

## INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

**The quality of this reproduction is dependent upon the quality of the copy submitted.** Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

# UMI

A Bell & Howell Information Company  
300 North Zeeb Road, Ann Arbor MI 48106-1346 USA  
313/761-4700 800/521-0600



## **NOTE TO USERS**

**The original manuscript received by UMI contains pages with indistinct and slanted print. Pages were microfilmed as received.**

**This reproduction is the best copy available**

**UMI**



Role of Phosphorylation of the Alpha One Subunit in Cyclic Adenosine  
Monophosphate Dependent Modulation of Skeletal Muscle Calcium  
Channels

by

Jeffrey P. Brousal


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

Doctor of Philosophy

University of Washington

Approved by \_\_\_\_\_

Chairperson of Supervisory Committee

 \_\_\_\_\_

Program Authorized to Offer Degree Pharmacology \_\_\_\_\_

Date 8-18-98 \_\_\_\_\_

**UMI Number: 9907882**

---

**UMI Microform 9907882  
Copyright 1998, by UMI Company. All rights reserved.**

**This microform edition is protected against unauthorized  
copying under Title 17, United States Code.**

---

**UMI**  
**300 North Zeeb Road**  
**Ann Arbor, MI 48103**

Doctoral Dissertation

In presenting this dissertation in partial fulfillment of the requirements for the Doctoral degree at the University of Washington, I agree that the Library shall make its copies freely available for inspection. I further agree that extensive copying of this dissertation is allowable only for scholarly purposes, consistent with "fair use" as prescribed in the U.S. Copyright Law. Requests for copying or reproduction of this dissertation may be referred to University Microfilms, 1490 Eisenhower Place, P.O. Box 975, Ann Arbor, MI 48106, to whom the author has granted "the right to reproduce and sell (a) copies of the manuscript in microform and/or (b) printed copies of the manuscript made from microform."

Signature *Jeffrey P. Brousal*

Date 8-18-98

University of Washington

ABSTRACT

Role of Phosphorylation of the Alpha One  
Subunit in Cyclic Adenosine  
Monophosphate Dependent Modulation of  
Skeletal Muscle Calcium Channels

by Jeffrey P. Brousal

Chairperson of the Supervisory Committee

Professor William A. Catterall, Ph.D.

Department of Pharmacology

Phosphorylation of the skeletal muscle  $\alpha_1$  subunit of the L-type calcium channel has been proposed to regulate the calcium current, allowing increased calcium flux during tetanus and during  $\beta$ -adrenergic activation. This stimulation has been shown to increase skeletal muscle contraction and calcium channel activity through cAMP-dependent phosphorylation of serine and threonine amino acid residues. The molecular mechanism of this phosphorylation has been studied by altering the serine and threonine residues to alanine to prevent phosphate transfer. These mutants have been

analysed biochemically and physiologically for changes in calcium channel regulation.

Oligonucleotide directed mutagenesis was used to alter serine and threonine amino acid residues to alanine in consensus sequences for phosphorylation by cAMP-dependent protein kinase. This prevented the incorporation of phosphate into this molecule. These wild type and mutant cDNAs were transiently transfected into a heterologous expression system with the rat brain L-type calcium channel  $\beta_{1b}$  subunit and the rabbit skeletal muscle L-type calcium channel  $\alpha_2/\delta$  subunit in human embryonic kidney cells expressing the large T antigen. This system allowed analysis of protein expression, protein phosphorylation and electrophysiology.

The calcium channels expressed dihydropyridine binding sites with  $K_d$  at 136 - 215 pM and  $\beta_{max}$  at 141 to 718 fmol/mg protein. The wild type protein had a molecular mass of 212 kDa and the truncated mutant protein had a molecular mass of 190 kDa in western blot analysis. The wild type protein had normal *in vivo* and *in vitro* cAMP-dependent protein kinase phosphorylation. A mutant protein containing 17 amino acid changes was not phosphorylated by cAMP-dependent protein kinase *in vivo* or *in vitro*.

The functional properties of wild-type and mutant channels and their regulation by cAMP-dependent protein kinase were studied by analysis of tail currents with barium as the permeant ion. These measurements showed no change in the wild type or mutant channel in the absence or presence of the activating agent, dcl-cBIMPS. This shows that the  $\alpha_1$  subunit of the rabbit skeletal muscle calcium channel is not the target of phosphorylation for the phenomena of increased calcium channel activity due to  $\beta$ -adrenergic stimulation.

## TABLE OF CONTENTS

LIST OF FIGURES .....	iii
LIST OF TABLES.....	v
LIST OF ABBREVIATIONS .....	vi
INTRODUCTION.....	1
Initiation of Skeletal Muscle Contraction : .....	1
Calcium Channel Subunits.....	1
cAMP-dependent Protein Kinase: .....	2
Phosphorylation: .....	3
Protein - Protein Interactions: .....	4
Hypothesis: .....	5
CHAPTER 1: CHANNEL MUTATIONS .....	6
INTRODUCTION: .....	6
Protein Phosphorylation: .....	6
Mutations:.....	11
RESULTS: .....	12
DISCUSSION: .....	12
CHAPTER 2: CHANNEL EXPRESSION AND MODULATION .....	14
INTRODUCTION: .....	14
Dihydropyridines: .....	14
Western Blot Analysis: .....	15
Phosphate Incorporation: .....	15

Electrophysiology: .....	16
RESULTS: .....	21
Calcium Channel Expression: .....	21
Protein Expression: .....	29
Phosphorylation Studies: .....	33
Functional Analysis of Wild-type and Mutant Calcium Channels: .....	36
DISCUSSION: .....	41
CHAPTER 3: PROTEIN - PROTEIN INTERACTIONS THAT MAY REGULATE CALCIUM CHANNEL EXPRESSION AND FUNCTION.....	44
INTRODUCTION: .....	44
RESULTS: .....	45
DISCUSSION: .....	49
CHAPTER 4:SUMMARY.....	47
CHAPTER 5: EXPERIMENTAL PROCEDURES .....	51
BIBLIOGRAPHY .....	67
APPENDIX A: Cardiac Calcium Channel .....	82

## I. LIST OF FIGURES

<i>Number</i>	<i>Page</i>
<b>Figure 1.1:</b> Structure of the DNA subcloning vectors for site directed mutagenesis	10
<b>Figure 2.1:</b> Structure of the DNA expression vectors for the heterologous expression system	18,19
<b>Figure 2.2:</b> Saturation binding of [ <sup>3</sup> H]-PN-200-110 to transiently expressed wild type and mutant skeletal muscle calcium channels and Scatchard analysis	23,24,25
<b>Figure 2.3:</b> Immunological characterization of rabbit skeletal muscle calcium channel $\alpha_1$ subunits expressed in tsA-201 cells	27
<b>Figure 2.4:</b> Immunological characterization of rabbit skeletal muscle calcium channel $\beta_{1b}$ subunits expressed in tsA-201 cells	28
<b>Figure 2.5:</b> <i>In vitro</i> western blot and PKA phosphorylation of the rabbit skeletal muscle calcium channel $\alpha_1$	

subunit, wild type and mutant	30
<b>Figure 2.6:</b> <i>In vivo</i> western blot and PKA phosphorylation of the rabbit skeletal muscle calcium channel $\alpha_1$ subunit, wild type and mutant	31
<b>Figure 2.7:</b> <i>In vivo</i> western blot and PKA phosphorylation of the rabbit skeletal muscle calcium channel $\beta_{1b}$ subunit	32
<b>Figure 2.8:</b> Electrophysiology analysis of wild type calcium channels expressed in tsA-201 cells	35
<b>Figure 2.9:</b> Tail currents in wild type and mutant calcium channel expressing in tsA-201 cells	38

## II. LIST OF TABLES

<i>Number</i>	<i>Page</i>
<b>Table 1.1:</b> Consensus sequences for serine/threonine to alanine mutations in the rabbit skeletal muscle calcium channel $\alpha_1$ subunit	8
<b>Table 1.2:</b> Primers for mutagenesis	9
<b>Table 2.1:</b> Bmax values for [ <sup>3</sup> H]-PN-200-110 binding to membranes from various tissues	20
<b>Table 2.2:</b> Wild-type and Mutant Calcium Channel Tail Current Increases	40

### III. LIST OF ABBREVIATIONS

ATP	Adenosine triphosphate
BSA	Bovine serum albumin
B <sub>max</sub>	Concentration of binding sites
cAMP	Cyclic adenosine monophosphate
cDNA	Copy deoxyribonucleic acid
Ci	Curie
CPM	Counts per minute
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytosine triphosphate
dGTP	Deoxyguanosine triphosphate
dNTP	Deoxyribonucleotide triphosphate
dTTP	Deoxythymidine triphosphate
DMEM	Dulbecco's minimal essential medium

DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EC50	Drug concentration producing half-maximal effect
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal bovine serum
fmol	Femtomole
Hepes	N-2-hydroxyethylpiperazine-N'-2 etanesulfonic acid
HPLC	High performance liquid chromatography
hr	Hour
kb	Kilobase
Kd	Dissociation constant
kD	Kilodalton
M	Molar

mg	Milligram
μg	Microgram
min	Minute
ml	Milliliter
μl	Microliter
mmol	Millimole
mM	Millimolar
μM	Micromolar
mRNA	Messenger ribonucleic acid
ms	Millisecond
nM	Nonomolar
pmole	Picomole
pM	Picomolar
PCR	Polymerase chain reaction

PKA	cAMP-dependent kinase
RT	Room temperature
SD	Standard deviation
SDS	Sodium dodecylsulfate
SDS-PAGE	SDS-Polyacrylamide gel electrophoresis
TCA	Trichloroacetic acid

## ACKNOWLEDGMENTS

The author wishes to thank several people without whom this work would not have been possible. Dr. William A. Catterall has provided ideas and advice invaluable in the completion of this work. Drs. Charles Chavkin, Karen DeJongh, Albert Gordon, Neil Nathanson, and Stanley McKnight have provided important insight and precious time as members of my thesis committee. The members of the Catterall laboratory and the entire Pharmacology Department have been very helpful in the day to day work necessary for such an endeavor.

This work was supported in part by a grant from the Muscular Distrophy Association.

Also my parents, Peter and Elsa Brousal, should be acknowledged for their careful guidance and stimulating efforts in my upbringing.

Finally, I would like to acknowledge my daughter, Caliope Brousal, for her untiring efforts to remind me of the need to finish this project and give her a chance to explore and enjoy the world.

## DEDICATION

The author wishes to dedicate this dissertation to his daughter, Caliope Ann Brousal.

## IV. INTRODUCTION

### **Initiation of Skeletal Muscle Contraction**

Skeletal muscle contraction is initiated by cytosolic free calcium released from the sarcoplasmic reticulum in response to the depolarization of the transverse tubule membrane as an action potential travels along the sarcolemma (Schneider, et al., 1973; Beam, et al., 1986; Rios, et al., 1987; Catterall, 1991). Calcium is released from ryanodine sensitive calcium channels (calcium release channels) in the sarcoplasmic reticulum which are thought to be linked to voltage sensitive calcium channels in the transverse tubules through a junctional foot region (Schneider, et al., 1973; Lai, et al., 1988), a process called excitation-contraction coupling. The voltage sensitive, dihydropyridine-binding calcium channels (L-type calcium channels) in the transverse tubule are composed of five subunits. There is a central  $\alpha_1$  subunit of 212 kD, an intracellular  $\beta$  subunit of 55 kD, a glycosylated transmembrane  $\gamma$  subunit of 30 kD, a glycosylated  $\alpha_2$  subunit of 130 kD disulfide linked to a glycosylated 28 kD  $\delta$  subunit (Takahashi, et al., 1987).

### **Calcium Channel Subunits**

The  $\alpha_1$  subunit, enriched in transverse tubules, forms the ion channel and contains the binding sites for calcium antagonist drugs (Curtis, et

al., 1984, 1986; Singer, et al., 1991; Flockerzi, et al., 1986; Takahashi, et al., 1987). Although the cDNA encodes a 212 kD protein (Tanabe, et al., 1987), two forms of the  $\alpha_1$  subunit can be immunoprecipitated from rabbit skeletal muscle, a 212 kD full length form and a 190 kD form truncated at the carboxyl terminus (De Jongh, et al., 1989). Purified, reconstituted calcium channel  $\alpha_1$  and  $\beta$  subunits are phosphorylated via a cAMP dependent protein kinase (PKA) *in vitro*, increasing calcium flux (Curtis and Catterall, 1985; Flockerzi, et al., 1986; Nunoki, et al., 1989).

#### **cAMP-Dependent Protein Kinase**

Activation of cAMP dependent protein kinase follows from epinephrine activation of  $\beta$ -adrenergic receptors to stimulation of adenylate cyclase to the production of cAMP.  $\beta$ -adrenergic receptors and adenylate cyclase exist in the T-tubule membranes of skeletal muscle (Caswell, et al., 1978). The activation of PKA by cAMP, cAMP analogues or direct application of catalytic subunits of PKA increases calcium channel activity, increases the amplitude of the peak current, and increases the open probability resulting in increased muscle tension (Hymel, et al., 1988; Arreola, et al., 1987; Flockerzi, et al., 1986). Long term activation of PKA increases the number of dihydropyridine receptors and decreases their affinity for dihydropyridines (Schmid, et al., 1985).

### **Phosphorylation**

Phosphorylation of the skeletal muscle  $\alpha_1$  subunit has been shown to occur at specific sites. Biochemical experiments have shown several to be preferentially phosphorylated. Serine 687 and 1617 have been shown to be phosphorylated in the 190 kD form of the channel (Rörhkasten, et al., 1988; De Jongh, et al., 1991). Serine 1757, 1772 and 1854 have been shown to be phosphorylated in the 212 kD form of the channel (Lai, et al., 1990; Rotman, et al., 1992; Rotman, et al., 1995).

Phosphorylation of these sites has been proposed to alter channel activity and protein - protein interactions. Calcium channel activity in rat and mouse skeletal muscle myotubes is increased by frequent, short depolarizations which mimics tetanic trains of action potentials (tetanic activity). This modulation includes shifts in voltage dependence to more negative membrane potentials and slowing of channel deactivation. This process is blocked by the PKA substrate inhibitor (PKI) and PKA anchoring inhibitors (Ht31) indicating a direct role for PKA in the process (Sculptoreanu, et al., 1993; Johnson, et al., 1994). Stimulation of PKA by the cAMP analogue Sp-5,6-DCI-cBIMPS increases calcium channel current two to four fold and slows deactivation. This effect is also blocked by PKI (Johnson, et al., 1997).

### **Protein - Protein Interactions**

Further evidence for essential protein-protein interactions in excitation-contraction coupling includes the following.

Mice with the lethal muscular dysgenesis mutation have an absence of skeletal muscle contraction in response to depolarization of the transverse tubule. This has been localized to the absence of the  $\alpha_1$  subunit in these mice (Knudson, et al., 1989; Chaudhari, et al., 1992). Using myotubes isolated from these mice, microinjection of the cDNA for the  $\alpha_1$  subunit of the calcium channel has restored voltage-gated calcium currents, coupling of membrane excitation with muscle contraction and intramembrane charge movements (Tanabe, et al., 1988; Adams, et al., 1990). A critical region for initiation of excitation-contraction coupling has been localized to the intracellular loop between transmembrane regions II and III containing serine 687 (Tanabe, et al., 1990).

Also, in mice homologous for disruption of the ryanodine receptor gene (RyR-1), L-type calcium channel activity is decreased 20 fold blocking excitation-contraction coupling. Injection of the RyR-1 cDNA into myotubes from these mice restores L-type calcium current and coupling (Nakai, et al., 1996). Purified, reconstituted calcium release channels are activated by peptides corresponding to the II-III loop of the  $\alpha_1$  subunit of the dihydropyridine receptor. This activity is blocked by phosphorylation of

serine 687 or mutation to alanine, indicating a requirement for the  $\beta$ -hydroxyl group on serine (Lu, et al., 1994; Lu, et al., 1995). Additionally, a peptide corresponding to a region of the dihydropyridine receptor's carboxyl terminus (amino acids 1487 to 1506) inhibits calcium release channel activity (Slavik, et al., 1997).

The calcium channel  $\beta$  subunit is also involved as the disruption of its gene also blocks excitation- contraction coupling (Gregg, et al., 1996).

### **Hypothesis**

These complex protein-protein interactions and phosphorylation events have led to the hypothesis that direct PKA phosphorylation of the  $\alpha_1$  subunit of the L-type calcium channel is responsible for the increase in calcium current resulting from  $\beta$ -adrenergic activation and tetanic stimulation. This regulatory process may be necessary in prolonged repeated muscle contractions that would deplete calcium stores of the muscle. In order to examine this hypothesis mutation of the relevant serines and threonines in the  $\alpha_1$  subunit was performed, and the resulting mutants were expressed in mammalian cells and analysed by biochemical and electrophysiological methods.

## V. CHAPTER 1: CHANNEL MUTATIONS

### A. INTRODUCTION:

#### **Protein Phosphorylation**

Protein phosphorylation at serine, threonine, and tyrosine amino acid residues alters protein function by direct and allosteric alteration of the protein structure or electrostatic modification of the charged surfaces of the protein. The  $\alpha_1$  and  $\beta$  subunits of the skeletal muscle calcium channel are substrates for PKA phosphorylation (Curtis and Catterall, 1985; Flockerzi, et al., 1986; O'Callahan, et al., 1988; De Jongh, et al., 1989; Nunoki, et al., 1989). This involves epinephrine or norepinephrine activation of the  $\beta$ -adrenergic receptor at the membrane. This signal is transduced to the cytoplasm and G protein activation occurs, allowing a GTP bound  $\alpha$  subunit of the G protein to interact with and stimulate adenylate cyclase, increasing concentrations of

cAMP which binds to the regulatory subunit of PKA allowing the catalytic subunit to interact with a protein and transfer a phosphate to a serine or threonine (Krebs, et al., 1979; Nukada, et al., 1987). The phosphate acceptor must be in an appropriate secondary and tertiary structure. In the primary protein sequence, certain amino acids have been evinced to be required for a phosphorylation event. These include motifs of the R/K-R/K-X-S/T or R/K-X-S/T followed by hydrophilic residues (Sonyang, et al., 1994). (See Table 1.1)

In many systems, alteration of the phosphate acceptor to a non-acceptor, has been shown to block phosphorylation and functional modulation of a protein (Xu, et al., 1996). In this system, the amino acid sequences shown in Table 1.1 have been chosen for alteration from serine or threonine to alanine in the  $\alpha_1$  subunit.

These alterations change the amino acid sequence to that of the peptide inhibitor of PKA, PKI (5-24) (R-R-X-A) thus preventing protein phosphorylation but not the binding of PKA to the peptide.

The nucleotide sequence of the cDNA for the skeletal muscle L-type calcium channel  $\alpha_1$  was used to design primers for the site directed mutagenesis. These primers had to have G-C content that would allow recognition and appropriate melting temperatures to allow annealing to the single stranded template. These primers are shown in Table 1.2.

**Table 1.1****Consensus sequences for serine/threonine to alanine mutations in the rabbit skeletal muscle calcium channel  $\alpha_1$  subunit.**

The amino acid residue(s) and its position in the primary protein sequence are indicated on the left. The amino acids that make up the consensus sequences for cAMP dependent protein kinase phosphorylation are shown. These amino acid substitutions were performed as described in the experimental procedures. A description of the sites' significance is added on the right.

