation; which is consistent with a lag before the sharp response in $[Ca^{++}]_i$ normally associated with platelet activation. Both studies show significant heterogeneity in the response among the population of adherent cells. Jen et.al. report that at the conclusion of the experiment, those platelets that were the most spread were generally correlated with the larger rises in platelet $[Ca^{++}]_i$ during the observation. Previous static experiments in this laboratory confirm this, but the short period of observation used in these flow studies meant that there were no fully spread platelets to attempt such a correlation here in these flow studies.

6.6 Conclusion

This chapter has presented the results of experiments demonstrating the feasibility of measuring platelet $[Ca^{++}]_i$ in cells during their attachment and adhesion to protein covered surfaces from a flowing mixed cell suspension. In these experiments cells can be seen to attach and adhere to the surfaces. There is a small gradual rise in platelet $[Ca^{++}]_i$ in most of the cells, although the $[Ca^{++}]_i$ stays below 150nM in most of the cells during the 5.5 minute observation period. The initiation of morphologic change can be seen some of the cells, as there appear to be the beginnings of psuedopod formation.

The experiments were done on two different surfaces, a polystyrene surface coated with the adhesive protein fibrinogen, and an ODCE-PEU surface coated with fibrinogen. While there were no striking differences between the platelet $[Ca^{++}]_i$ response on the two surfaces in these very preliminary experiments, it is premature to arrive at any conclusions regarding material differences (or lack thereof) in these types of studies.

Several of the observations made in this study are in agreement with previous published work. While a gradual rise in platelet $[Ca^{++}]_i$ was seen, there appeared to be no dramatic $[Ca^{++}]_i$ rise immediately associated with attachment of the platelet to the surface. There appears to be significant heterogeneity in the platelet $[Ca^{++}]_i$ values and responses among the adherent platelet population.

6.7 Future directions

As stated, the studies presented here indicate the need for several technical refinements which should enhance the usefulness of future experiments. First, a redesigned flow cell (or flow cell top) could be made to accommodate the lower power

Nikon 40X objective, thereby providing a larger field of view and presumably larger numbers of cells to follow during the experiment. Temperature control in these experiments would be improved by the use of an environmental enclosure. A Plexiglas enclosure system is available which surrounds the microscope stage and provides temperature control by circulating heated air. The length of the experimental runs should be extended as long as possible by increasing the size of the blood draw, and preparing a much larger volume of mixed cell suspension, or in the alternative, recirculating the mixed cell suspension. The latter suggestion is less attractive since proper controls would be needed to show that the reactivity of the platelets, as evidenced by the $[Ca^{++}]_i$ response, was not affected (enhanced) by the recirculation.

6.7.1 Time Resolution

The six second interval used in these studies is a limitation that derives from several aspects of our imaging system. Many different factors combine to determine the effective temporal resolution of the system. The intensity of the fluorescence signal from the cells is critical, and is affected by dye loading, cell thickness, illumination time and, of course, $[Ca^{++}]_i$. The binning used on the chip is a factor; more binning means more sensitivity to light, less spatial resolution, fewer pixels, and faster analog to digital conversion times. The analog to digital conversion speed (which may be improved in newer generation cameras and camera electronic units) directly affects the speed of data acquisition. Finally, the speed of the host computer and its storage devices is also a factor; e.g. temporary storage of the images in volatile memory may be required for increased speed. So, increased temporal resolution involves several of tradeoffs. In addition, attention must be paid to the total exposure time of the cells to illumination, to avoid the onset of the effects of photo-bleaching or other artifacts resulting from prolonged illumination.

6.7.2 Correlative Microscopy

A key improvement to the utility of these experiments would be the addition of a correlative microscopy technique to provide clearer images of the platelets and perform more accurate morphology. There are two choices: concurrent/intermittent observations during the flow experiment or end point observations. Observations during the flow experiment could be done in two ways: the fluorescence of the Ca^{++} indicator itself can be used to image the cells, as was, in fact, done in these studies, or transmitted light microscopy could be used (phase contrast, DIC, video-enhanced, etc.). End point observations would most likely involve SEM. The following discussion addresses how these techniques could be applied to the flow studies used here.

Currently the cells are imaged using the fluorescence from Fura Red™. However, epi-fluorescence images are inherently fuzzy, predominately due to the blurring caused by contributions from out of focus planes above and below the intended plane of focus. This is typically addressed in one of two ways: confocal techniques such as laser scanning confocal microscopy or deconvolution techniques. Laser scanning confocal microscopy provides excellent resolution but is typically associated with long scan times, so time resolution is poor. However, in more recent years scan times have improved and specialized adaptations of confocal, e.g. the slit-scanning Odessey instrument, have achieved video rates. Measurements of $[Ca^{++}]_i$ in confocal instruments are usually made with a single excitation - dual emission dye, typically Indo-1 or a dye mixture of Fura Red™ and Fluo-3. The uses of laser scanning microscopy in $[Ca^{++}]_i$ imaging have been reviewed by Schild [27].

Computational methods for deblurring fluorescence (as well as brightfield images) have also become very popular. This technique is known under a variety of names: computational confocal, optical sectioning microscopy, deconvolution microscopy,

computational deblurring, etc. The idea is to use a pre-determined or a deduced description of how light from the various focal planes is blurred in the optical system (the point spread function) to remove the contributions of out of focus light from the original blurry image. The technique has been applied to ratio fluorescence imaging (reviewed in [28]), and has found use in imaging $[Ca^{++}]_i$ gradients in cardiac myoctyes with time resolution on the order of milliseconds [29].

Transmitted light microscopy of the flow cell experiments would require that the flow cell be redesigned to provide optical access to both the top and bottom of the flow channel. Furthermore, the flow channel itself must be fairly thin, so most often such experiments are conducted using thin capillary tubes of various geometries. While certainly providing more clear images, transmitted light microscopy is less convenient than using the fluorescence directly. It requires the intermittent use of a separate illumination system, and phase contrast objectives have lower overall transmittance, making them less desirable for low light level fluorescence work. Furthermore, although cooled CCD cameras can be used for both low light fluorescence and brightfield microscopy, they do require changes in the operating parameters, (gain, exposure time, etc.) causing additional operational difficulty.

Morphology of the adherent cells at the end of the experiment can be done directly by scanning electron microscopy. This is reasonably straightforward, and involves the following considerations: At the end of the experiment, the non-adherent cells must be flushed from the system and the adherent cells fixed (e.g., using glutaraldehyde). Then the sample is removed and processed for SEM. Care must be taken to show that the fixation is quick and complete so that the structure is truly representative of that found at the end of the experiment and not affected by any later changes in the cells. Also, care must be taken in processing so as not to lose platelets that may be only marginally

attached. The fixatives used are extraordinarily toxic, so they must be carefully cleansed from any flow apparatus before it is reused. Finally, the electron micrographs must be later reconciled with the fluorescence images and measurements, so the measurements of $[Ca^{++}]_i$ in individual cells can be correlated with the structure. This can be done with the aid of fiducial marks or by eye with careful attention to detail [22].

6.7.3 Other Future Considerations

Any variety of substrates can be implemented in this system, as long as they can be cast as transparent thin films on a coverslip. It is not known if the platelet $[Ca^{++}]_i$ response during attachment and spreading will show a dependence upon the type of surface, although it would seem likely that different surfaces with differing adsorbed protein layers would elicit differing responses. It is believed that specific platelet receptors are important for adhesion to surfaces of various adhesive proteins: e.g., integrin $\alpha_2\beta_1$ on collagen, GP Ib-IX-V on von Willebrand factor, and integrin $\alpha_{IIb}\beta_3$ on fibrinogen. It would be useful to understand if different signal transduction pathways are utilized in the platelet responses to such surfaces.

Other important variables might include: the conformation, density and perhaps even the spatial organization of the adsorbed adhesive protein(s) on the surface, as well as the effect of secondary mediators: e.g., secreted ADP, thrombin, etc. Platelets are known to respond to shear forces, in part through the GP Ib-IX-V receptor. Therefore, studies like these utilizing flowing conditions are important so that any role that shear might play during attachment and adhesion (spreading) is not overlooked.

Lastly, the techniques developed here to follow platelet $[Ca^{++}]_i$ in single cells during attachment and adhesion from flowing suspensions, can be applied just as well to other important blood cells, for example monocytes, for whom attachment and activation are also primary aspects their functioning. The experiments can also be done

with whole blood, instead of a mixed cell suspension, which may provide a more relevant view of the blood-materials interactions.

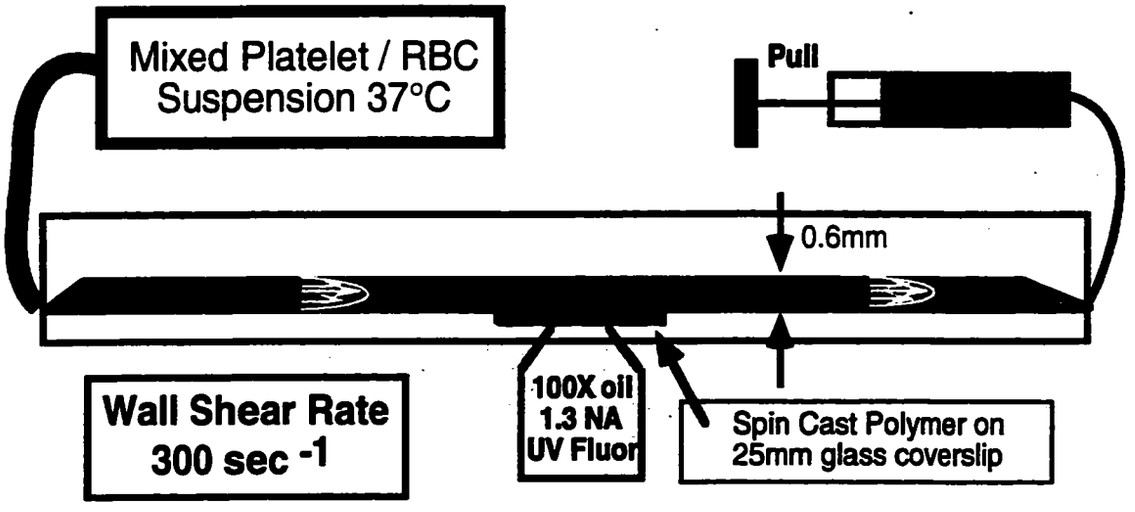

Figure 6.1 Schematic of the flow cell and apparatus used in the flow cell experiments. The flow cell was mounted on the stage of the microscope. The mixed cell suspension was held in tubes in a water bath adjacent to the flow cell. Silastic™, silicon rubber tubing connects the reservoir to the cell, and the cell to the syringe. A fixed speed, motorized syringe pump was used to operate pull the fluid through the flow cell at 1 1ml/min with a corresponding wall shear rate of 300 sec^{-1}.

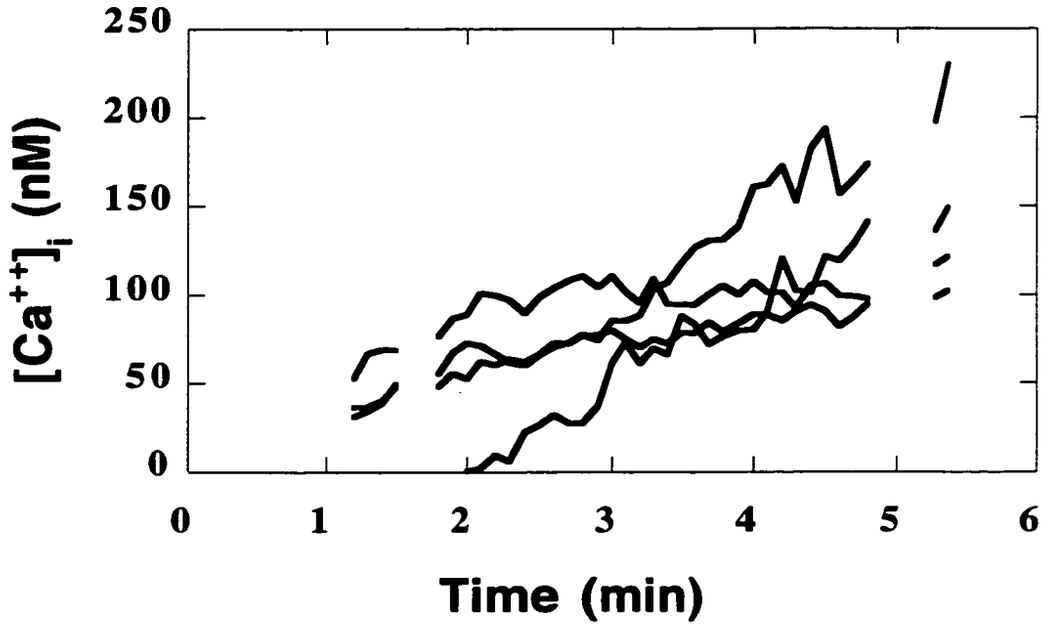

Figure 6.2. The time course of platelet $[Ca^{++}]_i$ in four platelets attaching and adhering to a fibrinogen coated polystyrene surface from a flowing mixed cell suspension.

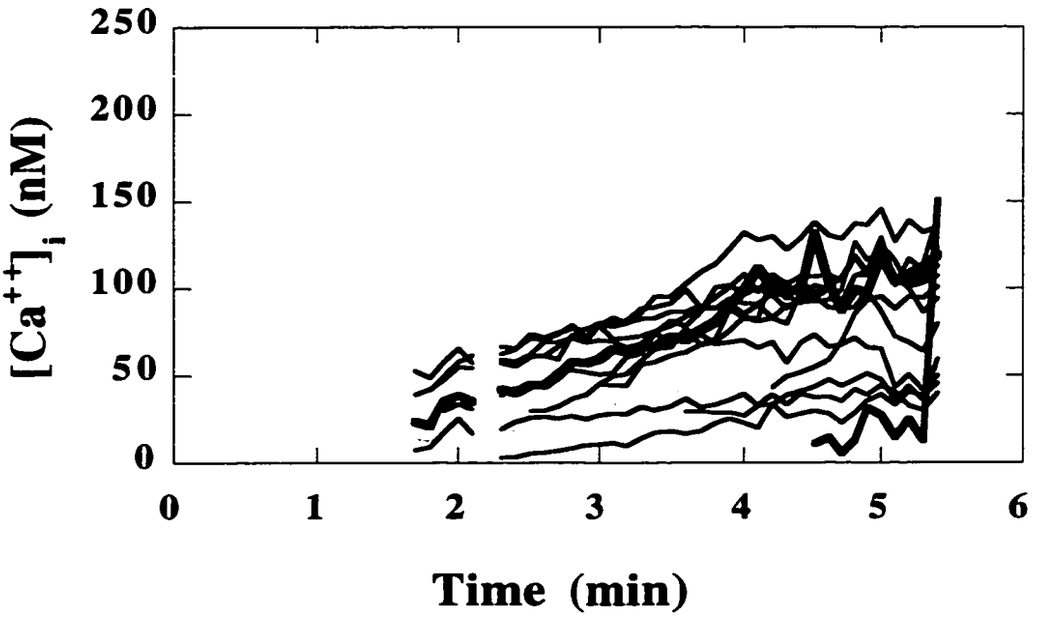

Figure 6.3. The time course of platelet $[Ca^{++}]_i$ in 15 platelets attaching and adhering to a fibrinogen coated ODCE polyurethane surface from a flowing mixed cell suspension. The heavy lines accentuate one platelet with a slow gradual rise over four minutes, and another with a sharp rise in platelet $[Ca^{++}]_i$ after less than a minute in contact with the surface.

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Chapter 7: Conclusions and Future Directions

7.1 Conclusions

The use of medical devices that come in contact with flowing blood continues to grow. Such devices are an integral part of the practice of modern medicine; some examples include catheters used for vascular access and procedures such as angioplasty, or prosthetic vascular grafts and heart valves. The thrombotic complications associated with such devices often place limitations on their use or their overall effectiveness and success. The clogging of catheters, formation of emboli that can lead to infarct, reduction in platelet number, and lifelong anticoagulation, are all consequences of concern that are associated with the use of various blood contacting devices. Thus, the development, design, and assessment of non-thrombogenic materials and surfaces continues to receive much attention from the providers, users, and recipients of these life-saving tools of modern medicine.

The activation of blood platelets is a key aspect to the blood compatibility of materials. The adhesion of platelets to surfaces, the agglomeration of platelets to one another, and the potent acceleration of blood coagulation promoted by activated platelets are some of the reasons they receive prominent attention. Quantification of platelet adherence can be achieved with a variety of methods, and the activation of platelets is often assessed using markers of granule release, e.g. P-selectin exposure, or serotonin release. The measurement of $[Ca^{++}]_i$ affords a novel measure of platelet activation. Furthermore, the use of ratio fluorescence microscopy to measure $[Ca^{++}]_i$ means that this novel measurement of platelet activation can be applied exclusively to those platelets in contact with the material

The work presented in this dissertation describes the application of ratio fluorescence microscopy to measure platelet $[Ca^{++}]_i$ during material contact. A novel fluorophore, Fura Red™, was chosen and characterized for use in the study. An *in situ* calibration of the system was performed. Platelet $[Ca^{++}]_i$ measurements were made under both static and flowing conditions. It was found in static experiments that platelets with elevated $[Ca^{++}]_i$ time courses were found to have spread during the experiment. In flowing studies there is evidence that some pseudopodia formation, the beginnings of shape change and spreading, occur without (or prior to) large increases in $[Ca^{++}]_i$.

Calibration of the fluorescence ratio measurement is important for several reasons. Since the relationship between the measured ratio and the quantity of biologic interest, $[Ca^{++}]_i$, is not linear but sigmodial, calibration aids in the correct interpretation of the data. Calibration of the system provides for important metrics in comparing the $[Ca^{++}]_i$ response with the other physiologic changes in the cell. Finally, calibration is, of course, critical for the comparison of results from different laboratories, and for the comparison of the results of $[Ca^{++}]_i$ measurements using other techniques. The properties of most ion indicators in buffered solution are readily available. However, the dissociation constant and other optical properties are often altered in the intracellular environment. Thus an *in situ* calibration of the fluorescent indicator as loaded in the cell and measured with the optics of the experimental apparatus is important. Due to the technical difficulty of making such *in situ* calibrations, they are often omitted from published studies of $[Ca^{++}]_i$ imaging, particularly in studies involving platelets.

An *in situ* calibration of the calcium indicator, Fura Red™, was performed in adherent platelets at 37°C using the ratio fluorescence microscopy system developed for this study. The R_{min} and R_{max} were found to be 0.77 and 5.9, respectively and the

$K_{1/2}$ was determined to be in the range of 550-650nM. The $K_{1/2}$ and R_{max} for intracellular Fura Red™ were found to be considerably higher than those measured for Fura Red™ in buffered solution.

The studies of platelet $[Ca^{++}]_i$ in single platelets during attachment and spreading on biomaterial surfaces presented in this work represent some of the first observations of their kind. The response was seen to be heterogeneous. Resting $[Ca^{++}]_i$ was found to be 50-100nM, and cells that attached to the surface demonstrated resting $[Ca^{++}]_i$ values initially. After a variable lag period some cells showed a rise in $[Ca^{++}]_i$ of 100-300nM or more. Oscillations were also seen to be a prominent feature of the $[Ca^{++}]_i$ response.

The many platelet $[Ca^{++}]_i$ time courses generated during an experiment were categorized by the peak $[Ca^{++}]_i$ observed. The platelets that had appeared to have spread during the experiment were identified, and the results compared. It was found that platelets that had spread during the experiment were associated with the time courses that had demonstrated a rise in $[Ca^{++}]_i$, while in the group that had not spread, the $[Ca^{++}]_i$ values remained lower. While the results do not prove that spreading is a result of an $[Ca^{++}]_i$ rise, they are indicative of a link between this step of signal transduction, and the ultimate morphologic response of the cell.

