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MODULATION OF KIR3 BY LIPIDS AND TYROSINE PHOSPHORYLATION

By Sherri Lynn Rogalski

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

Doctor of Philosophy

University of Washington

2000

Program Authorized to Offer Degree: Pharmacology

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Doctoral Dissertation

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Graduate School

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Chair of Supervisory Committee:

Charles Chavkin

Reading Committee:

Mont Juchau

Daniel Storm

Todd Scheuer

Charles Chavkin

Date: 11/17/2000

University of Washington

Abstract

Modulation of Kir3 by Lipids and Tyrosine Phosphorylation

by Sherri Lynn Rogalski

Chairperson of the Supervisory Committee
Professor Charles Chavkin
School of Medicine
Department of Pharmacology

Abstract

Neurotransmitters, hormones and growth factors act on ion channels to modulate excitable tissue. When a ligand binds a receptor it may activate ion channels in the membrane to initiate multiple intracellular events. One family of ion channels that are of particular interest is the G-protein gated-inwardly rectifying potassium channel (Kir3), the target of a variety of effectors.

Activation of Kir3 produces distinct downstream events that modulate cellular function. An understanding of these pathways may elucidate the molecular mechanisms of excitability.

Kir3 channel gating requires Gβγ, Na, PIP2 and ATP. I tested the hypothesis that Kir3 gating components have unique roles in downstream signal transduction events. Using *in vitro* expression systems I have determined that agonist-activation of the Gq-coupled human endothelin A receptor (HETAR) by endothelin-1 (Et-1) inhibits the Gi-coupled mu opioid receptor when coexpressed with Kir3 heteromultimers. Similarly, arachidonic acid mimics endothelin-inhibition of Kir3, suggesting that eicosanoids modulate Kir3. The response to MOR-activation was significantly inhibited by Et-1 activation of HETAR in homomeric channels composed of either Kir3.2 or Kir3.4. In contrast, homomeric channels of Kir3.1 were insensitive. Domain deletion and channel chimera studies suggested that the sites within the channel sensitive to Et-1-induced inhibition were within the region responsible for channel gating. Mutation of a single amino acid in the homomeric Kir3.1 to produce Kir3.1(F137S)(N217D) converted the channel from an arachidonic acid and endothelin insensitive channel to an eicosanoid-sensitive channel. The critical aspartate residue required for eicosanoid sensitivity is the same residue required for Na regulation of PIP2 gating. The results suggest a model of Kir3 gating

that incorporates a series of regulatory steps including Gβγ, PIP2, Na and arachidonic acid binding to the channel gating domain.

In another study, I identified specific tyrosine residues in the amino-terminus of Kir3 subunits that are the target of tyrosine kinase following BDNF activation of the TrkB receptor. I determined that electrostatic interactions may define a consensus site for tyrosine phosphorylation of Kir3.

My thesis provides a new framework for the molecular basis of how Kir3 regulation activates downstream signal transduction pathways to modulate excitability.

TABLE OF CONTENTS

LIST OF FIGURES

Number *Page*

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I appreciate the many thoughtful and engaging scientific discussions that I had with my advisor, Charles Chavkin. I value his knowledge and his interest in my training. I appreciate the thoughtful comments from my Reading Committee, Charles Chavkin, Daniel Storm, Todd Scheuer and Mont Juchau. I am indebted to Todd Scheuer for sharing his enthusiasm, expertise and friendship with me over the course of my graduate studies.

DEDICATION

I dedicate my thesis to my best friend and husband Bob, and to my wonderful children Robbie, Jessica and my new daughter, Maria. My family never complained about the changes in their lives because of my graduate work, including the institution of spaghetti and Hamburger Helper. My family made many trips to the lab with me to start experiments, to nurture oocytes or start cultures on nights and weekends, without protest. The love and support that I received at home helped me traverse the inevitable bumps in the graduate school road. Thirteen years ago, when my darling Jessica was born with multiple birth problems, I thought our family had lost everything of value. As I completed this work, I understood that by losing everything, we gained a precious appreciation of what truly matters in life.

CHAPTER I

INTRODUCTION

The graceful flight of the butterfly and the complex thought processes of human beings are linked by the events that comprise the intricate process termed neurotransmission. From the "all-or-none" responses dictated by some types of ion channel activity to the modulated responses that are produced by ion channels linked to second messenger systems, neurotransmission is a complex cascade of events. Inwardly rectifying potassium channels stabilize the resting membrane potential near the equilibrium potential (E_K) in both cardiac and neuronal tissue. The six subsets of inwardly rectifying potassium channels differ in both structure and in gating properties (Dascal 1997). G-protein activated potassium channels (GIRK, or Kir3) are one of the six groups that constitute the family of inwardly rectifying potassium channels. Although many ion channel families are activated by the action of G-protein-coupled receptors (GPCR) (Jan and Jan 1997), Kir3 channels are unique in that they are the only inwardly-rectifiying potassium channel family to be gated by direct interaction with $\beta\gamma$ subunits (Clapham and Neer 1997). Voltage gated channels and the inwardly rectifying potassium channel families Kir1 and Kir2 are constitutively active. In contrast, Kir3 channels are activated by ligand binding to GPCRs. A cell may convert a stimulus into a biological response by ligand-bound GPCRs activating Kir3. Kir3 channels are considered to have

anomalous rectification: potassium ions flow inward more readily than outward. Both Mg or polyamines can produce rectification in these channels. Kir3 channels share a common design characterized by two transmembrane domains M1 and M2, a pore region (P or H5) similar to that of voltage-dependent channels and cytoplasmic N and C termini (Fig. 1).

Figure 1. Diagram of Kir3.

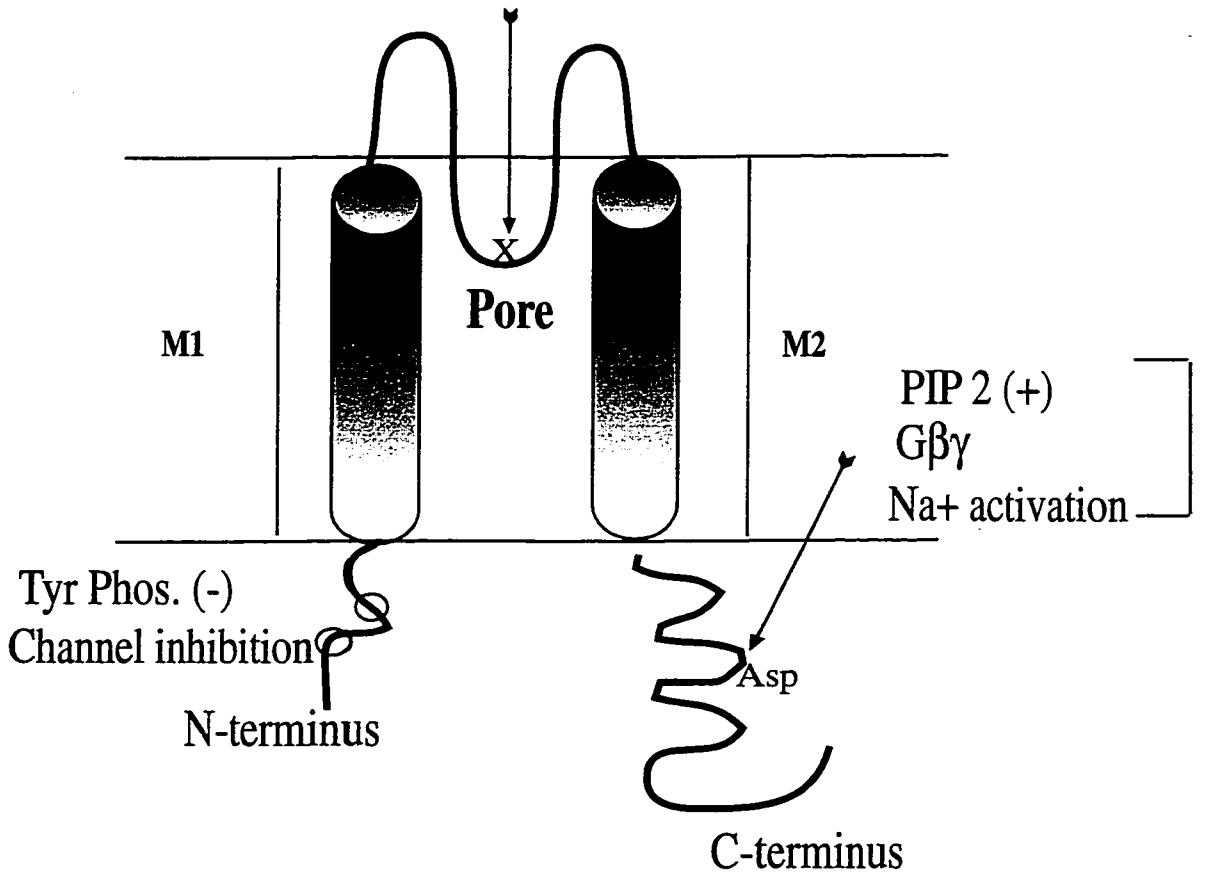
Kir3 channels share a common design characterized by a cytoplasmic N terminus (90 amino acids) and C terminus with two transmembrane domains M1 and M2, that surround the ion selective pore region (P or H5). Kir3 channels function as active heteromultimers (Kir3.1 pairs with other subtypes). Mutations in the P-region enhance the activity of homomers. This diagram indicates the P region with the site of specific point mutations that produce functional homomeric channels; Kir3.1(F137S), Kir3.2(S146T), and Kir3.4(S143T). The presumed PIP2-Na-Gβγ interaction site in the C-terminus is indicated. Kir3 is also inhibited by tyrosine phosphorylation (Rogalski et al 2000) with phosphorylation sites in the amino-terminus noted by the 'o'.

Homomultimers

Kir 3.1 (F137S)

Kir 3.2 (S146T)

Kir 3.4 (S143T)



G-proteins govern the exchange of information from the exterior of the cell to the interior of the cell activating downstream signal transduction pathways. Kir3 activation is modulated by the switch between active and inactive states by guanine nucleotide exchange and GTP hydrolysis with dissociation of the G-protein coupled receptor αßγ complex (Sui et al 1999; Wickman et al 1999). Activated Gβγ binds Kir3 to produce current by stabilizing the actions of the membrane phospholipid, PIP2 and Na (Huang et al 1998; Krapivinsky et al 1998; Zhang et al 1999).

The family of Kir 3 is composed of five subunits (Kir3.1-5). Subunits vary between 393 amino acids (Kir3.3) and 501 amino acids (Kir3.1), but are 60-80% homologous. The sequences of Kir 3.1-3.4 have been defined by cloning from a variety of human and animal species (Dascal 1997). Kir3.5 was cloned from the African frog *Xenopus* (Clapham and Neer 1997). The homology of Kir3 channels between species, is greater than 90%, e.g., human and rat Kir 3.1 share 99% identity, thus suggesting that research performed on homologues may have implication in the study of human disease.

Kir3 channels form heterotetramers by joining of two different Kir3 subunits (Krapivinsky et al 1995). Kir3.1/Kir3.2 and Kir3.1/Kir3.3 are found predominantly in neuronal tissue. A principal difference between these two neuronal inward rectifiers is that Kir3.1/Kir3.2 generates receptor-evoked currents,

whereas, Kir3.1/Kir3.3 does not (Dascal 1997). Because Kir3.3 has multiple splice variants, it may coassemble with other members of the family to produce neuronal diversity (Doupnik et al 1995).

Kir3.1 and Kir3.4 form the potassium channel that is predominantly found in cardiac tissue. Logothetis and colleagues noted that a specific point mutation in the pore of Kir3.1(F137S) or Kir3.4(S143T) would produce a functional homomer with large inward currents (Chan et al 1997; Vivaudou et al 1997). Kir3.2, found extensively in the brain, may form a functional homomer (Lesage et al 1995).

The coupling of GPCRs to ion channels produces direct actions on the ion channel and/or activation of second messenger systems to modulate cellular function in excitable tissue. The actions of these second messenger systems have implications for the modulation of both neuronal excitability and synaptic plasticity. For example, opioid peptide binding to their respective GPCR modulate excitatory transmission in the brain. Endorphin activation of mu opioid receptors inhibit pain responses (Cesselin 1995). Dynorphin activation of kappa opioid receptors may mediate anticonvulsant effects in epilepsy and inhibit long term potentiation (Simmons and Chavkin 1996).

Direct modulation of Kir3

G-proteins interact with Kir3 heteromultimers to modulate ion channel function. The cardiac Kir3.1/Kir3.4, termed IKACH or the muscarinic K channel, was the first G-protein activated ion channel and the first Gβγ effector discovered (Logothetis et al 1987; Kurachi 1995). Stimulation of the vagus nerve activates

IKACH to slow the heart when acetylcholine binds to the pertussis-toxin sensitive, muscarinic m2 receptor. Gβγ activates IKACH directly (Pfaffinger et al 1985). G-protein coupled receptor release of Gβγ binds Kir3 to activate the channel. More recently, it has been shown that IKACH activity is modulated by additional cofactors. Intracellular Na, ATP, phosphatidylinositol bisphosphate (PIP2) and fatty acids activate Kir3, although these cofactors are not required for Gβγ activation of the channel. Clapham and colleagues showed that treatment with the ser/thr phosphatase PP2A prevents Gβγ gating of the IKACH (Medina et al 2000). The presence of additional regulatory cofactors and phosphorylation/phosphatase events provides further support that Kir3 gating requires interactions at multiple sites on the channel.

Indirect modulation of Kir3

Activation of members of the pertussis toxin-sensitive family of Gi/Go coupled receptors (e.g. opioids, GABAB, somatostatin, 5HT, dopamine, adenosine, and muscarinic and α2 adrenergic (Jan and Jan 1997; Wickman et al 1999) releases Gβγ that directly activates Kir3 (Clapham and Neer 1997). In contrast, the activation of members of the pertussis toxin-insensitive family of Gq/11-coupled receptors (e.g. substance P and endothelin) inhibits Kir3 (Jan and Jan 1997). G-protein coupled receptor activation of Kir3 activates multiple signal transduction cascades. Signal transduction is a rapidly evolving field noted by

complex interrelationships between signaling cascades. This introduction will focus on the signal transduction cascades that may modulate Kir3 gating events.

Second messenger systems

Three second messenger pathways that may modulate excitatory tissue include the adenylate cyclase/cyclic AMP-dependent kinase system, guanylate cyclase/cyclic GMP-dependent protein kinase system (Ruth 1999), and the phospholipase mediated turnover of membrane phospholipids to produce inositol trisphosphate (IP3), diacylglycerol (DAG) and arachidonic acid.