1	Ser 387	KLS	near $\beta$ interaction domain
2	Ser 595	RRS	in pore loop, no expression
3	Ser 687	RRKMS	near ryanodine receptor interaction domain
4	Ser 1392	RDWS	conserved in cardiac channel, CP15 site
5	Thr 1501, Ser 1502	KRTS	conserved in cardiac channel
6	Thr 1552	KKDT	
7	Thr 1573, Ser 1575	RRTIS	conserved in cardiac channel
8	Thr 1601	RRRT	
9	Thr 1615, Ser 1617	RTNS	
10	Thr 1756, Ser 1757	RRTS	eliminated in truncation
11	Ser 1771, Ser 1772	RRSS	eliminated in truncation
12	Ser 1838	RES	eliminated in truncation
13	Ser 1853, Ser 1854	RRSS	eliminated in truncation

**Table 1.2****Primers for mutagenesis**

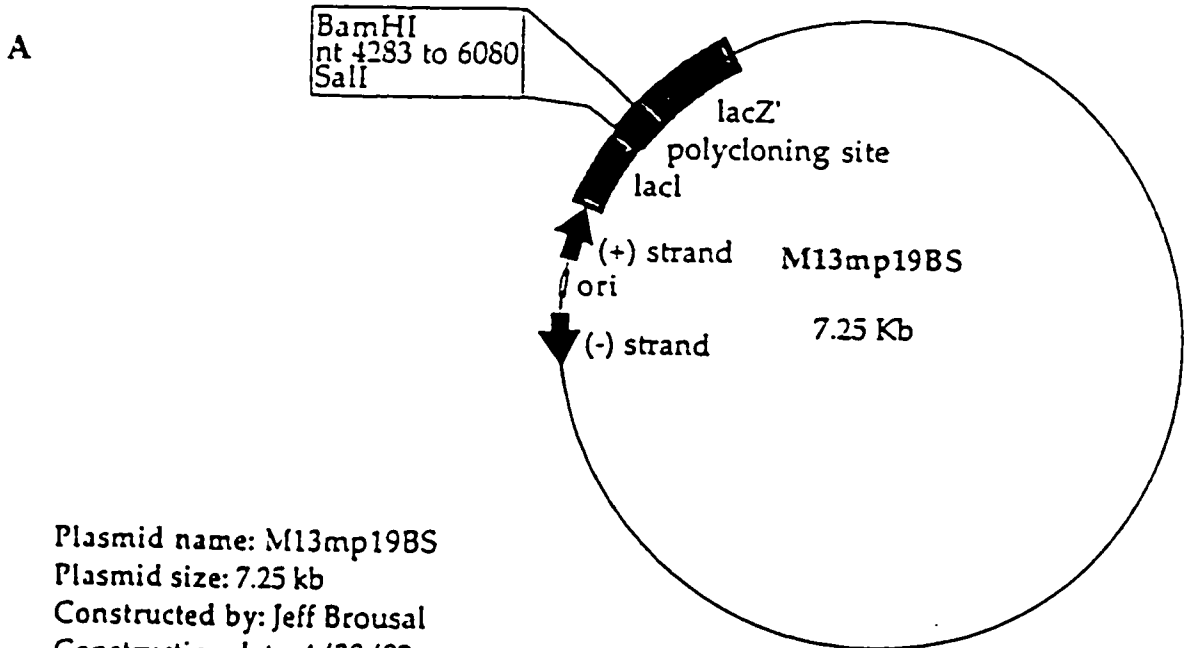
Oligonucleotides for site-directed mutagenesis are listed. On the left primer numbers are listed as JB with the # and sequencing primers are labelled as the # with S. This number is followed by the nucleotide sequence. This is followed by the order of amino acid in the primary sequence and the amino acid residue that is altered to alanine. Below the primer is given the original nucleotide.

JB1	AGGAAGATGGCCAGGGGTCTC	3	SER687
	T		
1S	AACCTGGCCGAGGCCGAGAGC		
JB2	TGGAAGAGAGCCGCCATGAAGCTAC	5	THR1501/SER1502
	A AG		
2S	AAGACAGAAGGTAACCTTCGAG		
JB3	AAGAAGGACGCCGTGCAGATCTHR1552	6	THR1552
	A		
JB4	ATCCGCCGCGCCATCGCAGGAGACCTG	7	THR1573/SER1575
	A T		
3/4S	GGGAAGTTCTACGCCACATTCC		
JB5	CAGAGGAGCCGCCCTGACAGGG	10	THR1756/SER1757
	A T		
JB6	GGAGGGCCGCCGAGGGG	11	SER1771/SER1772
	AG T		
5/6S	CCCATCAACCAGGCACCTCCTG		
JB7	AGCCGCAGGGCCGCCCTGGGCAGC	13	SER1853/SER1854
	T T		
7S	GCCACAGAGCTACTGAAAGAG		
JB8	AGCCTGCACTGAGAAAGGGAG		CYS1686-STOP
	T		
8S	CCAATACCAACAACGCAATGCC		
JB9	CCGGAGGGCCGGAGG	8	SER1601
	A G		
JB10	GGAAAGGGCCAACGCCCTGCCCCCG	9	SER1617
	A T		
9/10S	CCGCCGAGGAGGAGCTGG		
JB11	CGCGACTGGGCCATCCTGGGC	4	SER1392
	T		
11S	GCGCCTTCCTGATCATCAACC		
JB13	GGAAAGCTGGCCTTGGAAGAGGGG	1	SER387
	T		
13S	GGAGGAGCACCTTCGGGG		

**Figure 1.1****Structure of the DNA subcloning vectors for site directed mutagenesis.**

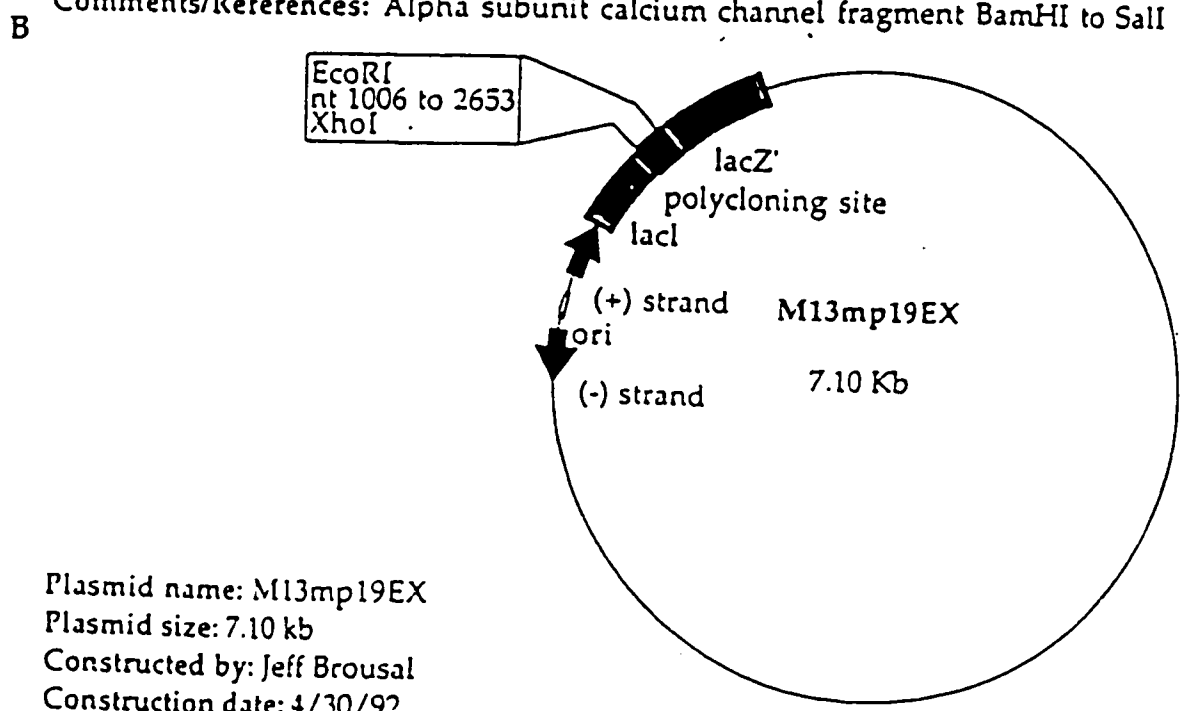
**A** M13mp19EX contains a EcoRI / XhoI fragment (nt 1006 to 2653) of the rabbit skeletal muscle calcium channel  $\alpha_1$  subunit used for site directed mutagenesis (see Experimental Procedures). This fragment contains the first three serines in the consensus sequences for PKA phosphorylation in the loop regions of the channel.

**B** M13mp19BS contains a BamHI / SalI fragment (nt 4283 to 6080) of the rabbit skeletal muscle calcium channel  $\alpha_1$  subunit. This fragment contains the last sixteen serines and threonines in the consensus sequences for PKA phosphorylation that lie in the carboxy tail region of the channel.



Plasmid name: M13mp19BS  
 Plasmid size: 7.25 kb  
 Constructed by: Jeff Brousal  
 Construction date: 4/30/92

Comments/References: Alpha subunit calcium channel fragment BamHI to SalI



Plasmid name: M13mp19EX  
 Plasmid size: 7.10 kb  
 Constructed by: Jeff Brousal  
 Construction date: 4/30/92

Comments/References: Alpha subunit calcium channel fragment EcoRI to XhoI

## Mutations

The mutations were made in the M13EX construct for sites 1 -3 and in M13BS for sites 4 - 13. M13BS also had a stop codon placed in the region where the short form of the calcium channel  $\alpha_1$  subunit would be proteolytically truncated, eliminating the last 4 sites in the clone. These mutations were performed serially so that they would occur together in each M13 subclone. The subclones were then digested with the appropriate restriction endonucleases and the purified fragments of calcium channel cDNA were reassembled by ligation into the expression vector, ZEM RVSP6  $\alpha_1$  CaCH. The phosphorylation deficient mutant, with all 18 serines and threonines mutated to alanine (not the non-expressing Ser 595) or removed by truncation was constructed as well as constructs with single residues altered or multiple residues altered (5,7,&9 or 10,11,&13) and no truncation. These expression clones were analysed by restriction endonuclease digestion to assure no spontaneous rearrangement occurred. Also, extensive sequence analysis was performed to determine all mutations to be included and to assure no spontaneous mutations might have occurred. The important clones for further analysis were the ones designated 3-13 or  $\Delta$  which has no sites for phosphorylation and 3,5-13 which has the serine 687 as a single site for phosphorylation.

## RESULTS:

All mutations were confirmed by sequencing (see Experimental Procedures, Chapter 5) in the M13 clone isolated after site directed mutagenesis protocols. After ligation into the expression vector, ZEM RVSP6 CaCH  $\alpha_1$ , all mutations were confirmed again by sequencing and the clone's integrity was determined by endonuclease restriction enzyme mapping.

The amino acids chosen to be mutated and expressed were based on protein biochemistry (Rörhkasten, et al., 1988; Lai, et al., 1990; De Jongh, et al., 1991; Rotman, et al., 1992; Rotman, et al., 1995). Thus the most rapidly phosphorylated sites *in vitro* were mutated first (serine 687 and 1854) and others followed as single mutations. Triple mutations were tried, as single mutations were found to have a wild type phenotype, and multiple mutations followed the triple mutations. When all reasonable consensus sequences for PKA phosphorylation were mutated and the wild type phenotype remained, protein phosphorylation studies were undertaken to assess whether phosphorylation had indeed been prevented.

## DISCUSSION:

The mutation of the skeletal muscle calcium channel was successful and as the following chapter will demonstrate phosphate incorporation was eliminated. The alteration of the serines and threonines to

alanine prevented phosphate transfer but not the interaction of PKA with the channel. The consensus sequence for the PKA inhibitor, PKI, is RRNA which comes from the regulatory subunit of the holoenzyme (Walsh, et al., 1990; Van Patten, et al., 1992). This subunit binds to the catalytic subunit in the absence of cAMP and blocks phosphorylation events. Thus, the mutational strategy would not address protein - protein interactions of the catalytic subunit and the substrate.

## VI. CHAPTER 2: CHANNEL EXPRESSION AND MODULATION

### INTRODUCTION:

#### **Dihydropyridines**

Voltage sensitive L-type calcium channels in skeletal muscle are characterized by pharmacological sensitivity to modulation by dihydropyridine calcium agonists and antagonists such as Bay K 8644 and isradipine (PN200-110) (Janis, et al., 1983; Hess, et al., 1984; Schramm, et al., 1985). Dihydropyridine antagonists promote null mode behavior; the channels are unavailable for opening. Dihydropyridine agonists prolong the open state of the channel (Kokubun, et al., 1984). Tritiated PN200-110, used as a radioactive ligand for saturation binding studies, determines channel protein expression levels. Dihydropyridine binding dissociation constants ( $K_d$ ) are an indication of the structural integrity of the protein and binding site concentrations ( $B_{max}$ ) are an indication of level of expression (Schmid, et al., 1985). Correlating this data with electrophysiological studies allows analysis of functional channels.

### **Western Blot Analysis**

Another method of determining protein expression is the western blot analysis. This entails preparing membrane microsomes or antibody precipitated protein complexes and separating these materials on a polyacrylamide gel in an electric field under reducing conditions (PAGE). Antibodies specific for subunits of the calcium channel will precipitate these proteins or recognize them when separated and transferred to a nitrocellulose membrane. The antibody recognition along with the migration on the gel are indications of the integrity and size of the protein and confirm expression. The antibodies used in this study were directed against the  $\alpha_1$  and  $\beta_{1b}$  subunits of the calcium channel.

### **Phosphate Incorporation**

Protein phosphorylation can be monitored by replacing the endogenous pool of phosphate with one that has had the radioisotope  $^{32}\text{P}$  incorporated into the phosphate. Cellular metabolism and its stimulation by PKA activating agents then creates radioactive phosphorylations that can be monitored by precipitating proteins with cognate antibodies and examining them in electrophoresis gels. This can give an idea of the amount of phosphate incorporated into a molecule.

Protein phosphorylation studies can be performed *in vitro* or *in vivo*. *In vitro* experiments involve purifying channel proteins, adding purified PKA, and initiating the phosphate transfer reaction with  $\gamma^{32}\text{P}$ -ATP. This method can be occluded by proteins that are already phosphorylated upon purification. The *in vivo* experiments involve a more exacting method which is to replace endogenous pools of ATP with  $^{32}\text{P}$  via the synthesis pathway. This allows the purification of endogenously phosphorylated proteins. Both of these methods were employed to collect proteins for PAGE analysis.

### **Electrophysiology**

L-type calcium channels derived from mouse skeletal muscle cells (129CB3 cells) have been analysed by electrophysiological methods and compared with heterologously expressed L-type calcium channels in human embryonic kidney cells (tsA-201 cells) (Johnson, et al., 1997). The subunits coexpressed in the tsA-201 cells are  $\alpha_1$ ,  $\beta_{1b}$ , and  $\alpha_2/\delta$  of the cloned L-type calcium channel (Ellis, et al., 1988; Pragnell, et al., 1991). This heterologous expression system allows comparison of wild type and mutant subunits of the calcium channel. The currents analysed are dependent on test potential. Peak currents are an indication of calcium entry into the cell and may affect the concentration of the calcium stores in repeated muscle use or tetanus

muscle contractions (Oz, et al., 1991). Even more calcium enters the cell upon repolarization from the test potential to the holding potential due to the increased driving force on calcium. This is seen in tail currents that occur upon repolarization. The regulation of these tail currents may be important in altering calcium concentrations in the cell. Phosphorylation of the channel may affect this calcium homeostasis (Sculptoreanu et al., 1993).

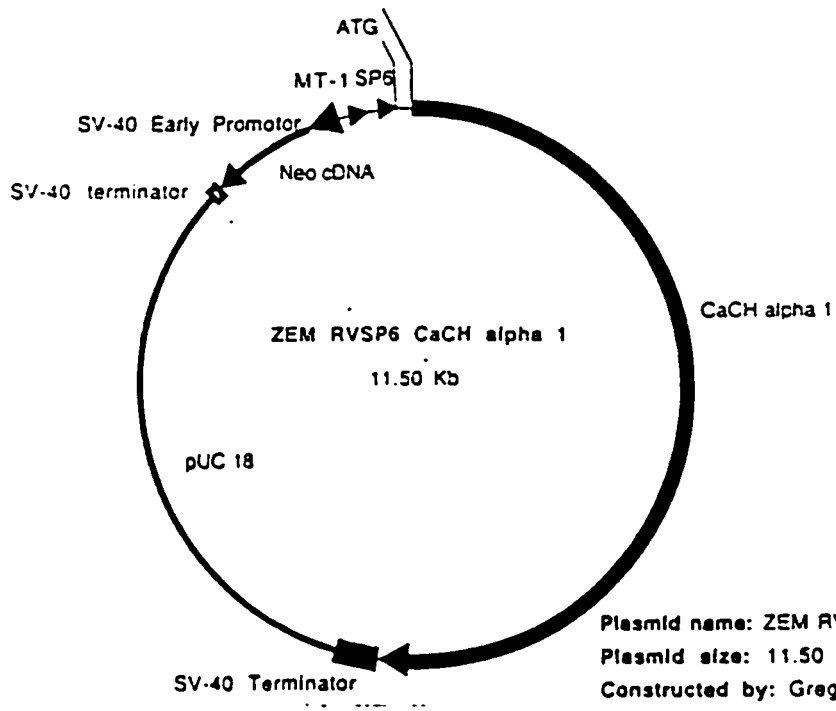
Dihydropyridine binding results, western blot analysis, protein phosphorylation studies and electrophysiological analysis together give information about specific protein expression and modulation by kinase in a physiological framework that allows understanding of the molecular events involved in channel regulation.

**Figure 2.1****Structure of the DNA expression vectors for the heterologous expression system**

**A** ZEM RVSP6 CaCH Alpha 1 contains the cDNA for the  $\alpha_1$  subunit of the rabbit skeletal muscle L-type calcium channel with a constitutive metallothionein promoter (MT-1) for in vivo RNA expression and a phage promoter for the SP6 RNA polymerase (SP6) for in vitro RNA production. RNA stability is conferred by an SV40 poly A tail. Ampicillin resistance is maintained and used for plasmid growth in the pUC 18 region of the plasmid and neomycin resistance is conferred under SV40 promotion and poly A tail.