Platelet $[Ca^{++}]_i$ was imaged in single platelets that attached to biomaterial surfaces from a flowing suspension of mixed washed labeled platelets and red blood cells. These observations are also some of the first observations of their kind. Over this short period after initial attachment, the cells demonstrated resting $[Ca^{++}]_i$ around 50nM, and gradual rises in $[Ca^{++}]_i$ with values generally well below what had been observed for activated platelets in the static experiments. The images revealed the formation of small pseudopodia on some of the cells. This occurred without a significant rise in $[Ca^{++}]_i$, an interesting observation that indicates that $[Ca^{++}]_i$ signaling in platelets may not be

involved in the initial attachment and shape change events. The $[Ca^{++}]_i$ response may still be associated with later events such as spreading, granule release, and membrane lipid scrambling.

Together, the work presented here represents an important and novel advancement in the ability to detect and measure the activation of platelets by contact with biomaterial surfaces. The techniques of ratio fluorescence microscopy that have been used in larger cell types to study signal transduction have been adapted and applied to the study of platelets at biomaterial surfaces. A new fluorophore was characterized for use in the studies. The measurement of $[Ca^{++}]_i$ provides insight into the process of signal transduction that is triggered by materal contact. This may lead to a better understanding of the biochemical process that control the activation of platelets, and a better understanding of how surfaces contribute to that activation. Currently there is wide interest in the modifcation of surfaces and other interventions, at the platelet material interface, designed to reduce platelet adhesion and activation. The tools developed here may help investigators to understand how such efforts are perceived by the platelets themselves.

7.2 Future Directions

7.2.1 Ca^{++}-Indicators

There are several issues to note with regard to the calcium indicators used in this work and their calibration.

Chapter 4 presents an *in situ* calibration of Fura Red in platelets. The results of these experiments raise several new questions, including the heterogeneity seen during the calibration, possible differences in the affinity and spectral properties of the indicator

in solution and in the intracellular environment, and the comparison of Fura Red™ and Fura 2.

First the *in situ* calibration depends on the use of the ionophore ionomycin and the use of thapsigargin, a calcium sequestering (SERCA) pump inhibitor, to bring an entire population of adherent platelets to a uniform, prescribed $[Ca^{++}]_i$, namely that $[Ca^{++}]$ found in the buffer bathing the cells. At some concentrations of calcium, this effort was not uniformly successful, as a biomodal distribution of measured ratios was seen in the population of adherent platelets with supposedly uniform $[Ca^{++}]_i$. This observed heterogeneity may reflect the heterogeneity of the platelets themselves; i.e. some cells may be more or less resistant to the actions of the ionophore and thapsigargin to defeat Ca^{++} homeostasis than others. Some authors have speculated on the possible heterogeneity of the platelet granules themselves with regard to their calcium pumps and sensitivity to the action of thapsigargin; and have suggested this to play a role in the spiking of platelet $[Ca^{++}]_i$. In part due to the manner in which the platelet are formed, namely by blebbing off of megakaryocytes, they are heterogeneous in size and in granule content from the very beginning. Thus, it is conceivable that some platelets may contain more or less of the thapsigargin sensitive granules than others, leading to the heterogeneity seen during the calibration efforts. The *in situ* calibration might be improved by choice of another SERCA pump inhibitor, increasing the concentration of thapsigargin, increasing the concentration of ionophore, or trying a different ionophore, e.g. A23187.

Second, the calcium indicator Fura Red™ has not received much use reported in the literature. The spectra we have measured for the dye in buffered solution were found to differ somewhat from that provided by the vendor. The affinity of the dye for calcium, and the dye's spectral characteristics may be altered in the intracellular environment.

Further characterization of Fura Red™ may be useful. Experiments could be conducted in a spectrofluorimeter, using Fura Red™ in various buffers made to mimic the intracellular environment of platelets. Similarly, such experiments could be performed to evaluate the effects of protein composition and concentration on the characteristics of the indicator. Finally, it would be interesting to compare an *in situ* calibration of Fura Red™ in platelets in stirred suspension using a spectrofluorimeter with the results obtained using ratio fluorescence microscopy.

Third, Fura Red™ was chosen for this work, in part due to the fact that the excitation wavelengths required are in the visible range, making possible the use of polymeric substrates. Fura 2 is excited with 340 and 380nm light, and care is usually required to ensure adequate transmittance at these wavelengths for all the components of the apparatus. Nonetheless, Fura 2 has received much wider use and characterization. It also has the benefit that the calcium sensitive fluorescence increases with increasing calcium, meaning that this signal would increase as the cell responds with a rise in $[Ca^{++}]_i$ and possibly spreads. For Fura Red™, conversely, the calcium sensitive fluorescence decreases with increasing calcium, therefore the calcium sensitive fluorescence is decreasing in spreading cells due to rises $[Ca^{++}]_i$ and decreasing cell thickness. Indeed activated platelets with high $[Ca^{++}]_i$ values, that became thinly spread often became impossible to accurately image or measure in the experiments presented here. So each indicator has certain drawbacks, and it may be beneficial to re-evaluate the choice of calcium indicator in some cases. To start, for example the transmittance of various spin cast substrates on glass coverslips could be measured at the excitation wavelengths required and their suitability for use with Fura 2 determined.

7.2.2 Low Magnification

The scarcity of adherent platelets to measure in the experiments reported in this work, is a significant problem. If the technique is to be used to assess and compare the platelet reactivity to various biomaterials including those that might be expected to be less reactive, then a wider field of view must be used to encompass a greater number of platelets. There are a number of tradeoffs involved in this decision. First, lower power objectives have much less light gathering capacity, and therefore will result in less signal. Second, in static platelet adhesion assays, the non-adherent cells are differentiated from the adherents by their characteristic ratio pattern. Under lower magnification this distinction may be much more difficult to discern. However, in a flowing regime, the non-adherent platelets are much less of a problem since they are flushed away in the flow. Finally, the uniformity of illumination across the wider field may be of issue. While the ratio measurement removes the effect of variations in illumination from the $[Ca^{++}]_i$ determination, nonetheless if portions of the field of view are poorly illuminated in comparison to others, this poses added difficulty in setting acquisition parameters, and may mean the loss of data from some adherent cells.

7.2.3 Static versus Flow

The bulk of the studies presented here were done using a static adhesion regime. The results presented in Chapter 6, describe some initial experiments conducted under flowing conditions. There are advantages and disadvantages to both techniques, however future work in a flowing regime may prove to be more productive and beneficial.

In the static adhesion system, a small number of washed labeled platelets are added to buffer in a temperature controlled well that uses a glass coverslip with a spin cast thin polymer film of the biomaterial of interest on the bottom. The platelets are imaged as they attach and spread onto the surface. There are several advantages to this system. First, a small number of prepared platelets is required, so the convenient and gentle procedure of gel filtration can be used to prepare the washed platelets from a small (20ml) donation of human whole blood. Second, a commercial temperature control system is available to conduct the experiment at 37°C. Third, transmitted light microscopy is possible with the static adhesion well.

The major disadvantage to using the static technique is that cells that are in motion near or on the surface are also imaged, and more importantly, cells that are in motion over, or land upon other adherent cells interfere with the measurement of $[Ca^{++}]_i$ in the adherents. The effort spent to determine which cells are in motion, and to identify and remove data compromised by such interference is painstaking and time consuming. This disadvantage may be eliminated in flow cell experiments (see below) or overcome by letting some cells from a very concentrated platelet suspension attach, then rinsing the sample and following the $[Ca^{++}]_i$ response in the adherent cells while bathed in platelet free buffer. This, of course, sacrifices the measurement of the initial $[Ca^{++}]_i$ response to attachment. Another disadvantage is that the platelets settle to the surface slowly over the first one to two hours, leading to long experimental runs. A third disadvantage is that secondary mediators of activation released by the adherent platelets, e.g. ADP, TxA2 , serotonin, may act locally to stimulate other nearby platelets. Apyrase is added to the suspension buffer to remove ADP, nonetheless, in a flowing regime, these agents would be swept away and would have less chance to accumulate locally to any significant concentration.

In the flow cell experiments, washed labeled platelets are prepared and mixed with washed red blood cells. This mixed cell suspension is passed through a parallel plate flow cell, where the biomaterial of interest, again coated as a thin film on a glass coverslip, forms a section of one wall. This technique has several key advantages. First, it provides uniquely different information, as the behavior of platelets is well known to be affected by shear, and a flowing regime is more representative of the conditions of platelet biomaterial interaction in vivo. Second, as indicated above the fact that non-adherent cells are washed away by the flow and are not imaged, greatly, greatly simplifies the image analysis. Third, the flowing regime provides a more meaningful setting in which to observe $[Ca^{++}]_i$ during transient encounters, i.e. the attachment and detachment of platelets can be studied under a well defined shear stress, as has been done using other epi-fluorescent microscopy techniques. Finally, it may be possible to extend the studies to anticoagulated whole blood, or even *ex vivo* experiments, which present a more complicated, but more relevant setting in which to observe the platelet biomaterial interactions.

There are several disadvantages to using the flowing regime. First, the preparation of the mixed cell suspension requires a much larger number of platelets and a larger donation of human whole blood, roughly 60-120ml per experimental run, as compared to 20ml of whole blood used for many experimental runs in the static experiments. This means that centrifugation must be used to prepare the washed platelets, a process which is more time consuming and may contribute to more adventitious platelet activation than the preparation of washed platelets by gel filtration. Using the 300 sec^{-1} shear rate flow cell, the flow requirement is 11 ml/min, so experimental run time, and the number of runs has been limited. An alternative is to recirculate the mixed cell suspension using a peristaltic pump, although it may be difficult to accomplish this without any evidence of

pre-activating the circulating platelets. Also, smaller flow cells can be used, particulary ones made from small capillaries, which require much less volumetric flow. Such capillary flow devices require more accurate control of volumetric flow rate, and can be difficult to coat with polymeric films of interest. Second, currently there is no temperature control of the flow chamber nor the tubing and syringe pump used to transport the suspension. The suspension is drawn from a reservoir at 37°C, but may drop in temperature slightly in the flow cell. This problem could be addressed with the use of a temperature controlled enclosure around the microscope and apparatus. Third, the currently available flow cell is compatible with the 100x objective only, meaning the field of view is reduced significantly compared to the 40x objective used in the static experiments. Using lower magnification, and imaging a wider field of view may afford certain advantages as listed above, especially in the flow regime where non-adherent cells are washed away. Finally the transient encounters of the platelets with the surface in the flowing regime occur much more quickly than in the static experiments. If such transient encounters were to be studied, images would have to be collected with much greater time resolution, preferably video rates, which would require much additional imaging hardware and probably the use of a dual emission indicator, such as Indo-1.

7.2.4 Time Resolution and Length of Experimental Run

The minimum time interval between image pairs used in the flow cell experiments was six seconds. In the static experiments, a 30 second interval was used. Spiking of platelet $[Ca^{++}]_i$ on fibrinogen, with ADP stimulation has been noted with intervals of 5-30 seconds. So increased time resolution may be required to fully characterize the oscillatory nature of the platelet $[Ca^{++}]_i$ response. There are many interrelated factors that are involved in determining the time resolution of the system, and some of these

have been discussed in Chapter 6. First, the intensity of the fluorescence signal is crucial; longer acquisition times (compare with the shutter speed on any camera) are required when the fluorescence is signal is small. Second, the slow-scan digital camera can be run in several modes with the characteristics of: high resolution, large image file size, and poor sensitivity to light; to low resolution, small image file size, and high sensitivity to light. Resolution may be critical in defining cell morphology especially at low magnification. File size has immediate impact on the time required to: digitize the image, transfer the image from camera to computer memory, operate on the image to calculate and display the ratio, and store the image to disk. Of course, the light sensitivity affects the exposure time required. Finally, the illumination must be controlled. The wavelength of illumination must be alternated and synchronized with sufficient speed. Also, the total exposure time of the cells to illumination should be minimized to avoid the onset of photo-bleaching or possible artifacts resulting from prolonged illumination.

The length of the experimental runs was typically 45 minutes in the static experiments and only 5.5 minutes in the flow cell work. In previous radiolabeled static platelet adhesion assays done in this laboratory, where the number of adherent platelets was measured at some endpoint, two hour adhesion times were typically used. The settling of platelets, which are very small cells, is slow; and in the static experiments presented here it was common not to have more than 10-15 cells, even on the most activating surfaces, arrive and attach over the first 20-30 minutes. The act of platelet spreading itself may take as long as 15 minutes to reach completion. Increased platelet concentration in the suspension may result in more rapid accumulation of platelets at the surface, however it would likely also increase the confounding problems associated with non-adherent cells and interfering cells. Future static experiments should be run

for longer times roughly 60-90 minutes. This hopefully would increase the number of cells that could be tracked, but, of course, doubles the number of frames which must still be analyzed individually. If acquisition intervals were also shortened to better catch oscillations and short transients in $[Ca^{++}]_i$, then the situation quickly becomes untenable. The problem of non-adherent cells that appear in the image, and sometimes interfere with the $[Ca^{++}]_i$ measurement of already adherent cells is a serious limitation to the use of the static regime. If one is willing to forgo the first five minutes of observation after attachment, then a protocol could be used where high concentrations of cells were applied to the surface and allowed to attach for three minutes, and then the non-adherents rinsed away and removed. The $[Ca^{++}]_i$ could then measured during the mid- to later stages of spreading. This may be useful in correlating later events, such as granule release, with $[Ca^{++}]_i$, however, this protocol is less than ideal. For these reasons, flow cell studies may prove to be more productive and deserve more attention in the future.

The short run times in the flow cell experiments stem from the volume of mixed cell suspension required. In the static experiments lag times of 3-5 minutes were often seen before $[Ca^{++}]_i$ response, so the 5.5 minute run time in the flow cell experiments is clearly too short, and should be extended to at least 20 minutes or longer if possible.

7.2.5 Correlating the $[Ca^{++}]_i$ Response with Platelet Functions

Clearly, the major extension of this work that is required is to correlate the $[Ca^{++}]_i$ response with other important physiologic events of platelet activation on surfaces, namely: shape change, granule release, and the membrane lipid scrambling that supports blood coagulation, known as procoagulant activity.

7.2.5.1 Morphology

Crude measures of cell spreading were applied to the static experiments as described in Chapter 5, and it was found that the platelets with the higher transient $[Ca^{++}]_i$ values were generally the ones seen to spread during the experiment, and the non-spread cells were seen to remain at low $[Ca^{++}]_i$. These measurements are clearly in need of improvement. Morphology of the adhering platelets can be done in two ways: as an end-point, i.e. at the end of the experiment, or concurrent with the $[Ca^{++}]_i$ measurements itself.

The easiest way to make assessments of shape change and spreading of the platelets at the end of the experiment is by scanning electron microscopy. Some of the small technical issues to be worked out are: a) a way of referencing locations on the surface so that the particular platelets studied in the ratio fluorescence microscopy experiments could be reconciled with the platelets in the electron micrographs; b) a way of breaking the coverslips into small enough pieces to fit in the critical point drying apparatus, without disturbing the area of interest; c) deciding on an appropriate metric to be used, e.g. spread area, circularity, or perhaps a semi-qualitative scoring index that is commonly used in such work.

However, in order to temporally correlate the $[Ca^{++}]_i$ response with shape change and spreading, the morphology must be performed concurrently, or at least alternately with the ratio fluorescence microscopy during the experiment. This can be done with the use of transmitted light microscopy, and the clever use of band pass filters and spilt path optics, as well as two detectors. Alternatively, it may be possible to use deconvolution techniques to make better use of the fluorescence images themselves. This would require the acquisition of several image pairs at various focal planes for each time point, and would require additional hardware and software. Again, in either case,

an appropriate scoring index would need to be applied to quantitate the changes in cell shape.

7.2.5.2 Granule Release

The release of granule contents may be detected in the microscope using a dye that is taken up into granules, such as mepacrine. Also, recent work by Heemskerk demonstrates the use of FITC-labeled antibodies to P-selectin to detect the exposure of this granule membrane protein on the surface of adherent platelets, as a marker of granule release. The release of platelet granules is believed to be an event dependent upon increased $[Ca^{++}]_i$, and the centralization and presumably the release of granule contents is believed to occur at much later times after attachment and spreading on a surface. Granule release is usually associated only with strong stimulation of the platelet, and may not take place at all in response to weaker agonists such as ADP. Collagen, and the combination of collagen and thrombin are particularly potent activators of granule release.

7.2.5.3 Procoagulant Activity

Finally, the use of FITC labeled annexin V, a protein which recognizes the exposure of phosphatidylserine on the platelet membrane indicative of membrane phospholipid scrambling, has been used as a marker for the onset of procoagulant activity in single adherent platelets. Such experiments might nicely compliment other studies of the procoagulant activity stimulated in adherent platelets using in situ thrombin generation assays.

The work presented here and the efforts of others to image $[Ca^{++}]_i$ responses in single platelets is still at an early stage of development. By far ,most investigators are

still interested only in the single platelet $[Ca^{++}]_i$ response to soluble agonists. Much work remains to be done to study the $[Ca^{++}]_i$ response during spreading on surfaces of including biomaterial surfaces of varying types. The extent to which adsorbed adhesive proteins are responsible for the platelet activation on surfaces might be profitably addressed in experiments using surfaces varying protein composition. In particular, the variations in the surface density of an adsorbed adhesive protein like fibrinogen, might be seen by the platelet as variations in the spatial density of ligands, available to engage and use for attachment and spreading. It would be interesting to know if different intracellular signals arise from interactions with such different surface ligand densities.

The correlation of the $[Ca^{++}]_i$ response with the physiologic outcomes of the platelet, such as granule release, is important. Ultimately, however, our understanding of the mechanisms of platelet activation and the role of $[Ca^{++}]_i$ signaling will be aided most by linking the $[Ca^{++}]_i$ rise with other distinct biochemical pathways in the platelet. Efforts to do so await further advancements in biochemistry and molecular biology. However, one example may not be far from the lab bench. Calmodulin is a calcium binding protein which is believed to bind calcium as a consequence of elevated $[Ca^{++}]_i$ and then mediate other downstream events; e.g. several transcription events in nucleated cells, and the activation of myosin light chain kinase in platelets. Recently Bolsover *et al* have constructed a fluorescently labeled calmodulin whose fluorescence is dependent upon the binding of calcium to this protein [1, 2]. They have used this new tool investigate the role of Ca^{++}:calmodulin in neuronal signaling. Such experiments might be used with single platelets to demonstrate, and in fact image and localize, the triggering of an important biochemical pathway as a consequence of $[Ca^{++}]_i$ mobilization.

Notes to Chapter 7

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Appendix A:

UNIVERSITY OF WASHINGTON
CONSENT FORM
CELL AND PROTEIN REACTIONS WITH BIOMATERIALS

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Investigator's Statement

Purpose and Benefits

This study will investigate the way in which proteins and cells interact with biomaterial surfaces to help design materials which can be used in contact with blood without the clinical complications presently encountered with these materials. Benefits to all persons whose tissue or blood must come in contact with materials during implantation of a biomedical device are anticipated.

Procedures

Before your donate, you may be asked if you have taken any medications in the past two weeks. Your blood will be drawn from a vein in the arm as is usually done for blood sampling in the clinic. This will be done by a trained practitioner at the University of Washington Medical Center. The amount of blood drawn will usually be 60cc (2oz.), but no more than 200cc (6.75oz.). This amount of blood will be drawn from you only once. The time needed to complete the procedure will be about 20 minutes. If you choose to donate again, you must wait four weeks between blood draws. You may choose not to answer a question, or decide not to participate at any time.

Risks, Stress or Discomfort

The risks, stress and discomfort are minimal and will be the same as with any routine blood sampling in the clinic. You may feel discomfort from the insertion of the needle. Possible side effects include a bruise on the arm, possible temporary lightheadedness or fainting. If you do feel faint, please tell the clinician right away so that the chair can be reclined for you.

Other Information

You are free to refuse to participate and to withdraw from the study at any time without penalty. You will receive $15.00 for your blood donation. In the unlikely event of an adverse effect, you will be referred for appropriate treatment at no cost to you, within the limits of the University's Compensation Plan.