Adenylate cyclase

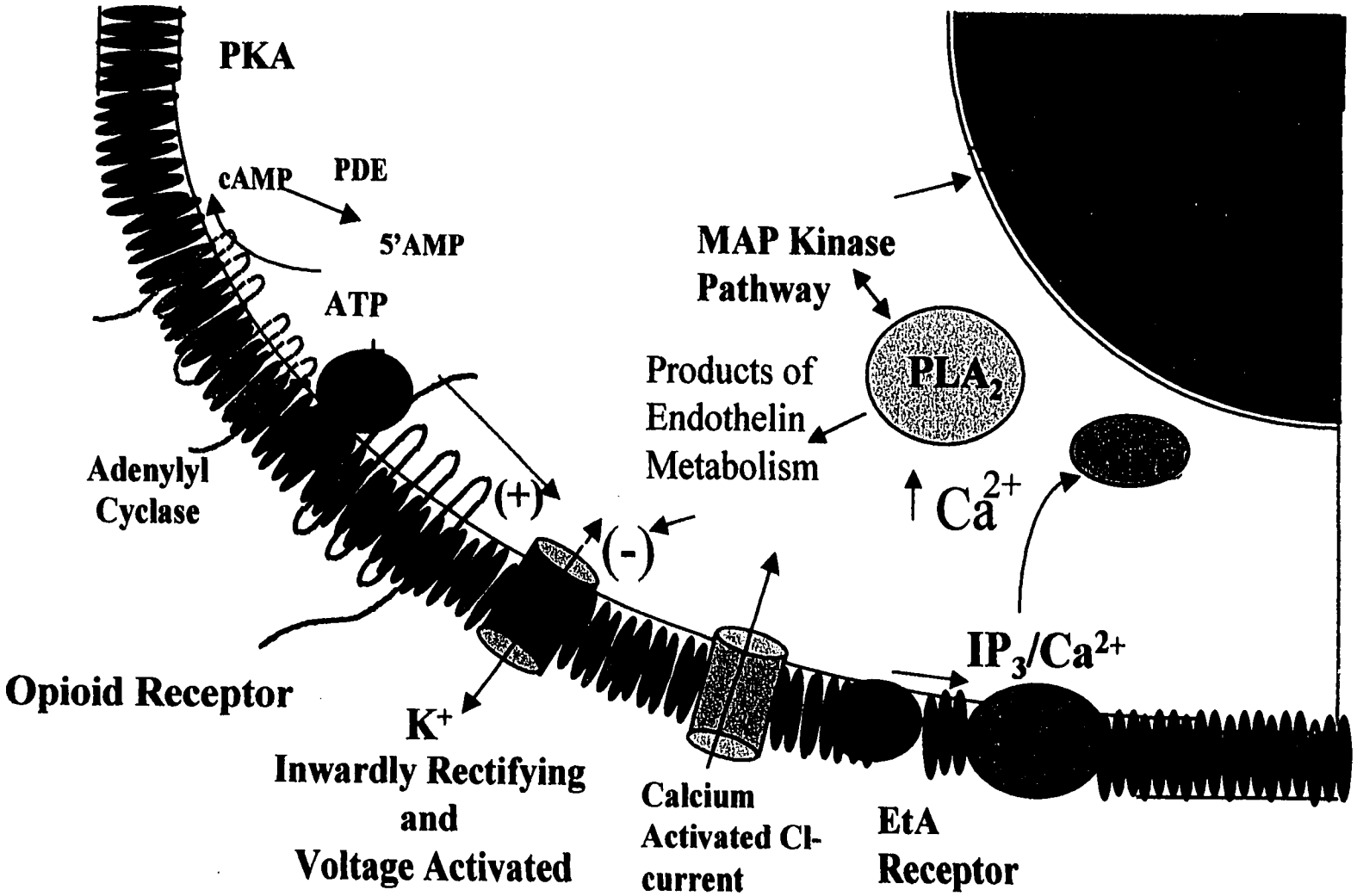
Phosphorylation of ion channels is one method of modulation of ion channel function. The cyclic AMP system mediates serine/threonine phosphorylation on target ion channels (Jonas and Kaczmarek 1996). Activation of the GPCR may activate the cyclic AMP cascade to catalyze the transfer of the terminal phosphate from ATP to the hydroxyl groups of serine or threonine residues on downstream targets (Choi et al 1993). ATP is involved in Kir3 gating, which may impact downstream signaling events. ATP hydrolysis enables both Gβγ and Na activation of Kir3. ATP activity enables intracellular Na to activate Kir3 by limiting the time the channel remains in the closed state (Sui et al 1996). Activation of PKA may modulate downstream signal transduction pathways to fine tune neuronal responses.

Membrane phospholipids as signal transducers

G proteins couple to the membrane enzyme phospholipase C (PLC) (Rhee and Choi 1992). PLC splits the minor membrane phospholipid, phosphotidlyinositol-4,5-bisphosphate (PIP2) into two products: soluble inositol-1,4,5-trisphosphate (IP3), and diacylglycerol (DAG), which remain in the membrane (Exton 1997). Both IP3 and DAG modulate signaling pathways (Carpenter and Cantley 1996) that have effects on Kir3. IP3 diffuses in the cytoplasm and binds to receptors that are located on the endoplasmic reticulum (ER) to activate a calcium channel in the membrane of the ER. Calcium channel activation leads to the release of calcium ions into the cytoplasm from the lumen of the ER. The increase in cytoplasmic calcium ions activates signal transduction cascades to indirectly modulate ion channel function or calcium ions can directly modulate ion channel function (Catterall 1997). Ligand-activation of Gq-coupled receptors expressed in *Xenopus* oocytes activates an endogenous chloride current (Barish 1983). The role of the calcium-activated chloride current on Kir3 function has not been established. Release of endoplasmic stores of calcium activates a lipid signal transduction cascade to modulate Kir3 (Berridge 1986; Rogalski et al 1999) (Fig. 2).

Figure 2. Signal transduction cascades that modulate Kir3.

Agonist activation of the opioid receptor leads to the release of Gβγ in the membrane to activate Kir3. Ligand-activation of Gq-coupled endothelin A receptors expressed in *Xenopus* oocytes couple to the membrane enzyme phospholipase C (PLC) and activate a chloride current endogenous to the oocyte. PLC splits the minor membrane phospholipid, phosphotidlyinositol-4,5-bisphosphate (PIP2) into two products: soluble inositol-1,4,5-trisphosphate (IP3), and diacylglycerol (DAG), which remain in the membrane. IP3 diffuses in the cytoplasm and binds to receptors that are located on the endoplasmic reticulum (ER) to activate a calcium channel in the membrane of the ER. Calcium channel activation leads to the release of calcium ions into the cytoplasm from the lumen of the ER and this may activate a cytosolic phospholipase A2 (cPLA2). PLA2 activation may produce endothelin metabolites that inhibit Kir3.


PKA
cAMP
PDE
5'AMP
ATP
Adenylyl Cyclase
(+)
(-)
Opioid Receptor
K+
Inwardly Rectifying and Voltage Activated
MAP Kinase Pathway
Products of Endothelin Metabolism
PLA2
↑ Ca2+
IP3/Ca2+
Calcium Activated Cl- current
EtA Receptor

DAG is produced by activation of phospholipase D (PLD). PLD-activation catalyzes release of phosphatidic acid from the major membrane phospholipid phosphotatidylcholine. (Exton 1999). The increase in the membrane-associated DAG leads to the translocation of protein kinase C (PKC) from the cytoplasm to the membrane (Rubanyi and Polokoff 1994). PKC is a protein kinase that catalyzes the phosphorylation of serine and threonine residues in substrate proteins and ion channels. PKC is active only when it associates with the membrane and binds DAG. Moreover, DAG and PKC may be activated independently of IP3. Although the IP3, DAG, PKC pathways are interdependent, they may have different actions on Kir3 that produce diverse signaling events.

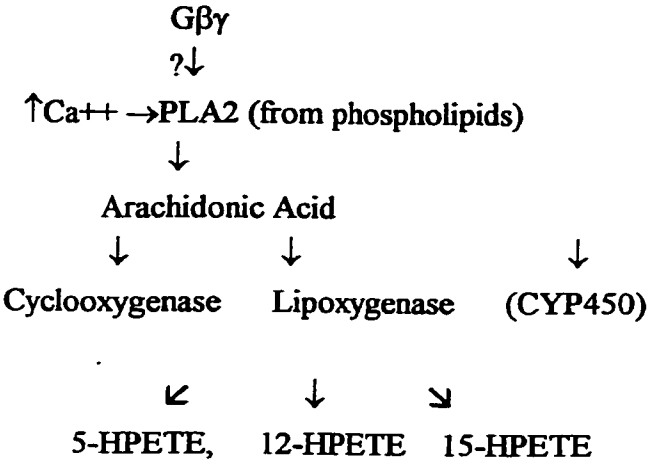
The role of arachidonic acid metabolism in cellular regulation

Arachidonic acid may act as an intracellular messenger in the brain (Axelrod 1990). Membrane phospholipids are hydrolyzed by phospholipases A2: a family of secretory and cytosolic enzymes that regulate arachidonic acid release (Bonventre 1992). It has been reported that Gβγ subunits activate PLA2 directly in a reconstituted system of purified retina (Jelsema and Axelrod 1987). In contrast, calcium-dependent cPLA2 may be activated indirectly by increased intracellular calcium. Activated cPLA2 may translocate to the phospholipid membrane to modulate cellular activity. It is presumed that cPLA2 translocation to the cellular membranes provides a potential link between agonist activated G-

protein linked receptors that mobilize calcium and the stimulation of arachidonic acid release (Clark et al 1991).

Eicosanoids may translocate to post-synaptic sites to modulate neuronal activity or serve as second messengers within neurons (Schweitzer et al 1990; Villarroel and Schwarz 1996). Eicosanoids may leave the cells in which they are produced and act as a first messenger at neighboring cells (Piomelli et al 1987).

PLA2 Metabolism



(Adapted from Shimizu and Wolfe 1990)

Agonist stimulation of cells activates phospholipase A2 to release arachidonic acid from esterified stores in the phospholipid bilayer. Arachidonic acid (5,8,11,14-cis-eicosatetraenoic acid), a polyunsaturated fatty acid (PUFA), is converted to prostaglandins, and thromboxanes by cyclooxygenase, to leukotrienes, lipoxins and related compounds by lipoxygenases, and to epoxyeicosatrienoic acid by cytochrome P-450. Lipoxygenases catalyze the

stereospecific insertion of oxygen into PUFAs, such as arachidonic acid. Arachidonic acid is susceptible to autooxidation to yield various hydroperoxy acids (HPETE). The lipoxygenase pathway yields 5-, 12-, and 15-, HPETEs. Arachidonic acid is converted to 5-HPETE. Importantly, arachidonic acid is metabolized to 12-HPETE by 12-lipoxygenase, the principal lipoxygenase in the brain. 12-lipoxyenase undergoes rapid transformations through at least six pathways to produce multiple signaling compounds (Piomelli et al 1989; Shimizu and Wolfe 1990; Piomelli 1994).

Arachidonic acid signals to ion channels

Kang and Leaf (1996) determined that polyunsaturated fatty acids (PUFA) bind directly to cardiac Na channel proteins. Electrophysiological evidence suggests that arachidonic acid and its metabolites modulate several ion channels including Cl (Anderson and Welsh 1990), delayed rectifier K (Villarroel and Schwarz 1996) and the inward rectifier in cardiac myocytes (Kurachi et al 1989). Arachidonic acid increases delayed-rectifying potassium conductance in cortical synaptasomes that are sensitive to both dendrotoxin and MCDP (Zoltay and Cooper 1994). Activation of G-protein coupled receptors is linked to voltage gated channels in intact preparations. It was shown that the mu opioid receptor signals a dendrotoxin-sensitive, delayed-rectifying potassium channel conductance in rat midbrain neurons. This conductance was blocked by inhibitors of arachidonic acid metabolism (Vaughan et al 1997). Arachidonic acid signals a

diverse variety of ion channels and this may imply that lipids have important regulatory roles in excitability.

Arachidonic acid effects on Kv channels are structure and concentration dependent. High concentrations of ω-6 arachidonate (20:4) directly activate Kv channels in cardiac culture (Kim and Clapham 1989) although lower concentrations of ω-6 arachidonate block delayed-rectifying voltage gated Kv channels cloned from cardiac muscle (Honore et al 1994). ω-6 arachidonate (20:4) has diverse effects on delayed-rectifying potassium channels expressed in oocytes. ω-6 arachidonate inhibits the Kv3 and Kv4 families of Kv channels and activates dendrotoxin-sensitive Kv1.1 (Villarroel and Schwarz 1996). Although ω-3 docosahexaenoic acid (22:6) (DHA), the most plentiful PUFA in both the rat and human nervous system (Salem et al 1996), is released from its esterified form in membrane phospholipids by PLA2, DHA inhibits the dendrotoxin-sensitive, delayed-rectifying potassium channel, Kv1.2 (Poling et al 1996). These data suggest that lipid regulation of ion channels is dependent on the structure of the lipid.

Arachidonic acid blocked Kir 1.1, an inwardly-rectifying K channel in *Xenopus* oocytes. Kir1.1 conductance was not inhibited by other fatty acids nor was this effect reversed by inhibitors of fatty acid metabolism (Macica et al 1996). It was found that mutation of ser in the amino terminus abolished arachidonic acid

inhibition of Kir 1.1. Furthermore, inhibition of Kir 1.1 was reversed by pretreatment with the protein kinase inhibitor staurosporine (Macica et al 1998).

In contrast, GPCR activation of Kir3 is inhibited by arachidonic acid in a channel-specific manner (Rogalski et al 1999) and this effect was not blocked by the ser/thr inhibitor staurosporine (unpublished observations Rogalski and Chavkin). In inside-out atrial patches arachidonic acid inhibits ATP-activated IKACH, suggesting a complex regulation between gating and eicosanoid modulation. Moreover, unsaturated free fatty acids also inhibit PIP2-induced changes in IKACH (Kim and Pleumsamran 2000). These results suggest that arachidonic acid may play a regulatory role in Kir3 activity.

Tyrosine phosphorylation regulates Kir3

In addition to the complex regulation of Kir3 by multiple signaling events, ion channels may be regulated by direct phosphorylation of tyrosine residues (Lev et al 1995; Jonas and Kaczmarek 1996). Several families of growth factors have been identified within the central nervous system (CNS), including the superfamily of nerve growth factor related neurotrophin factors (NGF). The NGF family is comprised of the growth factor receptor tyrosine kinases (RTKs). Receptor tyrosine kinases are a class of protein kinases that phosphorylate tyrosine residues. In contrast to ser/thr protein kinases, intracellular second messengers do not regulate the activity of the receptor tyrosine kinases (Lev et al 1995). Growth factors directly activate RTKs because these receptors are tyrosine kinases. Once

activated, RTKs phosphorylate themselves, other substrates and ion channels to produce a variety of cellular effects.

TrkA, TrkB, and TrkC are three known neurotrophin receptors (Schuman 1999). The TrkA receptor is found on sympathetic and sensory neurons and a small number of neurons in the brain. In contrast, TrkB and TrkC are found on the majority of neurons. TrkB binds both BDNF and NT-4/5, while Trk C binds NT-3 (Bothwell 1995).

Neurotrophins play a crucial role in the maintenance of various neuronal populations within both normal and diseased brain (Kaplan and Miller 2000). These signals also acutely modulate the activity of neurons and ion channels to control neuronal excitability (Kafitz et al 1999). Recent evidence suggests that neurotrophins may act to transduce signals in the nervous system (reviewed in Kaplan and Miller 2000). The role of neurotrophins as signal transducers is only now being defined (Segal and Greenberg 1996). The neurotrophin TrkB modulates Kir3 on specific phosphorylation sites in the amino terminus (Rogalski et al 2000), which may further define how neurons produce a rapid response to a stimulus.

Mitogen Activated Protein Kinases (MAPK)

The MAPK superfamily of protein ser/thr kinases is widely distributed and highly conserved. Diverse stimuli, including growth factors, hormones, stress, and elevated temperature, result in activation of phosphorylation cascades utilizing MAPK. Activation of MAPK allows a cell to regulate growth, proliferation, and

differentiation. Alternatively MAPK allows for cellular protective events against cytotoxic insult. Extracellular signal-regulated kinases, or (ERKS) comprise a family of related protein kinases that are activated by phosphorylation on threonine and tyrosine residues. Upstream kinases activate ERKs by phosphorylation of serine residues (Lin et al 1993; Alessi et al 1995; Brar et al 2000; Keyse 2000). Activation of the MAPK cascade occurs in pathophysiological conditions in cardiac cells (Sugden and Clerk 1997, 1998) and in learning and memory in the brain (Impey and Storm, 1999). Signal transduction events that regulate Kir3 excitability in neurons and cardiac cells may have the MAPK pathway as the common denominator. Kir3 is instrumental in cellular regulation because it is both the target of a variety of membrane bound receptors as well as downstream effectors of those receptors. As I elucidate the complex interactions that modulate Kir3, we may better understand the elegant series of events that determine animal behavior.

RESEARCH AIMS

The work presented in this dissertation was designed to address the mechanism of how Kir3 gating elements modulate downstream signal transduction pathways to regulate excitability. Previous results obtained in the Chavkin lab by Dr. Curt Cyr showed that agonist activation of the Gq-coupled human endothelin receptor A by Et-1 inhibits the Gi-coupled mu opioid receptor response to DAMGO. Moreover, Suzanne Appleyard noted that BDNF-activation of TrkB receptors inhibited the basal response in Kir3 heteromultimers stimulated with high

potassium buffer. The work described within this thesis will define the disparate molecular mechanisms of these two examples of Kir3 inhibition and explain how these two separate signal transduction pathways determine excitability.