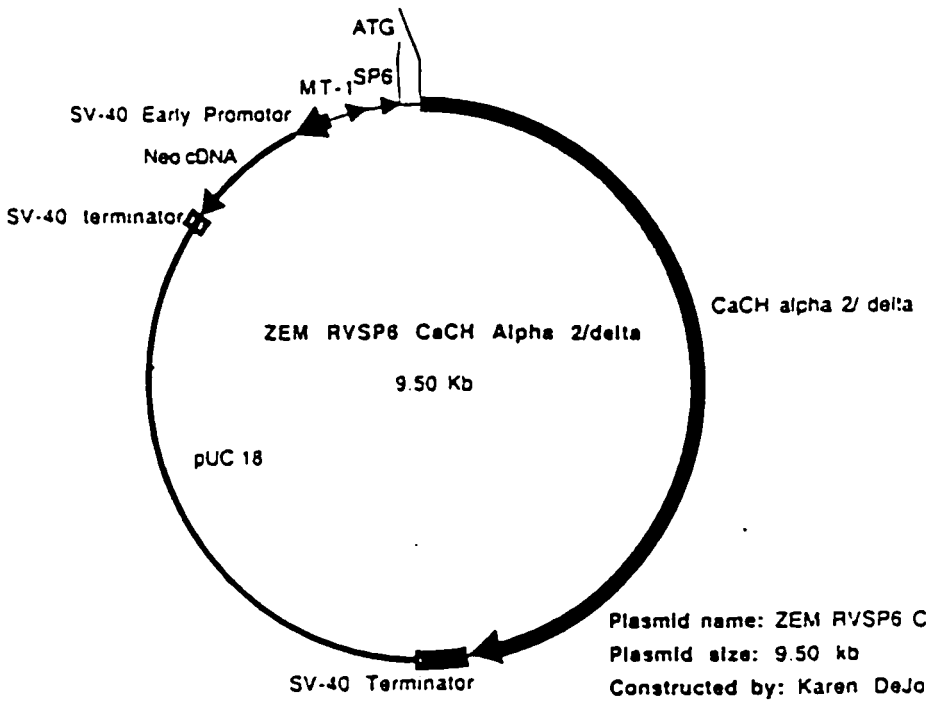
**B** ZEM RVSP6 CaCH Alpha 2 / delta contains the cDNA for the  $\alpha_2/\delta$  subunit of the rabbit skeletal muscle L-type calcium channel with a constitutive metallothionein promoter (MT-1) for in vivo RNA expression and a phage promoter for the SP6 RNA polymerase (SP6) for in vitro RNA production. RNA stability is conferred by an SV40 poly A tail. Ampicillin resistance is maintained and used for plasmid growth in the pUC 18 region of the plasmid and neomycin resistance is conferred under SV40 promotion and poly A tail.

**A**



Plasmid name: ZEM RVSP6 CaCH alpha 1  
Plasmid size: 11.50 kb  
Constructed by: Greg Hockerman

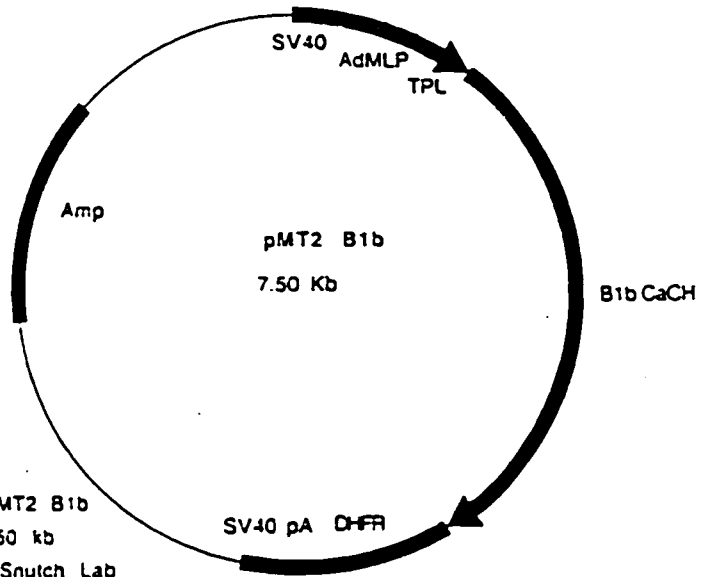
**B**



Plasmid name: ZEM RVSP6 CaCH Alpha 2/delta  
Plasmid size: 9.50 kb  
Constructed by: Karen DeJongh

C pMT2 B1b contains the cDNA for the  $\beta_{1b}$  subunit of the rat brain L-type calcium channel with an SV40 enhancer sequence in front of the adenovirus major late promoter and tripartite leader sequence cotranscribed with dihydrofolate reductase and an SV40 poly A tail. Ampicillin resistance is conferred by the beta-lactamase cDNA.

C



Plasmid name: pMT2 B1b  
Plasmid size: 7.50 kb  
Constructed by: Snutch Lab

**Table 2.1**

**Bmax values for [<sup>3</sup>H]-PN-200-110 binding to membranes  
from various tissues**

Concentrations of [<sup>3</sup>H]-PN-200-110 binding sites per milligram protein in different tissue preparations and cell lines. References are cited.

Skeletal muscle microsomes      2-5 pmol/mg protein

(Schramm et al., 1985; Kim et al., 1990)

Purified skeletal T tubules      15-18 pmol/mg protein

(Perez-Reyes et al., 1989)

L cells (selected)                      50-80 fmol/mg protein

(Perez-Reyes et al., 1989; Kim et al., 1990)

L cells (transient transfected)      130 fmol/mg protein

(Lacerda et al., 1991)

tsA-201 cells                              300-800 fmol/mg protein

(experimental results)

## RESULTS:

### Calcium Channel Expression

Several cell lines were examined for calcium channel expression. Chinese hamster lung cells (1610 cells) were used for expression of  $\alpha_1$  and  $\beta$  subunit RNA and protein. Expression of RNA was detected but dihydropyridine binding assays were near background levels. Dysgenic mouse fibroblast cells (TM38 cells) were used for  $\alpha_1$  subunit protein expression and current analysis. Endogenous calcium currents were found in these cells. Dysgenic mouse myocyte cells (DA3 cells) were used for  $\alpha_1$  subunit protein expression. These cells can be induced to differentiate to myotubes with reduced growth factors. Transfection protocols induced differentiation before  $\alpha_1$  subunit protein was expressed. Human embryonic kidney cells expressing the SV-40 large T antigen (tsA-201 cells) were found to be the best for protein expression and electrophysiology measurements. These cells were transfected successfully with molar ratio mixtures of  $\alpha_1$ ,  $\beta$ , and  $\alpha_2/\delta$  subunit cDNAs. A variant of these cells lacking the large-T antigen (HEK 293 cells) were also successfully transfected with wild type and mutant  $\alpha_1$  subunit cDNAs and selected for permanent cell lines.

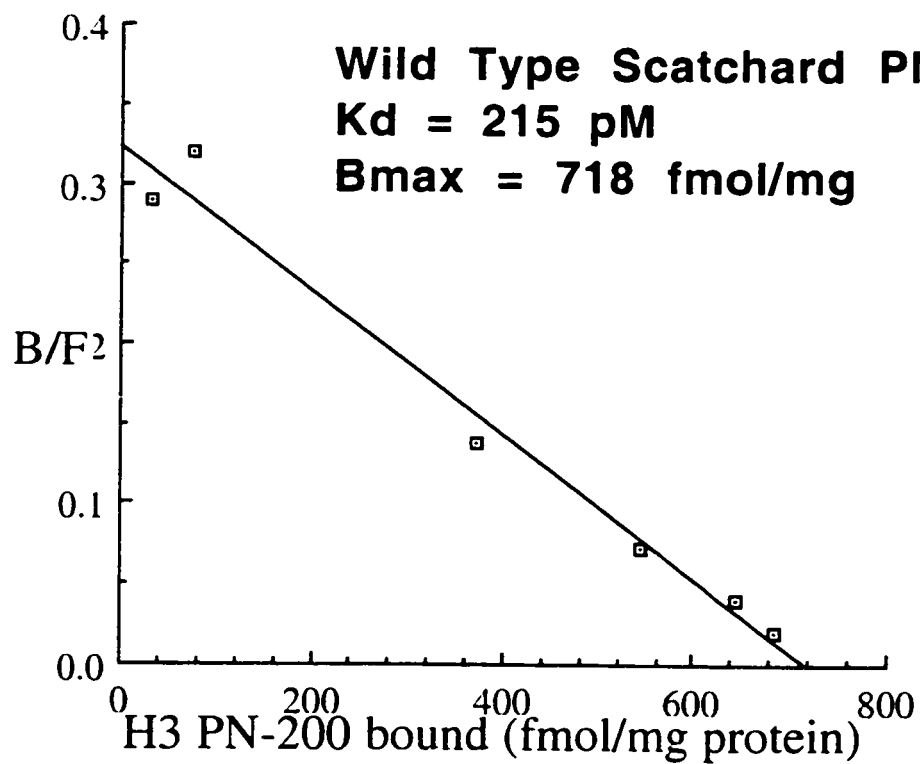
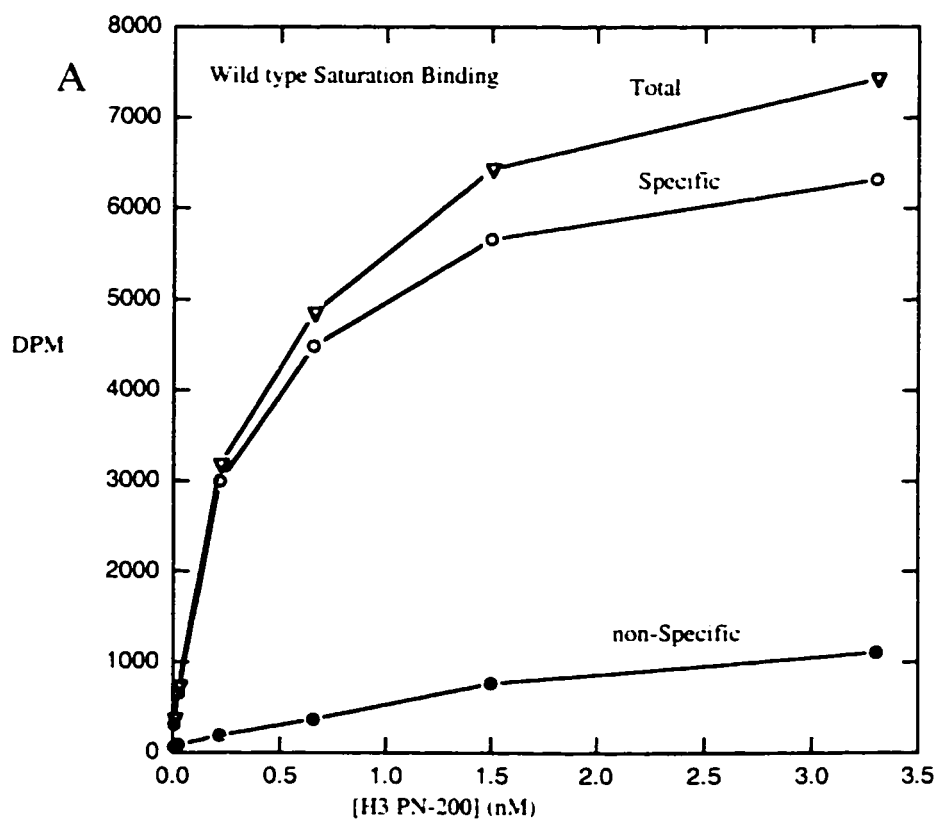
Wild type calcium channel transfections in tsA-201 cells resulted in transient expression of the dihydropyridine receptor with Bmax in the 500 to 750 fmol/mg protein range. Shown in Table 2.1 are expression levels of dihydropyridine receptors in various tissue preparations and expression systems. The heterologous system used here shows expression between that seen in L cells and that seen in muscle tissue. Mutant 3-13( $\Delta$ ) of the channel expressed Bmax of 130 to 350 fmol/mg protein. These expression levels are comparable for the ranges indicated with the mutant expressing one third to one half as well as the wild type channel. Affinity for the (+)-[<sup>3</sup>H]PN200-110 was similar but increased for the mutant with wild type being 215-245 pM and the mutant 3-13( $\Delta$ ) being 190-200 pM. This is consistent with Schmid's work on phosphorylation and expression (Schmid, et al., 1985).

**Figure 2.2**

**Saturation binding of [<sup>3</sup>H]-PN-200-110 to transiently expressed wild type and mutant skeletal muscle calcium channels and Scatchard analysis.**

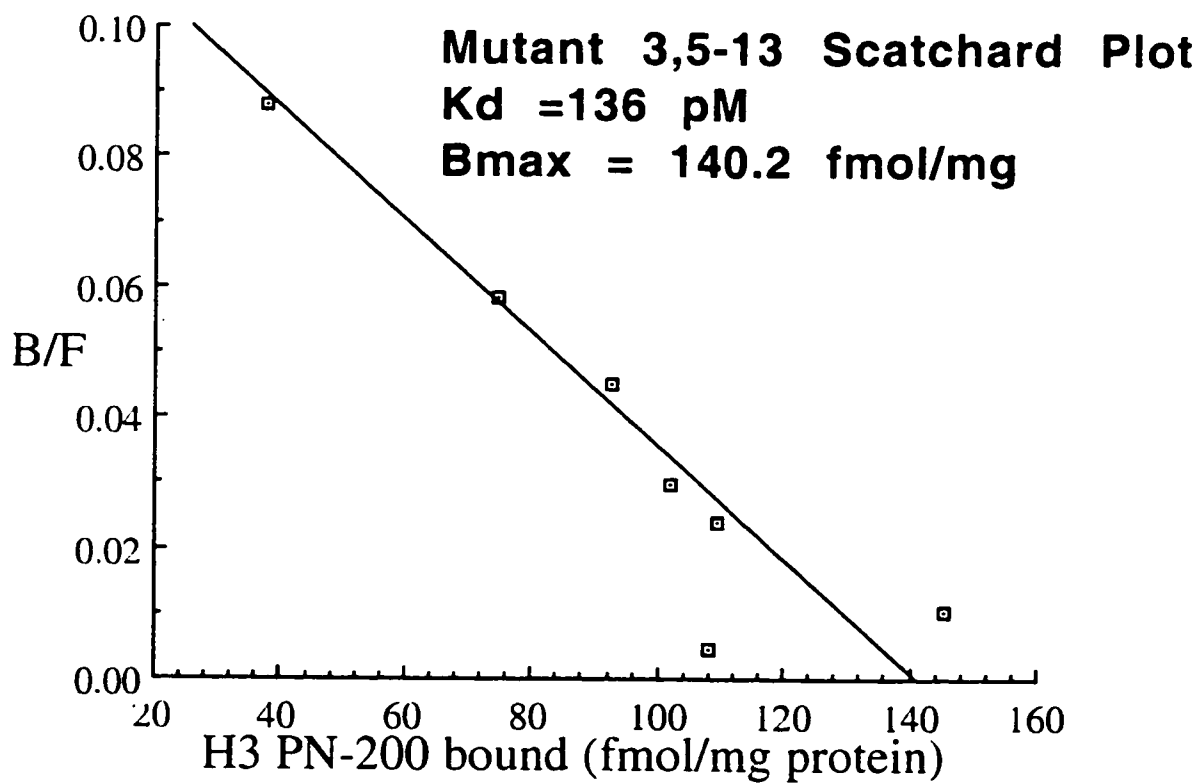
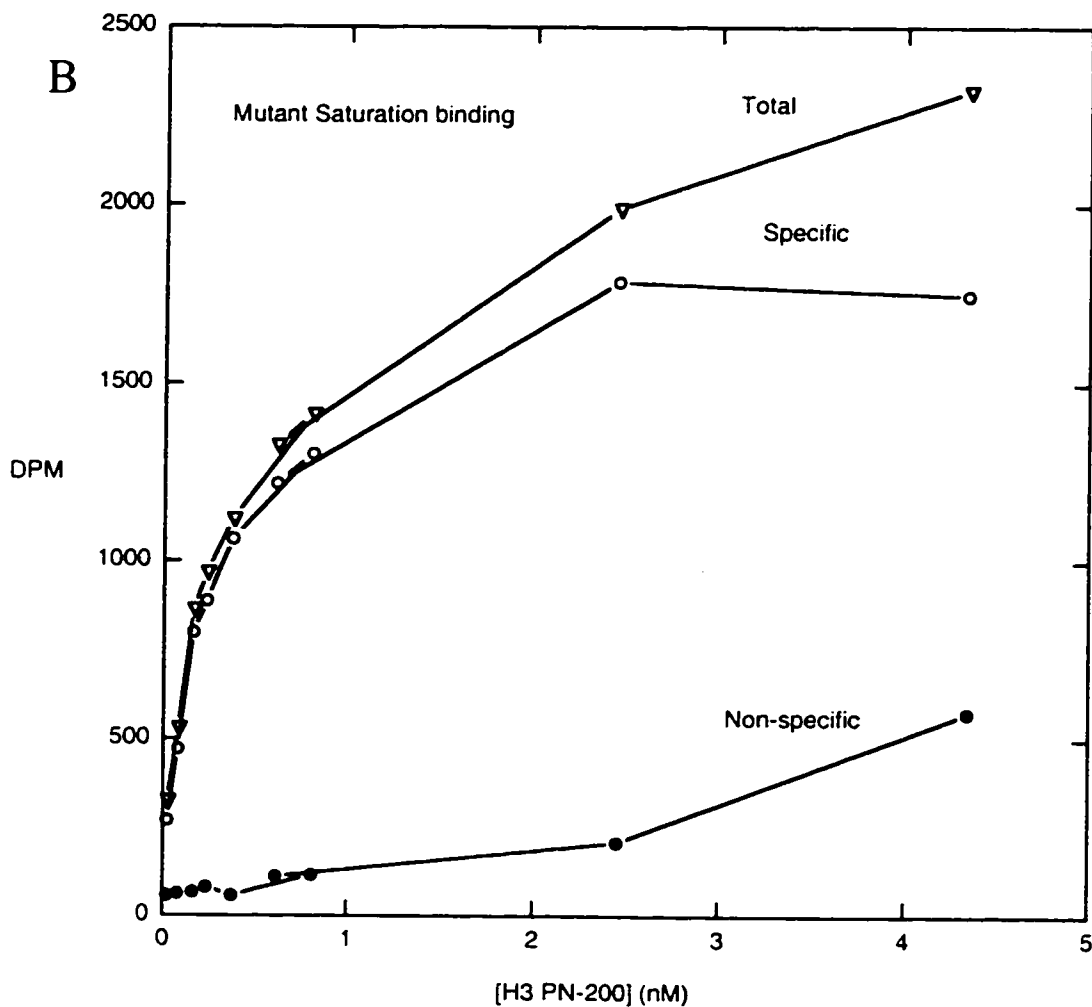
**A** Saturation binding isotherm for wild type  $\alpha_1$  calcium channel subunits coexpressed in tsA-201 cells using [<sup>3</sup>H]-PN-200-110 as the ligand (open triangles). Non-specific saturation binding of [<sup>3</sup>H]-PN-200-110 with excess cold ligand (filled circles). Specific binding of [<sup>3</sup>H]-PN-200-110 to membranes expressing wild type calcium channel subunits (difference between total and non-specific, open circles).

Shown below is the Scatchard transformation for the data above. Wild type membranes have a Bmax of 718 fmol/mg protein and a Kd of 215 pM.