Your identity will be kept confidential. The principal investigators and their staff are the only individuals who will have access to the donor records. The results of our experiments will be reviewed and published in the general scientific literature, but without any reference to the identity of the blood donors. The data generated will be kept indefinitely.

_____
Signature of Investigator

SUBJECT'S STATEMENT

The study described above has been explained to me. I voluntarily consent to participate in this activity. I have had an opportunity to ask questions. I understand that future questions I may have about the research or about my rights as a subject will be answered by one of the investigators listed above.

_____
Signature of Subject

Appendix B: Preparation, Display and Export of Ratio Images as a Single TIFFs or Time-Lapse Video

The data from a single ratio fluorescence microscopy experiment can comprise hundreds of detailed ratio fluorescence images. As described, the data can be reduced to quantitative metrics regarding the $[Ca^{++}]_i$ of single platelets. However, it is often useful to visually present this information as series of pseudocolored ratio images, especially when comparing $[Ca^{++}]_i$ transients with platelet morphology and movement or in flow cell experiments where platelets are seen to attach and then detach from the surface. For these reasons, efforts have been made to condense the ratio images from entire experimental runs to short movies and eventually video tape segments, which can be used present a summary of experimental data conveniently and portably on a variety of platforms, including computer displays, the internet (Web browsers) and conventional video tape players.

This section describes that process, which is rather specific for this laboratory but can in principle be adapted as software and hardware are advanced.

Ratio images

MetaFluor (version 2.5 Universal Imaging Corp.) is used to acquire and analyze the images in the ratio fluorescence experiments. Ratio images are calculated, displayed and stored using a variety of adjustable parameters. First for the calculation, background subtraction and threshold values for the individual wavelengths are specified. The full 12-16bit images (12 bit = 4096 levels) are ratioed using a full 32-bit intermediate calculation , resulting in a 16-bit ratio image. However in this system the ratio image, like all images must be scaled to an 8-bit (256 level) image for display. In the case of the ratio images, this is done by mapping a user specified range of ratio values to a user

specified (256 level) color scale ranging, in the default version, from deep blue, through green, to red; with black and white used to represent ratio values at or outside the bounds of the specified ratio range. Such an image is called a pseudocolor image, and is used because color is more useful for the human eye to distinguish the wide variety and subtlety of the gradations in ratio value, than a conventional grey scale. The proper selection of the ratio range is critical to the interpretation of the ratio images; unusually narrow ranges of ratio mapped to a wide rainbow of colors accentuates small, and possibly less meaningful, changes in ratio, while unduly large ranges, (e.g. chosen to include a single cell or particle demonstrating an abnormally high value,) can mask the moderate ratio changes of the majority of the cells. Typical values for platelet and monocyte experiments with Fura Red™ are a minimum displayed ratio of 0, and maximum of 2 - 4.

The color scale used comprises 256 separate colors and is known as a Look Up Table (LUT). A LUT may be constructed of varying gradations of just one hue, e.g. a deep red to a light pink, or contain all the colors of the rainbow. MetaFluor maintains two permanent LUTs for use, a standard grey scale (named "monochrome") and a standard rainbow (named "pseudocolor"); a variety of other LUTS are separately available, as well as a utility for creating novel LUTS from scratch. Different LUTS might be customized for use depending upon the ratio image's final destination, and color rendition capabilities thereof, e.g. a color slide made by a laser film recorder, a large format inkjet printer (poster making) or television monitor. In general the default, built-in "pseudocolor LUT" was used with good overall success.

Images for Export

The protocol used for data collection has been to permanently save only raw single wavelength fluorescence images, and to recreate the ratio images (using specified

threshold and display parameters) after the fact, as needed. As described above, MetaFluor maintains two internal, unchangeable LUTS for its own use in displaying images, one being the "pseudocolor" commonly used. In what charitably might be called an undocumented feature, when MetaFluor creates an ratio image using one of these LUTS it reduces the file size by placing a small tag in the file header specifying the use of the internal LUT, instead of including the entire LUT information in the actual file. While this is useful when these images are viewed using MetaFluor, it poses a problem when the files are destined for export to another platform, as the LUT information is missing and separate from the actual image file. Therefore, the first step in creating ratio images for export is to force MetaFluor to include the LUT information in the ratio image file. This is done any time a user-defined LUT (any LUT other than the two internal LUTS) are used in creating an image. Using the LUT utility, a exact duplicate of the built-in pseudocolor LUT is created, renamed a distinct name, and then specified as a user-defined LUT to be applied when creating any ratio image for export. Second, a color bar showing the range of the LUT and the ratio values associated with the colors is included with each image. This feature is set under the general preferences, and it is useful to have the program create the bar outside the image area to avoid obscuring items of interest in the image. Note that the final image created in now wider to allow space for the color bar. Finally, single or series of images can be created by specifying the first 5 characters of the ratio filename, and proceeding through the experiment. The ratio images will be stored as TIFF files, individually named as *****###.tif, where the first 5 characters are as above, the ### is taken from the frame number in the experiment, and the file extension is .tif. The byte order of TIFF files on Intel platforms is the reverse of that on Mac platforms; some newer Macintosh programs, e.g. Adobe Photoshop, are capable of determining the byte order from the

file header and reading either version correctly. A DOS conversion utility is available to manually swap byte order if necessary.

Ratio TIFF images so generated can then be exported to a variety of platforms and used in a variety of applications including color documents (Word), slides and transparencies (Photoshop) and large format posters (Photoshop, Pagemaker) as well as dye-sublimation prints, brochures, etc. To present the images as a movie or video segment, the approach is to compile a sequential series of images into a QuickTime™ (QT) movie which can then be played on various computer platforms or output to standard VHS videotape.

Compiling images into QT Movie or videotape segments:

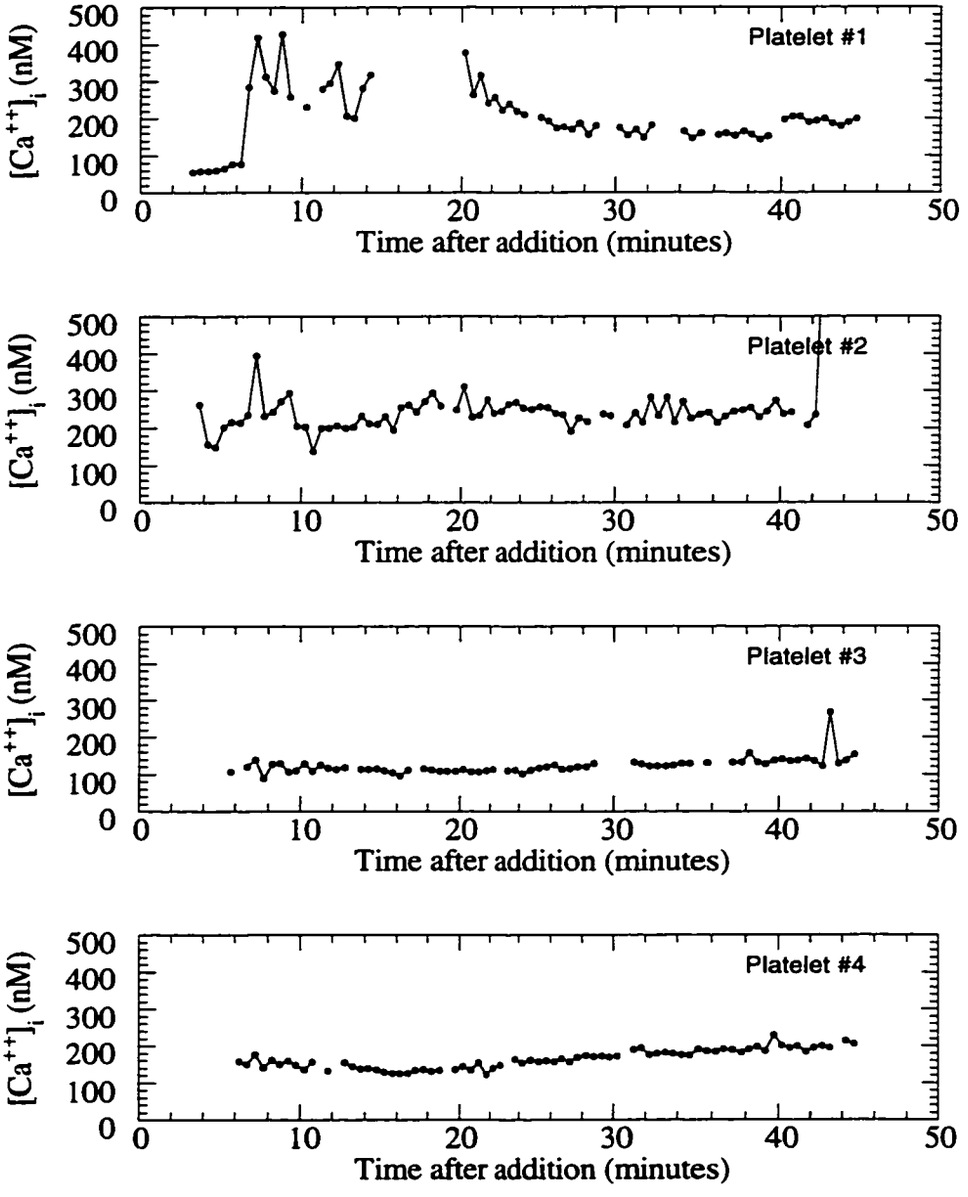
The process begins under DOS where the series of files are renamed from *****###.tif to *****###., i.e. the .tif extension is removed. Next the files are transferred to a Macintosh platform using FTP (binary protocol, FETCH). There, the files are converted to a PICT format in batch using the program GRAPHIC CONVERTER. The program SPARKLE will be employed to compile the images into a QT movie, and it requires the files to be in a single folder and named as filename.### where ### is the sequence or frame number. So, under the Macintosh Finder the files are renamed from *****###. to *****.###, manually (an AppleScript macro might be able to automate this process). Using SPARKLE, the folder with a series of PICT images is compiled into a QT movie. The speed with which the images are played (frame rate) is specified here (2 fps was useful in the initial work).

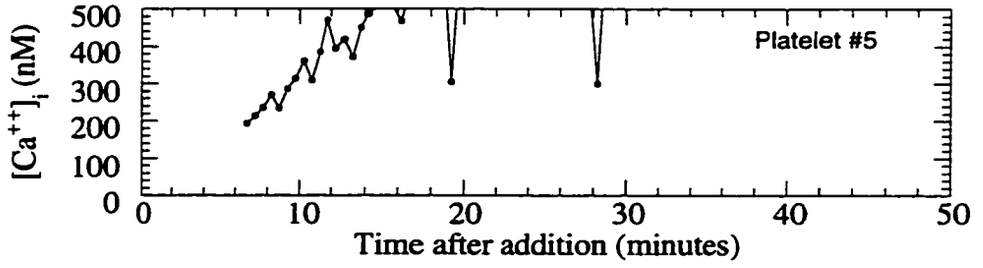
The QT movies can be displayed on computer displays, LCD data screens (overhead) or data projectors using SPARKLE or other QT movie players. For more detailed editing, annotating and sequencing of the segments and/or output to video tape, the QT movie can be imported into a digital video editor. This laboratory has used

AVID Videoshop from Apple. Director from Macromedia is another common application for this purpose. A key point here is that, while full frame NTSC video (VHS) is 640x480 pixels, many television monitors overscan, meaning that the usable region of the screen is decidedly smaller. To maintain resolution yet deal with this problem, we specified an output of full frame 640x480, but then imported the QT movie and proportionally scaled it to only 400 pixels in width and placed it in the center of the frame leaving black space around the edges. Annotations such as arrows, scale bars, text, or timers are added in the video editor as PICTS or additional QT movies. Finally, still/hold frames, transitions such as fades, and some titling can be arranged using the video editor. The final product is then "printed" to VHS videotape using a PowerMacintosh (older AV model or newer with video out capability) and a videotape recorder.

Appendix C: Individual Platelet $[Ca^{++}]_i$ Timecourses for Experiments Shown in Chapter 5.

Washed Platelets on Biospan™

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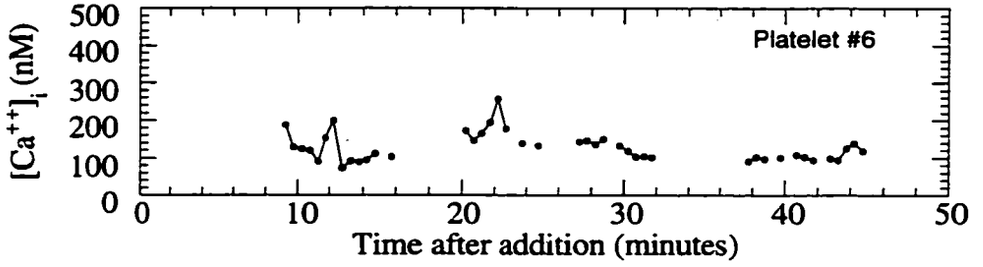
100
0
0
10
20
30
40
50
Time after addition (minutes)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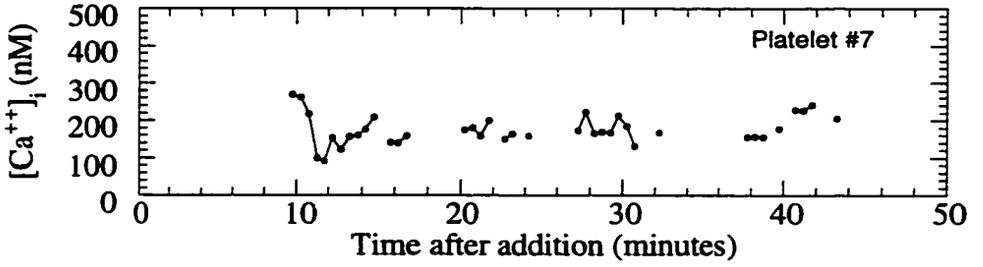
50
Time after addition (minutes)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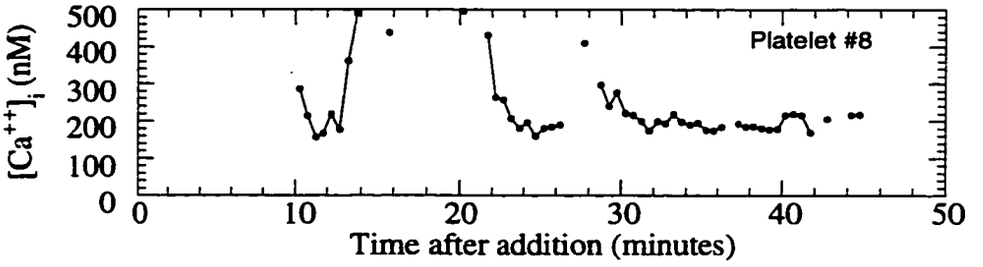
40
50
Time after addition (minutes)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30
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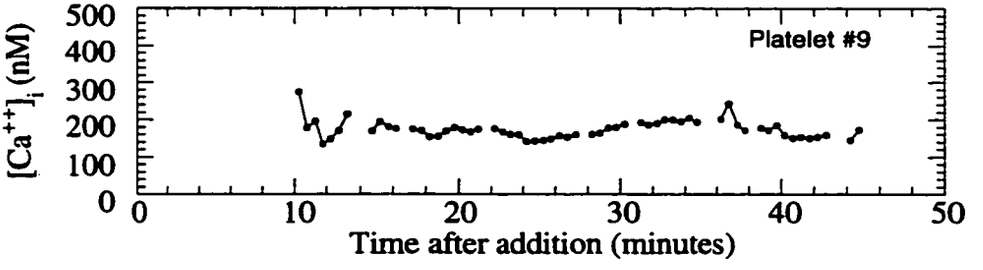
50
Time after addition (minutes)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200
100
0
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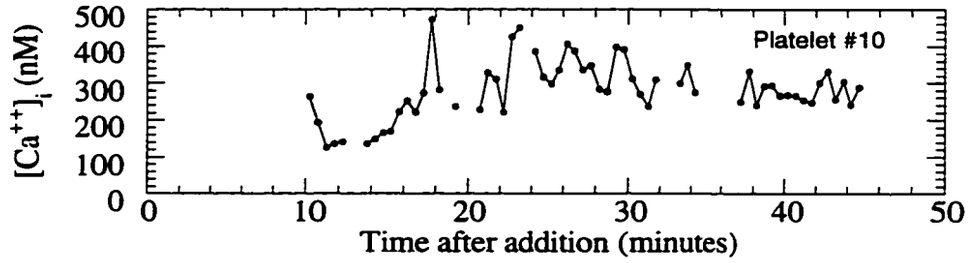
10
20
30
40
50
Time after addition (minutes)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$[Ca^{++}]_i$ (nM)
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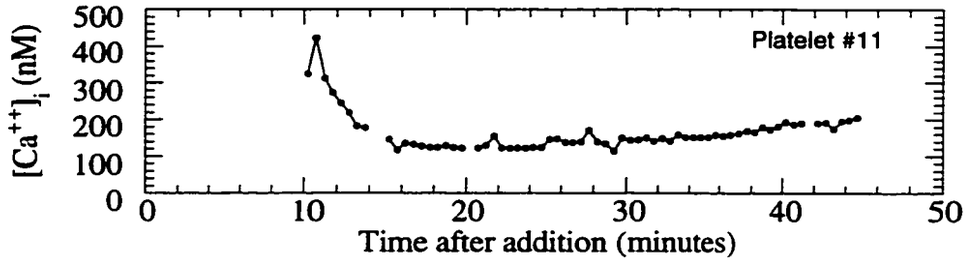
20
30
40
50
Time after addition (minutes)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100
0
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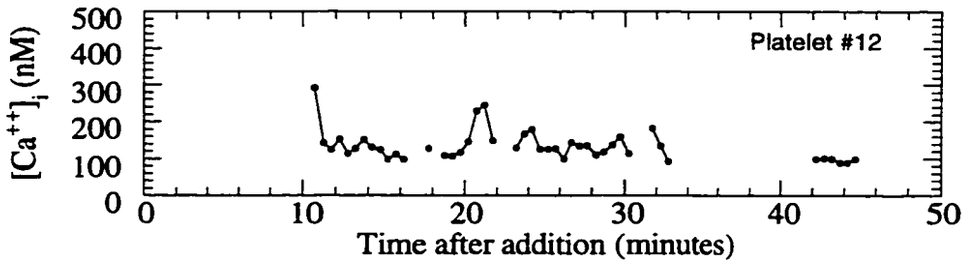
20
30
40
50
Time after addition (minutes)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0
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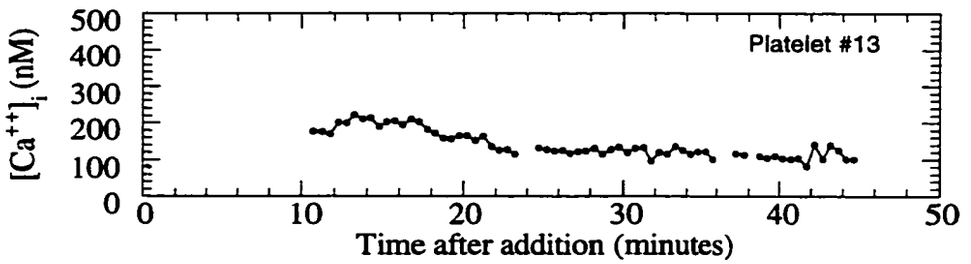
20
30
40
50
Time after addition (minutes)