Specific Aim 1. Investigate the mechanism of Gq-protein-coupled HETA receptor modulation of the mu opioid activation of the inwardly-rectifying potassium channel (Kir 3) expressed in *Xenopus* oocytes using two-electrode voltage clamp techniques.

A. Determine the signal transduction pathway that mediates the HETA inhibition of Kir 3. Effects of activators and inhibitors of arachidonic acid metabolism on *Xenopus* oocytes expressing the Gi-coupled mu-opioid receptor, the Gq-coupled receptor and Kir 3 heteromultimers will be investigated directly using standard two-electrode voltage clamp techniques to further define the effectors that allow diverse G-protein coupled receptors to modulate the same inwardly-rectifying potassium channel (Kir 3).

Specific Aim 2. Determine if arachidonic acid and the products of endothelin metabolism directly inhibit Kir 3 by affecting gating elements. Or alternatively, determine if arachidonic acid and the products of endothelin metabolism indirectly inhibit Kir 3 by channel phosphorylation.

A. Determine the putative channel binding site of arachidonic acid on Kir subunits by constructing channel mutants to determine how phospholipids modulate

Kir3. Determine the putative channel binding site for products of endothelin metabolism on channel constructs.

B. Determine the mechanism of eicosanoid inhibition of Kir 3 by site-directed mutagenesis of potential phosphorylation sites in Kir 3 subunits.

Specific Aim 3. Determine the mechanism of inhibition of Kir3 produced by BDNF-activation of TrkB receptors coexpressed with Kir3 heteromultimers in *Xenopus* oocytes.

A. Determine the mechanism of BDNF-inhibition of Kir 3 by site-directed mutagenesis of potential phosphorylation sites in Kir 3 subunits.

B. Produce functional homomultimers of Kir3 using the strategy of Chan et al (1996) to study individual subunits Kir3.1, Kir3.2 and Kir3.4.

C. Determine if electrostatic interactions are important in tyrosine phosphorylation of Kir3 subunits.

CHAPTER II

INHIBITION OF G-PROTEIN COUPLED INWARDLY RECTIFYING POTASSIUM CHANNEL IS MEDIATED INDIRECTLY BY PLA2[1]

INTRODUCTION

The current of the G-protein coupled inward rectifier potassium channel (Kir3) is regulated by signals generated by a large group of disparate G-protein coupled receptors. Activation of members of the pertussis toxin-sensitive family of Gi/Go coupled receptors (e.g. opioids, GABAB, somatostatin, 5HT, dopamine, adenosine, and others (Jan and Jan 1997), release Gβγ that directly activates Kir3 (Clapham and Neer 1997). In contrast, the activation of members of the pertussis toxin-insensitive family of Gq/11-coupled receptors (e.g. substance P and endothelin) inhibits Kir3 (Jan and Jan 1997). The mechanism(s) responsible for the

[1] This material has been published Rogalski, S. L., et al (1999). "Activation of the endothelin receptor inhibits the G protein-coupled inwardly rectifying potassium channel by a phospholipase A2-mediated mechanism." J Neurochem 72(4): 1409-16.

Gq/11-mediated inhibition of Kir3 is less clear. Substance P activates calcium-dependent, arachidonic acid production (Garcia et al 1994), and this production of arachidonic acid was suggested as the mechanism of Kir3 inhibition in locus coeruleus (Koyano et al 1994). Similarly, endothelin-1, activates a Gq/11-coupled receptor in glia, astrocytes, and neurons to stimulate phospholipase C-β (PLC), causes phosphoinositide hydrolysis, increases intracellular calcium, activates phospholipase A2, and produces arachidonic acid (Rubanyi and Polokoff 1994). In contrast, others (Kurachi et al 1989) have reported that arachidonic acid and its metabolites increase Kir3 current. Recently, metabotropic glutamate receptors (mGluR1a and mGluR5) were found to inhibit Kir3 expressed in *Xenopus* oocytes by a pertussis toxin-insensitive mechanism blocked by protein kinase C inhibitors (Sharon et al 1997). Thus, Gq/11 coupled receptors have been shown to affect Kir3 channel current, but the underlying mechanism(s) remains uncertain.

To further explore how diverse G-proteins signaling may interact to control Kir3 current, I used *Xenopus* oocytes to coexpress the Gq/11 coupled endothelin A receptor, the Gi/o-coupled mu opioid receptor and Kir3 to model the complex neuronal situation. Opioid receptors are coupled to pertussis toxin sensitive Gi/o proteins, and receptor activation inhibits adenylyl cyclase, decreases calcium channel current and increases both inwardly-rectifying and delayed-rectifying potassium channel currents (Kieffer 1995). In this study, I found that endothelin receptor activation does reduce the response evoked by opioid receptor activation.

The data obtained suggest that PLA2 antagonists blocked the endothelin-induced inhibition.

MATERIALS AND METHODS

Complementary DNA clones and mRNA synthesis

The rat mu opioid receptor clone was obtained from Dr. Lei Yu (GenBank accession number L13069). cDNA for the human endothelin A (HETA) receptor (Genbank accession no: S67127) was obtained from Dr. Richard Kris. cDNAs for the Kir 3.1 (GIRK1) (Genbank accession no: U01071) and Kir 3.2 (GIRK 2) (Genbank accession no: U11859) were obtained from Drs. Cesar Lebarca and Henry Lester. Dr. John Adelman provided the Kir 3.4 (GIRK4) clone (Genbank accession no: X83584). Dr. David Clapham provided the Kir 3.5 (XIR) clone (Genbank accession no: U42207). Plasmid templates for all constructs were linearized prior to *in vitro* mRNA synthesis as described (Dascal et al 1993). cRNA was prepared by *in vitro* synthesis from cDNA templates using mMessage Machine (Ambion Inc, TX).

Oocyte maintenance and injection

Healthy stage V and VI oocytes were harvested from mature anesthetized *Xenopus laevis* (Nasco, Ft. Atkinson, WI) and defolliculated enzymatically as described previously (Snutch 1988). The oocytes were maintained at 18°C in standard oocyte buffer, ND96 (96 mM NaCl, 2 mM KCl, 1 mM CaCl2, 1 mM MgCl2, 5 mM HEPES, pH 7.5) supplemented with 2.5 mM sodium pyruvate and 50 μg/ml gentamicin (Sigma Chemical Co.). One day after harvest, each oocyte

was injected with 50 nl of mRNA for the mu opioid receptor (MOR), human endothelin A receptor (HETA) and G-protein inwardly rectifying potassium channel heteromultimers, Kir 3.1, Kir 3.1 and Kir 3.2 or Kir 3.1 and Kir 3.5 (XIR) into the vegetal pole. Recordings were made at least 72 hr after cRNA injection.

Electrophysiological studies

An Axoclamp 2A amplifier was used for standard two-electrode voltage-clamp experiments. The pCLAMP program (Axon Instruments) was used for data acquisition and analysis. Oocytes were removed from incubation medium, placed in the recording chamber containing ND 96 medium, and clamped at –80 mV. Recordings were made in hK buffer (2 mM NaCl, 16 mM KCl or 24 mM KCl, 1 mM CaCl2, 1 mM MgCl.2, 5 mM HEPES, pH 7.5). Microelectrodes were filled with 3 M KCl and had resistances of 0.5 - 1.0 MΩ. Currents were measured without leak subtraction. Individual comparisons of drug effects on Kir 3 were conducted using oocytes from the same harvest and injection batch. Pharmacologic agents were perfused directly into the bath from freshly made stock solutions.

Materials

Stock solutions of arachidonic acid, BAPTA-AM and phorbol esters were dissolved in dimethylsulfoxide (DMSO). The final concentration of DMSO applied to the oocytes was ≤ 0.02%. AACOCF3 was dissolved in ethanol. The final concentration of ethanol applied to the oocytes was <0.01%. Nitrogen was bubbled through water prior to dissolving endothelin. All other drugs were dissolved in water. Alkaline phosphatase was obtained from New England Biolabs, Beverly, MA; DAMGO was obtained from Peninsula Laboratories,

Belmont, CA; endothelin 1-(human, porcine) was obtained from Research Biochemicals International, Natick, MA; Arachidonic Acid, AACOCF3, BAPTA-AM, and 4,4-diisothiocyanatostilbene-2,2-disulfonic acid (DIDS) were obtained from Calbiochem, La Jolla, CA; Quinacrine, genistein, staurosporine, phorbol, 12-myristate, 13-acetate (PMA), and DMSO were obtained from Sigma Chemical Co, St. Louis, MO.

Statistical analysis

Data are presented as means ± SEM. The statistical significance of differences between results was calculated using a paired T test. A probability of $p < 0.05$ was considered statistically significant.

RESULTS

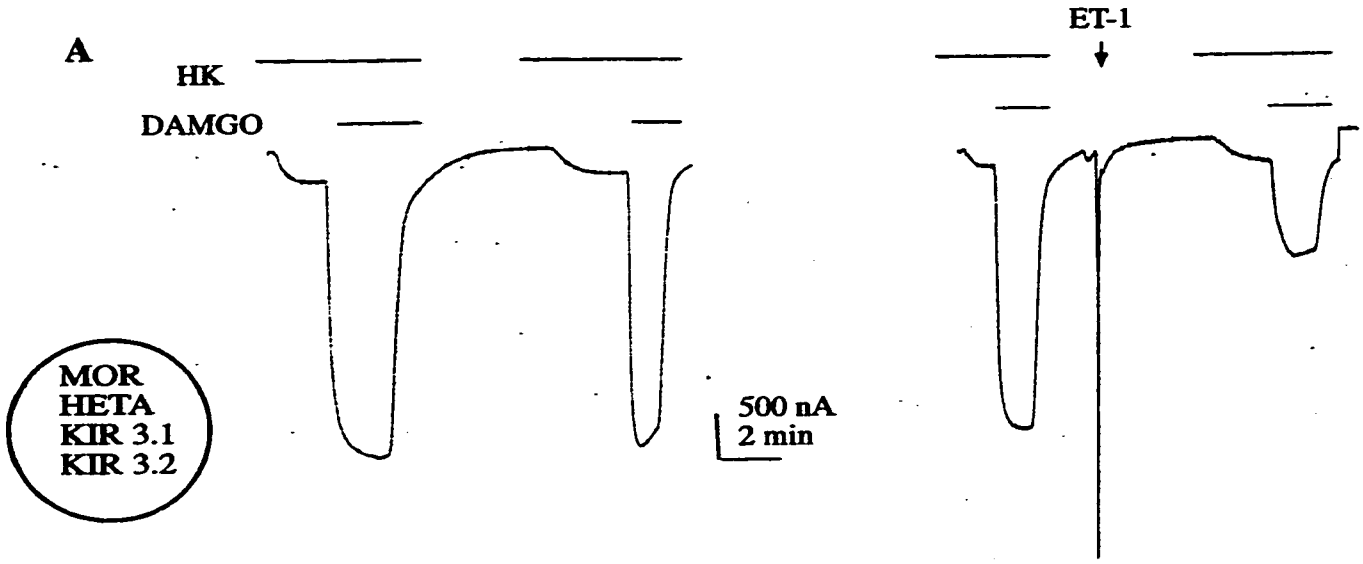
Effects of endothelin-1 on the DAMGO elicited mu opioid response mediated by the heteromultimer Kir 3.1 and Kir 3.2 expressed in Xenopus oocytes

Application of the mu opioid agonist DAMGO (100 nM) in oocyte buffer containing 16 mM KCl increased the inward current (Fig. 3A). Previous *in vitro* expression studies in *Xenopus* oocytes showed that the opioid-induced current was caused by activation of the expressed G-protein gated inwardly-rectifying potassium channel (Dascal 1993; Chen and Yu 1994), and this was confirmed by current-voltage analysis of the opioid activated current (data not shown). After the opioid-induced response reached steady state, oocytes were perfused with ND96 buffer for 4 min. Oocytes receiving a second application of 100 nM DAMGO showed a similar response to DAMGO (Fig. 3A); the amplitude of the second opioid response was 99 ± 2.9% (n=41) of the first response (Fig. 3B). The opioid

response was only observed in oocytes injected with both receptor and channel mRNA. Prior studies showed that the endothelin A receptor couples to phospholipase C through G-protein βγ subunits (Camps et al 1992) thereby increasing intracellular calcium (Shimada et al 1991; Cyr et al 1993) intrinsic to the oocyte (Barish 1983) (Fig. 3A, right). Oocytes treated with endothelin-1 prior to the second DAMGO challenge showed a marked inhibition of the second opioid response. The amplitude of the second opioid response after endothelin-1 treatment was 33 ± 3.2% (n=43) of the first opioid response ($p < 0.01$) (Fig 3B). The endothelin-induced activation of the endogenous chloride current was only observed in oocytes injected with the endothelin A receptor mRNA.

Endothelin-1-induced inhibition of the opioid activated potassium current was dose dependent. At 50 or 100 nM, endothelin-1 produced a large activation of the chloride current (4.1 ± 0.8 μA, n=28). The inhibition of the second opioid response produced by 100 nM endothelin-1 was the same as that produced by 1 μM endothelin-1 (n=20). The mechanism of the endothelin-1 modulation of the opioid response is not clear and may involve either the action of the second messengers diacylglycerol or inositol trisphosphate (Shubeita et al 1990), the elevation of intracellular calcium, or metabolites produced following the activation of phospholipase A2 (PLA2) (Nishizuka 1984). These possibilities were next explored.

Figure 3. Effects of endothelin-1 on the DAMGO elicited mu opioid response (MOR) mediated by the heteromultimer Kir 3.1 and Kir 3.2 expressed in *Xenopus* oocytes. Oocytes were injected with a mixture of the following mRNAs: 0.8 ng MOR, 1.7 ng human endothelin A receptor, 0.065 ng Kir 3.1 and 0.065 ng Kir 3.2 as summarized in the inset (circle). Recordings were performed 3-4 days post injection. Initially, oocytes were bathed in normal oocyte saline buffer containing 2 mM KCl and clamped at –80mV. Oocytes were then perfused with a saline buffer containing 16 mM KCl. Elevation in potassium concentration facilitates current measurement by increasing the basal current through the inwardly rectifying potassium channels. Application of the mu opioid agonist DAMGO (100 nM) in oocyte buffer containing 16 mM KCl increased the basal current. After the response reached steady state, normal oocyte saline buffer was washed on for 4 min. Buffer flow was decreased while an application of 100 nM endothelin-1 was applied directly to the oocyte. After the endothelin-1 induced calcium activated chloride current returned to baseline, the bath flow was resumed for four minutes followed by a second treatment with 100 nM DAMGO in 16 mM KCl. Measurement of endothelin induced inhibition of the second opioid response is expressed as a percentage of first peak opioid response. A. Representative current traces from oocytes stimulated by perfusion with the mu opioid agonist 100 nM DAMGO in oocyte buffer containing 16 mM KCl in two applications. The first panel shows the response without endothelin-1. The second panel includes a 25 μL pulse of 100 nM endothelin-1 in ND96 applied directly to the oocyte in the recording chamber. Application of vehicle had no effect and the responses to endothelin were absent in oocytes not expressing the receptor. For these traces, oocytes were clamped at -80 mV in normal saline buffer. Horizontal bars indicate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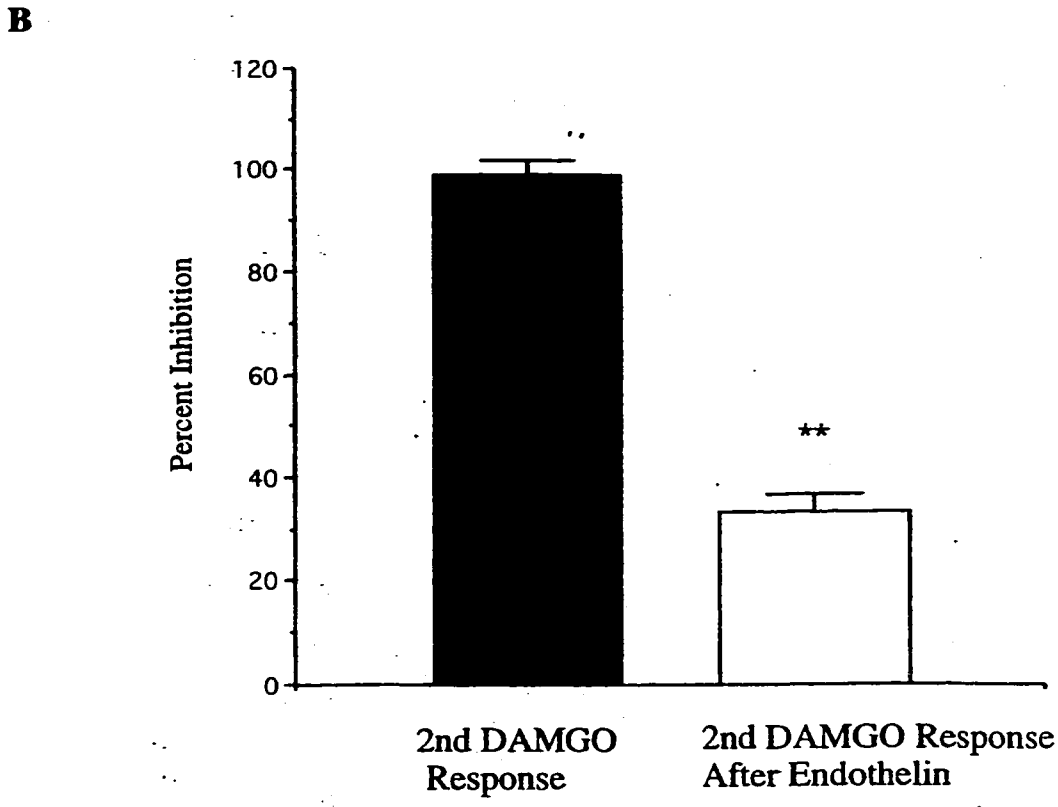
B
120
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Percent Inhibition
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2nd DAMGO Response
2nd DAMGO Response After Endothelin

the duration of drug perfusion. B. The amplitude of the second opioid receptor response was expressed as percent of first response. Bars represent mean ± SEM of 22 oocytes from three batches.