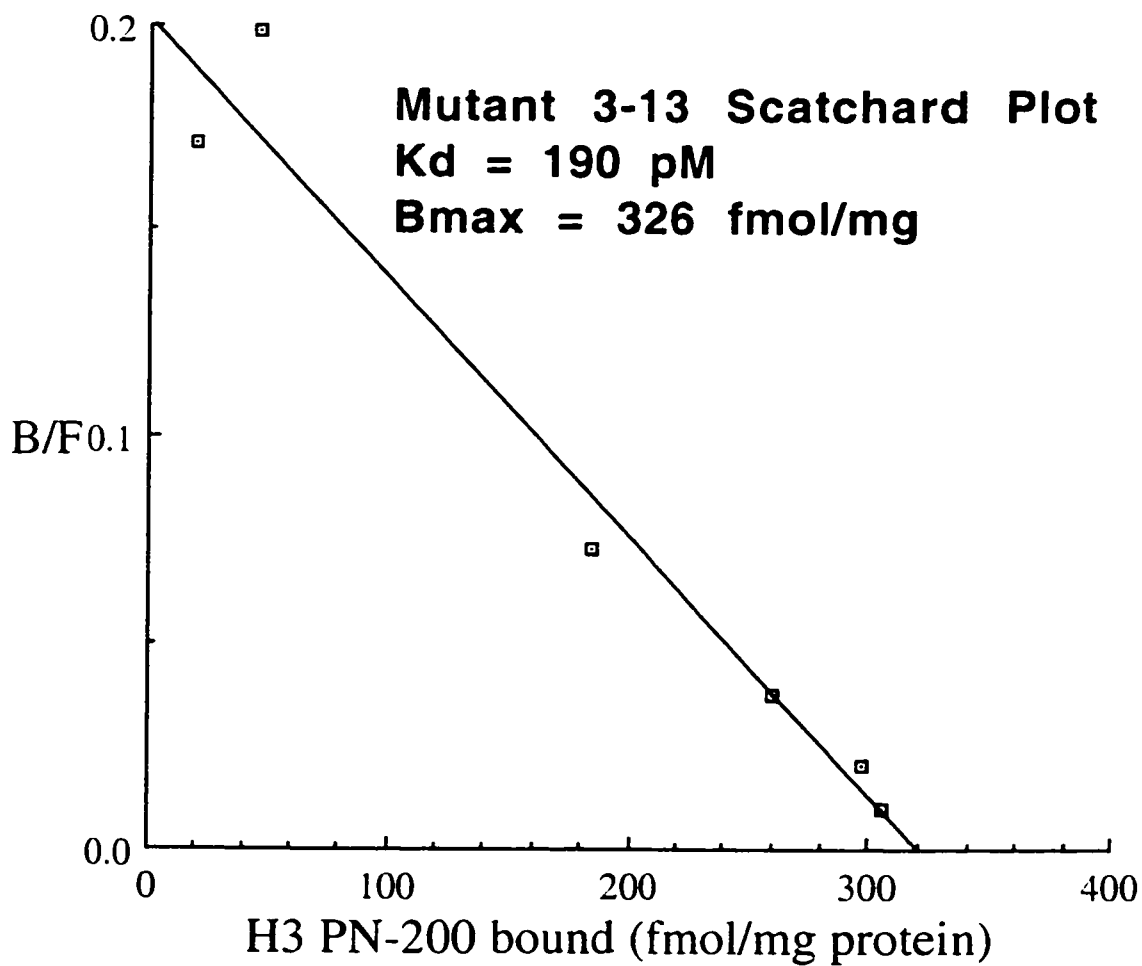
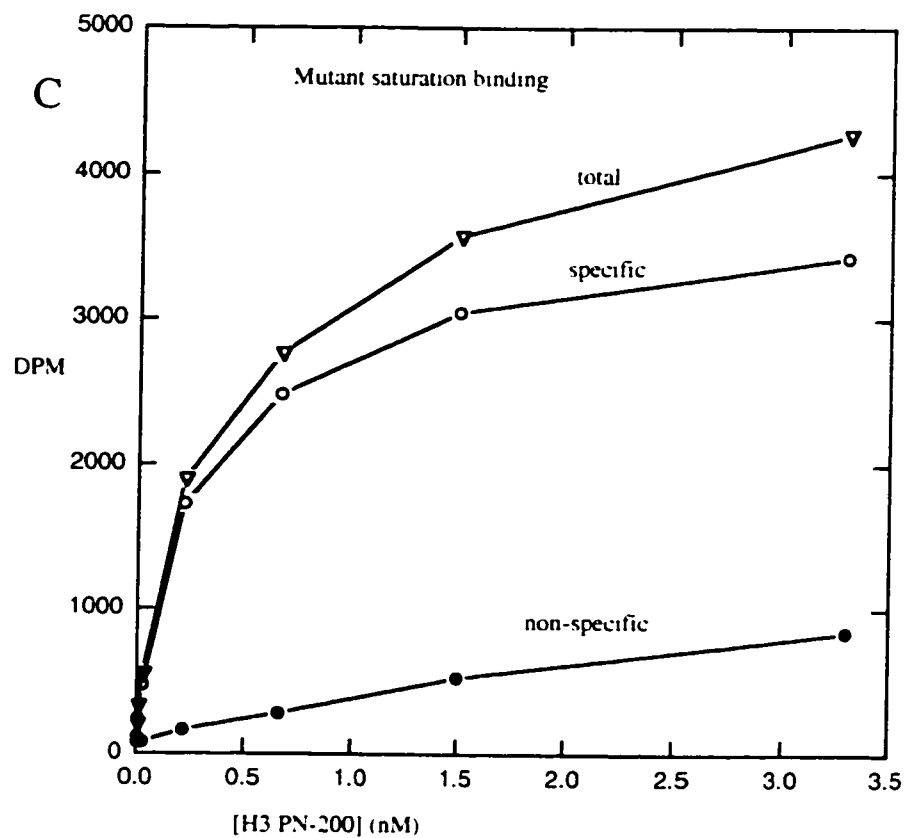
**B** Saturation binding isotherm for mutant 3,5-13  $\alpha_1$  calcium channel subunit coexpressed in tsA-201 cells with wild type  $\beta_{1b}$ ,  $\alpha_2/\delta$  subunits using [ $^3$ H]-PN-200-110 as the ligand (open triangles). Non-specific saturation binding of [ $^3$ H]-PN-200-110 with excess cold ligand (filled circles). Specific binding of [ $^3$ H]-PN-200-110 to membranes expressing mutant calcium channel  $\alpha_1$  subunit (difference between total and non-specific, open circles).

Shown below is the Scatchard transformation for the data above. 3,5-13 mutant expressing membranes have a Bmax of 140.2 fmol/mg protein and a Kd of 136 pM.



C Saturation binding isotherm for mutant 3-13( $\Delta$ )  $\alpha_1$  calcium channel subunit coexpressed in tsA-201 cells with wild type  $\beta_{1b}$ ,  $\alpha_2/\delta$  subunits using [ $^3$ H]-PN-200-110 as the ligand (open triangles). Non-specific saturation binding of [ $^3$ H]-PN-200-110 with excess cold ligand (filled circles). Specific binding of [ $^3$ H]-PN-200-110 to membranes expressing mutant calcium channel  $\alpha_1$  subunit (difference between total and non-specific, open circles).

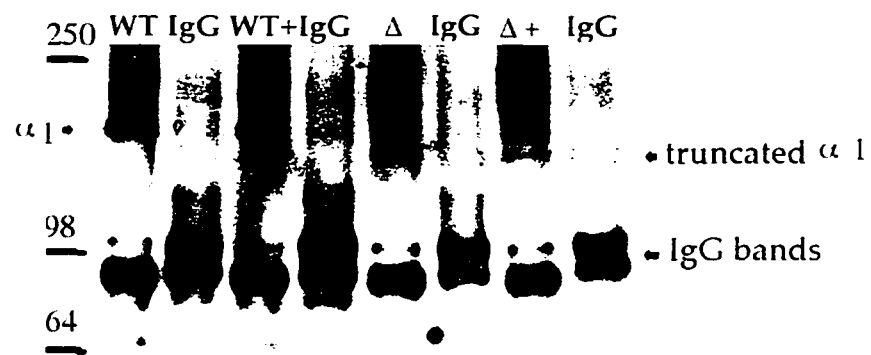
Shown below is the Scatchard transformation for the data above. 3-13 mutant expressing membranes have a  $B_{max}$  of 326 fmol/mg protein and a  $K_d$  of 190 pM.



Previous experiments in other cell types determined that tsA-201 cells were the best for biochemical and electrophysiological expression. The cell types tried included chinese hamster ovary cells (CHO), hamster kidney cells (BHK), chinese hamster lung cells (1610), dysgenic mouse myocyte cells transformed with SV40 large T antigen that can differentiate to myoballs and myotubes with reduced growth factors (DA3), another large T antigen immortalized dysgenic mouse myocyte cell line that can differentiate (GLT), and a dysgenic mouse fibroblast cell line transfected with an early gene in muscle development, Myo D, that can differentiate to myoballs and myotubes with reduced growth factors (TM38). These cell lines were found to be low expressors of dihydropyridine binding sites or had endogenous calcium channels that interfered with the L-type channel recordings. Multiple methods for transfection were examined including lipid mediated transfections and calcium phosphate based transfections on both plated cells and cells in suspension. Calcium phosphate precipitated DNA on plated cells was found to be the most effective. Stable transfections and transient transfections were tried and the transient expression system showed the greatest number of cells with current and the best biochemical data.

**Figure 2.3****Immunological characterization of rabbit skeletal muscle calcium channel  $\alpha_1$  subunits expressed in tsA-201 cells.**

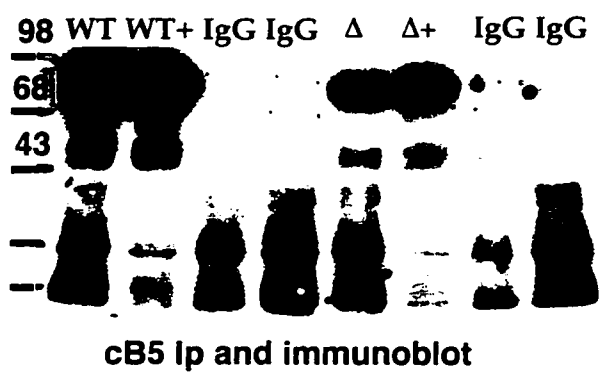
Membranes isolated from tsA-201 cells transiently transfected with wild type (WT) or mutant ( $\Delta = 3-13$ )  $\alpha_1$  subunits with wild type  $\beta_{1b}$ ,  $\alpha_2/\delta$  subunits were immunoprecipitated with an anti-peptide antibody directed to a region of the  $\alpha_1$  subunit, separated by PAGE and visualized with this anti-peptide antibody. This was the CP15 antipeptide antibody (see Experimental Procedures, Chapter 5). The 212 kD  $\alpha_1$  subunit is seen in the WT lanes and the 190 kD mutant 3-13( $\Delta$ )  $\alpha_1$  subunit is seen in the 3-13( $\Delta$ ) lanes. The + labelled lanes are from membranes isolated in the presence of 50  $\mu$ M Sp-5,6-DCL-cBIMPS which had no effect on protein expression. The lanes labelled IgG are controls using pre-immune serum. The mutant alpha 1 subunit is expressed at a lower concentration than the wild type corresponding to the Bmax data.



CP15 immunoblot

**Figure 2.4****Immunological characterization of rabbit skeletal muscle calcium channel  $\beta_{1b}$  subunits expressed in tsA-201 cells.**

Membranes isolated from tsA-201 cells transiently transfected with wild type (WT) or mutant ( $\Delta = 3-13$ )  $\alpha_1$  subunits with wild type  $\beta_{1b}$ ,  $\alpha_2/\delta$  subunits were immunoprecipitated with an anti-peptide antibody directed to a region of the  $\beta_{1b}$  subunit, separated by PAGE and visualized with the same anti-peptide antibody. The 74 kD  $\beta_{1b}$  subunit is seen most robustly in the WT lanes and 3-13( $\Delta$ ) lanes as a doublet. There is also a 55 kD band seen with less immuno-reactivity. The + labelled lanes are from membranes isolated in the presence of 50  $\mu$ M Sp-5,6-DCI-cBIMPS which had no effect or increased protein expression. The lanes labelled IgG are controls using pre-immune serum. As seen in Figure 2.2 there is less expression of  $\beta_{1b}$  protein when the mutant  $\alpha_1$  is coexpressed.



### Protein Expression

Analysis of the protein expression by antibody precipitation and western blotting showed a 212 kD size for the expressed wild type  $\alpha_1$  subunit and 190 kD size for the mutant 3-13( $\Delta$ ) truncated  $\alpha_1$  subunit similar to work of De Jongh (De Jongh, et al., 1991) as shown in Figure 2.3. Expression levels reflected the dihydropyridine binding results with a 2-fold higher level of wild type protein. The precipitation and the blotting by CP15 indicate a protein with functional epitopes for immunoreactivity.

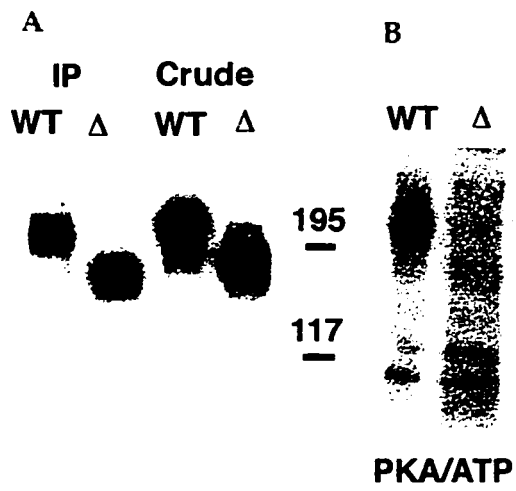
Beta subunit expression was seen for the  $\beta_{1b}$  clone with c $\beta$ 5 antibody as shown in Figure 2.4. The expression pattern showed doublet bands at the published size of 75 kD (Pragnell, et al, 1991; Chein, et al, 1996) but also doublet bands at a smaller size of 55 kD which may be a proteolysis product. The expression of  $\beta_{1b}$  protein was reduced by about 50% with coexpression of the mutant  $\alpha_1$  subunit indicating that  $\alpha_1$  and  $\beta_{1b}$  expression may be linked. For example, overexpression of the large  $\alpha_1$  subunit may saturate polyribosomes and reduce synthesis of  $\beta$ .

**Figure 2.5**

***In vitro* western blot and PKA phosphorylation of the rabbit skeletal muscle calcium channel  $\alpha_1$  subunit, wild type and mutant.**

**A** Immunoprecipitated wild type and mutant 3-13( $\Delta$ )  $\alpha_1$  subunits of the skeletal muscle L-type calcium channel expressed in tsA-201 cells are shown on the left and crude unprecipitated membranes are shown on the right of the PAGE analysis and visualized with the same antibody (CP15). A molecular mass of 212 kD and is seen for the wild type  $\alpha_1$  subunit and a 190 kD band is seen for the truncated mutant  $\alpha_1$  subunit.

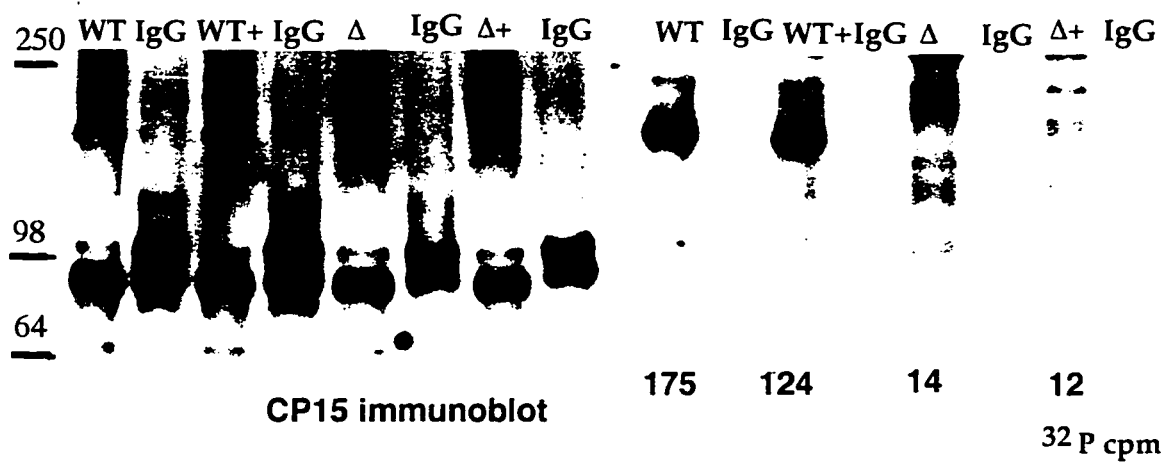
**B** Immunoprecipitated and *in vitro* phosphorylated wild type and mutant  $\alpha_1$  subunits are visualized by autoradiography after phosphorylation with  $\gamma^{32}\text{P}$ -ATP (see experimental procedures). A band is seen at the 212 kD size in the wild type lane. No phosphorylation is seen in the mutant lane.



**Figure 2.6*****In vivo* western blot and PKA phosphorylation of the rabbit skeletal muscle calcium channel  $\alpha_1$  subunit, wild type and mutant.**

**A** Figure 2.2 is repeated for comparison to B.

**B** The membrane used for antibody recognition of  $\alpha_1$  subunits in A was exposed to autoradiography film to determine orthophosphate  $^{32}\text{P}$  incorporation *in vivo*. Bands can be seen in the wild type  $\alpha_1$  lane at 212 kD while no band is seen in the mutant 3-13( $\Delta$ )  $\alpha_1$  lane. The effect of stimulating PKA activity by 50  $\mu\text{M}$  Sp-5,6-DCl-cBIMPS was a decrease in  $^{32}\text{P}$  incorporation (+ lanes). Quantification of the  $^{32}\text{P}$  incorporation was performed by cutting out the areas of the membrane corresponding to the immunostaining for the  $\alpha_1$  subunit. Cerenkov counting gave the values shown below the lanes after subtracting background as determined from the control IgG lanes. Background was 33 cpm.



**Figure 2.7**

***In vivo* western blot and PKA phosphorylation of the rabbit skeletal muscle calcium channel  $\beta_{1b}$  subunit.**

**A** Figure 2.3 is repeated for comparison to B.

**B** The membrane used for antibody recognition of  $\beta_{1b}$  subunits in A was exposed to autoradiography film to determine orthophosphate  $^{32}\text{P}$  incorporation *in vivo*. Doublet bands can be seen in the wild type  $\beta_{1b}$  lane at 75 kD and 55 kD while less intense bands can be seen in the mutant lanes roughly corresponding to the decreased protein expression seen in A. The effect of stimulating PKA activity by 50  $\mu\text{M}$  Sp-5,6-DCI-cBIMPS was a decrease in  $^{32}\text{P}$  incorporation (+ lanes). Quantification of the  $^{32}\text{P}$  incorporation was performed by cutting out the areas of the membrane corresponding to the immunostaining. Cerenkov counting gave the values shown below the lanes after subtracting background as determined from the control IgG lanes. Background was 30 cpm.



## Phosphorylation Studies

### *In Vitro*

The crude cell lysates from transfected cells were immunoprecipitated with CP11 and used for phosphorylation reactions. Protein phosphorylation of the  $\alpha_1$  subunit, as measured by incorporation of  $^{32}\text{P}$  in *in vitro* phosphorylation reactions, was seen for the wild type  $\alpha_1$  subunit but not for the mutant as shown in Figure 2.5. Both the wild type and mutant 3-13( $\Delta$ )  $\alpha_1$  subunits were expressed as seen in the CP11 Western blot. Dihydropyridine binding studies done in tandem with the phosphorylation studies showed expression of both proteins as well. The  $K_d$  of the mutant was lower than that of the wild type (data in Figure 2.2) and the  $B_{max}$  was also lower as stated in the Calcium Channel Expression section. This correlates well with the published work showing that long term stimulation of PKA activity, which phosphorylates the channel, causes an increase in expression levels and a decrease in affinity (Schmidt, et al., 1985). Thus, the mutant channel with no phosphorylation sites is expressed at lower levels but with higher affinity.