Platelet #13
$[Ca^{++}]_i$ (nM)
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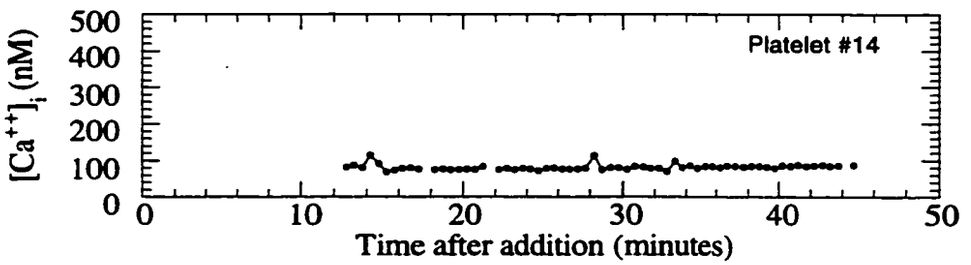
30
40
50
Time after addition (minutes)


Platelet #14
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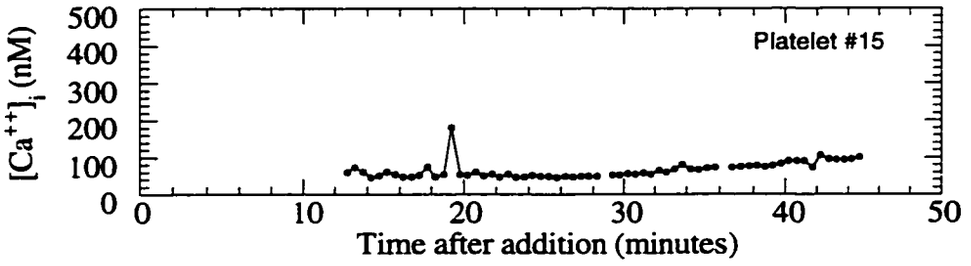
30
40
50
Time after addition (minutes)


Platelet #15
$[Ca^{++}]_i$ (nM)
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300
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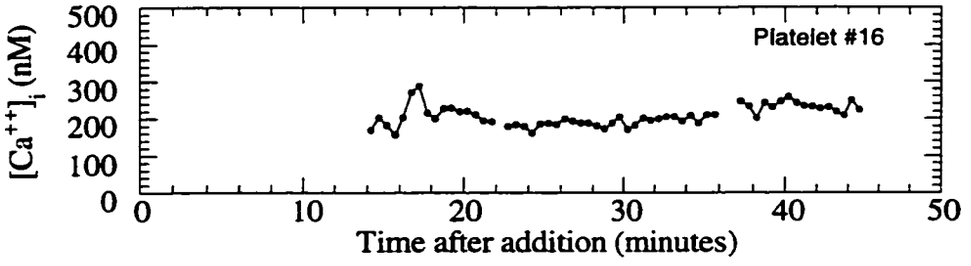
100
0
0
10
20
30
40
50
Time after addition (minutes)


Platelet #16
$[Ca^{++}]_i$ (nM)
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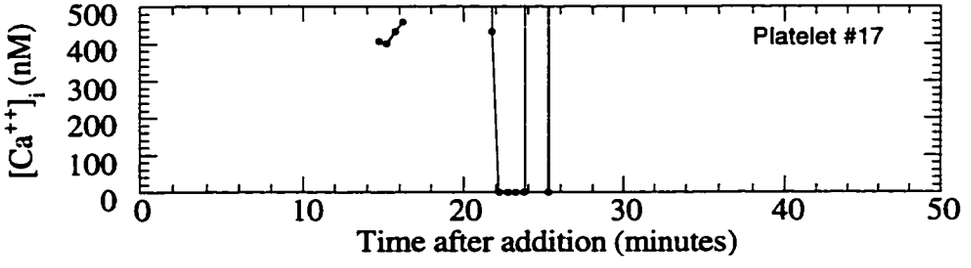
30
40
50
Time after addition (minutes)


Platelet #17
$[Ca^{++}]_i$ (nM)
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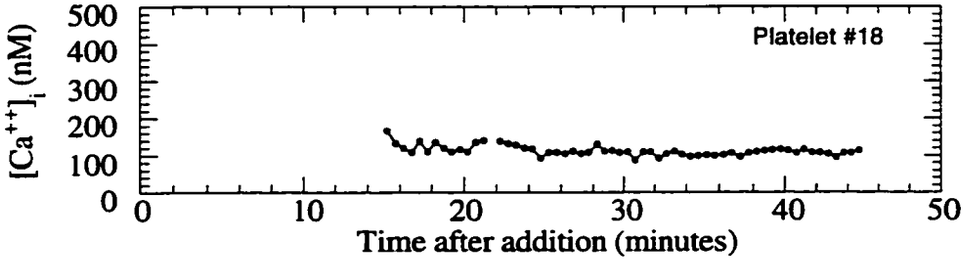
100
0
0
10
20
30
40
50
Time after addition (minutes)


Platelet #18
$[Ca^{++}]_i$ (nM)
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200
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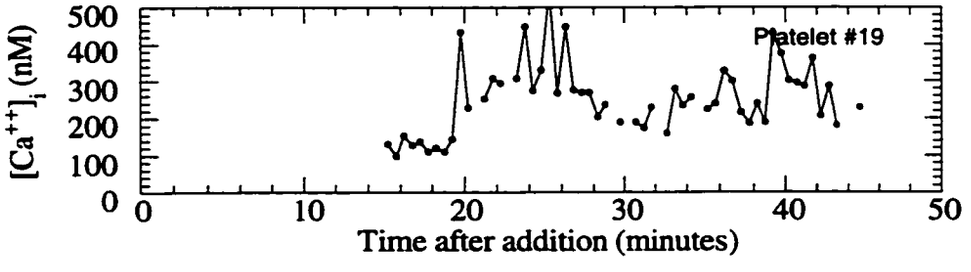
0
0
10
20
30
40
50
Time after addition (minutes)


Platelet #19
$[Ca^{++}]_i$ (nM)
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200
100
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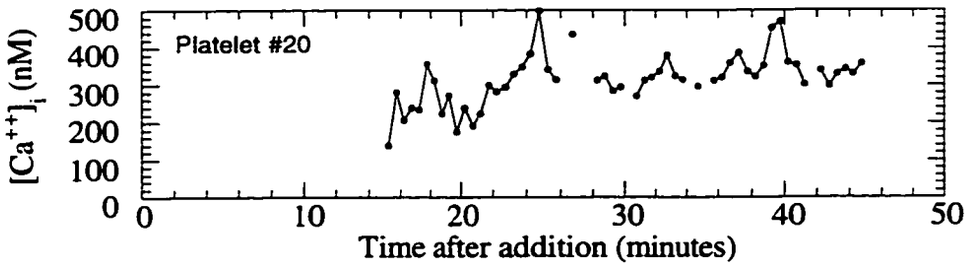
0
10
20
30
40
50
Time after addition (minutes)


Platelet #20
$[Ca^{++}]_i$ (nM)
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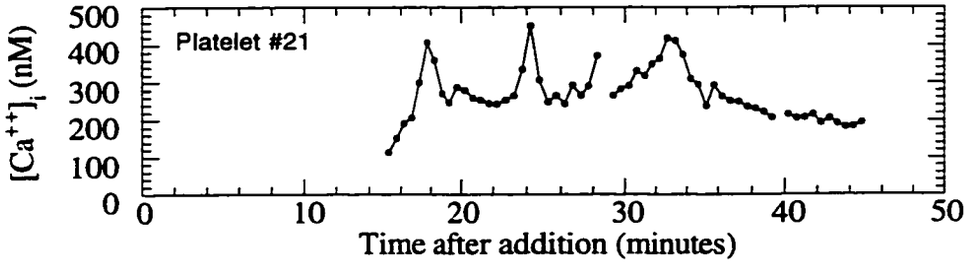
100
0
0
10
20
30
40
50
Time after addition (minutes)


Platelet #21
$[Ca^{++}]_i$ (nM)
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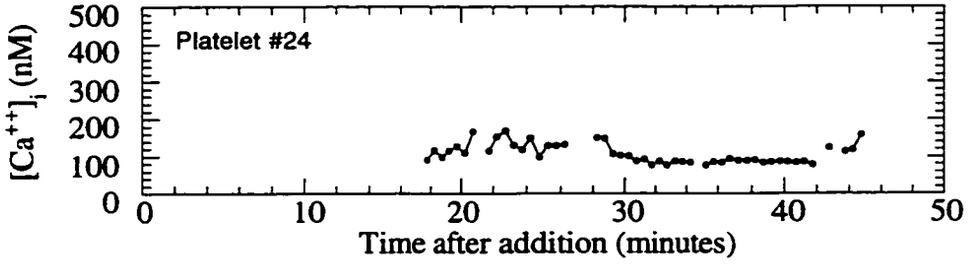
100
0
0
10
20
30
40
50
Time after addition (minutes)


Platelet #24
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0
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10
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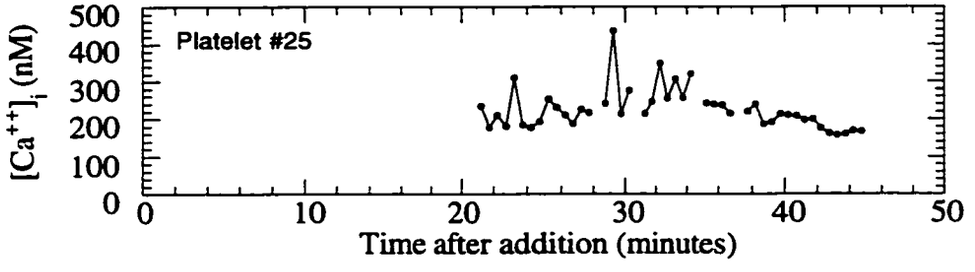
30
40
50
Time after addition (minutes)


Platelet #25
$[Ca^{++}]_i$ (nM)
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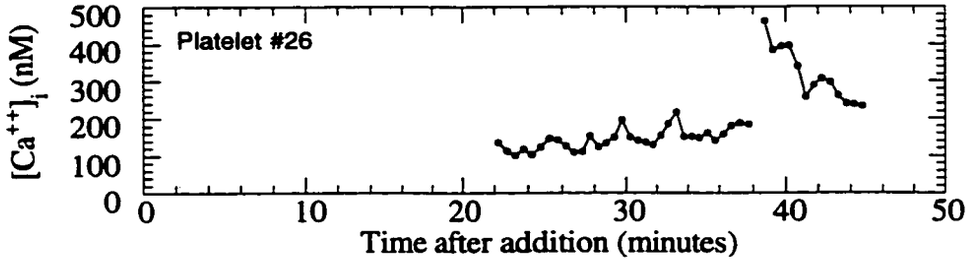
0
0
10
20
30
40
50
Time after addition (minutes)


Platelet #26
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0
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30
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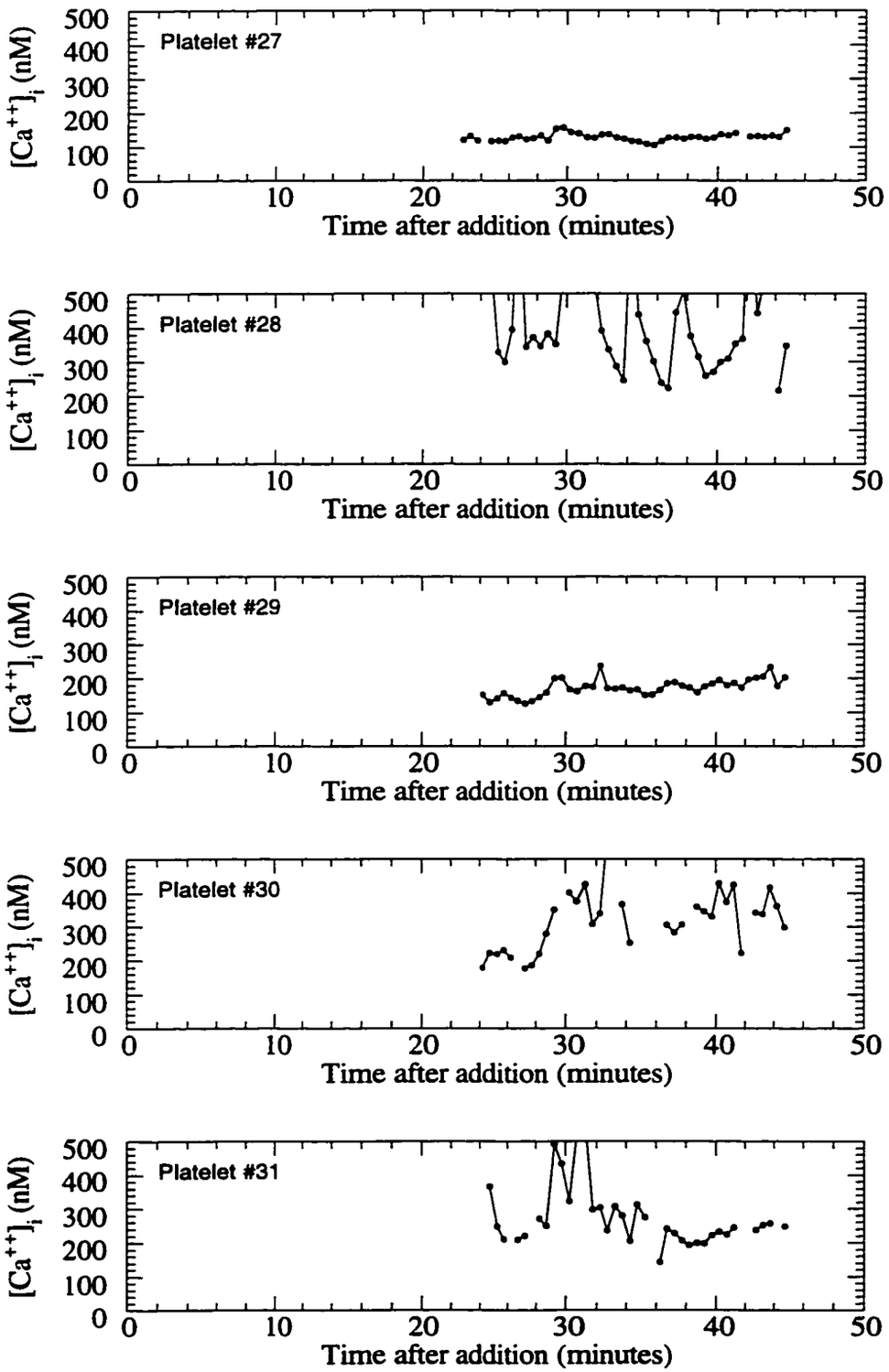
50
Time after addition (minutes)


Platelet #27
Platelet #28
Platelet #29
Platelet #30
Platelet #31
[Ca++]i (nM)
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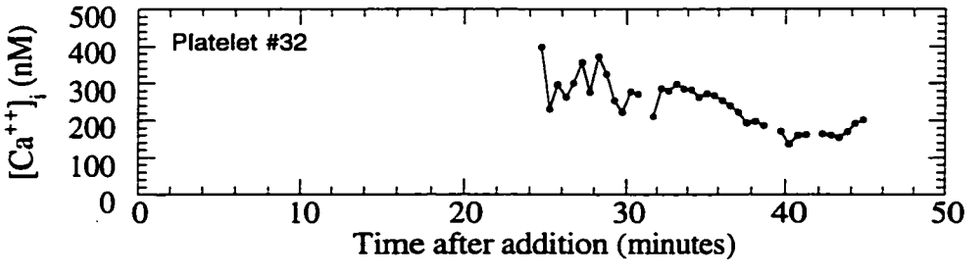
100
200
300
400
500
0
10
20
30
40
50
Time after addition (minutes)


Platelet #32
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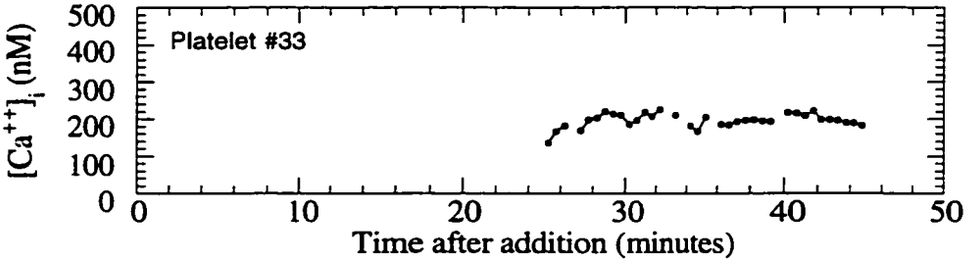
0
0
10
20
30
40
50
Time after addition (minutes)


Platelet #33
$[Ca^{++}]_i$ (nM)
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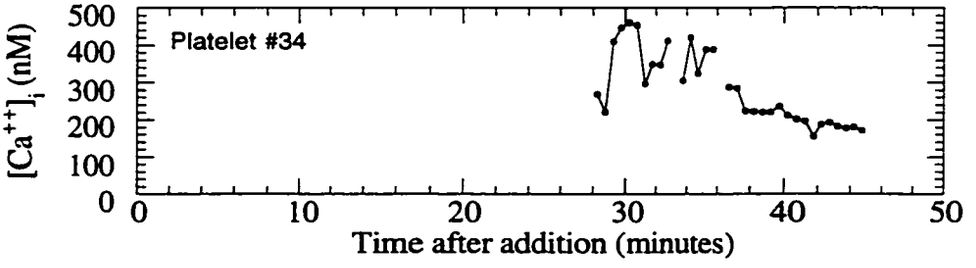
Time after addition (minutes)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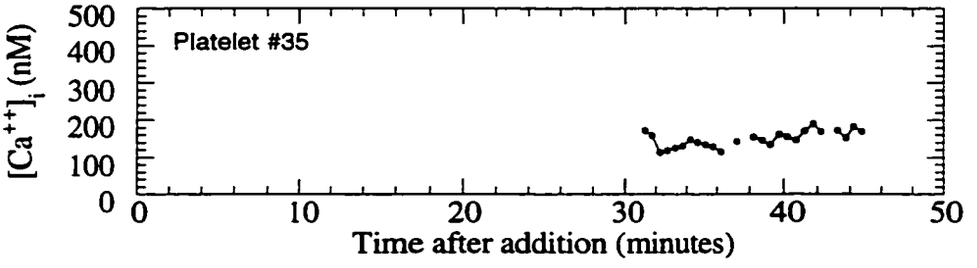
Time after addition (minutes)


Platelet #35
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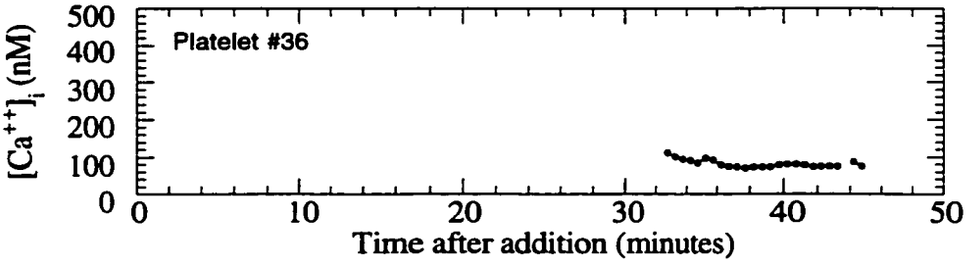
Time after addition (minutes)


Platelet #36
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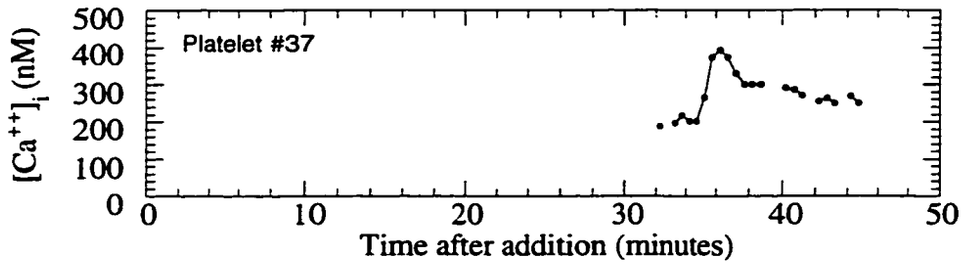
Time after addition (minutes)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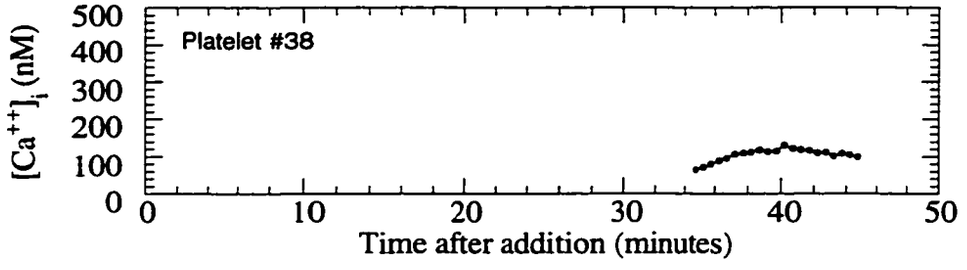
Time after addition (minutes)