Effects of anionic channel block

Because intracellular chloride has been reported to regulate G-protein gated channels in some cell types (Jentsch and Gunther 1997), I tested whether chloride flux mediated by the calcium-activated chloride channel produced the observed inhibition of the opioid response. The chloride channel blocker 4,4'-diisothiocyanatostilbene-2,2'disulfonic acid (DIDS) (100 nM) (Tzounopoulos et al 1995) was perfused into the recording chamber prior to endothelin-induced activation. Although the calcium-activated chloride current was significantly attenuated or blocked by DIDS, endothelin-1 still effectively inhibited the opioid response (Fig. 4). In oocytes pretreated with DIDS, the second opioid response was inhibited by 82 ± 10%, n=8 (p>0.05) compared with the first DAMGO response, an effect not significantly different from that in the absence of DIDS.

Effects of calcium chelation

To determine whether the observed inhibition of the opioid response was mediated by the endothelin-1-induced rise in cytoplasmic calcium, the effect of calcium chelators were next determined. In oocytes injected with EGTA (final intracellular concentration approximately 2.5 μM), the chloride channel response to endothelin was abolished (n=6); however, the endothelin-1-induced inhibition of the opioid response was only slightly reduced. In oocytes pre-injected with EGTA, endothelin-1 caused an 84% ± 9% (n=6) inhibition of the DAMGO response. This endothelin-1 effect was not significantly different from that

observed using matched oocytes not injected with EGTA. To assess the result of a more effective calcium chelation, BAPTA-AM, the membrane permeant analog was used (Dieter et al 1993). In oocytes soaked for 90-150 min in BAPTA-AM (50-100 μM) as previously described (Quamme 1997), the chloride current was reduced by >90% (n=12) (p<0.01). In BAPTA-AM treated oocytes the application of DAMGO after endothelin-1 produced only a 28 ± 6% (n=12) lower response as compared to the first DAMGO response (Fig. 4). Neither EGTA nor BAPTA-AM treatment altered the amplitude of the first opioid response to DAMGO, confirming that elevation of intracellular calcium was not required for the opioid-activated increase in potassium current. The greater effectiveness of BAPTA-AM was consistent with its more effective chelation of calcium in cytosolic and vesicular stores compared to EGTA (Tsien 1980) and with the higher concentration of chelator achieved.

Effects of inhibition of kinase and phosphatase activity

To determine whether the inhibition of the opioid response was the result of the action of an endothelin-activated kinase, oocytes were treated with the nonspecific kinase inhibitor staurosporine. Staurosporine (1 μM) treatment had no effect on resting membrane current, and the amount of endothelin-1 inhibition of the second opioid response was not significantly different in staurosporine treated oocytes (p>0.05) when compared to control (108 ± 6%, n=12) (Fig 4). To validate the negative result obtained with staurosporine, control oocytes were perfused with 100 nM of phorbol, 12-myristate, 13-acetate (PMA), for 15 minutes between DAMGO applications. PMA produced significant inhibition of both the basal and opioid response as previously reported (Dascal 1997), and the PMA-induced inhibition was blocked by pretreatment with 1 μM staurosporine (n=15).

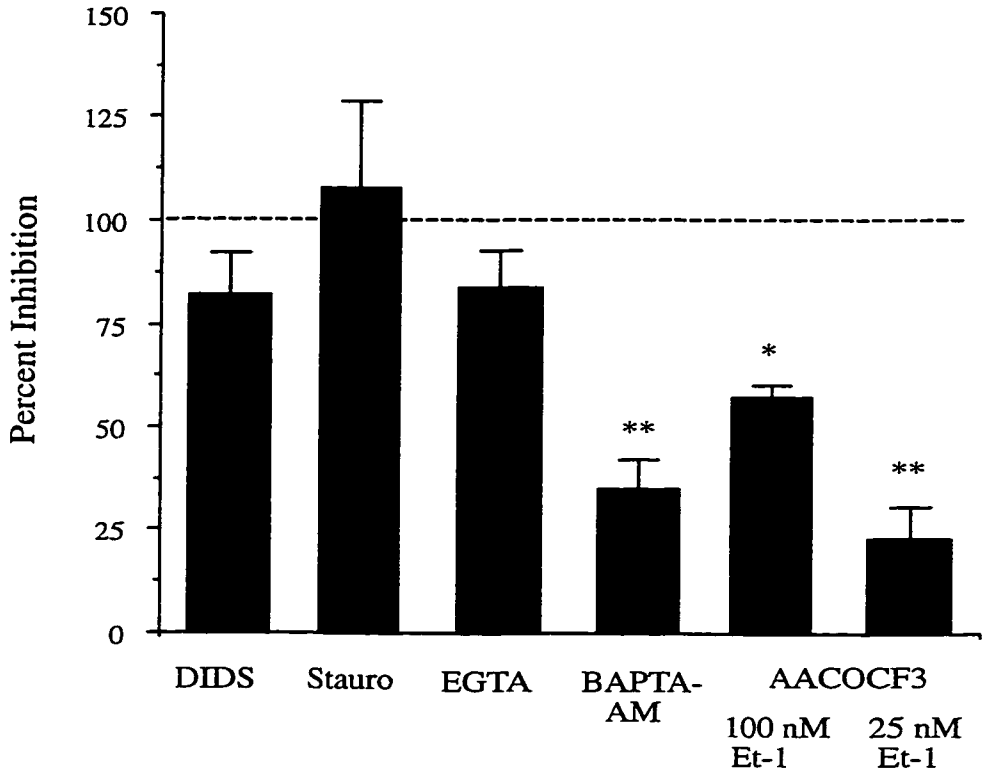
Treatment with the tyrosine kinase inhibitor, genistein (Akiyama et al 1987) (100 μM) also had no significant effect on the amount of endothelin-induced inhibition of the second opioid response (116 ± 8% of control, n=5). Neither the magnitude of the endothelin-1 effect nor the second opioid response was different compared to control oocytes ($p>0.05$). To determine if the endothelin-1 effects could be blocked by reduction in protein phosphorylation, alkaline phosphatase was injected into oocytes 30 min prior to recording (0.025 units/ml final concentration). Alkaline phosphatase did not effect the inhibition of the opioid response (99 ± 22% of control, n=6). The data failed to support the hypothesis that a kinase or phosphatase directly mediated endothelin-induced channel inhibition. However, the negative data do not exclude the possibility of kinase or phosphatase regulation.

Effects of block of phospholipase A2

To determine which calcium-dependent signal transduction pathway leads to endothelin-1-induced inhibition of the opioid response, oocytes were next treated with either quinacrine or AACOCF3 (Street et al 1993), two inhibitors of phospholipase A2 (PLA2). PLA2 activity leads to the direct release of arachidonic acid from phospholipids, which results in the formation of several biologically active arachidonate metabolites. In oocytes soaked in 15 μM AACOCF3 for 30 minutes, the inhibitory effects of 100 nM endothelin-1 were significantly reduced (Fig 4); the endothelin 1 effect was only 57 ± 3.1% (n=7) of the maximal effect in the absence of AACOCF3. AACOCF3 produced a more dramatic block of the effects of 25 nM than 100 nM endothelin-1 (Fig 4). Pretreatment with AACOCF3 alone did not affect the opioid activated Kir3 response (n=7). Quinacrine (50 μM) also blocked the endothelin-1 effect, but the interpretation of quinacrine's actions

was confounded by its direct inhibitory effects on Kir3 current (data not shown). Additionally, the endothelin-1 activation of the calcium-sensitive chloride channel was unchanged by the presence of AACOCF3. The chloride current evoked by 25 nM endothelin treatment was 930 ± 304 nA (n=4), and was 940 ± 340 (n=4) in the presence of 15 μM AACOCF3. These data suggest that AACOCF3 did not block phospholipase C or IP3-mediated calcium release from cytoplasmic stores.

Figure 4. Effects of pretreatment with DIDS, Staurosporine, EGTA, BAPTA-AM or AACOCF3 on endothelin-1 inhibition of the MOR response. The mechanism of the endothelin inhibition of the DAMGO response was determined using a series of pharmacological blockers of endothelin-activated second messenger systems. Bar graph summarizing the effect of DIDS, staurosporine, EGTA, BAPTA-AM and AACOCF3 on the endothelin inhibition of the second opioid response. To normalize for daily differences in receptor expression, data are presented as a percentage of the average maximal inhibition of the second opioid response produced by endothelin in control oocytes (100% equals maximum inhibition of the 2nd DAMGO response for that batch of oocytes). Each group was compared to its own control with that day's matched group. Aqueous EGTA was injected into the oocyte (46 nl of a 50 mM solution was injected to yield a final concentration of approximately 2.5 μM) 4 minutes before recording. EGTA blocked the endothelin-activated chloride current, increased the basal Kir3 current, but did not block the endothelin-induced inhibition of the 2nd DAMGO response. The non-specific anion channel blocker DIDS (4,4'-diisothiocyanato-stilbene-2,2'disulfonic acid) (100 nM) was perfused in ND 96 to the bath for 4 minutes following the first peak response to DAMGO as in Figure 1. DIDS blocked the endothelin-activated increase in the chloride current, but did not affect the endothelin-induced inhibition of the 2nd DAMGO response. Oocytes were presoaked for 90-150 min with the membrane permeant calcium chelator BAPTA-AM (50-100 μM) prior to recording. BAPTA-AM also blocked the endothelin-activated chloride current and blocked the endothelin-induced inhibition of the 2nd DAMGO response. Oocytes were presoaked for 30


Percent Inhibition
150
125
100
75
50
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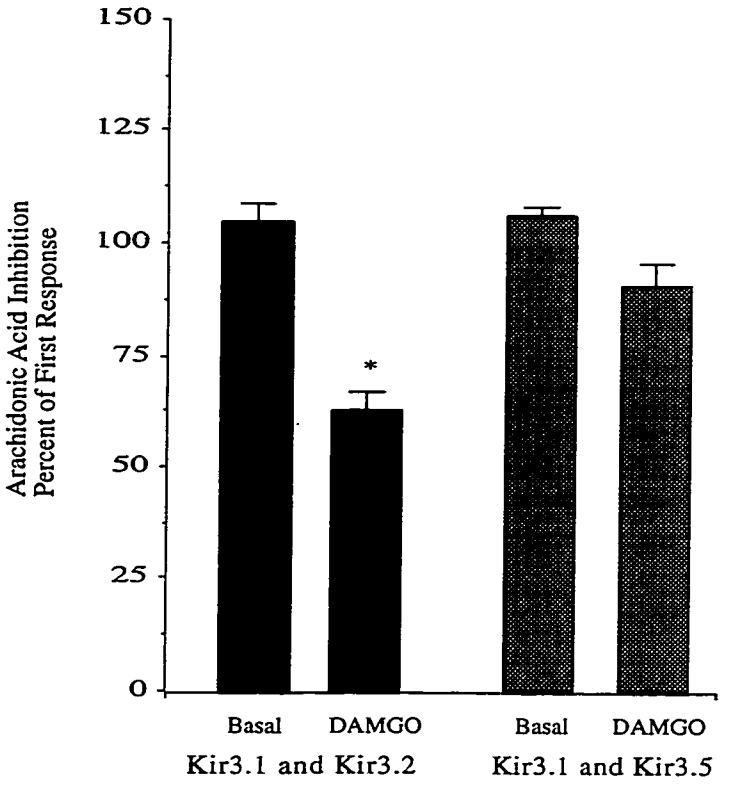
DIDS
Stauro
EGTA
BAPTA-AM
AACOCF3
100 nM Et-1
25 nM Et-1

min with 15 μM AACOCF3 prior to recording. (Data are means ± SEM * $p<0.05$, ** $p<0.01$).

Arachidonic acid effects on GIRK channel heteromultimers.

The partial reversal of the endothelin-1 effects by inhibition of PLA2 suggested that a product of PLA2 metabolism might be responsible for channel inhibition. Endothelin receptors have been shown to stimulate arachidonic acid synthesis (Rubanyi and Polokoff 1994). Moreover, ion channels, including chloride (Anderson and Welsh 1990), delayed rectifier K (Villarroel and Schwarz 1996) and the inward rectifier in cardiac myocytes (Kim and Clapham 1989) are targets of arachidonic acid action. To determine if a product of PLA2 metabolism reproduced the observed inhibition caused by endothelin-1, 20 μM arachidonic acid was perfused for 6 min prior to activation of the second opioid response. In oocytes expressing the channel heteromultimer composed of Kir3.1 and Kir 3.2, arachidonic acid produced an inhibition of the second opioid response; the second DAMGO response was 61 ± 3.9% of the first response ($p<0.05$) (Fig 5). Interestingly, in oocytes expressing the channel heteromultimer composed of Kir3.1 and Kir 3.5, arachidonic acid produced a significantly smaller inhibition. In matched oocytes treated with 20 μM arachidonic acid, the second opioid response was 88 ± 4.4% percent of first response (Fig. 5). Arachidonic acid did not affect the basal channel currents for either Kir 3.1-2 or Kir 3.1-5 combinations (Fig 5).

Figure 5. Arachidonic acid effects on GIRK channel heteromultimers. Oocytes were injected with a mixture of the following mRNAs: 0.8 ng MOR, 0.065 ng Kir 3.1 and 0.065 ng Kir 3.2 or 0.8 ng MOR, 0.13 ng Kir 3.1 and 0.13 ng Kir 3.5. Comparative RNA dose response studies were used to define the minimum concentrations required to elicit similar currents with each combination. Initially, oocytes were bathed in normal oocyte saline buffer containing 2 mM KCl and clamped at –80mV. Oocytes were then perfused with a saline buffer containing 16 mM KCl. Each bar represents the amplitude of the second response (after arachidonic acid application). Arachidonic acid did not affect the basal responses of either Kir3 channel. Application of the mu opioid agonist DAMGO (100 nM) in oocyte buffer containing 16 mM KCl increased the current. The peak opioid response to 100 nM DAMGO was followed by perfusion with 20 μM arachidonic acid for 6 min at a flow rate of 4-5 ml/min, a one minute wash in ND 96 and a second stimulation with DAMGO. Oocyte membrane stability was not maintained with concentrations higher than 20 μM arachidonic acid (data not shown); thus, higher doses of arachidonic acid were not evaluated. The second mu opioid response is expressed as percent of first response. Bars represent the mean ± SEM of 20 oocytes from four donors.