*In Vivo*

Channel proteins were immunoprecipitated from transfected cells grown in the presence of  $^{32}\text{P}$  orthophosphate. Western blotting with CP11, ECL labeling for  $\alpha_1$  subunit visualization, and autoradiography to detect  $^{32}\text{P}$  incorporation were performed. *In vivo* incorporation of  $^{32}\text{P}$  was seen in the CP11-labeled 212 kD band correlating to the size of the full length  $\alpha_1$  subunit in the wild-type lanes with no stimulation of incorporation and with bath application of DCI-cBIMPS. No incorporation of phosphate was seen in the CP11 labeled 190 kD band correlating to the truncated mutant 3-13( $\Delta$ ) channel with no stimulated incorporation in the presence of DCI-cBIMPS as shown in Figure 2.6. This data indicates that the mutant protein is expressed but cannot be phosphorylated *in vitro* or *in vivo*.

*In vivo* protein phosphorylation as measured similarly to by incorporation of  $^{32}\text{P}$  was seen for  $\beta_{1b}$  at the 55 and 75 kD sizes. Incorporation correlated to protein expression as seen with antibody analysis and included incorporation in doublet bands as shown in Figure 2.7.

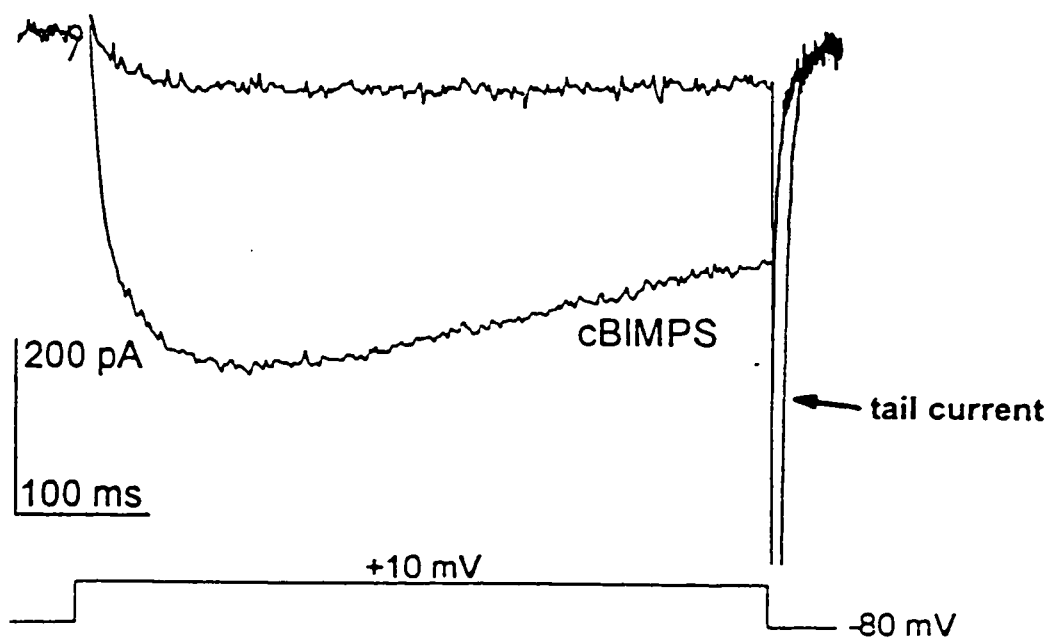
**Figure 2.8**

**Electrophysiology analysis of wild-type calcium channels expressed in tsA-201 cells**

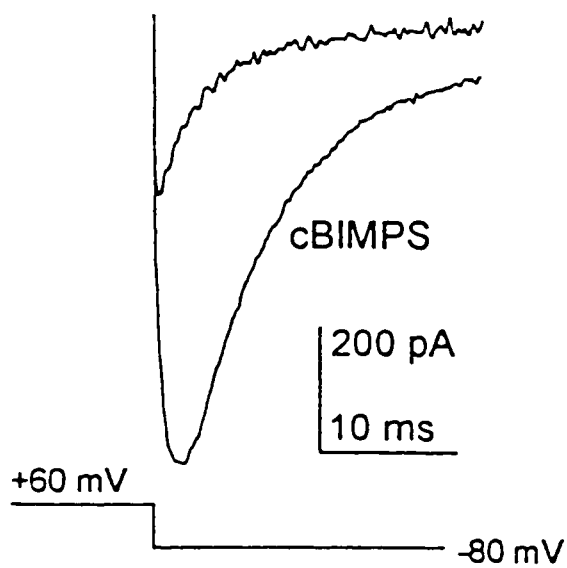
**A** Pulse current traces during a depolarization from a holding potential of -80 mV to a test potential of +10 mV before and after a bath application of 50  $\mu$ M DCI-cBIMPs, final concentration. Tail currents are shown upon repolarization to -80 mV.

**B** Tail current traces upon repolarization to -80 mV after a test pulse to +60 mV before and after bath application of 50  $\mu$ M DCI-cBIMPs, final concentration.

# A Pulse current



# B Tail current



## Functional Analysis of Wild-type and Mutant Calcium Channels

Bay K 8644 is a dihydropyridine agonist of L-type calcium channels. Bath application of 10  $\mu\text{M}$  of this drug increase tail currents 7-fold and slows their decay 6-fold (Johnson et al., 1997).

DCI-cBIMPs is a membrane permeant, non-hydrolysable analogue of cAMP that activates PKA. When DCI-cBIMPS is bath applied to tsA-201 cells, expressed calcium channel currents and tail currents increase and tail current decay is slowed (Johnson et al., 1997). The addition of the peptide inhibitor of PKA, PKI, blocks this increase.

TsA-201 cells expressing L-type calcium currents from skeletal muscle show the following current parameters. Upon depolarization from a holding of -80 mV to a test potential of +10 mV, an average peak current of 24 +/- 5 pA (+/- SE) is seen in the presence of Bay K 8644 for 35% of the cells tested. Tail currents, averaging 722 +/- 176 pA (+/- SE) are seen upon repolarization of the cell to the holding potential of -80 mV in 95% of the cells tested. This tail current is seen due to the increased driving force on ions at this potential as they pass through the deactivating channel. Figure 2.8 shows the pulse current and tail current from a typical recording with wild type calcium channel transfected cells, both currents are increased by application of the PKA stimulating agent DCI-cBIMPS. This current flow may provide the

calcium replenishment necessary in prolonged and repeated muscle contraction and modification of this current by  $\beta$  adrenergic stimulation through PKA may enhance this process.

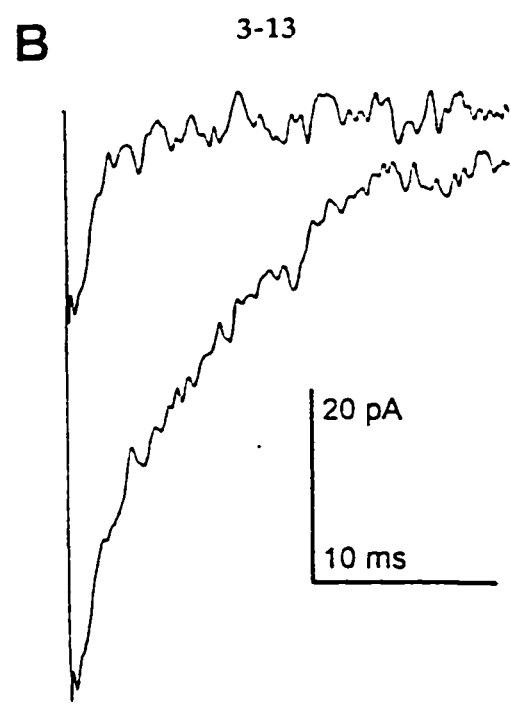
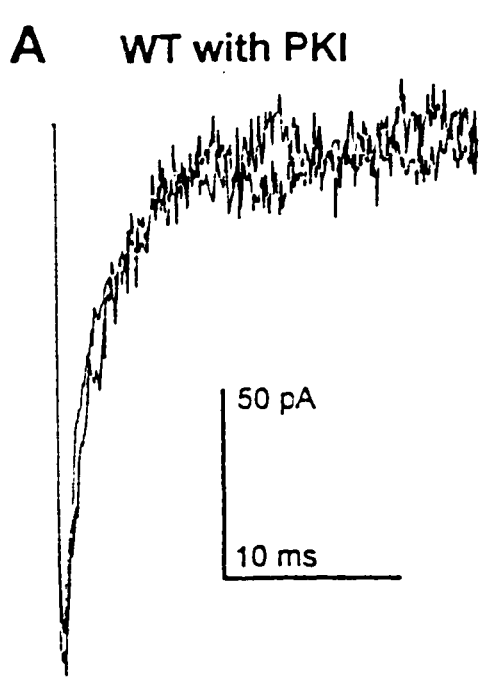
More evidence for the involvement of PKA is shown in Figure 2.9 where in panel A there is a block of the increase in wild type tail current amplitude during DCI-cBIMPs application when PKI is pre-applied through the pipette. The tail current parameter was chosen for the analysis of the effect of mutations in the channel since tail currents could be recorded in most transfected cells. Tail current amplitude increase is seen in the phosphorylation-deficient mutant-transfected tsA-201 cells as shown in Figure 2.9, panel B. Tail current amplitude increases recorded from wild type and mutant calcium channel transfected cells are shown in Figure 2.9, panel C. The percentage increases after DCI-cBimPs application are show for wild type, two phosphorylation-deficient mutants (3,5-13 and 3-13), and the control PKI block. There is no significant difference between the wild type and the mutant transfected cells.

**Figure 2.9****Tail currents in wild type and mutant calcium channel expressing tsA-201 cells**

**A** Tail current traces from wild type calcium channels expressed in tsA-201 cells upon repolarization to -80 mV from a test potential of +10 mV before and after perfusion of the PKA peptide inhibitor PKI from the pipette.

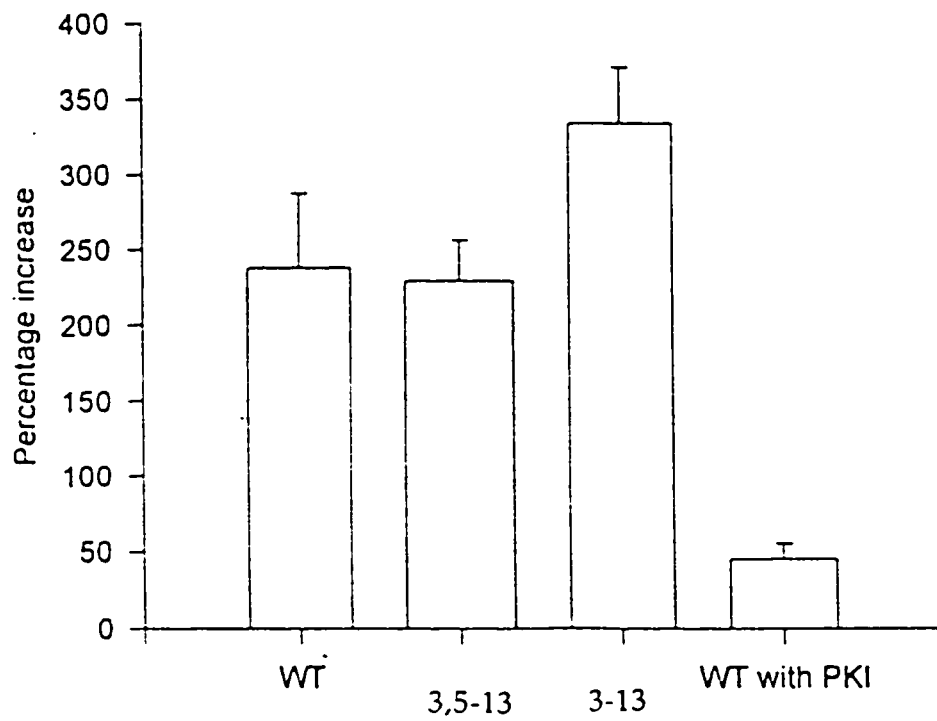
**B** Tail current traces from mutant 3-13 ( $\Delta$ ), which is not phosphorylated *in vitro* or *in vivo*, upon repolarization to -80 mV from a test potential of +10 mV before and after bath application of 50  $\mu$ M DCI-cBIMPs, final concentration.

**C** Percentage increase and standard error in tail currents upon repolarization to -80 mV after a test potential of +60 mV after a bath application of 50  $\mu$ M DCI-cBIMPs, final concentration. Data is shown for wild type, mutant 3,5-13, 3-13 ( $\Delta$ ), and wild type after perfusion of the peptide inhibitor of PKA, PKI, via the pipette.



**C**

Stimulation of tail currents by cBIMPS



PKA stimulation increased current recordings in tsA-201 cells expressing the wild type calcium channel subunits by  $127 \pm 55\%$  ( $n=10$ ). Shown in Table 2.2, wild type tail currents increased in response to PKA stimulation by  $3.37 \pm 0.71$  fold ( $n=13$ ). Mutant tail currents in the 1,3-9 mutant increased  $3.29 \pm 0.4$  fold ( $n=4$ ) and in the 1,3-10 mutant  $4.34 \pm 0.48$  fold ( $n=3$ ). These data do not vary significantly. Data for the other mutants produced and analysed are also given in Table 2.2. Mutations removing any number of phosphorylation sites, up to 17 serines and threonines, all failed to prevent tail current increases upon PKA stimulation.

**Table 2.2 Wild-type and Mutant Calcium Channel Tail Current Increases**

Fold increase was measured for calcium channel tail current before and after application of 50  $\mu$ M DCI-cBIMPS. Tail currents were measured at -80 mV after a 100 ms voltage step to +60 mV. Designations on the left correspond to sites numbered in Table 1.1. Fold increase and the standard error of the mean are shown in the center. Number of cells analysed is shown on the right

	Fold increase ( $\pm$ SEM)	number
WT	3.37 $\pm$ 0.71	13
WT with PKI	1.45 $\pm$ 0.32	4
3 (S687A)	2.45 $\pm$ 0.17	2
6	2.46 $\pm$ 0.58	2
8	4.70 $\pm$ 1.32	4
4	2.33 $\pm$ 0.31	2
1	3.58	1
5,7,9	2.25 $\pm$ 0.20	4
10,11,13	2.44 $\pm$ 0.33	2
5-13	5.75	1
3,5-13	3.29 $\pm$ 0.39	4
3-13	4.34 $\pm$ 0.48	3

## DISCUSSION:

The hypothesis that direct phosphorylation of the  $\alpha_1$  subunit of the L-type calcium channel from rabbit skeletal muscle is responsible for the increase in current seen with the activation of PKA by cAMP analogues has been examined by expressing the cloned cDNA in human embryonic kidney tsA-201 cells. This system allowed the determination of protein expression, phosphate incorporation and channel function. These parameters were compared for wild type and phosphorylation-deficient mutant calcium channels. The results of these studies indicate that even though the phosphate incorporation into the  $\alpha_1$  subunit has been eliminated, the channel is still regulated by PKA. The *in vitro* and *in vivo* phosphorylation data, compared with protein expression data show that the channel protein is expressed but no phosphate is incorporated in the mutant channel 1,3-9 and the increase is still seen in current profiles upon PKA stimulation. That PKA is involved is evidenced by the blocking of this effect by PKI in wild type transfected cells.

The results of this study indicate that other proteins may mediate the effect of PKA on this system. Other channel subunits may be phosphorylated and mediate this effect. The  $\beta$  subunit, which is a PKA substrate, is one candidate. The evidence against this is channel modulation

seen in the absence of expression of the  $\beta$  subunit for cardiac calcium channels (Yoshida, et al., 1992; Sculptoreanu, et al., 1993). Also, another protein such as a cytoskeletal anchor or cytoskeletal structural protein could be phosphorylated and alter calcium current. This idea is supported by the requirement for an anchoring protein shown by the work of Johnson et al. (Johnson, et al., 1997) where the peptide Ht-31 blocked the current increase by cAMP analogues. Cytoskeletal disrupting and stabilizing agents also altered the calcium current increases seen with PKA stimulation (Johnson et al., unpublished results).

Another, less likely, explanation that should be discussed is the idea that PKA interacts with the channel directly in a protein - protein modification. This type of interaction and effect on current is seen cardiac and brain calcium channels for the  $\beta$  and  $\alpha_2/\delta$  subunits (Stea, et al., 1993; Chien, et al., 1995; Strube, et al., 1996). In these systems, coexpression of the  $\beta$  and  $\alpha_2/\delta$  subunits increased whole cell current and shifted voltage dependence of inactivation, coexpression of the  $\beta$  subunit increased surface expression, and lack of the  $\beta$  subunit decreased current.

The alteration of the consensus sequences for phosphorylation by PKA to that of the regulatory subunit does not prevent the interaction of

the kinase with the channel, it only prevents the transfer of phosphate. The protein - protein modification may still persist and effect current parameters. The alteration to amino acids that block PKA binding may be required. One other way to test this idea is to alter the PKA catalytic subunit to be unable to bind to its substrate, possibly the mutation of lysine 72 in the catalytic domain would allow this analysis.

Protein - protein interactions with components of the excitation-contraction pathway will be addressed again in Chapter Three.

## VII. CHAPTER 3: PROTEIN - PROTEIN INTERACTIONS THAT MAY REGULATE CALCIUM CHANNEL EXPRESSION AND FUNCTION

### INTRODUCTION:

The skeletal muscle dihydropyridine receptor interacts with several known proteins. Purification of the channel coprecipitates other proteins including the  $\beta$ ,  $\alpha_2/\delta$ , and  $\gamma$  subunits (Curtis, et al., 1984) and AKAPs (Gray, et al., 1997). The dihydropyridine receptor interacts with the ryanodine receptor (RyR) (Schneider, et al., 1973; Lai, et al., 1988; Takeshima, et al., 1989; Takekura, et al., 1995) and there are both orthograde and retrograde signals with this molecule (Suda, et al., 1997; Nakai, et al., 1998). The nature of these interactions alter calcium current parameters and protein expression levels.

The interaction with the ryanodine receptor in excitation-contraction coupling is the basis for skeletal muscle contraction, so the interaction with this protein is of special interest. There are regions of the skeletal muscle dihydropyridine receptor that interact with the ryanodine receptor (Lu, et al., 1994; Lu, et al., 1995; El-Hayek, et al., 1995) and regions of the ryanodine receptor that interact with the dihydropyridine receptor

(Leong, et al., 1998). These data led to the idea to coexpress the ryanodine receptor with the calcium channel subunits in the heterologous tsA-201 cell system. The expectation was increased calcium currents as the dyspedic mouse system showed a requirement for the ryanodine receptor for calcium currents (Nakai, et al., 1996). The analysis of calcium currents during the depolarization pulse may reveal an effect of the phosphorylation-deficient mutants if the mechanism by which phosphorylation affects calcium channel function is by modifying its interaction with the ryanodine receptor.