Platelet #38
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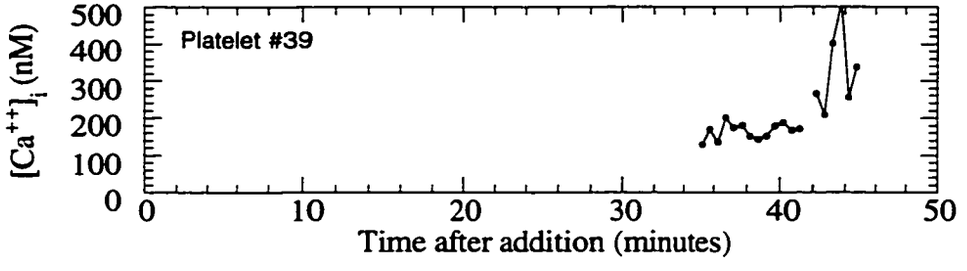
30
40
50
Time after addition (minutes)


Platelet #39
$[Ca^{++}]_i$ (nM)
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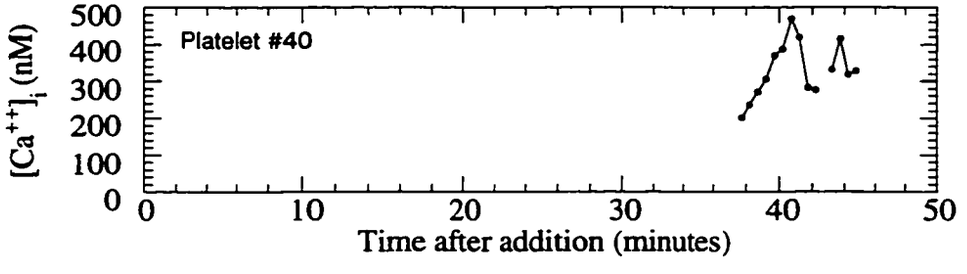
30
40
50
Time after addition (minutes)


Platelet #40
$[Ca^{++}]_i$ (nM)
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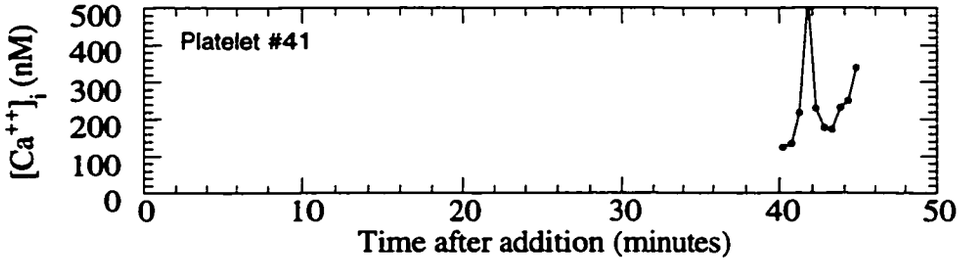
20
30
40
50
Time after addition (minutes)


Platelet #41
$[Ca^{++}]_i$ (nM)
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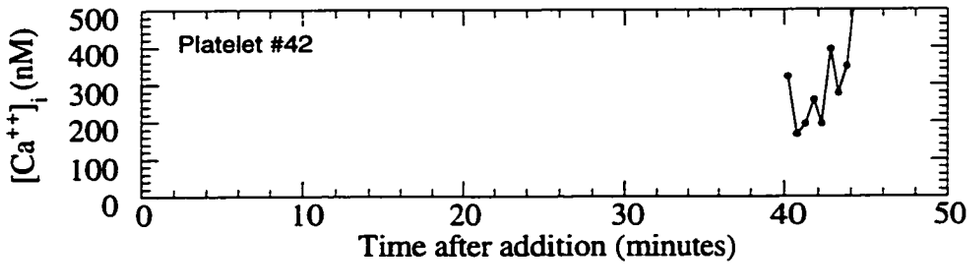
0
0
10
20
30
40
50
Time after addition (minutes)


Platelet #42
$[Ca^{++}]_i$ (nM)
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400
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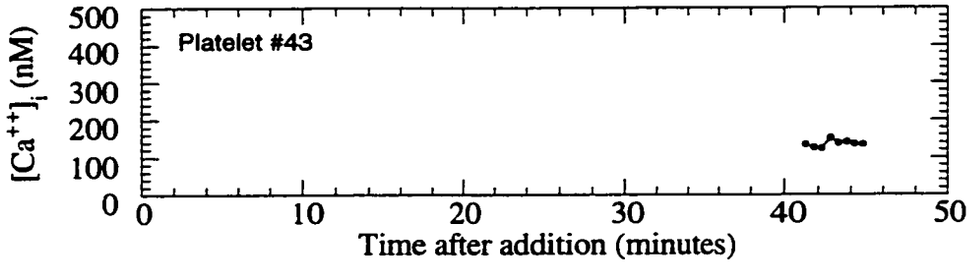
200
100
0
0
10
20
30
40
50
Time after addition (minutes)


Platelet #43
$[Ca^{++}]_i$ (nM)
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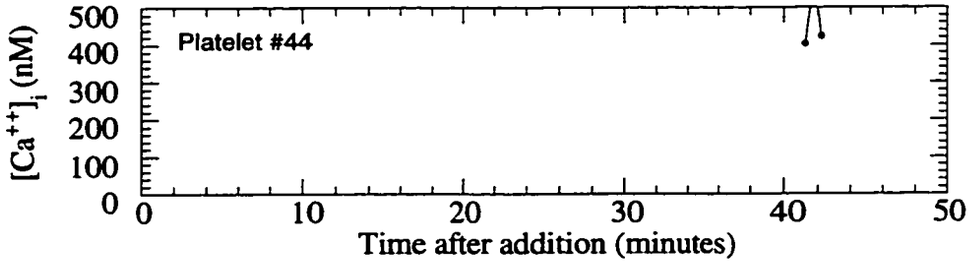
400
300
200
100
0
0
10
20
30
40
50
Time after addition (minutes)


Platelet #44
$[Ca^{++}]_i$ (nM)
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400
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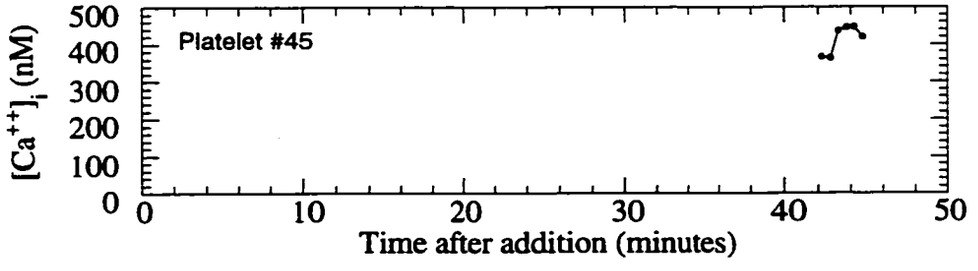
200
100
0
0
10
20
30
40
50
Time after addition (minutes)


Platelet #45
$[Ca^{++}]_i$ (nM)
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400
300
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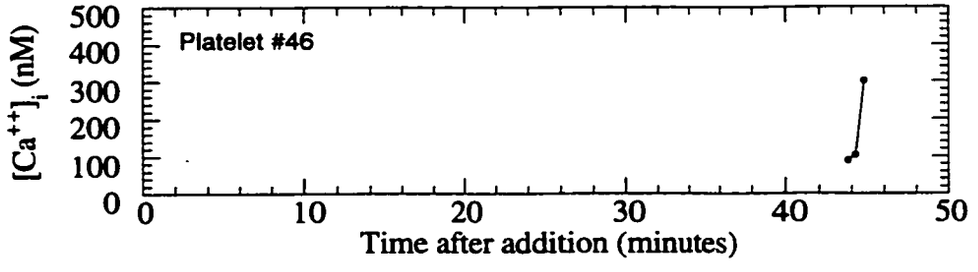
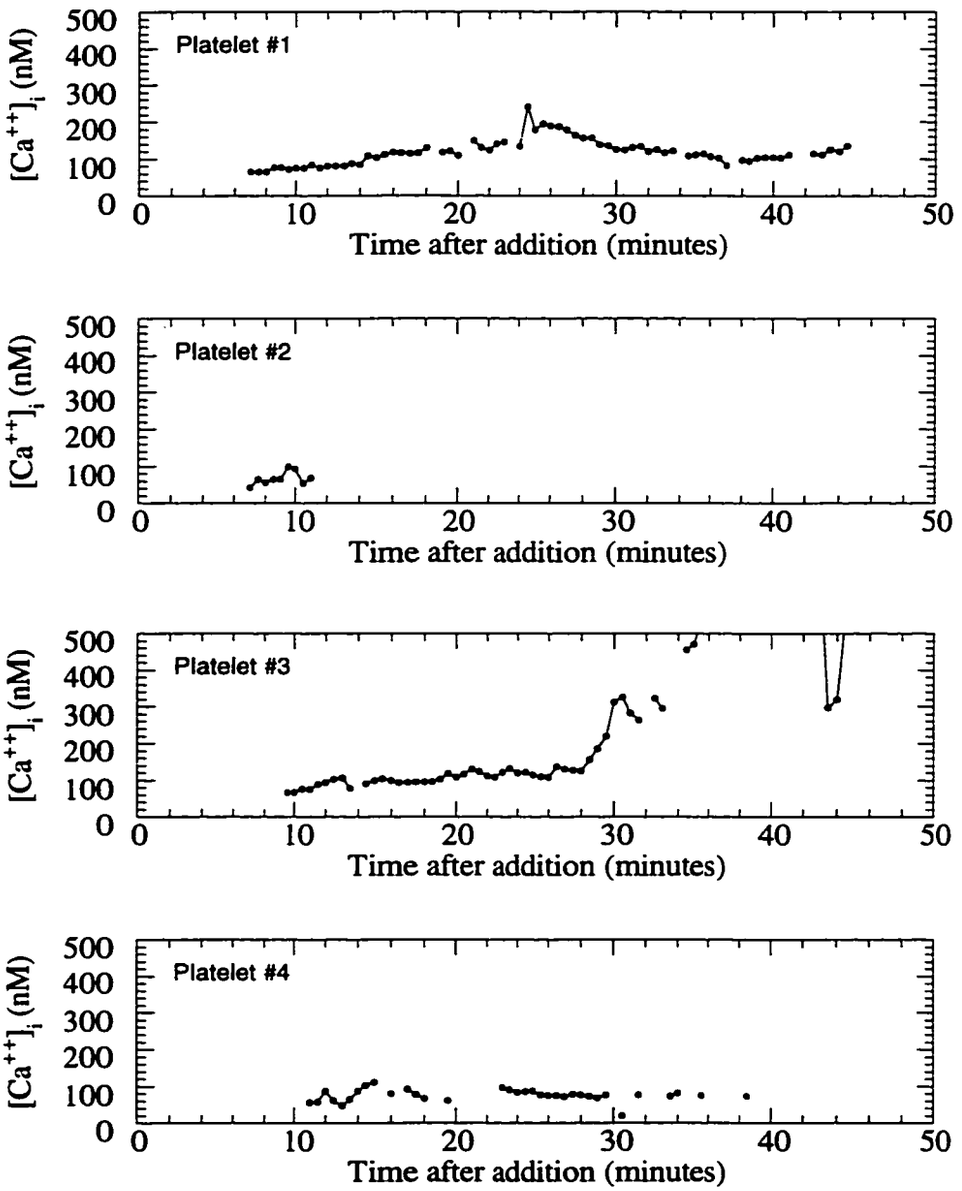
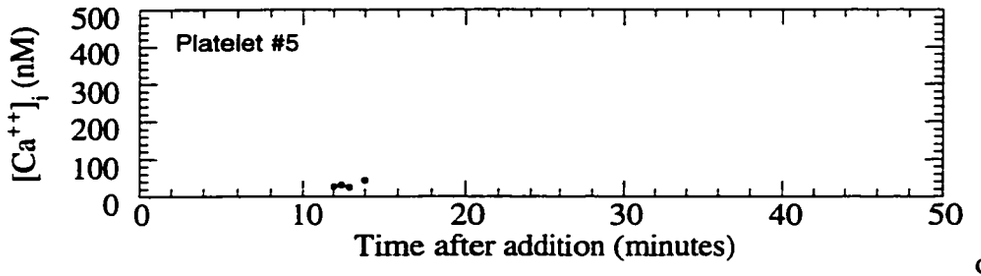
0
0
10
20
30
40
50
Time after addition (minutes)


Platelet #46
$[Ca^{++}]_i$ (nM)
500
400
300
200
100
0
0
10
20
30
40
50
Time after addition (minutes)

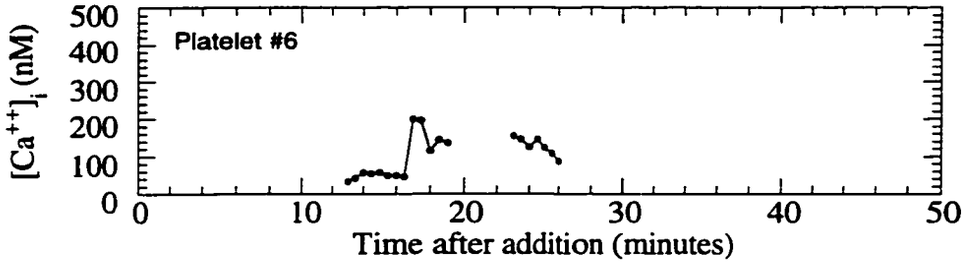
Platelets on ODCE-PEU

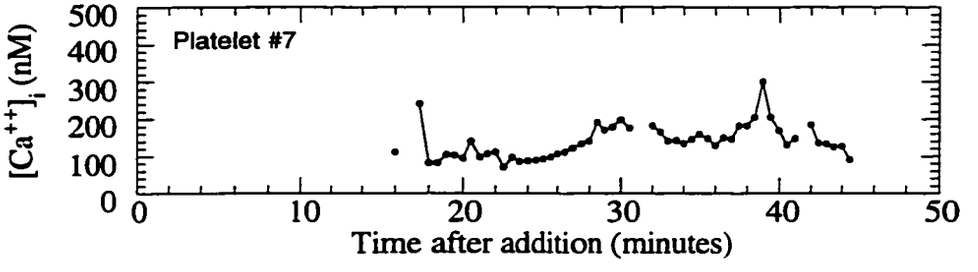


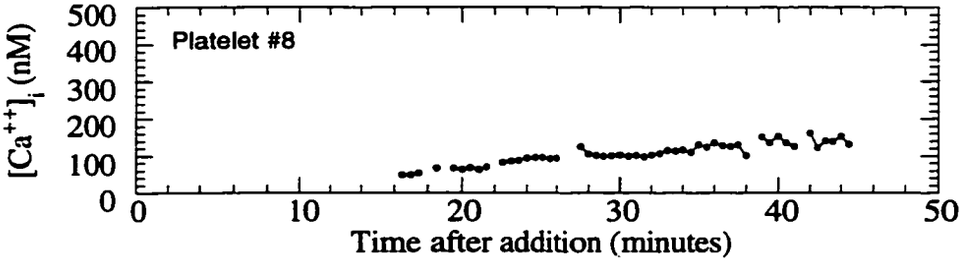
Omit

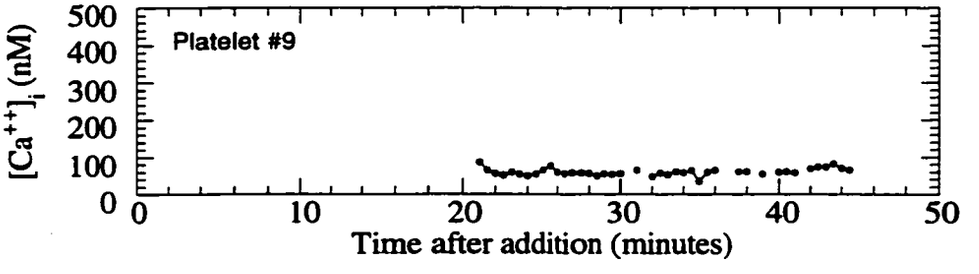

Platelet #5
$[Ca^{++}]_i$ (nM)
500
400
300
200
100
0
0
10
20
30
40
50
Time after addition (minutes)

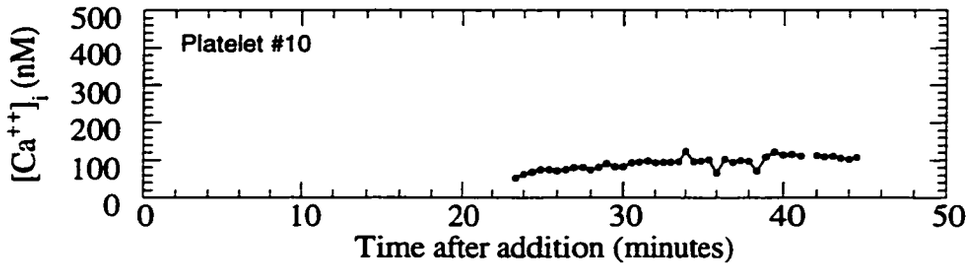
omit

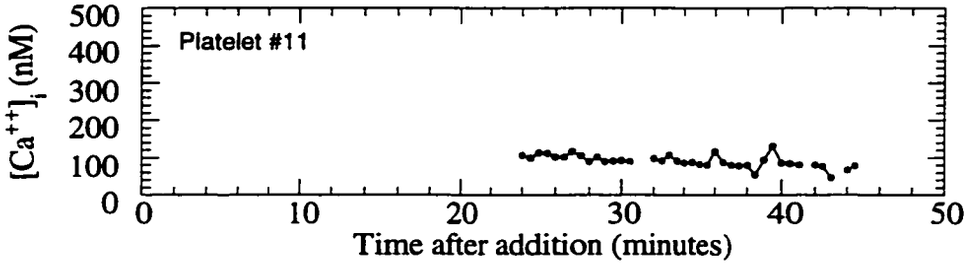

Platelet #6
$[Ca^{++}]_i$ (nM)
500
400
300
200
100
0
0
10
20
30
40
50
Time after addition (minutes)

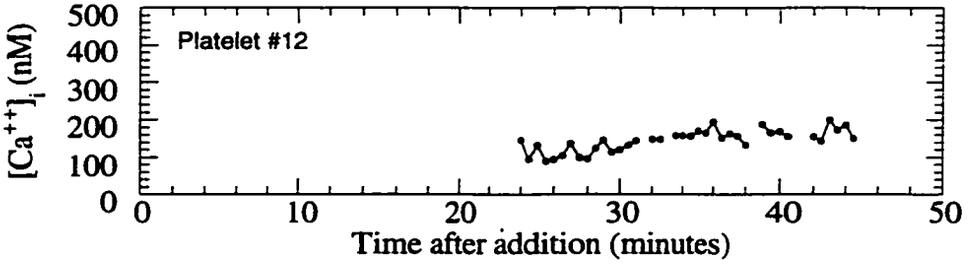

Platelet #7
$[Ca^{++}]_i$ (nM)
500
400
300
200
100
0
0
10
20
30
40
50
Time after addition (minutes)