150
125
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Arachidonic Acid Inhibition
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Basal
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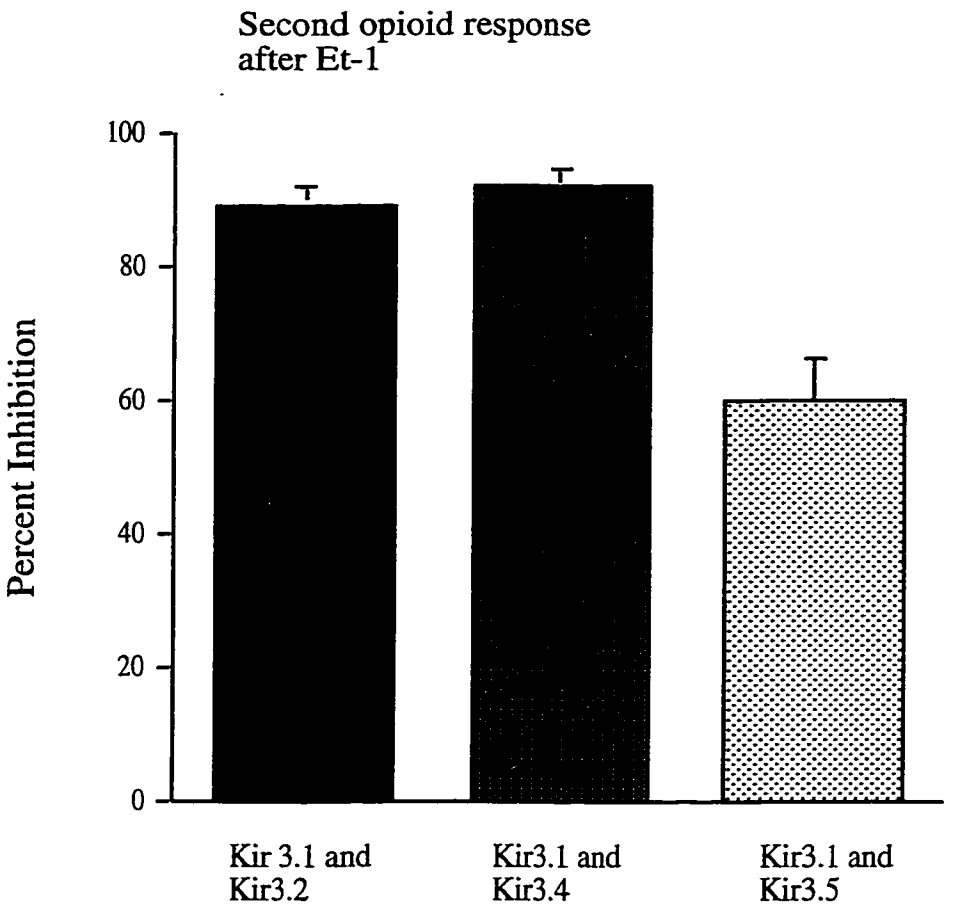
Kir3.1 and Kir3.2
Kir3.1 and Kir3.5

Endothelin effects on Kir3 channel heteromultimers

Endothelin-1 significantly inhibited the opioid response in oocytes expressing channel heteromultimers composed of Kir 3.1 and Kir 3.2. To determine whether the magnitude of the endothelin effect also depended on the subunit composition of the Kir3 channel, oocytes from the same batch were injected with the combination of A) Kir3.1 and Kir3.2, or B) Kir3.1 and Kir 3.4, or C) Kir3.1 and Kir3.5. Channel expression was controlled by adjusting the RNA doses injected so that the amplitudes of both basal Kir3 currents and opioid-activated responses were similar among the groups. For the combination of Kir 3.1 and Kir3.2, the second opioid response after endothelin-1 treatment expressed as percent of first response was 89 ± 3.1% (n=10) (Fig 6). Similarly, for the heteromultimer consisting of Kir 3.1 and Kir 3.4, the second opioid response after endothelin-1 treatment expressed as percent of first response was 94 ± 2.8% (n=12). For the heteromultimer consisting of Kir 3.1 and Kir 3.5, the second opioid response after endothelin-1 was 60 ± 7.4 % (n=9). Expression of Kir 3.1 alone in *Xenopus* oocytes showed a reduced response to endothelin-1 treatment, with the second opioid response 63 ± 6.3% (n=7) of first response (Fig. 6). The marked differences in endothelin-1 sensitivity among the channel heteromultimers indicated that endothelin-1 inhibition of Kir3 was channel subtype specific. Furthermore, the observed differences in Kir 3 sensitivity to endothelin-1 suggest that endothelin effects may have direct effects on the channel. Since endothelin-1 and arachidonic acid both produce an inhibition of the channel current that depends on the channel subtype expressed, the observed inhibition of the opioid activated response may occur by a similar mechanism. These observations are consistent with the observation that PLA2 antagonists inhibited endothelin-1 effects.

Figure 6. Endothelin effects on GIRK channel heteromultimers.

All data are presented as a comparison of the second peak opioid response to the first peak opioid response. Oocytes were injected with 0.8 ng MOR, 1.7 ng human endothelin A receptor , 0.065 ng Kir 3.1 and 0.065 ng Kir 3.2, or 0.8 ng MOR, 1.7 ng endothelin A receptor, 0.13 ng Kir 3.1 and 0.13 ng Kir 3.5, or 2 .6 ng, MOR, 2.6 ng endothelin A receptor and 8 ng Kir 3.1. All data are presented as the percent inhibition of the second opioid response n=15 from three batches.


Second opioid response
after Et-1
Percent Inhibition
100
80
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Kir 3.1 and
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Kir3.4
Kir3.1 and
Kir3.5

DISCUSSION

In this study, I expressed the mu opioid receptor, the human endothelin A receptor, Kir3 channel heteromultimers in *Xenopus* oocytes and used two-electrode voltage clamp recording techniques to characterize common signals that allow diverse G-protein coupled receptors to regulate Kir3. Treatment of oocytes with the mu opioid agonist DAMGO increased Kir3 current. Oocytes treated with endothelin-1 prior to a second DAMGO challenge showed a marked inhibition of the second opioid response. Although alternatives have not been excluded, my data suggest that the endothelin inhibition of Kir3 was mediated by calcium-dependent PLA2. PLA2 antagonists blocked the endothelin effect. Endothelin-1 had different effects on Kir3 heteromultimer combinations and that paralleled the differences in channel sensitivity to arachidonic acid. The results suggest that activation of the endothelin receptor activates PLC, to increase cytosolic calcium, and thus increasing PLA2 activity. PLA2 may produce an arachidonic acid metabolite that directly blocks Kir3 current.

Since protein kinases and phosphatases also regulate neuronal excitability, I used pharmacological inhibitors to determine if a phosphatase or kinase mediated the endothelin-induced inhibition of Kir 3. The non-specific kinase inhibitor staurosporine; the tyrosine kinase inhibitor genistein; and phosphatase inhibitor alkaline phosphatase were used to block intracellular kinase and phosphatase activity. The data suggested that a kinase or phosphatase did not directly mediate channel inhibition. Others (Sharon et al 1997) and I note that treatment of the oocytes with the phorbol ester PMA to activate PKC mimicked the endothelin-induced inhibition of Kir3. In my study, PMA inhibition of Kir3 was reversed by staurosporine, but pretreatment with staurosporine did not block the endothelin-

induced inhibition of the opioid response. I suggest that PKC is not directly involved in the endothelin-induced inhibition of the opioid response. However, the data do not exclude a possible role of kinase or phosphatase regulation of Kir3.

Several studies using phorbol esters and inhibitors of PKC provide evidence that PKC may be involved in endothelin-induced nuclear signaling mechanisms in other cell types (Rubanyi and Polokoff 1994). AT-1 cells treated with endothelin-1 were shown to be selective for the calcium-insensitive isoform, PKC-ε. In this system, endothelin stimulation of phosphoinositide hydrolysis increased the membrane association of PKC-ε and increased cytosolic calcium in a sub-population of cells (Jiang et al 1996). These studies argue that endothelin-activation of PKC initiates multiple events that may indirectly contribute to the observed endothelin-induced inhibition of the opioid response in other cell types.

Endothelin-1 activation leads to the release of intracellular calcium as is evident from the transient calcium-activated chloride current after agonist treatment. The BAPTA-AM data suggest that endothelin-1 induced increase in intracellular calcium was necessary for signals that lead to the inhibition of the opioid response. In addition to its role as a calcium chelator, BAPTA-AM was shown to down-regulate PKC in liver macrophages after prolonged exposure (Dieter et al 1993). I presume that BAPTA-AM treatment did not down-regulate all of the isoforms of PKC expression in the oocyte, since the staurosporine data suggests that PKC is not directly involved in the endothelin-induced inhibition of Kir3. However, alternative mechanisms of endothelin regulation of Kir3 are not excluded by these data.

In C6 glioma, endothelin mobilization of intracellular-calcium activated arachidonic acid release was reversed by quinacrine, a nonspecific inhibitor of

PLA2 (Dunican et al 1996). In a culture of human pericardium smooth muscle cells, it was shown that endothelin-1 evoked arachidonic acid release was dependent upon the elevation of intracellular calcium (Wu et al 1996). Phospholipases A2 comprise a family of secretory and cytosolic enzymes that regulate arachidonic acid release through hydrolysis of membrane phospholipids (Bonventre 1992). Increased cellular calcium may selectively activate the 85 KD, calcium-sensitive, cytosolic phospholipase A2 (cPLA2) (Clark et al 1991). I determined that endothelin-induced inhibition of Kir3 was calcium-dependent and partially reversed by treatment with the AACOCF3, the trifluoromethyl ketone analogue of arachidonic acid in which the COOH group is replaced with COCF3 to form a selective, high affinity inhibitor of cPLA2 (Street et al 1993). Stimulation by calcium mobilizing ligands may lead to cPLA2 translocation to the phospholipid membrane. cPLA2 may be the intermediate between agonist activated G-protein linked receptors that mobilize calcium and the stimulation of arachidonic acid release (Clark et al 1991). Because the specificity of AACOCF3 has not been previously tested in *Xenopus* oocytes, potential inhibitory effects on other phospholipases cannot be determined. My results suggest that cPLA2 or a related enzyme may be the mediator of endothelin-induced inhibition of Kir3. Although, AACOCF3 did not inhibit the magnitude of the endothelin-induced chloride current, I do not exclude the possibility of nonspecific effects on isoforms of PLC.

Gβγ subunits may act as signaling molecules to downstream effectors providing one potential mechanism to activate downstream signals. It has been reported that Gβγ subunits activate PLA2 in a reconstituted system of purified retina (Jelsema and Axelrod 1987). Gβγ subunits positively regulate many

effectors, including PLCβ, (Clapham and Neer 1997), and it has been suggested that Gβγ may directly activate phospholipase A2 (Axelrod 1990; Axelrod 1995). My data suggests that a PLA2 activation may occur by an indirect mechanism, whereby an increase in intracellular calcium leads to activation of PLA2. However, as suggested by Jelesma and Axlerod, Gβγ subunits may also directly activate PLA2.

These results obtained using the *Xenopus* oocyte expression system suggest a possible means by which Gq/11 receptor signaling may influence Gi/o in brain. Examples of this type of receptor cross-talk have been noted previously in neuronal recordings (Jan and Jan 1997), although the underlying mechanisms have not been elucidated. Furthermore, the suggestion that an arachidonic acid analog may directly inhibit Kir current will be important to explore in future studies. Understanding the signal transduction mechanisms that regulate the inwardly-rectifying potassium channel provide further insights concerning how disparate G-proteins regulate Kir3 in neurons and myocytes.

I have determined that endothelin receptor activation may modulate the mu opioid response by a direct effect on Kir 3 in a channel subtype-specific fashion.

CHAPTER III

TRKB ACTIVATION BY BDNF INHIBITS THE G PROTEIN GATED INWARD RECTIFIER KIR3 BY TYROSINE PHOSPHORYLATION OF THE CHANNEL[2]

INTRODUCTION

Neurotrophins are a family of growth factors that include NGF, BDNF, NT3, and NT-4/5 (Segal and Greenberg 1996) and which activate receptor tyrosine kinases (Trk) to regulate neuronal survival and differentiation during brain development (Bothwell 1995). Neurotrophins also rapidly modulate neuronal excitability to regulate synaptic plasticity in the hippocampus (Kang and Schuman 1995; Levine et al 1995; Kang et al 1997; Kafitz et al 1999; Schuman 1999) plasticity of spinal cord neurons in models of chronic pain (Kerr et al 1999) and excitability of cortical neurons (Desai et al 1999). The mechanisms of these neuronal effects on excitability are not yet known; however, BDNF was shown to rapidly modulate sodium channels in the CA1 region of the hippocampus (Kafitz et al 1999) and to enhance synaptic currents in hippocampal postsynaptic neurons

[2] This material has been published Rogalski, S. L et al (2000). "TrkB Activation by BDNF Inhibits the G Protein Gated Inward Rectifier Kir3 by Tyrosine Phosphorylation of the Channel." J Biol Chem. 275:25082-25088.

(Levine et al 1995). These studies suggest that BDNF has direct effects on ion channel properties and modulates synaptic activity.

The neurotrophin receptors are transmembrane tyrosine kinases. BDNF activation of the TrkB receptor is known to initiate a cascade of phosphorylation events that activate a complex of signaling proteins (Greene and Kaplan 1995). Tyrosine kinases directly phosphorylate ion channels to provide rapid regulation of neuronal excitability (Holmes et al 1996; Jonas et al 1996; Hilborn et al 1998; Jonas and Kaczmarek, 1996 Timpe and Fantl 1994; Lev et al 1995; Wilson and Kaczmarek 1993; Wischmeyer et al 1998).

Tyrosine kinase activation by G-protein coupled receptors (Luttrell et al 1999) also suppresses delayed rectifying potassium channels by phosphorylation of a tyrosine residue in the amino terminus of Kv1.2 (Huang et al 1993). Similarly, phosphorylation of serine residues in the amino terminus of a different delayed-rectifying potassium channel Kv3.4 causes channel inactivation (Covarrubias et al 1994). Additionally, tyrosine phosphorylation of other potassium channels may regulate neuronal excitability. Because G-protein coupled receptor activation of Kir3 type potassium channels is one of the major mechanisms controlling neuronal excitability, I explored the hypothesis that BDNF regulation of Kir3 may control neuronal excitability by modulation of these channels. My results show that BDNF inhibits basal Kir3 channel activity and define specific tyrosine phosphorylation

sites in the amino terminus of Kir3 that are important for channel inhibition caused by TrkB activation.

MATERIALS AND METHODS

Complementary DNA clones and mRNA synthesis

cDNAs for the Kir3.1 (GIRK 1) (Genbank accession no: U01071) and Kir3.2 (GIRK 2) (Genbank accession no: U11859) were obtained from Drs. Cesar Lebarca and Henry Lester. Dr. John Adelman provided Kir3.4 (GIRK 4) (Genbank accession no: X83584). TrkB (Genbank accession no: M55293) was obtained from Dr. Mark Bothwell. Rat Kappa opioid receptor (KOR) was obtained from Dr. David Grandy (Genbank accession no: D16829). Point mutations to produce functional homomeric channels; Kir3.1(F137S) (Chan et al 1996), Kir3.2(S146T) and Kir3.4(S143T) (Vivaudou et al 1997) were produced. Mutations were introduced by polymerase chain reaction amplification using PFU turbo DNA polymerase with complementary oligonucleotide primers incorporating the desired mutation. Positive clones were confirmed by automated sequencing. Plasmid templates for constructs were linearized prior to *in vitro* mRNA synthesis (Dascal et al 1993) using mMessage Machine (Ambion Inc, TX).

Oocyte maintenance and injection.