Another protein-protein interaction that may modify calcium channel activity is the A-Kinase Anchoring Protein (AKAP), AKAP-15. This protein binds to the regulatory subunit of PKA and through an amphipathic helix, anchors PKA to the calcium channel (Gray, et al., 1997; Gray, et al., 1998). Voltage-dependent potentiation in skeletal muscle cells is disrupted by the addition, via the recording pipette, of a peptide that blocks this interaction (Johnson, et al., 1994, Gray, et al., 1998). The AKAP 15 protein was cotransfected with the calcium channel subunits in tsA-201 cells. Stable cell lines expressing the AKAP 15 protein were also created.

## RESULTS:

Binding studies using tsA-201 cells cotransfected with calcium channel subunits and the ryanodine receptor gave dihydropyridine binding

levels of 125 to 1355 fmol/mg protein and ryanodine binding levels of 48 to 152 fmol/mg protein. Expressing ryanodine receptors alone gave higher binding levels of 219 fmol/mg protein.

In physiological studies a total of 21 cells were analysed that had been transfected with the dihydropyridine receptor subunits and the ryanodine receptor in 6 separate transfections. There were 14 cells with no calcium channel currents and 6 cells with currents which were not larger than currents seen in transfections without the ryanodine receptor.

In control transfections with the calcium channel subunits average tail currents were 2614 +/- 725 pA (=/- SE) (n = 11). In transfections using the ryanodine receptor and the calcium channel subunits average tail currents were 1737 +/- 1365 pA (+/- SE) (n = 6). These data were recorded after a test pulse of 100 ms at +80 mV and are not significantly different. Current amplitude was increased approximately 2 fold with DCI cBIMPS application in cells cotransfected with the ryanodine receptor.

Cotransfection of the AKAP 15 protein with calcium channel subunits in tsa-201 cells failed to provide calcium currents in any cells examined. Co-immunoprecipitation experiments using the antibody directed against the  $\alpha_1$  subunit, CP11, were performed. Material was precipitated that was shown by western blot analysis with CP11 to contain the  $\alpha_1$  subunit and

by RII overlay analysis to contain AKAP 15, indicating that both these proteins were expressed.

#### DISCUSSION:

The results of cotransfection of the ryanodine receptor with the dihydropyridine receptor in this heterologous system were negative. The dihydropyridine receptor, although expressed in internal compartments, is not processed completely to the cell surface for complete physiological effects. Evidence in tsA-201 cells and primary muscle cell cultures with immunostaining for surface expression of the dihydropyridine receptor shows very low expression at the surface of the cells without  $\beta$  subunits and only limited surface expression with  $\beta$  subunits (Chien, et al., 1995; Gregg, et al., 1996). The concept is that protein processing and trafficking of the dihydropyridine receptor complex requires cotranslation of the multiple subunits and the insertion into the membrane requires proteins found in skeletal muscle such as ryanodine receptors and triadin (Dulhunty, et al., 1996; Guo, et al., 1996). The complex membrane architecture found in the triads of skeletal muscle where dihydropyridine and ryanodine receptors interact and the surface membrane depolarization activates the sarcoplasmic reticulum calcium release cannot be created in transiently transfected cells using methods employed to date. Attempts to create this architecture by coexpressing AKAP's were not successful, as stated in the results section,

transient coexpression of AKAP's and calcium channel subunits failed to provide cells with any calcium current. Future experiments in selected cell lines may allow this architecture to occur (Suda, et al., 1997) but preliminary reports at meetings indicate that this approach also is not successful. Evidently, unknown proteins present in the muscle cell are needed for successful formation of triad junctions.

## VIII. CHAPTER 4: SUMMARY

The hypothesis examined in this research work is that the direct PKA phosphorylation of the  $\alpha_1$  subunit of the skeletal muscle L-type calcium channel is the molecular mechanism by which current parameters are altered and calcium current is increased by  $\beta$ -adrenergic regulation or during prolonged and repeated muscle contraction. Phosphorylation-deficient mutant  $\alpha_1$  subunits were constructed and tested in an expression system that allowed analysis of current increases and phosphorylation events.

Multiple mammalian cell lines were examined for the ability to express calcium currents, and the tsA-201 human embryonic kidney cell line was found to express calcium channel subunits with current parameters altered by PKA activation. Tail currents increased in channels in response to application of DCI cBIMPS and this effect was blocked by PKI. This cell line also produced levels of protein that could be analysed biochemically. Protein immunopurification methods allowed the analysis of *in vitro* and *in vivo* phosphorylation events in the channel  $\alpha_1$  subunit.

Oligonucleotide-directed mutagenesis produced  $\alpha_1$  subunits with serines and threonines altered to alanines. These mutants were shown to be deficient in phosphate incorporation and the tail currents, analysed electrophysiologically, were shown to still be regulated by PKA stimulation. Thus, the  $\alpha_1$  subunit of the skeletal muscle calcium channel was shown to not be the target of phosphate transfer modification for the PKA-mediated alteration in current parameters. The idea that interaction with ryanodine receptors or AKAP's may be necessary for regulation was explored. New directions of research towards other subunits and PKA substrates has been initiated and should lead to eventual identification of the site(s) of regulation of the skeletal muscle calcium channel by PKA.

## IX. CHAPTER 5: EXPERIMENTAL PROCEDURES

**Plasmids and Cells.** The bacteriophage, M13mp19, was obtained from Gibco/BRL. cDNAs encoding the  $\alpha_1$  and  $\alpha_2/\delta$   $\text{Ca}^{2+}$  channel subunits cloned from rabbit skeletal muscle (Ellis, et al., 1988) were provided by Drs. Steven B. Ellis, Michael M. Harpold (Salk Institute Biotechnology/Industrial Associates, Inc.) and Arnold Schwartz (University of Cincinnati College of Medicine). Two overlapping fragments of  $\alpha_1$  cDNA (pSKM $\text{CaCH}\alpha$ 1.7 and 1.8) were assembled, and the entire 5'-noncoding region was excised to yield the construct inserted into the expression plasmid ZEM RVSP6 (West, et al., 1992) derived from the ZEM 228 (Dr. Eileen Mulvihill, Zymogenetics Corp) to yield the ZEM RVSP6  $\alpha_1$  CaCH expression vector. The  $\alpha_2/\delta$  cDNA was subcloned into the BamH1 site of ZEM RVSP6 to create the expression vector, ZEM RVSP6  $\alpha_2\delta$  CaCH. The cDNA for  $\beta_{1b}$  (Pragnell, et al., 1991) in the pMT-2 expression vector (Genetics Institute, Cambridge, MA) was provided by Dr. Terry P. Snutch (University of British Columbia, Vancouver, B.C.).  $\beta_{1a}$  was cloned by Dr. Peter Gallombardo into the BamH1 site of pRc-CMV (Invitrogen, San Diego, CA). *E. coli* strains JM103, CJ236, and pEBO-pCD-

leu2, a vector expressing the CD8 antigen, were obtained from American Type Culture Collection (Rockville, MD). The expression plasmid for the cDNA of the ryanodine receptor and truncated pieces of the cDNA (Takeshima, et al., 1989) were obtained from Dr. David H. MacLennan. The cDNA for the cardiac L-type calcium channel in the expression vector pCCAR was obtained from Dr. Masami Takahashi. *E. coli* strain XL1-Blue was obtained from Statagene (La Jolla, CA). Anti-CD8 antibody coated microspheres (Dynabeads M-450 CD8) were from Dynal (Great Neck, NY). The cell line tsA-201 was obtained from Bob Durbridge (Cell Genesis, Foster City, CA).

**Antibodies.** The peptides, C $\beta$ 1, C $\beta$ 2, C $\beta$ 5, CP11 and CP15, used in producing antibodies, were synthesized by the Molecular Pharmacology Facility, Pharmacology Department, University of Washington. The peptides were purified on reverse phase HPLC, injected into rabbits and the anti-sera collected. IgG antibodies from uninjected rabbits (pre-immune) or from rabbits injected with the peptides were purified from the antisera by protein-A Sepharose chromatography. The anti-peptide antibodies were further purified by affinity chromatography using the immobilized cognate peptide. These antibodies were used for immunoprecipitation and western blot analysis.

## Antibody information:

Designation			Sequence	Residues
-----Subunit---Tissue				
C $\beta$ 1	$\beta$	rab skm	KYRPTPPASGNEnITNLAFE	203-219
C $\beta$ 2	$\beta$	rab skm	KSNTRSSLAEVQSEIER	338-353
C $\beta$ 5	$\beta$	rat brain	KHEYPGELGQPPGLYPS	466-481
CP11	$\alpha_1$	rab skm	KTGGLFGQVDFTLERTNSL	1601-1618
CP15	$\alpha_1$	rab skm	KDNFDYLTRDWSILGPHHLD	1382-1400

**Materials.** Radionucleotides were obtained from DuPont New England Nuclear (Boston, MA). The catalytic subunit of cAMP dependent protein kinase was prepared by Dr. B. Murphy according to the procedure of Kaczmarek et al. (1980). Sp-5,6-DCI-cBIMPS was obtained from BioLog Life

Sciences Institute. Cyclic AMP-dependent protein kinase inhibitor 5-24 was obtained from LC Laboratories. Enzymes were purchased from New England Biolabs (Beverly, MA), Promega Inc. (Madison, WI), Gibco/BRL (Gaithersburg, MD) Boehringer Mannheim Biochemica (Indianapolis, IN), or USB (Cleveland, OH).

**Construction of bacteriophage vectors for mutagenesis protocols.** M13BS and M13EX were prepared by digestion of 2mg of M13mp19 bacteriophage vector with BamH1 and Sal1 or EcoR1 and Xho1 restriction enzymes in the appropriate buffers in 20mL volumes. The reaction was electrophoretically separated on a 1% agarose gel to obtain the 7250bp fragments of the vector. These fragments were cut from the gel and separated from the agarose by spin column (Spin-X, Fisher, Pittsburgh, PA) according to the manufacturer's specifications. Similarly, 5mg of the expression plasmid ZEMRVSP6 containing the  $\alpha_1$  cDNA was digested with BamH1 and Sal1 or EcoR1 and Xho1 restriction enzymes and the 1805bp BS (site 4283 to site 6088) and 1647bp EX (site 1006 to site 2653) fragments were purified. These fragments and vectors were combined at equimolar ratios and a vector amount of 200ng in 20mM TrisHCl (pH 7.5), 5mM MgCl<sub>2</sub>, 10mM DTT, 5mM ATP, and 1 unit T4 DNA ligase and incubated one hour at room temperature. Five  $\mu$ l of the ligation reaction was used to transform *E. coli* XL1Blue cells. Briefly, 100  $\mu$ l

of competent cells are incubated with the ligation reaction for 30 minutes on ice. The cells are then heat shocked for 90 seconds at 42° C and plated on agar plates. Clones are picked and grown up and the replicative form of the bacteriophage DNA is analysed by restriction enzyme digest to determine insertion of the calcium channel cDNA fragment. Uracil-containing single stranded DNA templates were prepared as described by Kunkle et al. (1985). One hundred mL YT broth, containing 50 µg/mL uridine, was inoculated with *E. coli* CJ236 and bacteriophage at a multiplicity of infection of 1. After 6 hours of growth at 37° C, bacterial cells were pelleted by centrifugation. Supernatant was used to determine the titer of bacteriophage for infection of CJ236 and XL1Blue cells and thereby determine the efficiency of uridine substitution. Single-stranded template DNA was prepared from bacteriophage solutions with CJ236/XL1Blue titer ratios greater than 10<sup>6</sup>. Single-stranded template DNA was prepared from polyethylene glycol precipitated bacteriophage according to Ausubel, et al. (1989). Uridine-containing single-stranded DNA was quantified by absorbance at 260 nm and used in subsequent mutagenesis reactions.

**Oligonucleotide-directed mutagenesis.** Site-directed mutagenesis was carried out using a modification of the Kunkel protocol (Ausubel, et al., 1987). The mutagenic oligonucleotide was phosphorylated in a 20 µL

volume containing 2  $\mu\text{L}$  of 10X polynucleotide kinase buffer (700mM TrisHCl (pH 7.6), 100mM  $\text{MgCl}_2$ , and 50mM DTT), 2 mL of 10mM ATP (pH 7.0), 10 pmol of mutagenic oligonucleotide, 0.5  $\mu\text{L}$  T4 polynucleotide kinase (5 units, New England Biolabs), and sterile distilled water. This was incubated at 37°C for 15 minutes then the enzyme inactivated by 10 minutes at 65°C.

Annealing the oligonucleotide to the single-stranded, uracil containing template DNA was accomplished with 4  $\mu\text{L}$  of the phosphorylation reaction, 0.2 pmol of template, 1.25  $\mu\text{L}$  of 20X SSC (3M NaCl/ 0.3M sodium citrate, pH 7.0) in a volume of 10  $\mu\text{L}$ . This reaction was heated to 95°C for 5 minutes and slowly cooled to 30°C.

Extension of the mutagenic oligonucleotide and ligation of the extended product to form a plasmid was performed by adding 4  $\mu\text{L}$  sterile, distilled water and 5X T4 DNA polymerase mix (100mM TrisHCl (pH 8.8), 10 mM DTT, 50 mM  $\text{MgCl}_2$ , 2.5 mM each of dATP, dTTP, dCTP, dGTP (Pharmacia), and 5 mM ATP), and 1  $\mu\text{L}$  each of T4 DNA polymerase (3 units) and T4 DNA ligase (10 units, New England Biolabs). The reaction was incubated for 5 minutes each on ice and at room temperature and for 2 hours at 37°C. The reaction (7  $\mu\text{L}$ ) was used to transform 100  $\mu\text{L}$  of competent XL1Blue cells, which were combined with a plating culture of XL1Blue cells

(200  $\mu$ L, log phase) and 5 mL of 45°C 0.6% agarose in Luria Broth, and plated onto a Luria Broth agar plate for overnight incubation at 37°C. DNA was prepared from the resulting bacteriophage plaques and the mutations confirmed by sequencing. This process was repeated for several rounds to obtain multiple mutation-containing clones.

Mutagenesis of the serine 1928 to alanine in pCCAR was performed by Sculptor mutagenesis using a kit from Promega as per the manufacturer's directions.

**Identification of mutations.** Sequencing was performed using a dideoxy terminator sequencing kit (Sequenase 2.0, US Biochemicals). Double-stranded DNA from M13 bacteriophage (4-5  $\mu$ g) was denatured by heating to 95°C for 5 minutes. The denatured DNA was sequenced with <sup>35</sup>S-ATP as the radioactive label according to the manufacturer's specifications. Reactions were separated on a 0.5 mm 6% acrylamide-urea sequencing gel, fixed and dried, and exposed to XAR-5 X-ray film (Kodak).

**Construction of the mutant ZEM RVSP6 CaCH expression plasmid.** Double stranded DNA from clones containing the appropriate mutation reactions were restriction endonuclease-digested (BamH1 and Sal1 or EcoR1 and Xho1) and the fragments purified by agarose gel electrophoresis. These fragments were ligated into the expression vector (saved from previous digests and

purifications) and the reactions transformed into XL1Blue cells. Cells were plated on agar plates containing ampicillin and kanamycin and the resulting colonies were restriction endonuclease mapped to ascertain plasmid integrity. Sequence analysis was performed again on all clones used for expression of mutants in electrophysiology and protein experiments.

**Cell culture.** Human embryonic kidney cells transformed with the large T antigen (tsA-201 cells) were maintained in RPMI 1640 (Gibco/BRL, Gaithersburg, MD) containing 10% fetal bovine serum (Hyclone Laboratories, Inc., Logan, UT), 20  $\mu\text{g}/\text{mL}$  penicillin, 10  $\mu\text{g}/\text{mL}$  streptomycin, and 200  $\mu\text{g}/\text{mL}$  G418 (Geneticin, Gibco/BRL). Cells were subcultured weekly by treatment with 0.05% (w/v) trypsin, resuspension and reseeded at desired densities. Cells were maintained at 37°C in a 10% CO<sub>2</sub> incubator.

**Transient transfection of tsA-201 cells for calcium channel expression.** Expression of calcium channels for dihydropyridine binding assays was performed by plating 150 mm tissue culture plates the day before transfection at 50% confluency. The cells were fed with fresh medium the morning of transfection and placed in a 3% CO<sub>2</sub> incubator. A calcium phosphate/ DNA precipitate was made by combining 55  $\mu\text{g}$  of an equimolar mixture of ZEM RVSP6  $\alpha_1$  CaCH (wild type or mutant), ZEM RVSP6  $\alpha_2/\delta$  CaCH, and pMT2  $\beta_{1b}$  CaCH with a 250 mM CaCl<sub>2</sub> (final) solution in 1.125 mL. This was added

dropwise to 1.125 mL of 2X HEPES-buffered saline (274 mM NaCl, 40 mM HEPES, 17 mM dextrose, 10 mM KCl, 1.4 mM Na<sub>2</sub>HPO<sub>4</sub> adjusted to pH 7.125 prior to filtering). This solution was added to the cell media after 2 minutes and the plates returned to the 3% CO<sub>2</sub> incubator for 8 hours. Fresh medium was added and the cells were allowed to grow in a 5% CO<sub>2</sub> incubator for 36 to 48 hours before harvesting membranes.

Calcium channels were similarly transiently transfected for <sup>32</sup>P incorporation experiments in a 100 mm plate using 20 µg of an equimolar mixture of the channel subunits and 1 mL final precipitate volume. The cells were treated as described below at 36 to 48 hours with radioactive phosphate.

Transient expression for electrophysiological analysis of calcium channels was done in a 35 mm plate using 4 µg of DNA mixture supplemented with 0.8 µg of the CD8 expression plasmid, pEBO-pCD-leu2. This was done in a final precipitate volume of 166 µL. These cells were removed from the transfection plate at 24 hours by washing once with phosphate-buffered saline and once with phosphate-buffered saline containing 2 mM EGTA to disrupt cell attachment but not digest calcium channels. These cells were plated at a 1:1000 dilution on additional 35 mm plates and incubated at 5% CO<sub>2</sub> for recordings during the following 24 to 48

hours. Cells expressing the CD8 antigen were identified by incubation with polystyrene microspheres precoated with anti-CD8 antibodies (Jurman, et al., 1994) and used for recordings. Expression of the ryanodine receptor cDNA was performed similarly with molar ratios maintained by addition of the vector without the cDNA insert.

**Membrane Preparation.** All extract incubations and manipulations were carried out at 4°C or on ice to prevent proteolysis. Also, the following protease inhibitors were included in all buffers: 1 mM pepstatin A, 2µg/mL leupeptin, aprotinin (4 µg/mL), 0.2 mM phenylmethanesulfonyl fluoride (PMSF), 0.1 mg/mL benzamidine. Membranes were prepared from 150 mm plates by washing the cells 2X with ice cold PBS (50 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.4, 150 mM NaCl), addition of lysis buffer (50 mM TrisHCl, pH 8.0, 1 mM CaCl<sub>2</sub> and protease inhibitors) at a volume of 6 mL, and incubation on ice for 5 minutes. The lysate was scraped from the plate with a cell harvester and glass pipetted into a glass/teflon homogenizer (10 strokes at approximately 1000 RPM). The homogenate was separated at 2000 RPM for 5 minutes in 2059 tubes (Falcon) in a Beckman J-6B centrifuge. The membranes in the supernatant were pelleted by centrifugation in a Ti-70 rotor in a Beckman L8-70M ultracentrifuge at 45K RPM for 30 minutes. The pellet was resuspended in lysis buffer by glass/teflon homogenization (10 strokes at 1000 RPM) in a

volume so that the protein concentration as determined by the Peterson assay (Peterson, 1983) was approximately 1 mg/mL.