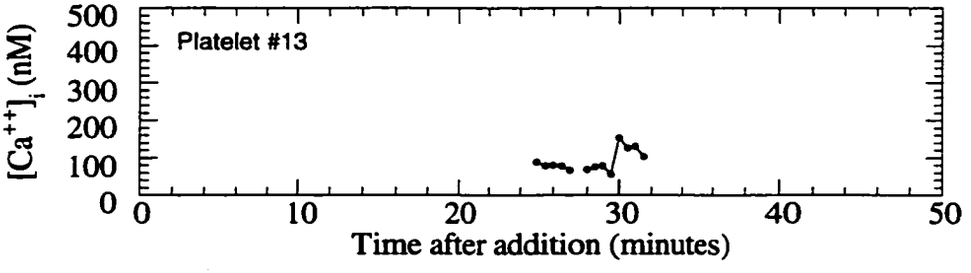

Platelet #8
$[Ca^{++}]_i$ (nM)
500
400
300
200
100
0
0
10
20
30
40
50
Time after addition (minutes)

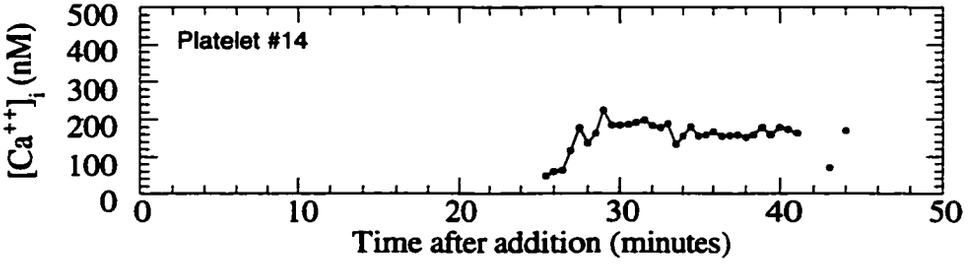

Platelet #9
$[Ca^{++}]_i$ (nM)
500
400
300
200
100
0
0
10
20
30
40
50
Time after addition (minutes)

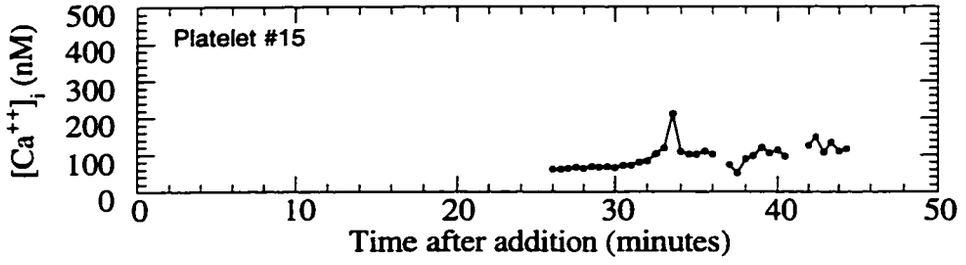

Platelet #10
$[Ca^{++}]_i$ (nM)
500
400
300
200
100
0
0
10
20
30
40
50
Time after addition (minutes)

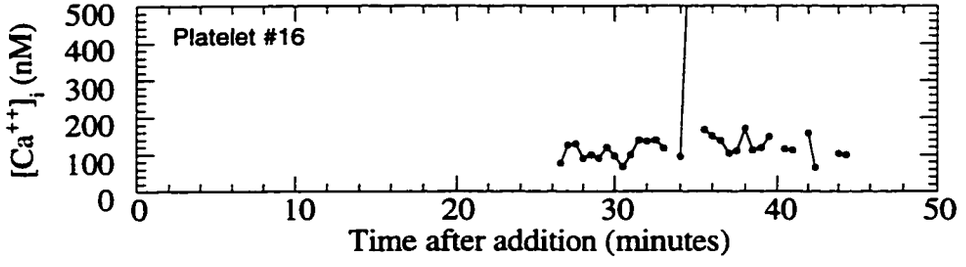

Platelet #11
$[Ca^{++}]_i$ (nM)
500
400
300
200
100
0
0
10
20
30
40
50
Time after addition (minutes)

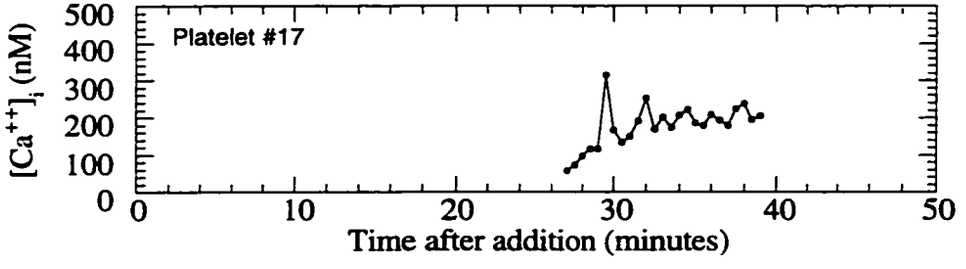

Platelet #12
$[Ca^{++}]_i$ (nM)
500
400
300
200
100
0
0
10
20
30
40
50
Time after addition (minutes)

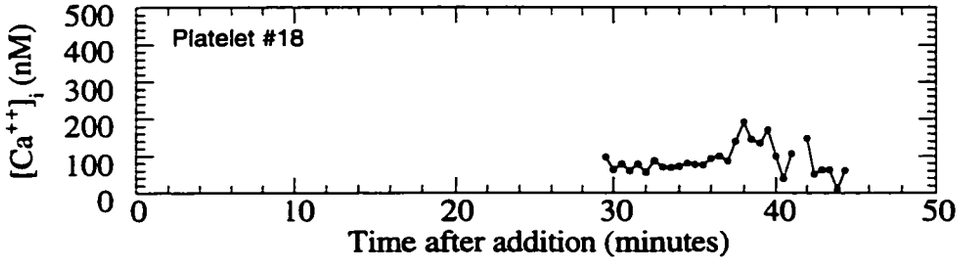

Platelet #13
$[Ca^{++}]_i$ (nM)
500
400
300
200
100
0
0
10
20
30
40
50
Time after addition (minutes)

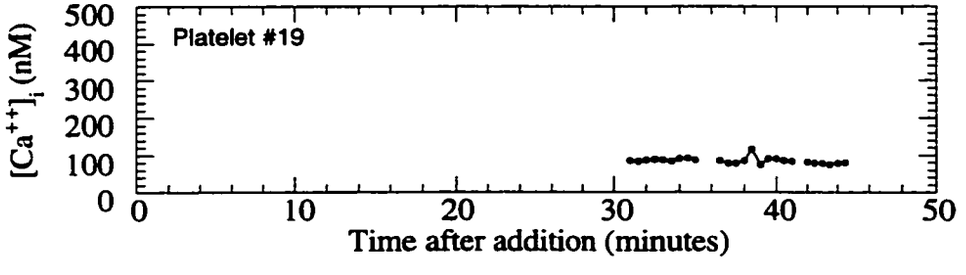

Platelet #14
$[Ca^{++}]_i$ (nM)
500
400
300
200
100
0
0
10
20
30
40
50
Time after addition (minutes)

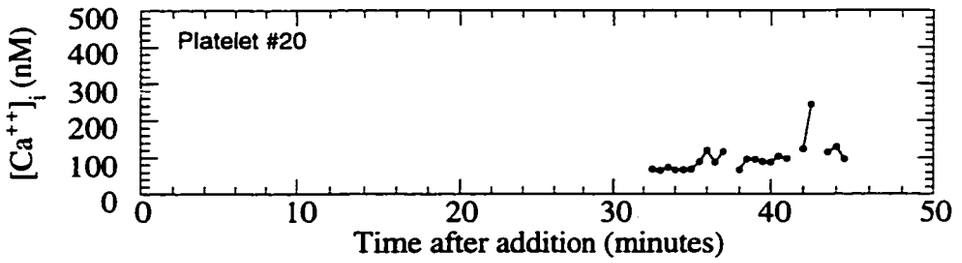

Platelet #15
$[Ca^{++}]_i$ (nM)
500
400
300
200
100
0
0
10
20
30
40
50
Time after addition (minutes)

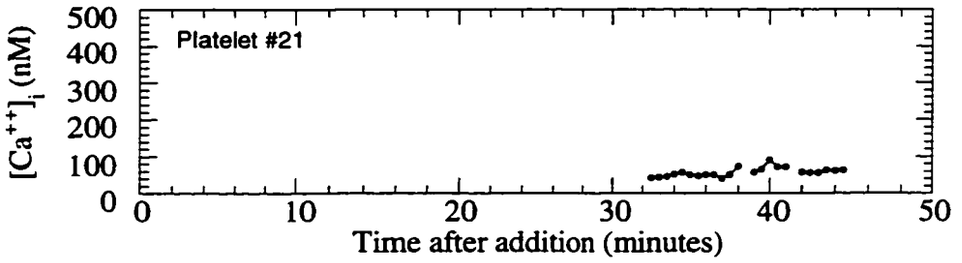

Platelet #16
$[Ca^{++}]_i$ (nM)
500
400
300
200
100
0
0
10
20
30
40
50
Time after addition (minutes)

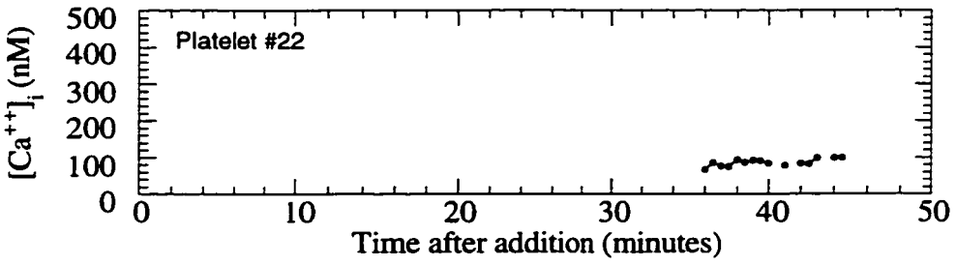

Platelet #17
$[Ca^{++}]_i$ (nM)
500
400
300
200
100
0
0
10
20
30
40
50
Time after addition (minutes)

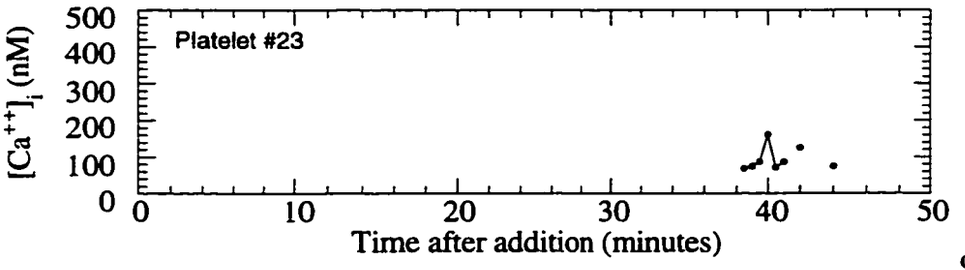

Platelet #18
$[Ca^{++}]_i$ (nM)
500
400
300
200
100
0
0
10
20
30
40
50
Time after addition (minutes)


Platelet #19
$[Ca^{++}]_i$ (nM)
500
400
300
200
100
0
0
10
20
30
40
50
Time after addition (minutes)

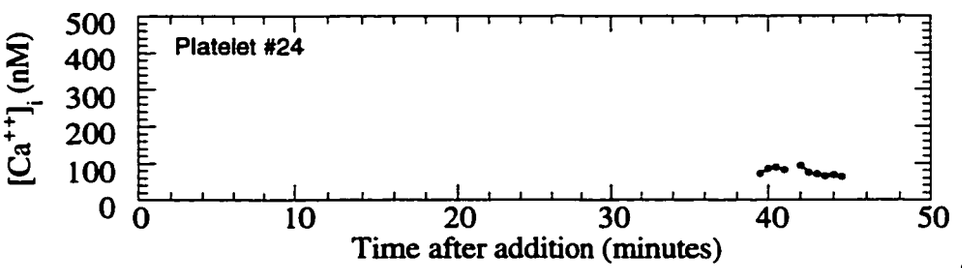

Platelet #20
$[Ca^{++}]_i$ (nM)
500
400
300
200
100
0
0
10
20
30
40
50
Time after addition (minutes)


Platelet #21
$[Ca^{++}]_i$ (nM)
500
400
300
200
100
0
0
10
20
30
40
50
Time after addition (minutes)

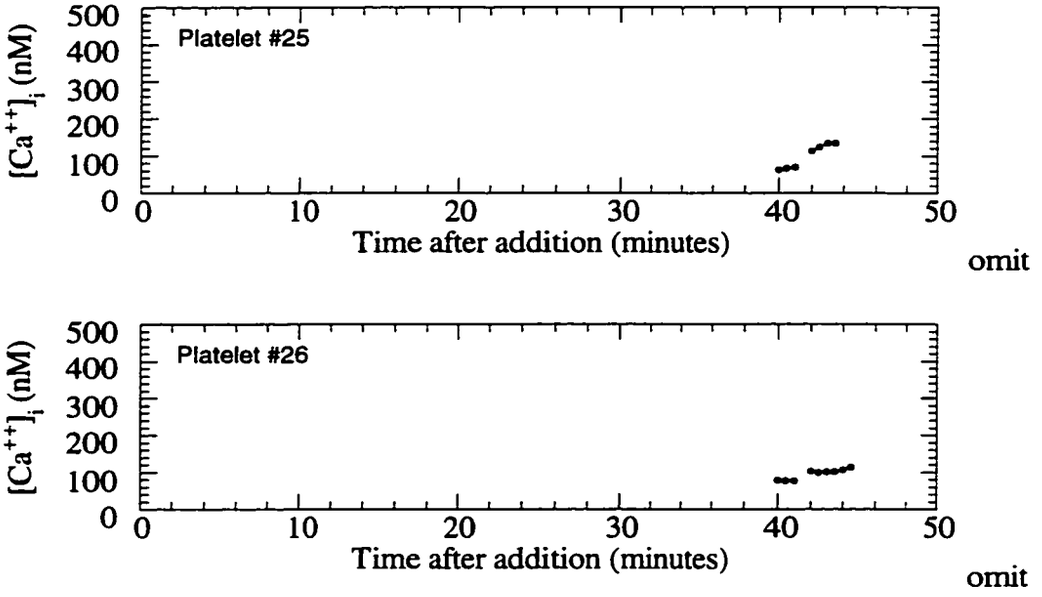

Platelet #22
$[Ca^{++}]_i$ (nM)
500
400
300
200
100
0
0
10
20
30
40
50
Time after addition (minutes)


Platelet #23
$[Ca^{++}]_i$ (nM)
500
400
300
200
100
0
0
10
20
30
40
50
Time after addition (minutes)

omit

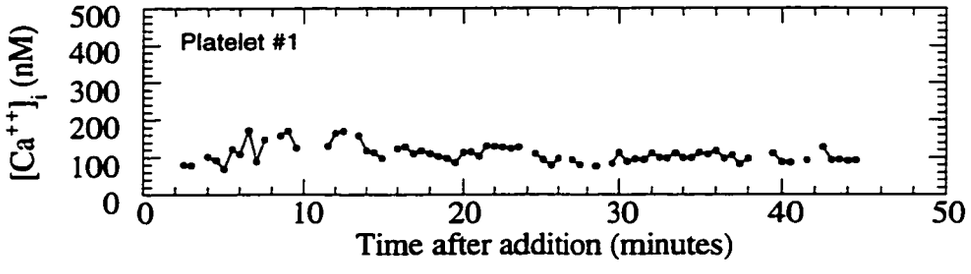

Platelet #24
$[Ca^{++}]_i$ (nM)
500
400
300
200
100
0
0
10
20
30
40
50
Time after addition (minutes)

omit

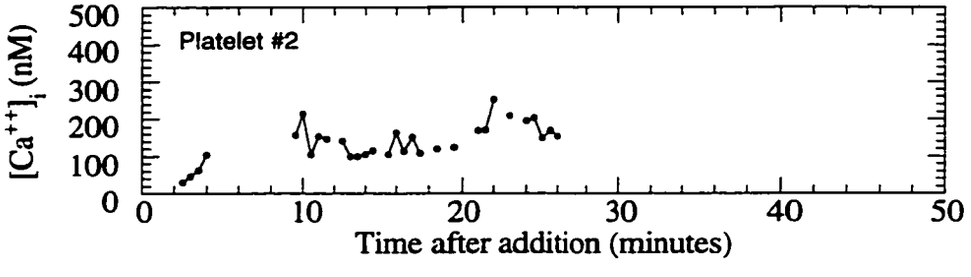

Platelet #25
$[Ca^{++}]_i$ (nM)
500
400
300
200
100
0
0
10
20
30
40
50
Time after addition (minutes)
omit

Platelet #26
$[Ca^{++}]_i$ (nM)
500
400
300
200
100
0
0
10
20
30
40
50
Time after addition (minutes)
omit