Healthy stage V and VI oocytes were harvested from mature anesthetized *Xenopus laevis* (*Xenopus* Express, Gainesville, FL) and defolliculated enzymatically as described (Dascal et al 1993). The oocytes were maintained at

18°C in standard oocyte buffer, ND96 (96 mM NaCl, 2 mM KCl, 1 mM CaCl2, 1 mM MgCl2, 5 mM HEPES, pH 7.5) supplemented with 2.5 mM sodium pyruvate and 50 μg/ml gentamicin (Sigma Chemical Co.). One day after harvest, each oocyte was injected with 50 nL of mRNA for the TrkB receptor, and either G-protein inwardly rectifying potassium channel heteromultimers Kir3.1 and Kir3.2, Kir3.1 and Kir3.4, or homomers Kir3.1(F137S), Kir3.2(S146T) or Kir3.4(S143T) into the vegetal pole. Recordings were made at least 48 hr after cRNA injection. Two electrode voltage clamp recordings were made at –80 mV in hK buffer (2 mM NaCl, 24 mM KCl or 96 mM KCl, 1 mM CaCl2, 1 mM MgCl2, 5 mM HEPES, pH 7.5).

Immunodetection of tyrosine phosphorylation of Kir3.1 after BDNF treatment.

Xenopus laevis oocytes were injected with 10 ng Kir3.1, 1 ng Kir3.4 and 0.08 ng TrkB mRNA. After four days of protein expression, groups of 25 oocytes received either ND96 or BDNF treatment (400 ng/ml) for 15 min then were frozen at -70°. Oocyte membranes were extracted with 100 μl solubilization buffer [1% SDS, 25 mM Tris (7.4), 150 mM NaCl, 2 mM EDTA], plus general protease inhibitors (1 μg/ml leupeptin, 2 μg/ml apoprotin, 200 mM phenylmethylsulfonylfluoride) and the tyrosine phosphatase inhibitor perorthovanadate (1 mM). The preheated (55°C) solubilization solution was applied to frozen intact oocytes for oocyte membrane solubilization and agitated until the outer surface pigment layer was removed. To immunoprecipitate Kir3.1, the oocyte membrane protein was incubated with 35 μg of an affinity purified antibody directed against carboxy-terminal residues 482-498 of rKir3.1 (Snutch

1988). The membrane/antibody complex was precipitated with 7.5 mg Protein A-Sepharose, washed 3X with RIA buffer (25 mM Tris pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% Triton-X) and once with 15 mM Tris pH 7.4, each containing protease inhibitors and perorthovanadate as described above. The immunoprecipitate was extracted using sample buffer (50 mM Tris pH 6.8, 500 mM β-mercaptoethanol, 2% SDS, 0.1% bromopheno-blue, 10% glycerol). The protein samples were resolved by SDS-PAGE, transferred to 0.2 μm nitrocellulose membranes, and then blocked overnight in 5% nonfat dry milk in TBS. Membranes were first blotted with a biotin-conjugated PY:20 anti-phosphotyrosine antibody (1:7500 dilution, ICN Biomedical), followed by a HRP-conjugated Avidin secondary antibody (ICN Biomedical). Immunoreactivity was visualized using ECL detection methods (NEN). The results shown were replicated three times.

Materials

Genistein, staurosporine, phorbol 12-myristate 13-acetate (PMA), and DMSO were obtained from Sigma Chemical Co, St. Louis, MO. Gö 6976 and K252a were obtained from Calbiochem, San Diego, CA. U69,593 was obtained from Research Biochemicals International. Pfu Turbo was from Stratagene, La Jolla, CA. PMA, Gö 6976, genistein and K252a were dissolved in dimethylsulfoxide (DMSO); the final concentration of DMSO applied to the oocytes was $\leq$ 0.02%. BDNF (Amgen, Thousand Oaks, CA) was dissolved in water and stored at –70°C until use.

Statistical analysis.

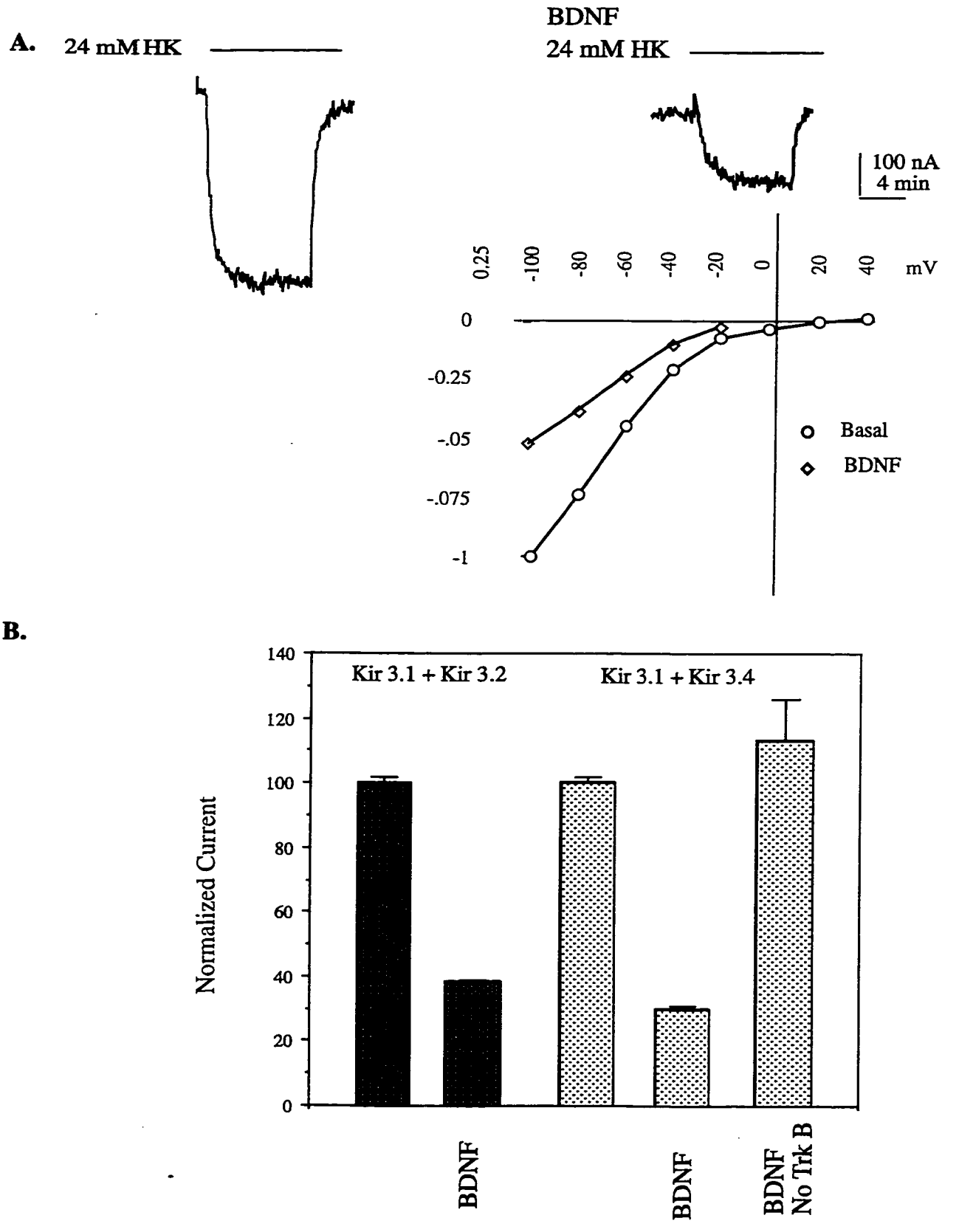
Data are presented as means ± SEM. Statistical analysis was carried out using an unpaired t test. A probability of $p < 0.05$ was considered statistically significant.

RESULTS

BDNF effects on Kir3 channels

Brief treatment of *Xenopus* oocytes expressing TrkB and Kir3 heteromultimers composed of either Kir3.1/3.2 or Kir3.1/3.4 with BDNF (200 ng/ml) produced a strong depression of the channel current (Fig 7). BDNF suppressed Kir3.1/3.2 by 62 ± 1% (n=17) and Kir3.1/3.4 by 70 ± 1% (n=16). As shown in the current-voltage curve (Fig 7A inset), BDNF reduced the total conductance through Kir3.1/3.2 channels; similar results were found for Kir3.1/3.4 (not shown). The effect of BDNF required co-expression of the TrkB receptor (Fig 7B). The dose response analysis (Fig 7C) showed that the potency of BDNF in this system (EC50 = 20 ng/ml) was consistent with prior findings (Sadick et al 1997).

Figure 7. BDNF treatment suppresses potassium currents in oocytes expressing TrkB with either Kir3.1 and Kir3.2 or Kir3.1 and Kir3.4. Oocytes were injected with a mixture of the following mRNAs: group 1 [0.004 ng TrkB, 0.05 ng Kir3.1 and 0.05 ng Kir3.2], group 2 [0.004 ng TrkB, 0.05 ng Kir3.1 and 0.05 ng Kir3.4], or group 3 [0.05 ng Kir3.1 and 0.05 ng Kir3.4, no TrkB]. Recordings were performed 3-4 days post injection. Initially, oocytes were bathed in normal oocyte saline buffer (ND96) containing 2mM KCl and clamped at –80mV. Oocytes were then perfused with a saline buffer containing 24 mM KCl. Elevation in potassium concentration facilitates current measurement by increasing the basal current through the inwardly rectifying potassium channels. A. Representative current traces from oocytes expressing Kir3.1/3.2 and TrkB during perfusion with the 24 mM KCl. The same oocyte was then perfused with BDNF (200 ng/ml) for 10-15 min then retested with 24 mM KCl. The insert shows the current voltage relationship (-120 to +20 mV) before and after BDNF treatment for this representative oocyte recording. The basal current in ND96 was subtracted from both curves. B. Potassium currents were measured in separate oocytes either pretreated with 200 ng/ml BDNF for 10-15 min or not. Responses were then normalized as a percentage of the mean response produced in untreated oocytes from the same batch and injection day. Bars represent mean ± SEM from three independent experiments (n=4-6). C. The inhibitory effects of BDNF on Kir3.1/3.4 were characterized by constructing a dose-response curve. Groups of oocytes


A.
24 mM HK
BDNF
24 mM HK
100 nA
4 min
0.25
-100
-80
-60
-40
-20
0
20
40
mV
0
-0.25
-.05
-.075
-1
Basal
BDNF
B.
Kir 3.1 + Kir 3.2
Kir 3.1 + Kir 3.4
Normalized Current
140
120
100
80
60
40
20
0
BDNF
BDNF
BDNF
No Trk B

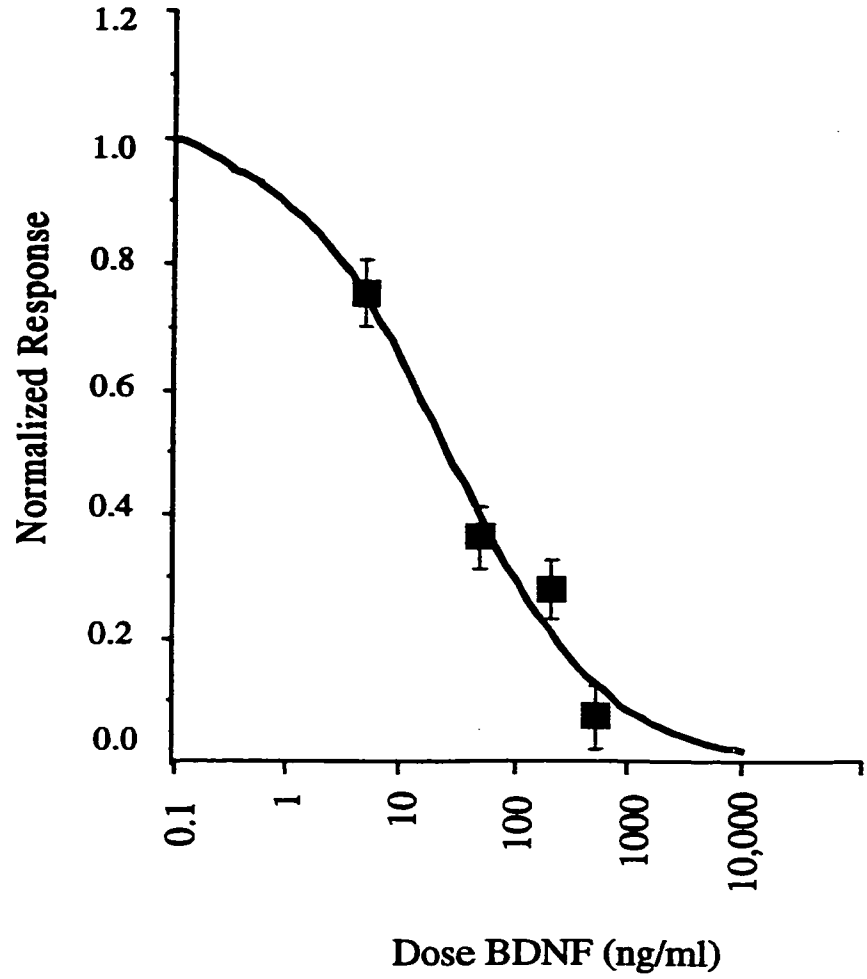


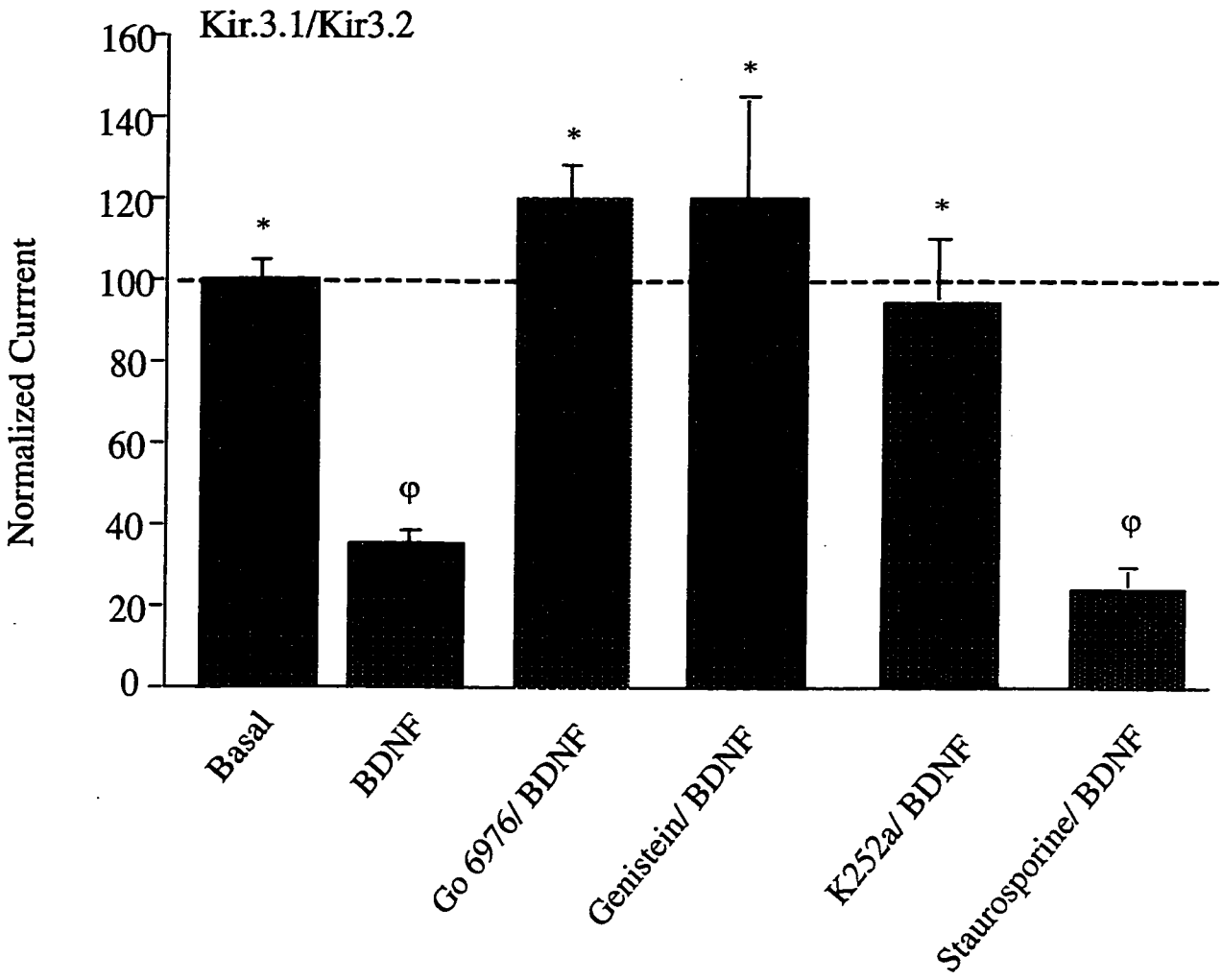
Figure 7 continued.

received either no treatment or treatment for 15 min. in BDNF (5, 50, 200, or 500 ng/ml) (n=3-4, each dose). Potassium currents in 24 mM KCl buffer were normalized as a percentage of the average response obtained from untreated oocytes of the same group.