**Radioligand binding assays.** Equilibrium binding assays were performed in 50 mM TrisHCl (pH 8.0) and 1 mM CaCl<sub>2</sub> and protease inhibitors using 50 - 100 µg of membrane protein and 0.1 - 20 nM (+)-[<sup>3</sup>H]PN200-110 (isradipine) at 22°C for 90 minutes. Non-specific binding was determined in the presence of 1 µM (+/-)-PN200-110, and bound ligand was separated by vacuum filtration over GF/C glass fiber filters (Whatman). Filters were washed using ice-cold wash buffer (10 mM TrisHCl (pH 8.0), 1% polyethylene glycol 8000, and 0.1% bovine serum albumin), and bound radioactivity was detected by liquid scintillation counting. Dissociation constants (K<sub>D</sub>) were determined using the program LIGAND (Munson, et al., 1980).

**Immunoprecipitation of calcium channels.** All procedures were performed on ice or at 4°C and in the presence of protease inhibitors. Calcium channels were concentrated from the crude membrane preparations using 10 µg anti-peptide affinity-purified antibody or control rabbit IgG for 2 hours on a rotating mixer. Protein A-Sepharose beads, 2.5 mg, prewashed in PBS with 0.5% BSA were added and incubated an additional 2 hours. These calcium channels on beads could then be used for phosphorylation studies or western blot analysis (see below).

**Incorporation of  $^{32}\text{P}$  phosphate ( *in vitro* and *in vivo*).** Calcium channels as prepared above were washed 2X in 1 mL of radioimmunoassay buffer (RIA, 25 mM TrisHCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1 mg/mL BSA, 0.05% sodium azide, 50 mM NaF, 1 mM EGTA) containing 1% Triton TX-100 with protease inhibitors (see above) and phosphatase inhibitors (20 mM  $\beta$ -glycerophosphate, 50 mM sodium pyrophosphate, 1 mM *p*-nitrophenylphosphate, and 1 mM sodium vanadate), 3X in 1 mL of phosphorylation buffer (50 mM TrisHCl, pH 7.5, 10 mM  $\text{MgCl}_2$ ) with 0.1% Triton TX-100 with protease inhibitors and phosphatase inhibitors. Exogenous PKA (type II) prepared from bovine heart at a concentration of 1 mg/mL (using 2  $\mu\text{g}$ ) was added to the reaction in the presence of 0.2  $\mu\text{M}$   $\gamma^{32}\text{P}$ -ATP. This was incubated at 37°C for 5 minutes, and the reaction was terminated by addition of 1 mL phosphorylation buffer with 0.1% Triton TX-100. Two additional washes in this buffer were performed and then one wash in 10 mM TrisHCl, pH 7.4 before loading on polyacrylamide gel (see below).

Labelling *in vivo* or metabolic labelling was performed as follows: All protocols were appropriately shielded. Cells transfected as above were used 48 hours post transfection. The medium was changed using phosphate- and serum-free medium including 2 washes.  $^{32}\text{P}$ -Orthophosphate

(3.125 mCi) was added dropwise around plate. After 4 hours incubation at 37°C at 5% CO<sub>2</sub>, PKA-activating agents were added directly to the media (50µM Sp-5,6-DCI-cBIMPS, final) and incubated for 30 minutes. Cells were harvested for membranes by aspirating the media off carefully, washing the cells 2X in 10 mL PBS, and adding 2 mL lysis buffer. The lysed cells were then hand-homogenized 10 strokes with gentle turning to prevent aspiration. The membranes were then centrifuged for 30 minutes at 16 K RPM in an SS-34 rotor in a Beckman J2-21 centrifuge. The pellet was resuspended in 1 mL lysis buffer and the membranes immunoprecipitated as above.

**Western immunoblot analysis.** Protein A-sepharose beads containing immunoprecipitated proteins, were washed 3X in RIA with 1% Triton TX-100, 2X in RIA with 0.1% Triton TX-100 and 1X in 10mM TrisHCl, pH 7.4. Samples were extracted from the beads in sample buffer (125 mM Tris HCl, pH 6.8, 2% sodium dodecyl sulfate (SDS), 2 mM EDTA, 10% sucrose, 20 mM DTT, 1 µM pepstatin A, 2 µg/mL leupeptin, 4 µg/mL aprotonin, 1 µM *p* - nitrophenylphosphate) for 30 minutes at 55-60°C with mixing. Samples were loaded onto a 6% SDS polyacrylamide gel for α subunits or a 10% gel for β subunits with a 3% stacking layer (Laemmli, 1970) and electrophoresed at 50 mA/gel for 2 - 3 hours. The separated proteins were transferred electrophoretically to nitrocellulose filters and blocked for 1 hour in buffer

consisting of 10% nonfat milk in TBS (10 mM TrisHCl, pH 7.4, 150 mM NaCl) with 0.1% tween. Filters were then incubated with affinity purified antibody (50  $\mu\text{g}/\text{mL}$  in blocking buffer) for 1 hour. Filters were then washed 3X for 10 minutes each, incubated 1 hour with protein A-horseradish peroxidase, diluted 1:1000 in blocking buffer. Washing continued in TBS 6X for 10 minutes and then 4X for 30 minutes. They were labeled with ECL reagents and examined with autoradiogram film for a few minutes at room temperature for antibody data or for hours to days at  $-80^{\circ}\text{C}$  with intensifying screens for  $^{32}\text{P}$  data.

**Electrophysiology.** Barium currents through skeletal muscle calcium channels were recorded using the whole cell patch-clamp technique. Patch electrodes were pulled from VanWaters and Rogers micropipettes and fire polished to produce an inner tip diameter of 4-6  $\mu\text{m}$ . Currents were recorded using a List EPC-7 patch-clamp amplifier and filtered at 2kHz (8-pole Bessel filter, -3dB). Data were acquired using Fastlab software (Indec Systems). Voltage-dependent currents have been corrected for leak using an on-line P/4 subtraction paradigm. The extracellular (bath) saline contained 150 mM TrisHCl, 2 mM  $\text{MgCl}_2$ , 10 mM  $\text{BaCl}_2$ ; pH was adjusted to 7.3 with methanesulfonic acid. The intracellular (patch electrode) saline contained 130 mM *N*-methyl-D-glucamine, 10 mM EGTA, 60 mM HEPES, 2 mM

MgATP, 1 mM MgCl<sub>2</sub>; pH was adjusted to 7.3 with methanesulfonic acid. All experiments were performed at room temperature (20-23°C). No nonlinear outward currents were detected under these conditions.

Calcium activated chloride currents are a potential concern in measurements of tail current in skeletal muscle cells and possibly in tsA-201 cells. Contamination of calcium channel tail current measurements by calcium activated chloride currents was prevented by using low chloride intracellular salines yielding a calculated chloride reversal potential (zero-current potential) of -63 mV, near the potential (-80 mV) at which calcium channel currents were measured. The reversal potential of barium tail currents was more than +30 mV, consistent with relatively pure barium permeation, and identical tail currents were observed when sodium carried current through the channel or when the calcium buffer EGTA (10 mM) in the intracellular was replaced with 10 mM BAPTA.

Sp-5,6-DCI-cBIMPS was stored frozen in a 100 mM DMSO stock. The appropriate amount of this stock was either added directly to the recording chamber or first diluted to 6X its final concentration in extracellular saline before addition. Ht-31 peptide (Carr, et al., 1992) was synthesized and purified by HPLC in the University of Washington Molecular Pharmacology Facility with the following sequence: Asp-Leu-Ile-Glu-Glu-Ala-Ala-Ser-Arg-Ile-Val-Asp-Ala-Ala-Val-Ile-Glu-Gln-Val-Lys-Ala-

Ala-Gly-Ala-Tyr. Ht-31P peptide containing proline residues substituted for the isoleucine residues at positions 10 and 16 was synthesized and purified using the same methods. Ht-31 peptides and PKI (5-24) amide were stored frozen in 1 mM stocks made from intracellular saline without MgATP and diluted to final concentrations (100 or 500 nM, respectively) in intracellular saline containing MgATP.

## X. BIBLIOGRAPHY

Adams, B.A., Tanabe, T., Mikami, A., Numa, S., and Beam, K.G. (1990) Intramembrane charge movement restored in dysgenic skeletal muscle by injection of dihydropyridine receptor cDNAs. *Nature* 346 , 569-572.

Arreola, J., Calvo, J., Garcia, M.C., and Sanchez, J.A., (1987) Modulation of calcium channels of twitch skeletal muscle fibres of the frog by adrenaline and cyclic adenosine monophosphate. *J. Physiol.* 393 , 307-330

Ausubel, F.M., Brent, R., Kingston, R.E., More, D.D., Seidman, J.G., Smith, J.A., and Struhl, K. (1987) Current protocols in molecular biology , Wiley and Sons, N.Y., N.Y..

Beam, K.G., Knudson, C.M., and Powell, J.A. (1986) A lethal mutation in mice eliminates the slow calcium current in skeletal muscle cells. *Nature* 320, 168-170

Carr, D.W., Stofko-Hahn, R.E., Fraser, I.D.C., Cone, R.D., and Scott, J.D. (1992) Localization of the cAMP-dependent protein kinase to the postsynaptic densities by A-Kinase Anchoring Proteins. *J. Biol. Chem.* 267 , 16816- 16823

Caswell, A.H., Baher, S.P., Boyd, H., Potter, L.T., and Garcia, M. (1978)  $\beta$ -Adrenergic receptor and adenylate cyclase in transverse tubules of skeletal muscle. *J. Biol. Chem.* 253 , 3049-3054

Catterall, W.A. (1991) Excitation-contraction coupling in vertebrate skeletal muscle: A tale of two calcium channels. *Cell* 64 , 871-874

Chaudhari, N. (1992) A single nucleotide deletion in the skeletal muscle-specific calcium channel transcript of muscular dysgenesis ( *mdg* ) mice. *J. Biol. Chem.* 267 , 25636-25639

Chien, A.J., Zhao, X., Shirokov, R.E., Puri, T.S., Chang, C.F., Sun, D., Rios, E., and Hosey, M. (1995) Roles of a membrane-localized  $\beta$  subunit in the formation and targeting of functional L-type  $\text{Ca}^{2+}$  channels. *J. Biol. Chem.* 270 , 30036-30044

Chien, A.J., Carr, K.M., Shirokov, R.E., Rios, E., and Hosey, M.M. (1996) Identification of palmitoylation sites within the L-type calcium channel  $\beta_{2a}$  subunit and effects on channel function. *J. Biol. Chem.* 271 , 26465-26468

Curtis, B.M., and Catterall, W.A. (1984) Purification of the calcium antagonist receptor of the voltage-sensitive calcium channel from skeletal muscle transverse tubules. *Biochem.* 23, 2113-2118

Curtis, B.M., and Catterall, W.A. (1985) Phosphorylation of the calcium antagonist of the voltage-sensitive calcium channel by cAMP-dependent protein kinase. *P.N.A.S.* 82, 2528-2532

Curtis, B.M., and Catterall, W.A. (1986) Reconstitution of the voltage-sensitive calcium channel purified from skeletal muscle transverse tubules. *Biochem.* 25 , 3077-3083

De Jongh, K.S., Merrick, D.K., and Catterall, W.A.Q. (1989) Subunits of purified calcium channels: A 212-kDa form of  $\alpha_1$  and partial amino acid sequence of a phosphorylation site of an independent  $\beta$  subunit. *Proc. Nat. Acad. Sci.* 86 , 8585-8589

De Jongh, K.S., Warner, C., Colvin, A.A., and Catterall, W.A. (1991) Characterization of the two size forms of the  $\alpha_1$  subunit of skeletal muscle L-type calcium channels. *Proc. Nat Acad. Sci.* 88 , 10778-10782

El-Hayek, R., Antoniu, B., Wang, J., Hamilton, S.L., and Ikemoto, N. (1995) Identification of calcium release-triggering and blocking regions of the II-III loop of the skeletal muscle dihydropyridine receptor. *J. Biol. Chem.* 270, 22116-22118

Ellis, S.B., Williams, M.E., Ways, N.R., Brenner, R., Sharp, A.H., Leung, A.T., Campbell, K.P., McKenna, E., Koch, W.J., Hui, A., Schwartz, A., and Harpold,

M.M. (1988) Sequence and expression of mRNAs encoding the  $\alpha_1$  and  $\alpha_2$  subunits of a DHP-sensitive calcium channel. *Science* 241 , 1661-1664

Dulhunty, A.F., Junankar, P.R., Eager, K.R., Ahern, G.P., and Laver, D.R. (1996) Ion channels in the sarcoplasmic reticulum of striated muscle. *Acta. Physiol. Scand.* 156, 375-385

Flockerzi, V., Oeken, H-J., Hofmann, F., Pelzer, D., Cavalié, C., and Trautwein, W. (1986) Purified dihydropyridine-binding site from skeletal muscle t-tubules is a functional channel. *Nature* 323 , 66-68

Gao, T., Yatani, A., Dell'Acqua, M. L., Sako, H., Green, S. A., Dascal, N., Scott, J.D., and Hosey, M.M. (1997) cAMP-dependent regulation of cardiac L-type  $\text{Ca}^{2+}$  channels requires membrane targeting of PKA and phosphorylation of channel subunits. *Neuron* 19, 185-196

Gregg, R.G., Messing, A., Strube, C., Beurg, M., Moss, R., Behan, M., Sukhareva, M., Haynes, S., Powell, J.A., Coronado, R., and Powers, P.A. (1996) Absence of the  $\beta$  subunit ( *cchb1* ) of the skeletal muscle dihydropyridine receptor alters expression of the  $\alpha_1$  subunit and eliminates excitation-contraction coupling. *Proc. Nat. Acad. Sci.* 93 , 13961-13966

Gray, P.C., Tibbs, V.C., Catterall, W.A., and Murphy, B.J. (1997) Identification of a 15-kDa cAMP-dependent protein kinase-anchoring protein associated with skeletal muscle L-type calcium channels. *J. Biol. Chem.* 272, 6297-6302

Gray, P.C., Johnson, B.D., Westenbroek, R.E., Hays, L.G., Yates, J.R.III, Scheuer, T., Catterall, W.A., and Murphy, B.J. (1998) Primary structure and function of an A kinase anchoring protein associated with calcium channels. *Neuron*, 20, 1017-1026

Guo, W., Jorgensen, A.O., and Campbell, K.P. (1996) Triadin, a linker for calsequestrin and the ryanodine receptor. *Soc. Gen. Physiol. Ser.* 51, 19-28

Hess, P., Lansman, J.B., and Tsien, R.W. (1984) Different modes of Ca channel gating behavior favoured by dihydropyridine Ca agonists and antagonists. *Nature* 311, 538-544

Hymel, L., Striessnig, J., Glossmann, H., and Schindler, H. (1988) Purified skeletal muscle 1,4-dihydropyridine receptor forms phosphorylation-dependent oligomeric calcium channels in planar bilayers. *Proc. Nat. Acad. Sci.* 85, 4290-4294

Janis, R.A., and Triggle, D.J. (1983) New developments in Ca<sup>2+</sup> channel antagonists. *J. Med. Chem.* 26, 775-785

Johnson, B.D., Scheuer, T., and Catterall, W.A. (1994) Voltage-dependent potentiation of L-type  $\text{Ca}^{2+}$  channels in skeletal muscle cells requires anchored cAMP-dependent protein kinase. *Proc. Nat. Acad. Sci.* 91 , 11492-11496

Johnson, B.D., Brousal, J.P., Peterson, B.Z., Gallombarido, P.A., Hockerman, G.H., Lai, L., Scheuer, T., and Catterall, W.A. (1997) Modulation of the cloned skeletal muscle L-type  $\text{Ca}^{2+}$  channel by anchored cAMP-dependent protein kinase. *J. Neurosci.* 17 , 1243-1255

Jurman, M.E., Boland, L.M., Liu, Y., and Yellen, G. (1994) Visual identification of individual transfected cells for electrophysiology using antibody coated beads. *Biotechniques* 17 , 876-881

Kaczmarek, L.K., Jennings, K.R., Strumwasser, F., Nairn, A.C., Walter, U., Wilson, F.D., and Greengard, P. (1980) Microinjection of catalytic subunit of cyclic AMP-dependent protein kinase enhances calcium action potentials of bag cell neurons in cell culture. *Proc. Nat Acad. Sci.* 77 , 7487-7491

Kim, H.S., Wei, X., Ruth, P., Perez-Reyes, E., Flockerzi, V., Hofmann, F., and Birnbaumer, L. (1990) Studies on the structural requirements for the activity of the skeletal muscle dihydropyridine receptor/ slow  $\text{Ca}^{2+}$  channel. *J. Biol. Chem.* 265 , 11858-11863

Knudson, C.M., Chaudhari, N., Sharp, A.H., Powell, J.A., Beam, K.G., and Campbell, K.P. (1989) Specific absence of the  $\alpha_1$  subunit of the dihydropyridine receptor in mice with muscular dysgenesis. *J. Biol. Chem.* 264 , 1345-1348

Kokubun, S., and Reuter, H. (1984) Dihydropyridine derivatives prolong the open state of Ca channels in cultured cardiac cells. *Proc. Natl. Acad. Sci.* 81 , 4824-4827

Krebs, E.G., and Beavo, J.A. (1979) Phosphorylation-dephosphorylation of enzymes. *Ann. Rev. Biochem.* 48 , 923-959

Kunkel, T.A. (1985) Rapid and efficient site-specific mutagenesis without phenotypic selection. *Proc. Nat. Acad. Sci.* 82 , 488-492

Lacerda, A.E., Kim, H.S., Ruth, P., Perez-Reyes, E., Flockerzi, V., Hofmann, F., Birnbaumer, L., and Brown, A.M. (1991) Normalization of current kinetics by interaction between the  $\alpha_1$  and  $\beta$  subunits of the skeletal muscle dihydropyridine-sensitive  $\text{Ca}^{2+}$  channel. *Nature* 352 , 527-530

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227 , 680-685

- Lai, F.A., Erickson, H.P., Rousseau, E., Liu, Q-Y., and Meissner, G. (1988) Purification and reconstitution of the calcium release channel from skeletal muscle. *Nature* 331 , 315-319
- Lai, Y., Seagar, M.J., Takahashi, M., and Catterall, W.A. (1990) Cyclic AMP-dependent phosphorylation of two size forms of  $\alpha_1$  subunits of L-type calcium channels in rat skeletal muscle cells. *J. Biol. Chem.* 265 , 20839-20848
- Leong, P., and MacLennan, D.H. (1998) A 37-amino acid sequence in the skeletal muscle ryanodine receptor interacts with the cytoplasmic loop between domains II and III in the skeletal muscle dihydropyridine receptor. *J. Biol. Chem.* 273 , 7791-7794
- Lu, X., Xu, L., and Meissner, G. (1994) Activation of the skeletal muscle calcium release channel by a cytoplasmic loop of the dihydropyridine receptor. *J. Biol. Chem.* 269 , 6511-6516
- Lu, X., Xu, L., and Meissner, G. (1995) Phosphorylation of dihydropyridine receptor II-III loop peptide regulates skeletal muscle calcium release channel function. *J. Biol. Chem.* 270 , 18459-18464
- Munson, P.J., and Rodbard, D. (1980) LIGAND: A versatile computerized approach for characterization of ligand-binding systems. *Anal. Bioch.* 107 , 220-239