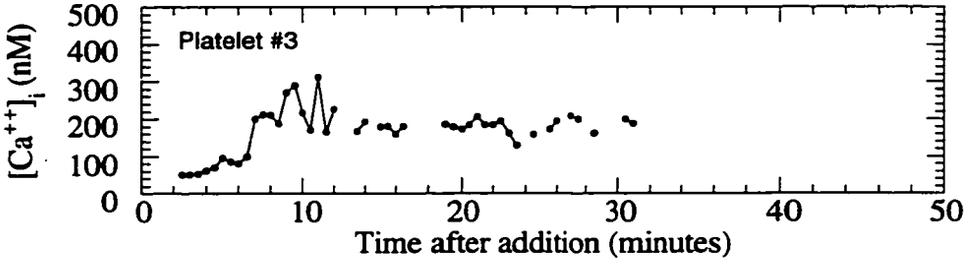
Platelets on Polystyrene



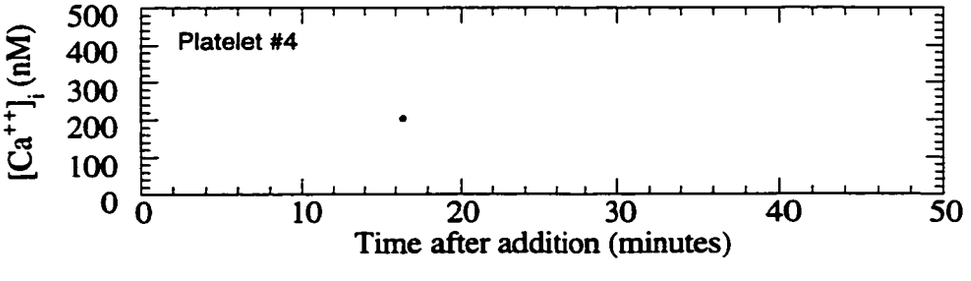
spread



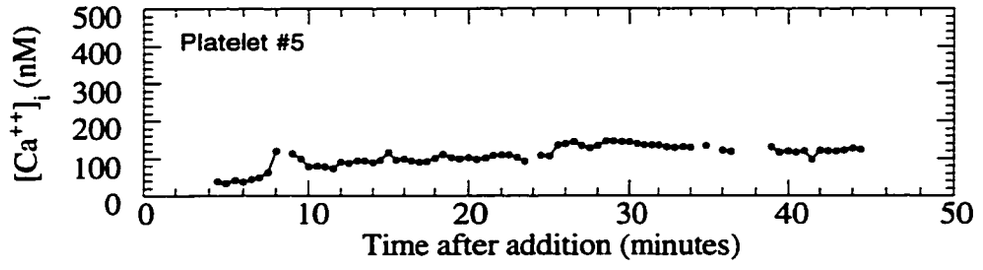
spread



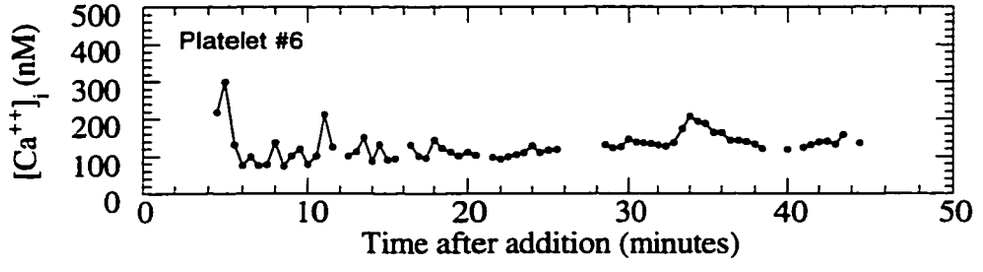
spread



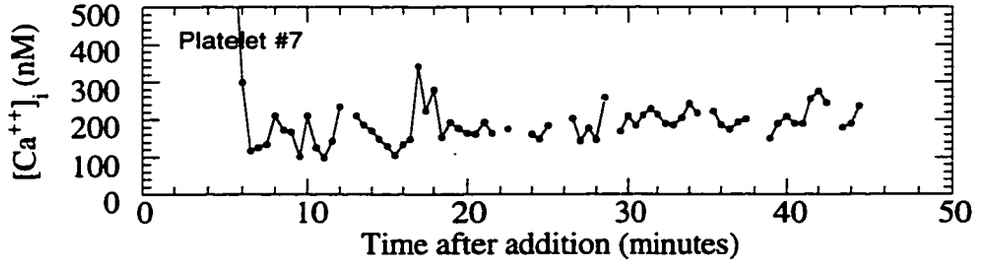
omit



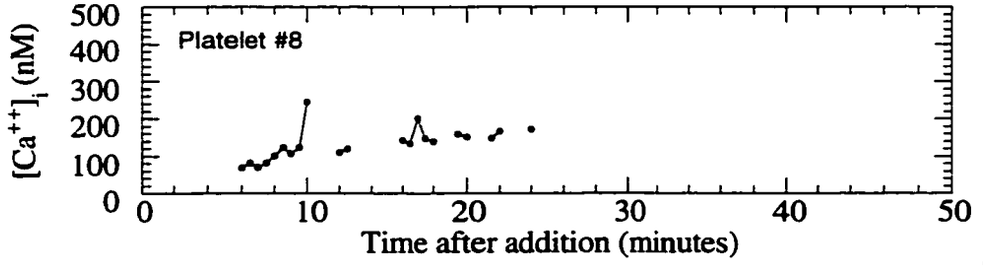
no change



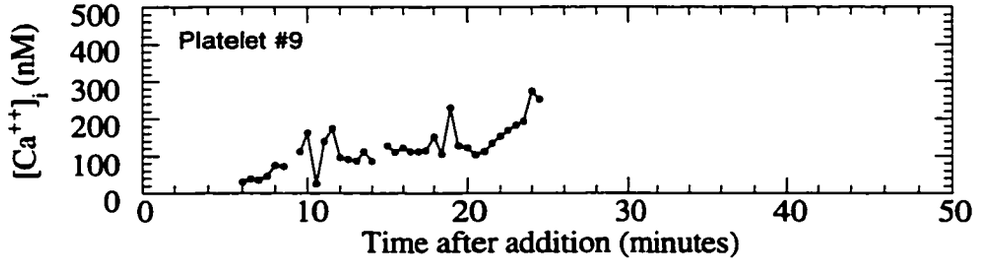
spread



spread

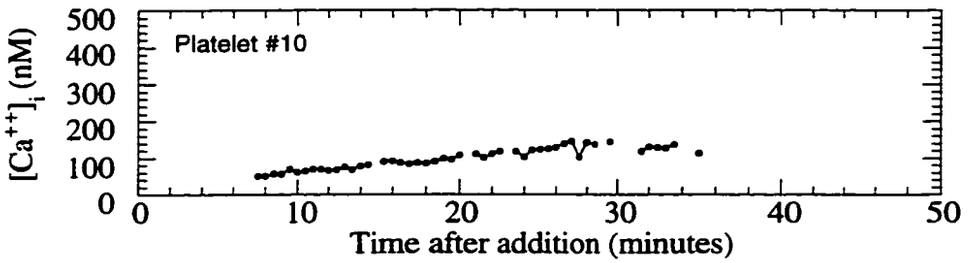


omit dim

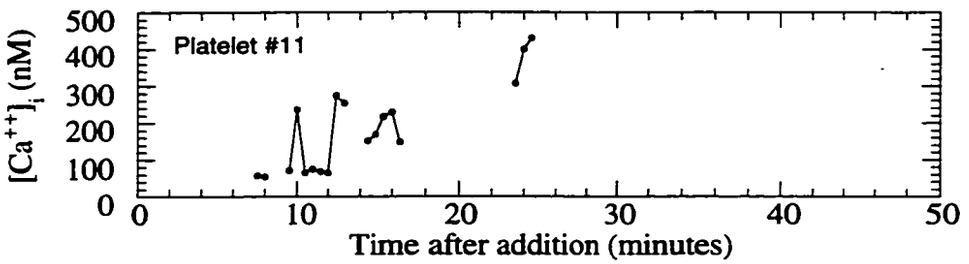


omit -leaves

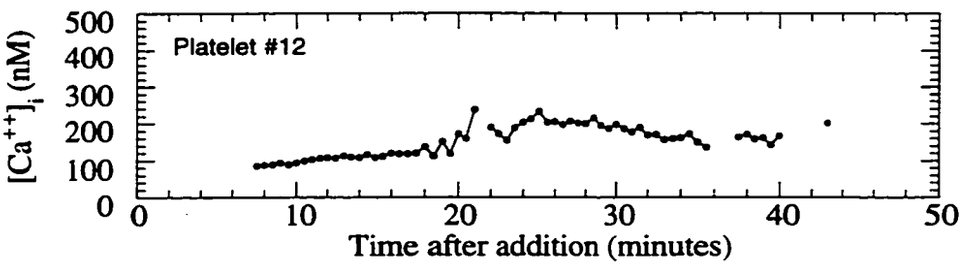
Figure 5. continued



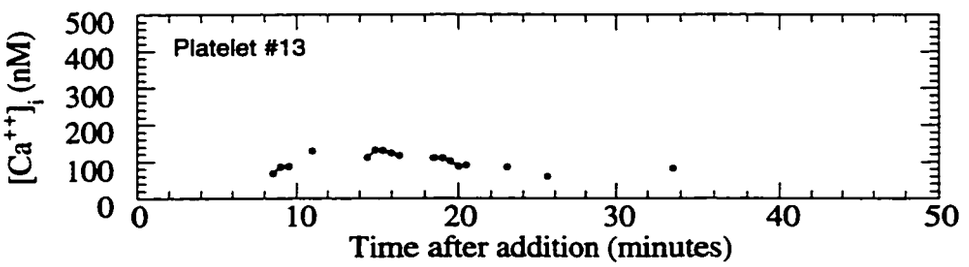
no change



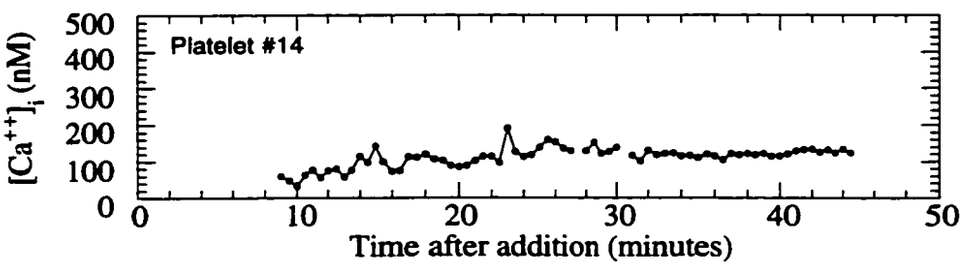
omit



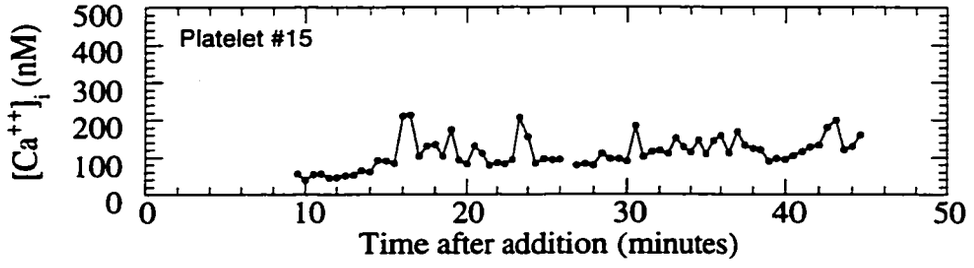
no change



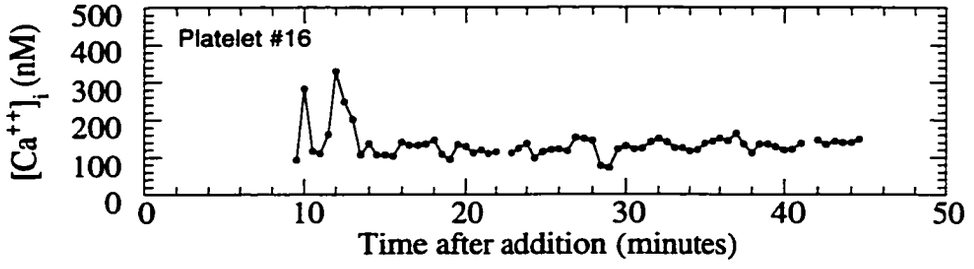
omit



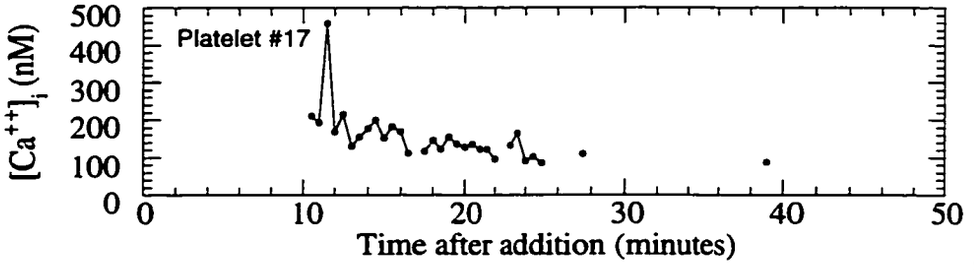
no change



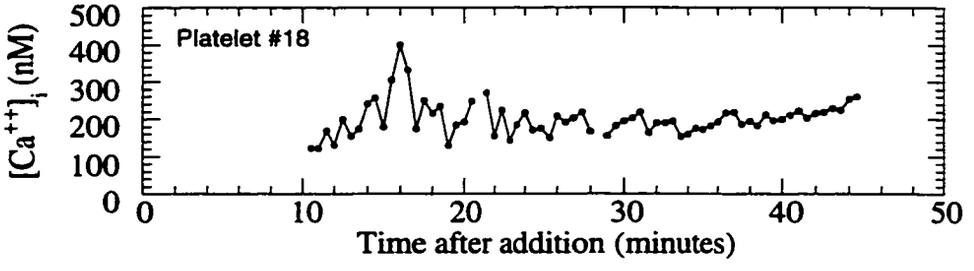
spread



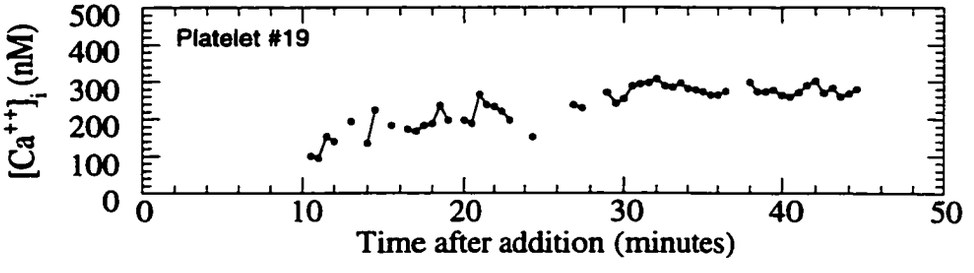
no change



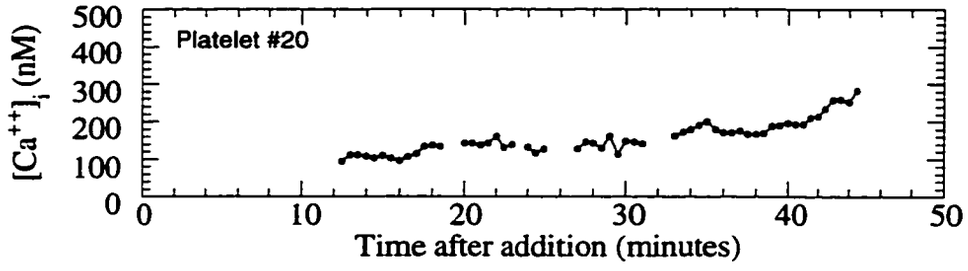
spread



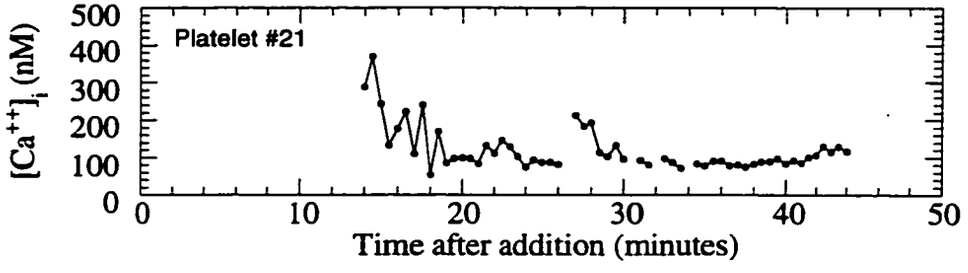
spread



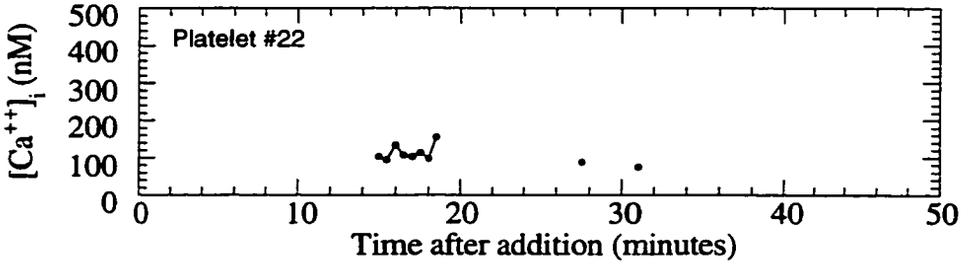
v. spread



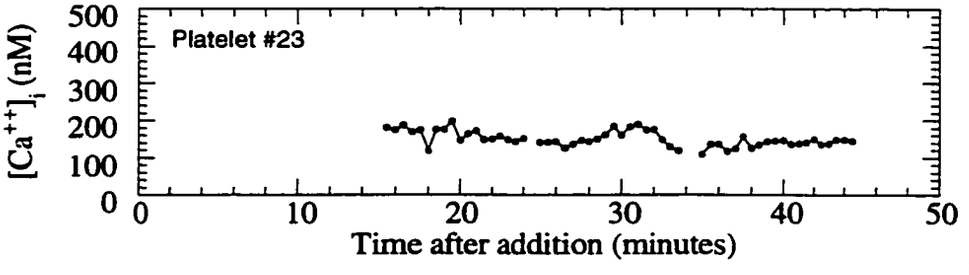
no change



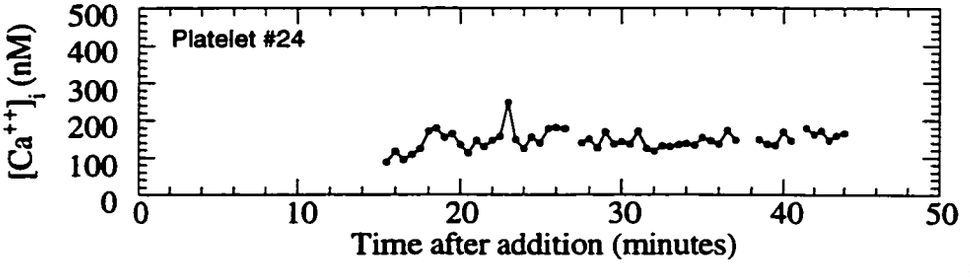
no change



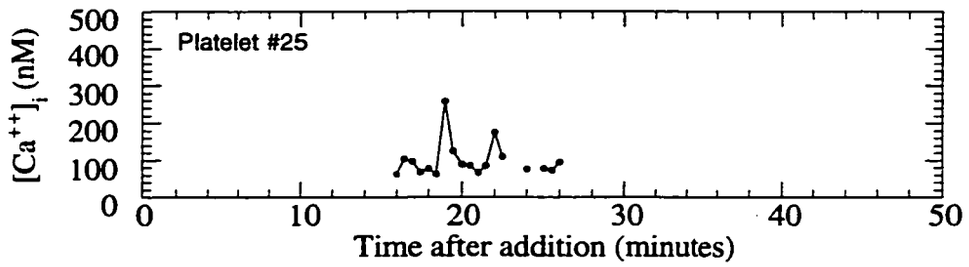
omit



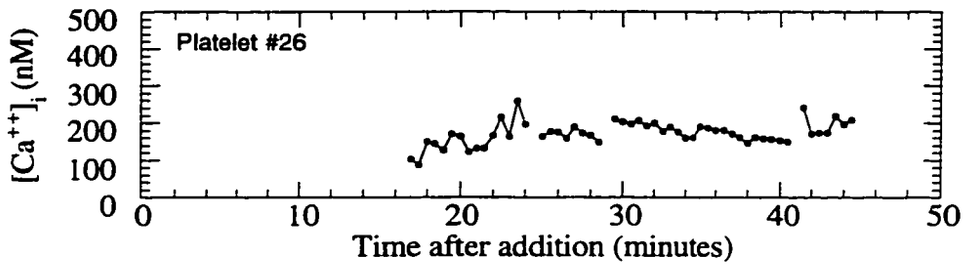
no change



spread



spread



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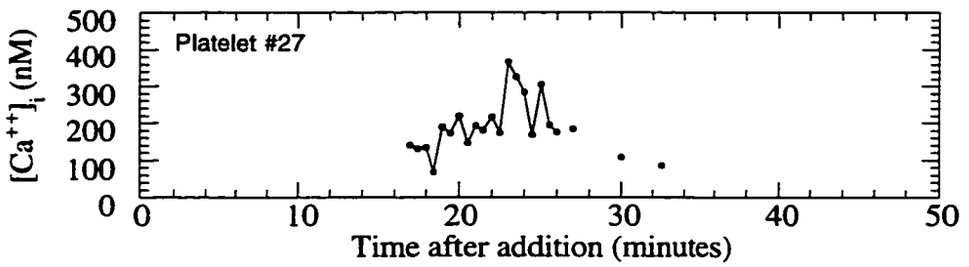

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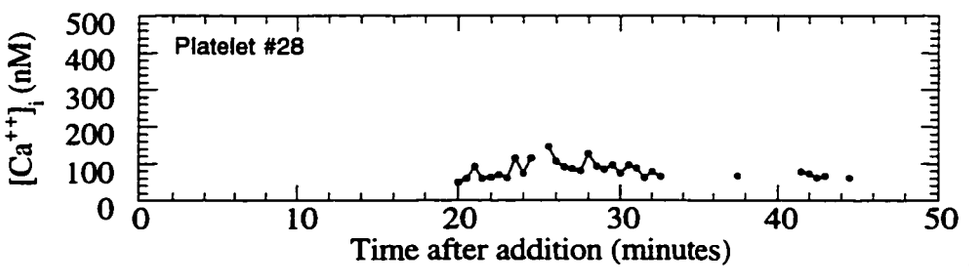