Tyrosine Kinase and Ser/Thr Kinase Inhibitor Studies

TrkB activation results in an increase in tyrosine kinase activity and the subsequent activation of a cascade of kinase effectors (Greene and Kaplan 1995). To determine whether the suppression of the Kir3 current is initiated by the tyrosine kinase activity, oocytes were pretreated for 25 minutes with either the nonspecific tyrosine kinase inhibitor Gö 6976 (100 nM) (Behrens et al 1999), genistein (100 μM) (Akiyama et al 1987), or K252a (20 μM) (Ueda et al 1994) prior to treatment with BDNF and inhibitor. Pretreatment with Gö 6976 completely blocked the effects of BDNF. Similarly, genistein or K252a pretreatment also completely blocked the effects of BDNF treatment. In contrast, the serine/threonine kinase inhibitor staurosporine (1 μM) was ineffective (Fig 8). The results suggest that the BDNF-induced suppression of the Kir3 current requires activation of the tyrosine kinase, but not an intervening serine/threonine phosphorylation event.

Figure 8. Tyrosine kinase inhibitors Gö 6976, genistein and K252a blocked the BDNF induced suppression of Kir3 channel currents, whereas the ser/thr kinase inhibitor staurosporine did not. Oocytes injected with TrkB, Kir3.1 and Kir3.2 mRNA were treated with either ND96 or kinase inhibitor for 25-30 min then treated with ND96 (Basal) or 200 ng/ml BDNF in the continued presence of inhibitor for an additional 10-15 min. Data are the percentage of mean Kir3 current measured in untreated oocytes from the same batch. Pretreatment with the tyrosine kinase inhibitors, Gö 6976 (100 nM), genistein (100 μM), or K252a (20 μM) for 25 min significantly blocked the BDNF effect ($p<0.05$; n= 8-10), whereas staurosporine (1 μM) did not (n=7) ($p>0.05$). The effects of BDNF on oocytes after treatment with Gö 6976, genistein, or K252a were not significantly different from control oocytes treated with inhibitor alone. Bars represent mean ± SEM from two independent experiments. As a control for the effectiveness of staurosporine, control oocytes were treated with 100 nM of phorbol, 12-myristate, 13-acetate (PMA) for 10 minutes. PMA produced significant inhibition of the Kir3.1/3.2 current, and the PMA-induced inhibition was blocked by pretreatment with 1 μM staurosporine (n=7). (*$p<0.05$).


Kir.3.1/Kir3.2
Normalized Currrent
160
140
120
100
80
60
40
20
0
*
φ
Basal
BDNF
Go 6976/ BDNF
Genistein/ BDNF
K252a/ BDNF
Staurosporine/ BDNF

BDNF and PMA phosphorylate Kir3 differentially

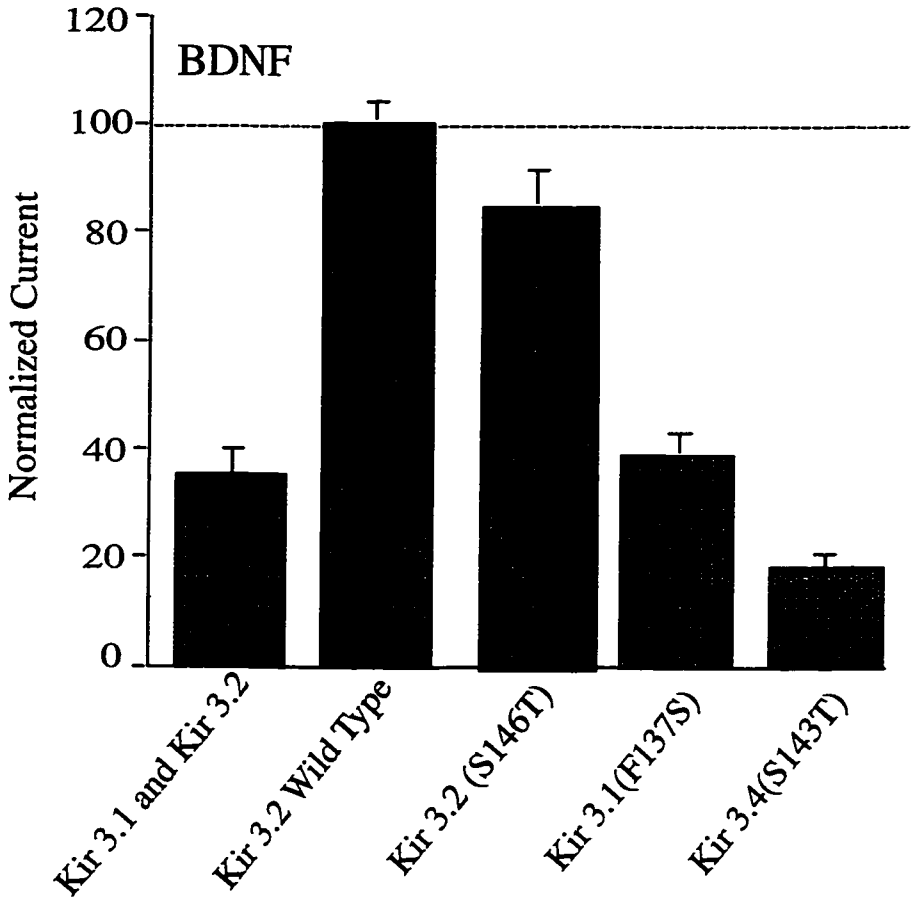
The BDNF-induced suppression could either result from a direct phosphorylation of Kir3 or an indirect effect on channel activation mechanisms. The Kir3 channels normally assemble as heteromultimers. To advance my analysis of the mechanism, I next determined whether the channels formed from subunits of single Kir3 isoforms were sensitive to the BDNF effect. To do this, I used the strategy developed by Logothetis and colleagues (Chan et al 1997; Vivaudou et al 1997) to generate functional homomeric Kir3 channels. Mutations in the putative pore of these channels, Kir3.1(F137S) and Kir3.4(S143T) greatly increased expression and activity of the channel homomers. I generated a point mutation in Kir3.2(S146T), and I found that this change also increased current compared to wild-type Kir3.2, which normally forms a homomeric channel. Moreover, the point mutation of Kir3.2(S146T) served as a control as this change was similar to Kir3.1(F137S) and Kir3.4(S143T). The homomeric Kir3.1(F137S) was as strongly inhibited by BDNF treatment as was the Kir.3.1/3.2 heteromeric channel (Fig 9A). The homomeric Kir3.4(S143T) was also highly sensitive to BDNF treatment. In contrast, neither wild-type Kir 3.2 nor Kir3.2(S146T) were affected by BDNF treatment. The channel type selectivity evident from this experiment supports the hypothesis that the BDNF-induced suppression of the current is caused by a direct modification of the channel.

Because Protein Kinase C (PKC) activation also suppresses Kir3 conductance (Sharon et al 1997; Rogalski et al 1999), I compared the effects of BDNF with those of phorbol myristate ester (PMA). Control oocytes expressing Kir3.1(F137S), or Kir3.2(S146T) or Kir3.4(S143T) homomeric channels were

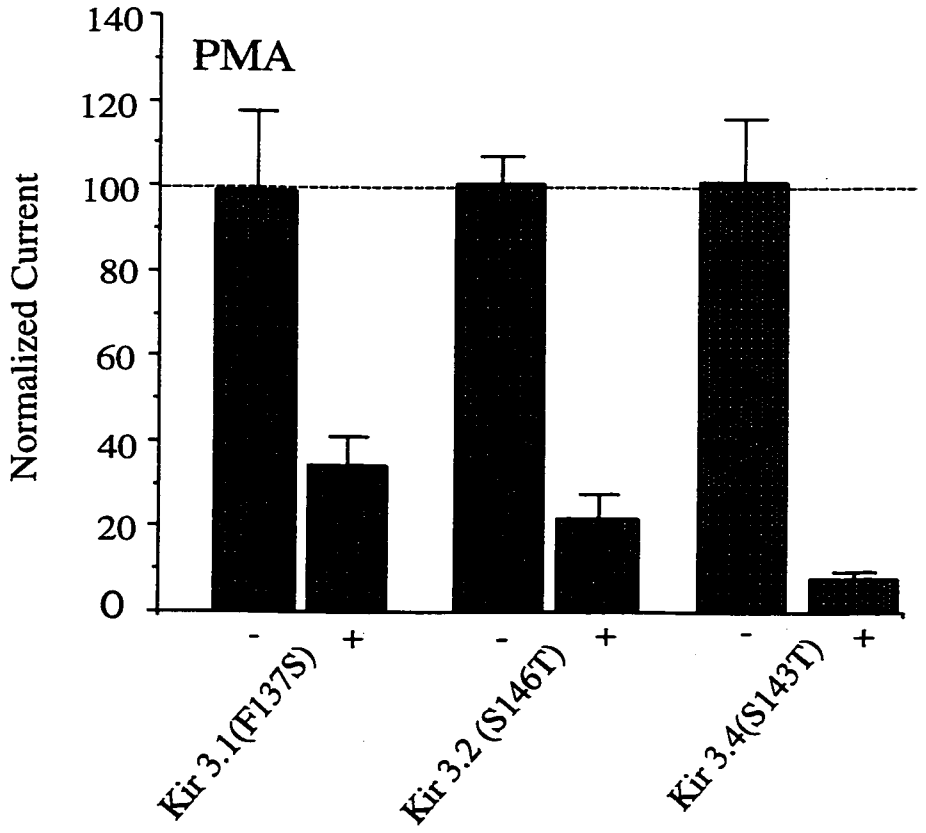
compared to matched oocytes preincubated in PMA (100 nM, 10-15 min). PMA produced significant inhibition of the potassium current for all three channels (Fig 9B). The lack of selectivity of PMA effects contrasts sharply with BDNF. Thus, I conclude that the channel subtype specific effects of TrkB activation were not mediated by PKC activation. This is consistent with the lack of effect of staurosporine described above.

Figure 9. BDNF and PMA effects on different combinations of Kir3 channel subunits. A. The effects of BDNF treatment were measured in oocytes injected with 0.004 ng TrkB mRNA and 0.05 ng Kir3.1 and 0.05 ng Kir3.2; or 1.0 ng wild type Kir3.2 alone; or 1.0 ng of Kir3.1(F137S); or 1.0 ng Kir3.2(S146T); or 1.0 ng Kir3.4(S143T). In each case, the effects of BDNF treatment were compared to untreated oocytes injected with the same mRNA. The effects of treatment with 200 ng/ml BDNF on Kir3.1/3.2 served as a positive control. BDNF treatment effectively reduced the Kir3 current for each channel except Kir3.2 and Kir3.2(S146T). For the Kir3.1 and Kir3.2 heteromultimer, the current was suppressed 65 ± 5% (n=10) by BDNF treatment compared to untreated oocytes. Kir3.1(F137S) was suppressed 61± 4% (n=16) by BDNF, a result not significantly different from the BDNF effect on the Kir3.1 and Kir3.2 combination. Kir3.2(S146T) was only reduced by 16 ± 5% (n=61) after BDNF treatment. Because the wild type Kir3.2 also forms homomeric channels in *Xenopus* oocytes, I tested its sensitivity to BDNF. For wild type Kir3.2, the potassium current after BDNF treatment was 100± 3% (n=10) of control. BDNF treatment suppressed Kir3.4(S143T) current by 85 ± 2% (n=11). B. In contrast to the selective effects of BDNF on Kir3 channel subtypes, activation of Protein Kinase C by PMA reduced the potassium currents mediated by each of the Kir3 channels. Oocytes were pretreated for 15 min in 100 nM PMA and then the potassium current in 96 mM hK buffer was measured. Significant inhibition of the basal response was observed in all three homomeric channels: PMA treatment suppressed Kir3.1(F137S) by 65± 13% (n=9), suppressed Kir3.2(S146T) by 78 ± 6% (n=7); and suppressed Kir3.4(S143T) by 91 ± 2% (n=14). Each group was compared to its own control

A.


BDNF
Normalized Current
120
100
80
60
40
20
0
Kir 3.1 and Kir 3.2
Kir 3.2 Wild Type
Kir 3.2 (S146T)
Kir 3.1(F137S)
Kir 3.4(S143T)

B.


PMA
Normalized Current
140
120
100
80
60
40
20
0
-
+
-
+
-
+
Kir 3.1(F137S)
Kir 3.2 (S146T)
Kir 3.4(S143T)

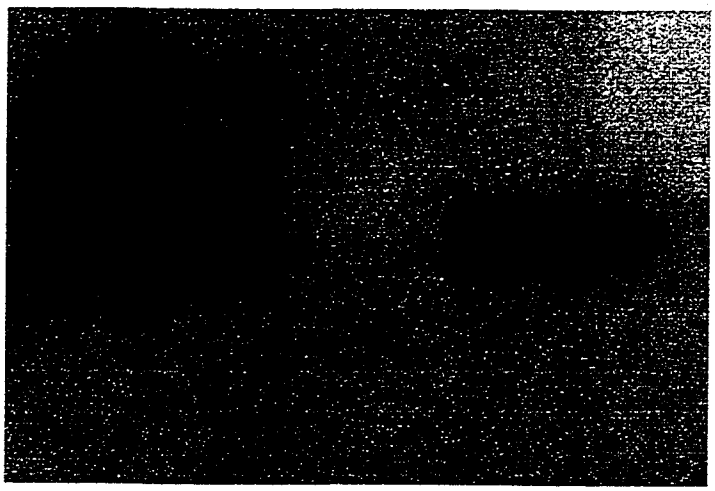
with that day's matched group. Bars represent mean ± SEM from two independent replicates.

Immunodetection of tyrosine phosphorylation of Kir3.1 after BDNF treatment

To test the hypothesis that the activated TrkB had a direct effect on the Kir3 channel, cells expressing TrkB and Kir3.1/3.4 were either pretreated with BDNF (400 ng/ml, 15 min) or pretreated in vehicle. Membrane proteins were solubilized and equivalent amounts of protein were immunoprecipitated with an affinity-purified antibody against Kir3.1 (Bausch et al 1995). A 45kD protein band corresponding to the immunoprecipitated Kir3.1 showed a substantial increase in phospho-tyrosine immunoreactivity following BDNF treatment. The 45 kD band was not evident in the lane loaded with an equal amount of Kir3.1 isolated from untreated oocytes expressing Kir 3.1/Kir 3.4 and Trk B (Fig 10). These results suggest that BDNF-induced TrkB activation causes direct tyrosine phosphorylation of the Kir3.1 subunit of the channel.