Nakai, J., Dirksen, R.T., Nguyen, H.T., Pessah, I.N., Beam, K.G., and Allen, P.D. (1996) Enhanced dihydropyridine receptor channel activity in the presence of ryanodine receptor. *Nature* 380 , 72-75

Nakai, J., Sekiguchi, N., Rando, T.A., Allen, P.D., and Beam, K.G. (1998) Two regions of the ryanodine receptor involved in coupling with L-type Ca<sup>2+</sup> Channels. *J. Biol. Chem.* 273, 13403-13406

Nukada, T., Mishina, M., and Numa, S. (1987) Functional expression of cloned cDNA encoding the  $\alpha$ -subunit of adenylate cyclase-stimulating G-protein. *FEBS LETTERS* 211 , 5-9

Nunoki, K., Florio, V., and Catterall, W.A. (1989) Activation of purified calcium channels by stoichiometric protein phosphorylation. *Proc. Nat. Acad. Sci.* 86 , 6816-6820

O'Callahan, C.M., and Hosey, M.M. (1988) Multiple phosphorylation sites in the 165-kilodalton peptide associated with dihydropyridine-sensitive calcium channels. *Biochemistry* 27, 6071-6077

Oz, M., and Frank, G.B. (1991) Decrease in the size of tetanic responses produced by nitrendipine or by extracellular calcium ion removal without blocking twitches or action potentials in skeletal muscle. *J. Pharm. Exp. Ther.* 257 , 575-581

Perez-Reyes, E., Kim, H.S., Lacerda, A.E., Horne, W., Wei, X., Rampe, D., Campbell, K.P., Brown, A.M., and Birnbaumer, L. (1989) *Nature* 340 , 233-236

Peterson , G.L. (1983) Determination of total protein. *Meth. Enzy.* 91 , 95-119

Powers, P.A., Liu, S., Hogan, K., and Gregg, R.G. (1992) Skeletal muscle and brain isoforms of a  $\beta$ -subunit of human voltage-dependent calcium channels are encoded by a single gene. *J. Biol. Chem.* 267 , 22967-22972

Pragnell, M., Sakamoto, J., Jay, S.D., and Campbell, K.P. (1991) Cloning and tissue-specific expression of the brain calcium channel  $\beta$ -subunit. *FEBS* 291 , 253-258

Rios, E., and Brum, G. (1987) Involvement of dihydropyridine receptors in excitation-contraction coupling in skeletal muscle. *Nature* 325, 717-720

Röhrkasten, A., Meyer, H.E., Nastainczyk, W., Sieber, M., and Hofmann, F. (1988) cAMP-dependent protein kinase rapidly phosphorylates serine-687 of the skeletal muscle receptor for calcium channel blockers. *J. Biol. Chem.* 263 , 15325-15329

Rotman, E.I., De Jongh, K.S., Florio, V., Lai, Y., and Catterall, W.A. (1992) Specific phosphorylation of a COOH-terminal site on the full-length form of

the  $\alpha_1$  subunit of the skeletal muscle calcium channel by cAMP-dependent protein kinase. *J. Biol. Chem.* 267 , 16100-16105

Rotman, E.I., Murphy, B.J., and Catterall, W.A. (1995) Sites of specific cAMP-dependent phosphorylation of the L-type calcium channel  $\alpha_1$  subunit from intact rabbit skeletal muscle myotubes. *J. Biol. Chem.* 270 , 16371-16377

Ruth, P., Röhrkasten, A., Biel, M., Bosse, E., Regulla, S., Meyer, H.M., Flockerzi, V., and Hofmann, F. (1989) Primary structure of the  $\beta$  subunit of the DHP-sensitive calcium channel from skeletal muscle. *Science* 245 , 1115-1118

Schmid, A., Renaud, J., and Lazdunski, M. (1985) Short term and long term effects of  $\beta$ -adrenergic effectors and cyclic AMP on nitrendipine-sensitive voltage-dependent  $\text{Ca}^{2+}$  channels of skeletal muscle. *J. Biol. Chem.* 260 , 13041-13046

Schneider, M.F., and Chandler, W.K. (1973) Voltage dependent charge movement in skeletal muscle: a possible step in excitation-contraction coupling. *Nature* 242 , 244-246

Schramm, M., and Towart, R. (1985) Modulation of calcium channel function by drugs. *Life Sci.* 37 , 1834-1860

Sculptoreanu, A., Scheuer, T., and Catterall, W.A. (1993) Voltage-dependent potentiation of L-type  $\text{Ca}^{2+}$  channels due to phosphorylation by cAMP-dependent protein kinase. *Nature* 364 , 240-243

Singer, D., Biel, M., Lotan, I., Flockerzi, V., Hofmann, F., and Dascal, N. (1991) The roles of the subunits in the function of the calcium channel. *Science* 253 , 1553-1557

Slavik, K.J., Wang, J-P., Aghdasi, B., Zhang, J-Z., Mandel, F., Malouf, N., and Hamilton, S.L. (1997) A carboxyl-terminal peptide of the  $\alpha_1$ -subunit of the dihydropyridine receptor inhibits  $\text{Ca}^{2+}$ -release channels. *Am. J. Physiol.* 272 , C1475-C1481

Songyang, Z., Blechner, S., Hoagland, N., Hoekstra, M.F., Piwnica-Worms, H., and Cantley, L.C. (1994) Use of an oriented peptide library to determine the optimal substrates of protein kinases. *Curr. Biol.* 4 , 973-982

Stea, A., Dubel, S.J., Pragnell, M., Leonard, J.P., Campbell, K.P., and Snutch, T.P. (1993) A  $\beta$ -subunit normalizes the electrophysiological properties of a cloned N-type  $\text{Ca}^{2+}$  channel  $\alpha_1$ -subunit. *Neuropharm.* 32, 1103-1116

Strube, C., Beurg, M., Powers, P.A., Gregg, R.G., and Coronado, R. (1996) Reduced  $\text{Ca}^{2+}$  current, charge movement, and absence of  $\text{Ca}^{2+}$  transients in

skeletal muscle deficient in dihydropyridine receptor  $\beta_1$  subunit. *Biophys. J.* 71, 2531-2543

Suda, N., Franzius, D., Fleig, A., Nishimura, S., Bödding, M., Hoth, M., Takeshima, H., and Penner, R. (1997)  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release in chinese hamster ovary (CHO) cells co-expressing dihydropyridine and ryanodine receptors. *J. Gen. Physiol.* 109, 619-631

Takahashi, M., Seagar, M.J., Jones, J.F., Reber, B.F., and Catterall, W.A. (1987) Subunit structure of dihydropyridine-sensitive calcium channels from skeletal muscle. *P.N.A.S.* 84, 5478-5482

Takekura, H., Takeshima, H., Nishimura, S., Takahashi, M., Tanabe, T., Flockerzi, V., Hofmann, F., and Franzini-Armstrong, C. (1995) Co-expression in CHO cells of two muscle proteins involved in excitation-contraction coupling. *J. Muscle Res. & Cell Motility* 16, 465-480

Takeshima, H., Nishimura, S., Matsumoto, T., Ishida, H., Kangawa, K., Minamino, N., Matsuo, H., Ueda, M., Hanaoka, M., Hirose, T., and Numa, S. (1989) Primary structure and expression from complementary DNA of skeletal muscle ryanodine receptor. *Nature* 339, 439-445

Tanabe, T., Takeshima, H., Mikami, A., Flockerzi, V., Takahashi, H., Kangawa, K., Kojima, M., Matsuo, H., Hirose, T., and Numa, S. (1987)

Primary structure of the receptor for calcium channel blockers from skeletal muscle. *Nature* 328 , 313-318

Tanabe, T., Beam, K.G., Powell, J.A., and Numa, S. (1988) Restoration of excitation-contraction coupling and slow calcium current in dysgenic muscle by dihydropyridine receptor complementary DNA. *Nature* 336 , 134-139

Tanabe, T., Beam, K.G., Adams, B.A., Niidome, T., and Numa, S. (1990) Regions of the skeletal muscle dihydropyridine receptor critical for excitation-contraction coupling. *Nature* 346 , 567-569

Van Patten, S.M., Howard, P., Walsh, D.A., and Maurer, R.A. (1992) The  $\alpha$ - and  $\beta$ -isoforms of the inhibitor protein of the 3',5'-cyclic adenosine monophosphate-dependent protein kinase: characteristics and tissue- and developmental-specific expression. *Mol. Endo.* 6 , 2114-2122

Walsh, D.A., Angelos, K.L., Van Patten, S.M., Glass, and D.B. Garetto, L.P. (1990) The inhibitor protein of the cAMP-dependent protein kinase. Kemp, B.E. (ed) Peptides and Protein Phosphorylation. CRC Press, Boca Raton, pp 43-84

West, J.W., Patton, D.E., Scheuer, T., Wang, Y., Goldin, A.L., and Catterall, W.A. (1992) A cluster of hydrophobic amino acid residues required for fast Na<sup>+</sup>-channel inactivation. *Proc. Nat. Acad. Sci.* 89 , 10910-10914

Xu, Z., Yang, Y., and Hebert, S., C. (1996) Phosphorylation of the ATP-sensitive, inwardly rectifying K<sup>+</sup> channel, ROMK, by cyclic AMP-dependent protein kinase. *J. Biol. Chem.* 271 , 9313-9319

Yoshida, A., Takahashi, M., Nishimura, S., Takeshima, H., and KoKubun, S. (1992) Cyclic AMP-dependent phosphorylation and regulation of the cardiac dihydropyridine-sensitive Ca channel. *FEBS* 309, 343-349

## XI. APPENDIX A: CARDIAC CALCIUM CHANNEL

The plasmid pCCAR (Yoshida, et al., 1992) containing the cDNA for the cardiac L-type calcium channel was mutated at serine 1928, a site of PKA phosphorylation, and coexpressed with the  $\beta_{1b}$  and  $\alpha_2/\delta$  subunits of the brain and skeletal muscle calcium channel in the tsA-201 expression system. This channel has been shown to be regulated by PKA and membrane targeting (Gao, et al., 1997). The expression in this system showed no alteration of regulation in the mutant channel. We were unable to reproduce the results seen in other laboratories. Average current amplitude upon depolarization from a holding potential of -80 mV to a test potential of +10 mV was 401 +/- 139 pA (+/- SE) (n = 10) for wild type cardiac channel. Average current amplitude under the same conditions was 356 +/- 224 pA (+/- SE) (n = 10) for the mutant cardiac channel. This is not a significant difference. Current amplitude increased approximately 2 fold with DCI cBIMPS application.

## VITA

### **JEFFREY P. BROUSAL**

550 Scott Circle  
Decatur, Ga. 30033  
(404) 634-5980

Pharmacology Department  
University of Washington  
Seattle, Wa. 98195  
(206) 685-1542

425 Malden Ave. E. #302  
Seattle, Wa. 98112  
(206)323-1991

#### **EDUCATION:**

B.S. Chemistry,  
University of Massachusetts, Amherst, Ma. 1980  
Graduated cum laude

M.S. Biochemistry,  
Stevens Institute of Technology, Hoboken, N.J. 1987  
Thesis work examined the effect of 2-aminofluorene on lipid biosynthesis.

Ph.D. Pharmacology, expected 1998  
University of Washington  
Dissertation on the modulation of skeletal muscle calcium channels by phosphorylation.

#### **PROFESSIONAL EXPERIENCE:**

University of Washington, Seattle, Wa.  
Graduate Student in the Pharmacology Department  
Expressed cDNA in mammalian cells and performed biochemical, pharmacological and electrophysiological analyses.  
(October 1991 to present)

Centers for Disease Control, Atlanta, Ga.  
Research Scientist in the Bacteriology Department  
Developed genetic probes for bacterial mutants using RNA hybridization techniques  
(March to August 1991)

Emory University, Atlanta, Ga.  
Research Scientist in the Physiology Department  
Performed Southern analysis of human genomes for putative urea transporter linked to the Kidd antigen. (May to December 1990)

Merck, Sharp, & Dohme Research Laboratories, Rahway, N.J.  
Staff Biochemist in the Biochemical Endocrinology Department.

Managed cell based assay that screened for novel anti-diabetic agents. Led efforts to determine the effects of point mutations on insulin receptor function.  
(August 1988 to January 1990)

Immunomedics (Affiliated with UMDNJ), Newark, N.J.  
Radiochemist in the Organic Chemistry Department  
Developed a radiolabelled monoclonal antibody for early detection of carcinoembryonic antigen. Managed chromatography laboratory for diagnostic and quality control.  
(October 1987 to July 1988)

Consolidated Astronautics, Saddle Brook, N.J.  
Production Chemist in the Metals Division  
Responsibilities included raw materials acquisition and purification, alloy production and quality control, shipping and receiving.  
(March 1982 to August 1985)

### **Internships:**

U.S. Peace Corps, Cameroun, West Africa  
Technical Advisor in the agriculture and business sector. (1980 to 1982)

University of Kentucky, Lexington, Ky.  
Research Intern in the Department of Experimental Pathology. (June to August 1979)

### **HONORS:**

Robert Crooks Stanley Fellow, Stevens Institute  
Awarded competitive Molecular and Cell Biology Predoctoral Fellowship, University of Washington

### **AFFILIATIONS:**

American Chemical Society  
Graduate Activities Board Officer, Stevens Institute

### **PERSONAL:**

Married with family. Willing to relocate and travel. Enjoy music, poetry, and farming. Fluent in French.

**PUBLICATIONS:**

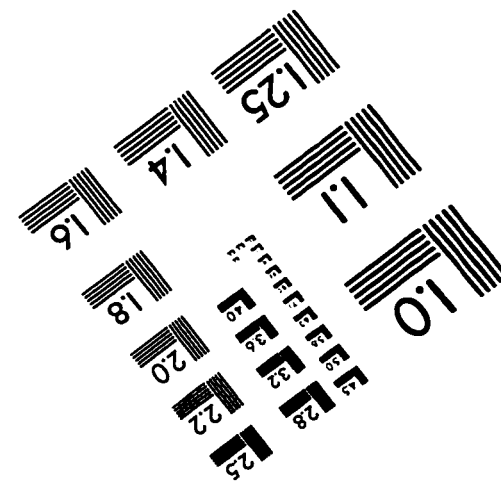
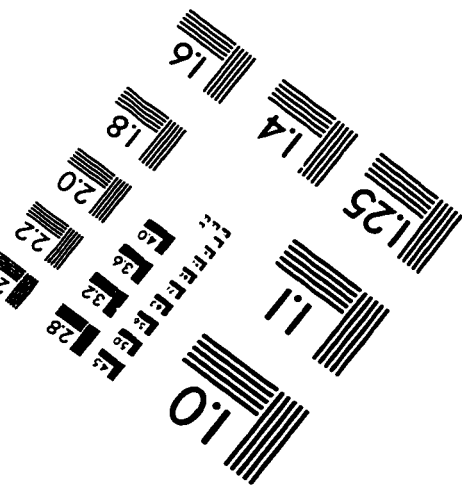
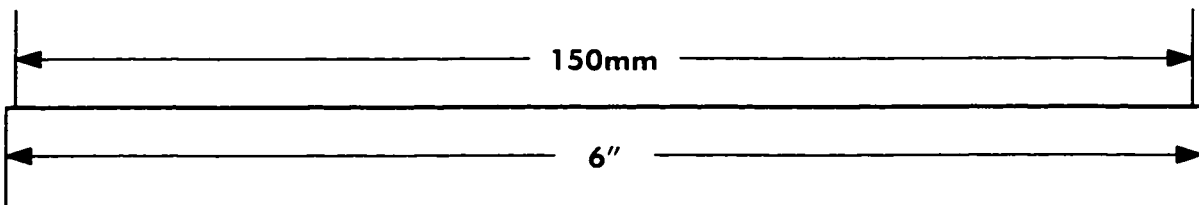
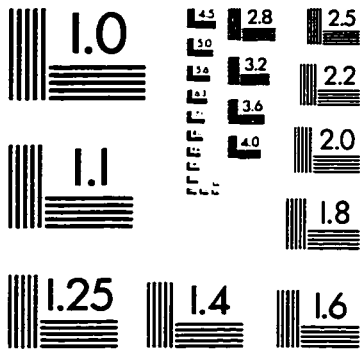
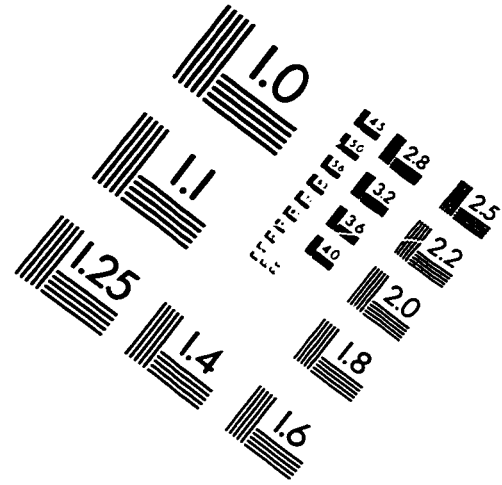
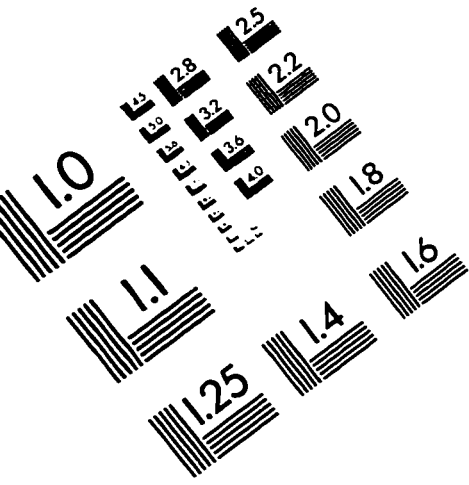
Utt, E.A., Brousal, J.P., Kikuta-Oshima, L.C., and Quinn, F.D. (1995) The identification of bacterial gene expression differences using mRNA-based isothermal subtractive hybridization. *Can. J. Microbiol.* **41**:152-156.

Johnson, B.D., Brousal, J.P., Peterson, B.Z., Gallombardo, P.A., Hockerman, G.H., Lai, Y., Scheuer, T., and Catterall, W.A. (1997) Modulation of the cloned skeletal muscle L-Type  $\text{Ca}^{2+}$  channel by anchored cAMP-dependent protein kinase. *Journal of Neuroscience* **17**: 1243-1255

Brousal, J.P., Johnson, B.D., Scheuer, T., and Catterall, W.A. (1998) Role of phosphorylation of the  $\alpha_1$  subunit in cAMP-dependent modulation of skeletal muscle calcium channels. In preparation.

University of Washington

# IMAGE EVALUATION TEST TARGET (QA-3)



**APPLIED IMAGE, Inc**  
1653 East Main Street  
Rochester, NY 14609 USA  
Phone: 716/482-0300  
Fax: 716/288-5989

© 1993, Applied Image, Inc., All Rights Reserved