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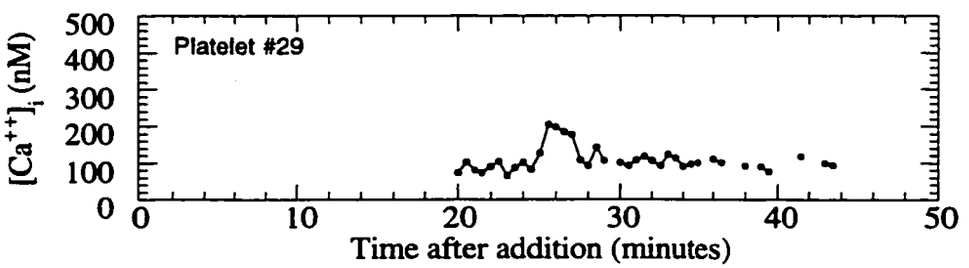

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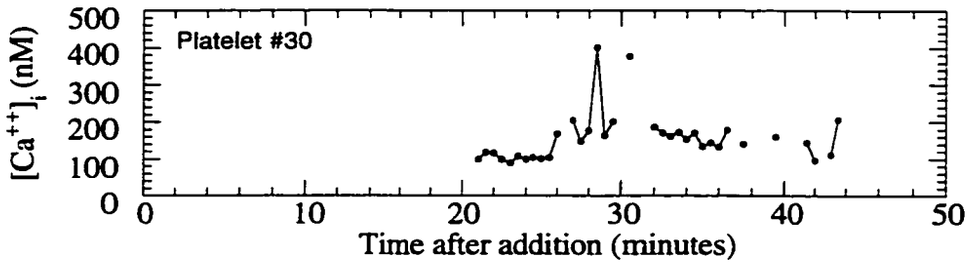

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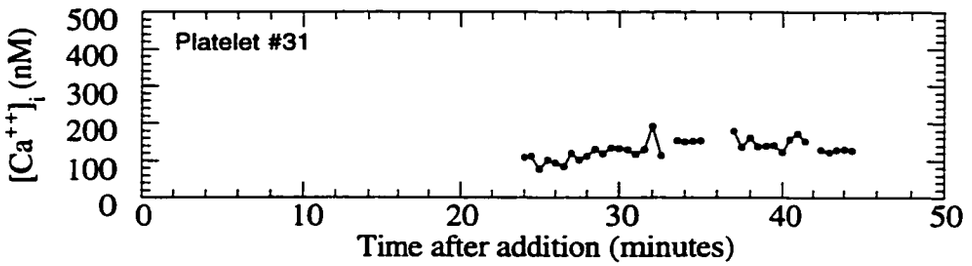

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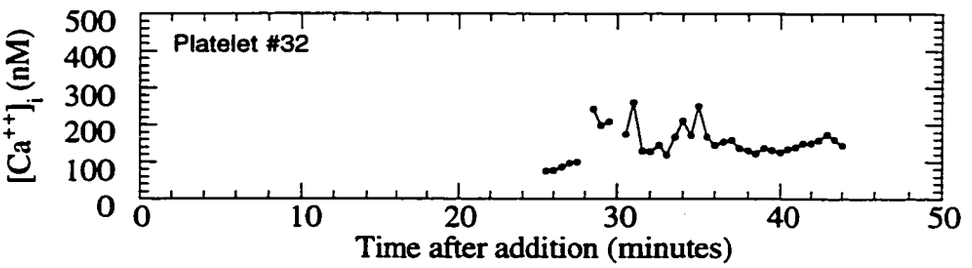

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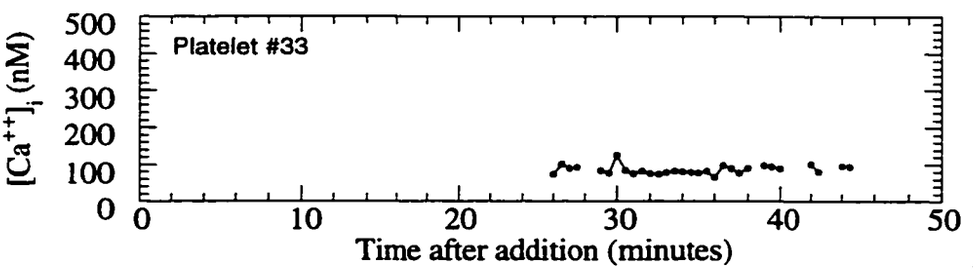

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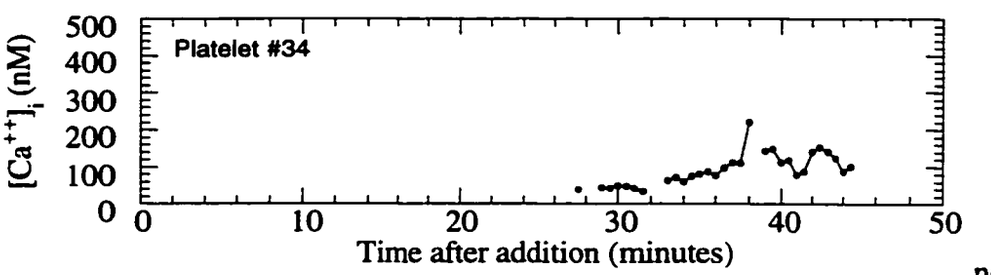

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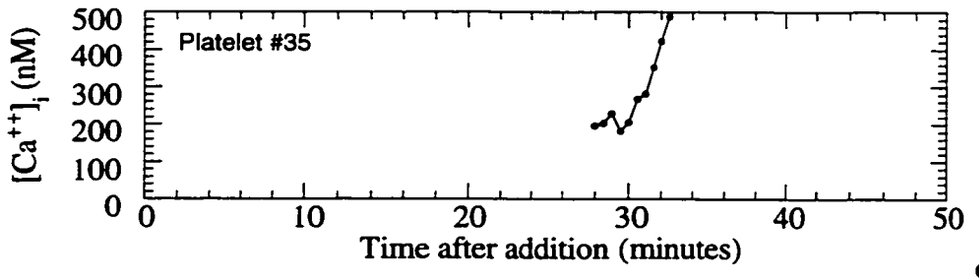

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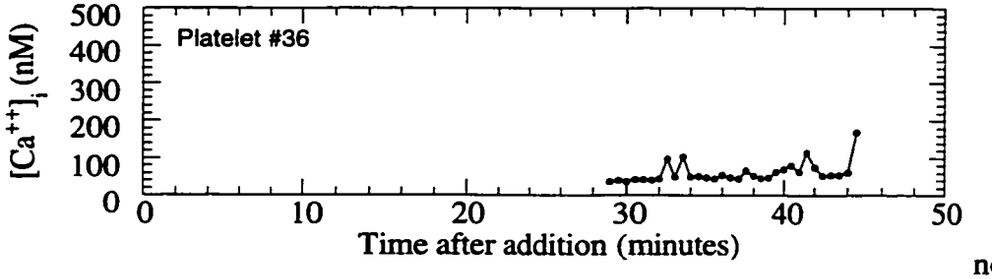

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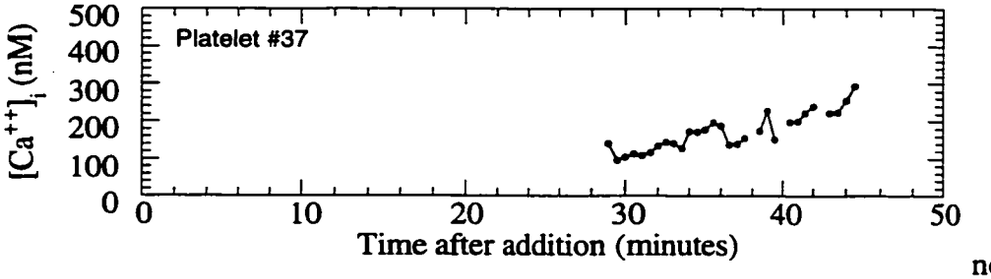

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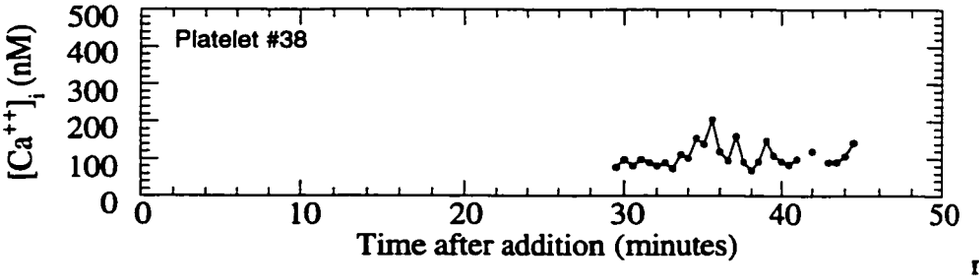

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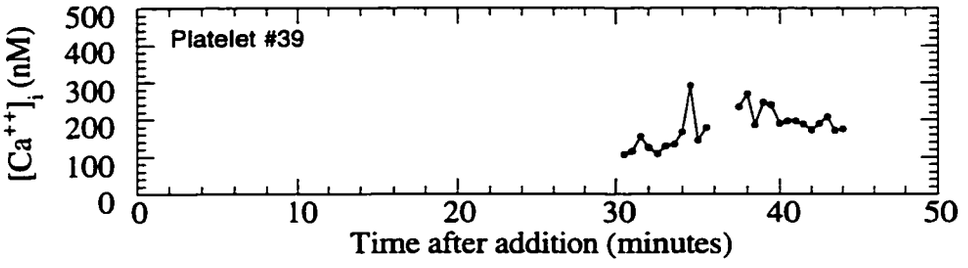

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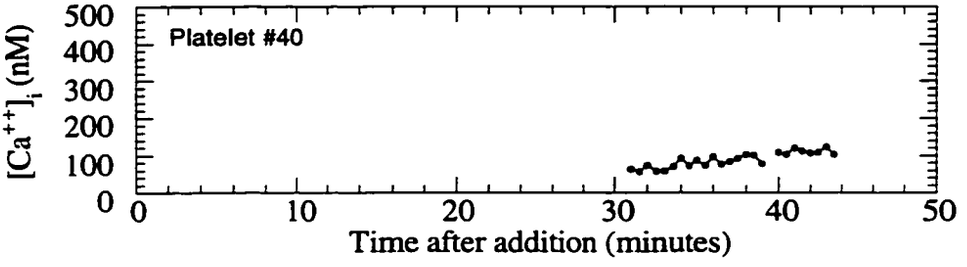

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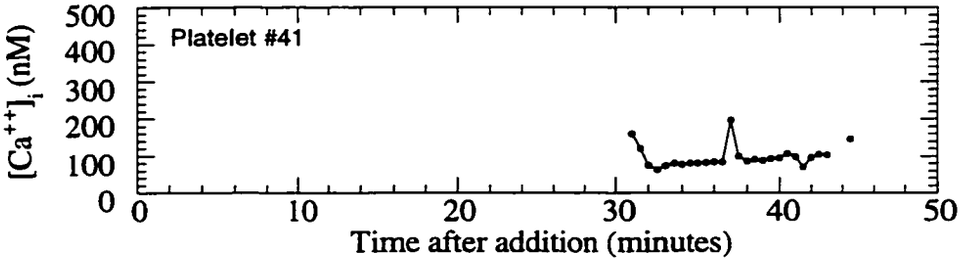

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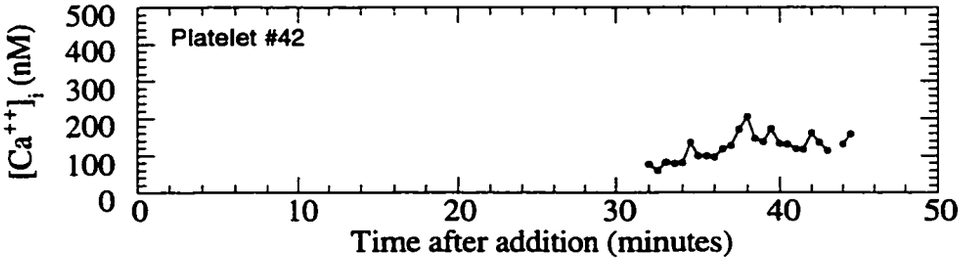

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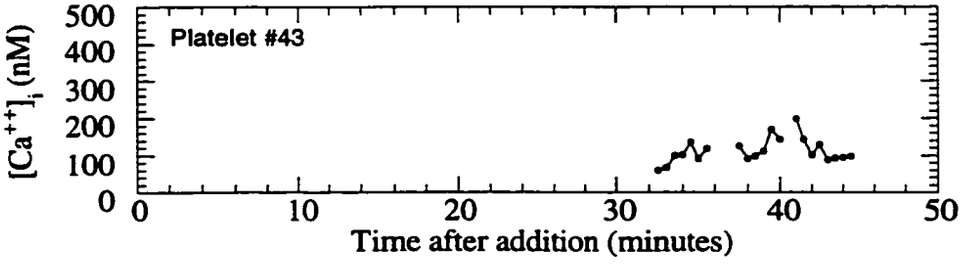

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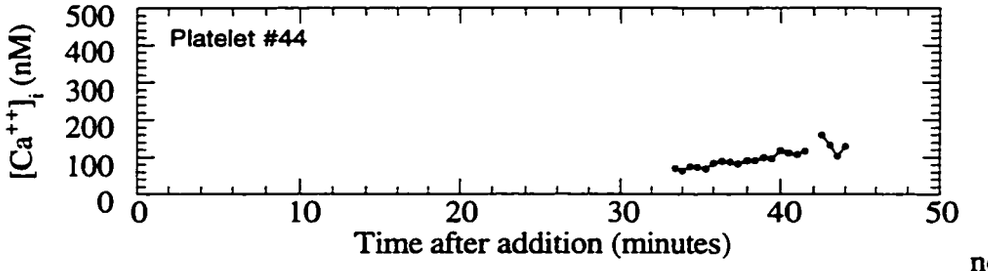

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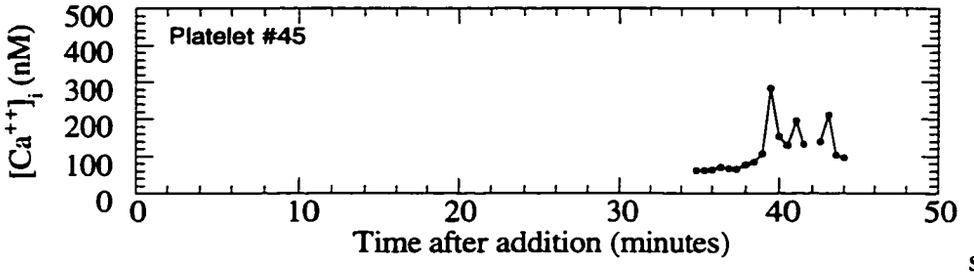

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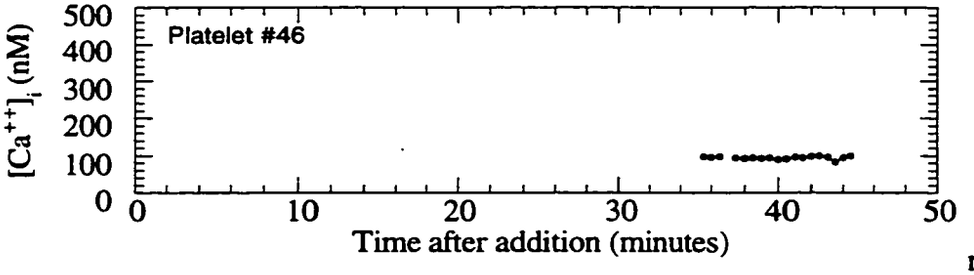

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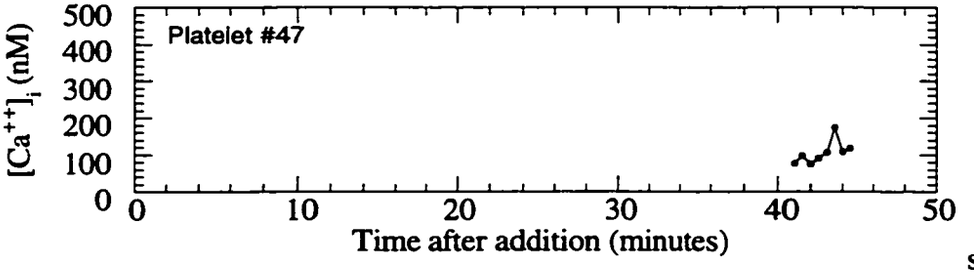

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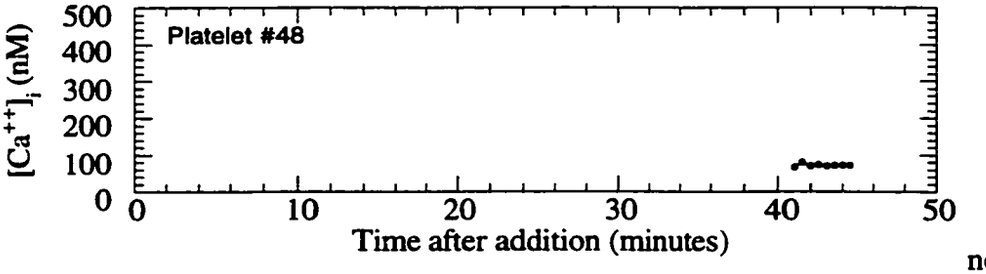

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Vita

Kip Dale Hauch was born in Minneapolis, MN on May 3, 1964 to Dale C. and Carol M. Hauch. He graduated from Grinnell High School, Grinnell, IA in 1982. He studied at the Univeristy of Iowa in the Department of Biomedical Engineering and transferred to the University of Minnesota in 1984, where he received a B.S. in Chemical Engineering in 1987. He then entered the Graduate Program in Department of Chemical Engineering at the University of Washington, where he joined the Biomaterials Group and conducted research on the activation of platelets by biomaterials. He received his Ph.D. from the University of Washington in 1997.

Awards and Honors

President, National Student Section, Society for Biomaterials: 1991-93.

McCarthy Award (Departmental) for Excellence in Graduate Teaching:1990, 1993,1994

Refereed Publications:

Hauch, KD and Horbett, TA: In Situ Calibration of the Calcium Ion Indicator Fura Red in Human Platelets, *submitted to Cell Calcium 9/97.*

Werthen, M. Hauch, KD, Shen, M and Horbett, TA: Calcium Mobilization in Monocytes as a Marker for Activation During Adhesion to Biomaterial Surfaces, *submitted to J. Biomat Sci Polymer Edn.*

Feuerstein IA, Buchan SM, Horbett TA, Hauch KD: Platelet Adherence and Detachment: a Flow Study with a Series of Hydroxyethyl Methacrylate-ethyl methacrylate Copolymers using Video Microscopy, *J Biomed Mater Res,* 25(2):185-198, 1991.

Abstracts and Presentations

Direct Observation of Cytosolic Free Calcium in Platelets During Material Contact: Epi-fluorescence Microscopy using Fura Red
K.D. Hauch and T.A. Horbett
Fourth World Biomaterials Congress, Berlin, 1992.

Platelet Activation by Biomaterials Indicated by Oscillations in Intracellular Free Calcium Concentration
K.D. Hauch and T.A. Horbett
Fifth World Biomaterials Congress, Toronto, 1996.

Ratio Fluorescence Imaging of Ca^{++} in Adherent Platelets Using Fura Red Suggests a Threshold in $[Ca^{++}]i$ Required for Spreading on Fibrinogen Coated Polyetherurethane Urea.
K.D. Hauch and T.A. Horbett
Annual Meeting of the American Institute of Chemical Engineers, Chicago, 1996

Ratio Fluorescence Imaging of Platelet Intracellular Calcium During Attachment to Biomaterials from Flowing Suspensions
K.D. Hauch and T.A. Horbett
Spring Meeting of the American Chemical Society, San Francisco, 1997.

Signal Transduction and Morphology in Individual Platelet-Biomaterial Interactions
K.D. Hauch and T.A. Horbett
Annual Meeting of the Society for Biomaterials, 1997 - (Poster)