Figure 10. Immunodetection of tyrosine phosphorylation of Kir3.1 after BDNF treatment. 45 kD protein band corresponding to the immunoprecipitated Kir3.1 showed a substantial increase in phospho-tyrosine immunoreactivity following BDNF treatment

Untreated BDNF



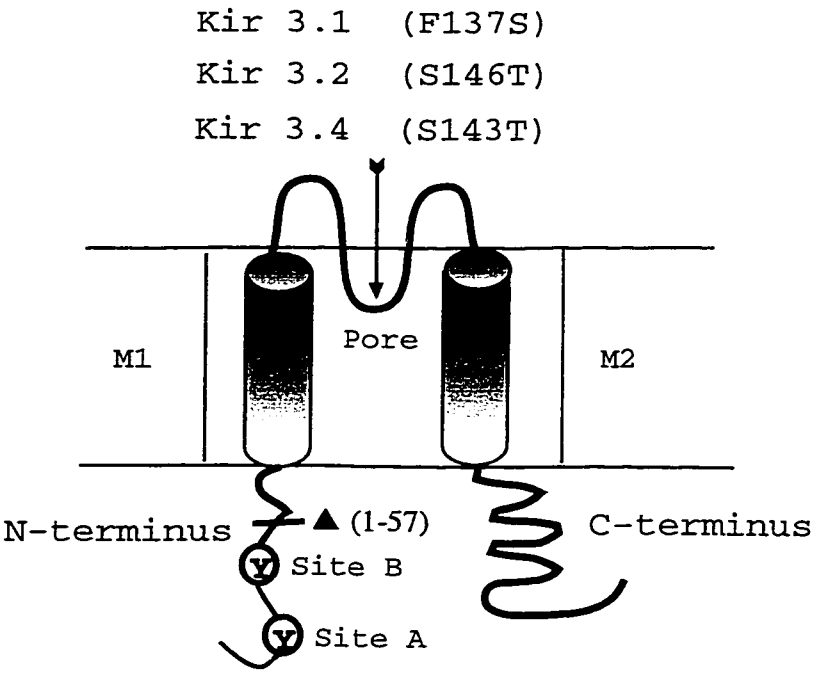
← 45kD

Identification of potential phosphorylation sites

Potential sites of tyrosine phosphorylation in the Kir3 sequence were first studied by making an amino-terminal truncation of Kir3.4(S143T) (Fig 11). Using a PCR based amplification, I prepared a cDNA template for mRNA encoding a Kir3.4(S143T) lacking amino acid residues 1-57. Oocytes expressing the truncated Kir3.4(S143T) produced strong potassium currents that were not significantly ($p > 0.05$) suppressed by BDNF treatment. After BDNF treatment, the mean current was $90 \pm 11\%$ ($n=8$) of control compared to untreated oocytes expressing truncated Kir3.4(S143T) (Fig 11A). Two tyrosine residues within the Kir 3.4 are affected by the amino-terminal truncation and are noted as potential phosphorylation Site 'A' and Site 'B' (Fig. 11B).

Figure 11 Potential amino-terminal phosphorylation sites of Kir3.1, Kir3.2 and Kir3.4 A. Kir3.1, Kir3.2 and Kir3.4 with the position of Asp and Tyr highlighted suggest potential sites of phosphorylation in the amino terminus of Kir3 homomers. **B.** The amino acid sequences adjacent to the two putative phosphorylation sites (A and B) are shown. Numbers in brackets to the right of the sequences are the residue numbers shown.

A.



B.

	Site A		Site B	
Kir 3.1	AL**RRK**FGDD**Y**QVV	(3-15)	GSETSR**Y**LSDLF	(61-72)
Kir 3.2	**PK**LPKQARD**D**LPR	(32-44)	KRKIQR**Y**VRKDG	(52-62)
Kir 3.4	**KK**IPKQARD**Y**IPI	(23-35)	KKPRQR**Y**MEKTG	(47-58)

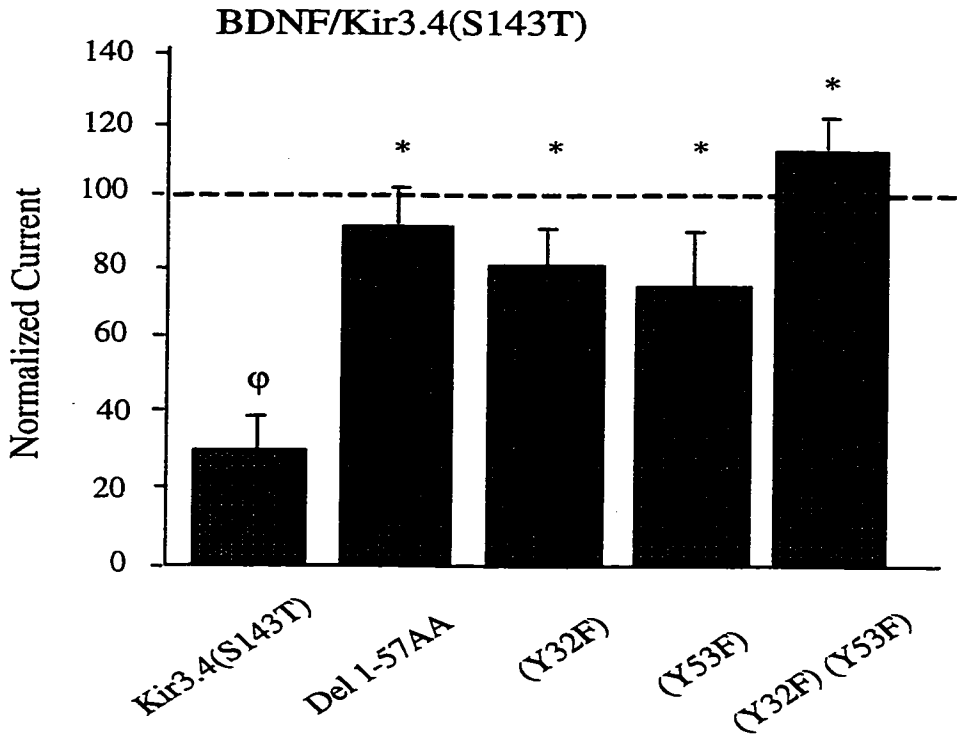
Mutation of Tyr in the amino terminus of Kir 3.4(S143T) and Kir 3.1(F137S).

Using a sequence alignment program (BCM Search Launcher, Baylor College of Medicine), I compared the sequence motifs surrounding Site A of Kir3.1, 3.2 and 3.4. Sequence alignment analysis indicated that Kir3.1 and 3.4 both have tyrosine residues in the amino terminus that align with aspartic acid 41 of Kir3.2 (Fig 11B). A tyrosine was present in all three channels in Site B (Fig 11B). To test whether either or both tyrosine residues of Kir3.1 and Kir3.4 were sites of BDNF-induced tyrosine phosphorylation, tyrosine residues 12 and 67 in the Kir3.1 sequence and 32 and 53 in the Kir3.4 sequence were mutated to phenylalanine. In contrast to the Kir3.4(S143T), BDNF did not significantly ($p<0.05$) inhibit the conductance of Kir3.4(S143T)(Y32F) (Fig 12A). After BDNF treatment, the current was $80 \pm 14\%$ ($n=14$) of the untreated current, not significantly different from control currents $100 \pm 23\%$ ($n= 8$) ($p>0.05$). The same loss of BDNF sensitivity was observed with channels having tyrosine 32 mutated to aspartic acid to produce Kir3.4(S143T)(Y32D) (data not shown). Mutation of the other tyrosine in the amino terminus Kir3.4(S143T)(Y53F) also significantly ($p<0.05$) reduced the sensitivity to BDNF; BDNF treatment caused only a reduction to $74 \pm 5\%$ ($n=11$) of the control, untreated currents. As expected, the deletion of both amino terminal tyrosines to produce Kir3.4(S143T)(Y32F)(Y53F) also significantly blocked ($p<0.05$) the effect of BDNF treatment $112 \pm 8\%$ ($n= 15$) (Fig 12A). These results suggest that tyrosine residues in both of these sites in the amino terminus of Kir3.4 (S143T) are critical for sensitivity to BDNF and are likely sites of tyrosine phosphorylation on Kir3.4.

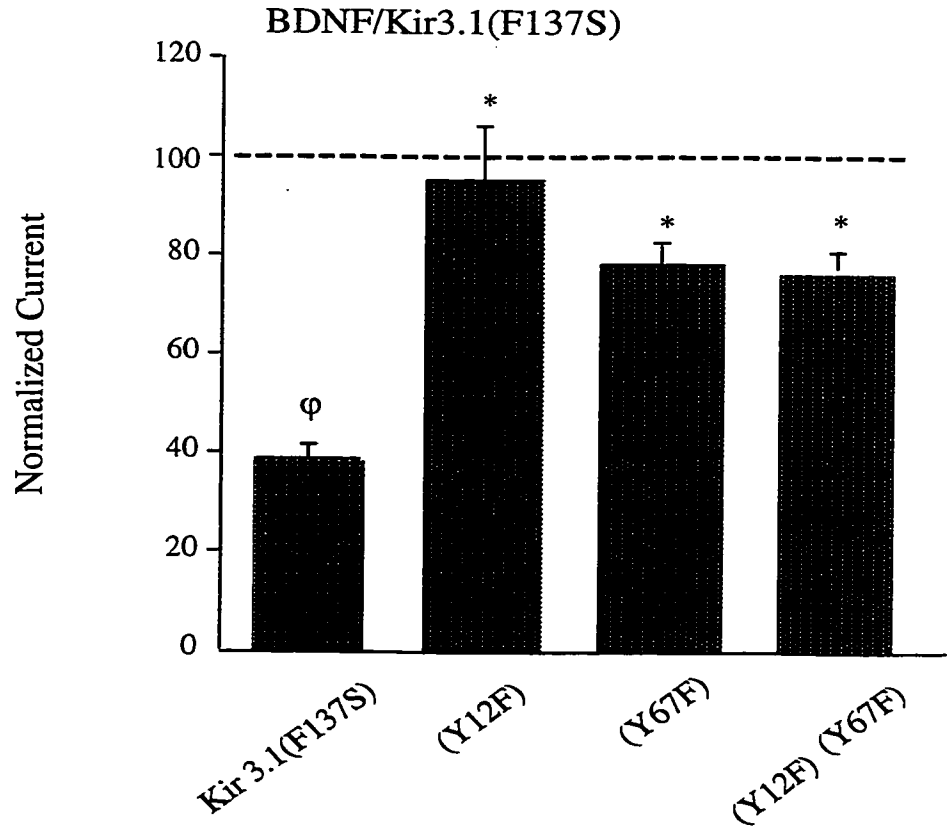
For the Kir3.1(F137S) homomeric channel, mutation of tyrosine 12 to phenylalanine, Kir3.1(F137S)(Y12F), completely blocked the BDNF effect $95 \pm 12\%$ (n= 23). (Fig 12B). Mutation of tyrosine 67, Kir3.1(F137S)(Y67F), effectively reduced the BDNF effect. After BDNF treatment, the current through Kir3.1(F137S)(Y67F) was $78 \pm 4\%$ (n=16) ($p<0.05$). Deletion of both tyrosines from the amino terminus to produce Kir3.1(F137S)(Y12F)(Y67F) also completely blocked the BDNF effect; current was $75 \pm 7\%$ (n=12) after treatment with BDNF, a result not significantly different than the single point mutations ($p<0.05$) (Fig. 12B). The results suggest that TrkB-induced phosphorylation of either Tyr 12 or Tyr 67 in Kir3.1 mediated the BDNF effect on the channel. Removal of the tyrosines 12 and 67 of Kir 3.1 did not block the substantial increase in phospho-tyrosine immunoreactivity after BDNF treatment (data not shown). Because Kir 3.1 has 14 potential sites for tyrosine phosphorylation, the data suggest that alternative tyrosine phosphorylation events must be occurring that does not affect channel conductance.

Figure 12. The effect of mutation of Tyr in the amino terminus of Kir 3.4(S143T) and Kir 3.1(F137S). **A.** Oocytes were injected with either 0.004 ng TrkB, and either 0.05 ng Kir3.4(S143T), 0.05 ng Kir3.4(S143T)(Del 1-57), 1 ng Kir3.4(S143T)(Y32F), 1 ng Kir3.4(S143T)(Y53F) or 1 ng Kir3.4(S143T)(Y32F) (Y53F). Oocytes were pretreated for 10-15 min with BDNF (200 ng/ml). Bar graph summarizing the effects of BDNF compared to untreated controls from the same batch. **B.** Oocytes were injected with either 0.004 ng TrkB and either 0.05 ng Kir3.1(F137S), 1 ng Kir3.1(F137S)(Y12F), 1 ng Kir3.1(F137S)(Y67F) or 1 ng Kir3.1(F137S)(Y12F) (Y67F). Oocytes were presoaked for 10-15 min BDNF (200 ng/ml). Bar graph summarizing the effect of BDNF on homomeric channels compared to its own control from that day's matched group. Data are means ± SEM from 4-7 oocytes and 2-3 independent experiments. ($p<0.05$) (φ T-test with value compared to 100% $p<0.05$).

A.


BDNF/Kir3.4(S143T)
Normalized Current
140
120
100
80
60
40
20
0
φ
*
*
*
*
Kir3.4(S143T)
Del 1-57AA
(Y32F)
(Y53F)
(Y32F) (Y53F)

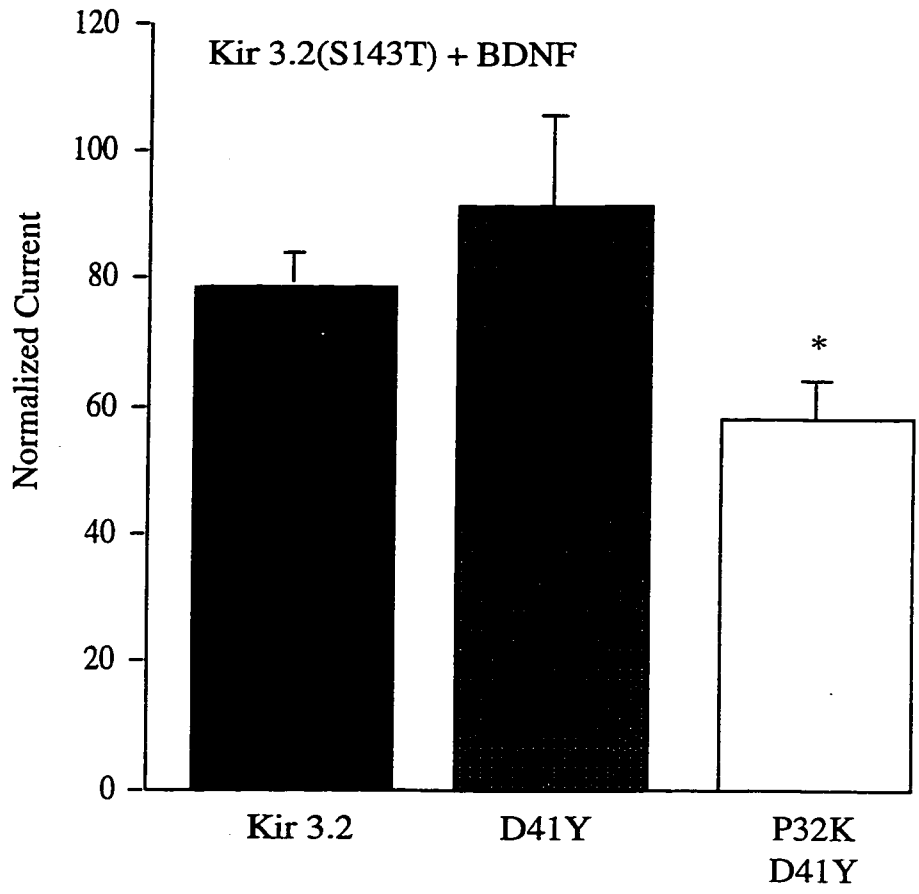
B.


BDNF/Kir3.1(F137S)
Normalized Current
120
100
80
60
40
20
0
φ
*
*
*
Kir 3.1(F137S)
(Y12F)
(Y67F)
(Y12F) (Y67F)

Amino acid charge is a determinant of Kir3 tyrosine phosphorylation

To determine the basis for Kir 3.2(S146T) insensitivity to BDNF, the sequences of Kir 3.2 and Kir 3.4 were compared (Fig 11B). An aspartic acid in the BDNF-insensitive channel Kir 3.2(S146T)(D41) aligned with the tyrosine in the BDNF-sensitive Kir 3.2(S146T)(Y32). Unexpectedly, the mutation of aspartic acid to tyrosine to produce Kir 3.2(S146T)(D41Y) did not confer sensitivity to BDNF (Fig. 13). The presence of a proline 32 in Site A of Kir3.2 instead of a lysine as in Kir3.4 (Fig 11B) was a potentially significant difference between the two channels. Consistent with other tyrosine phosphorylation motifs (Songyang et al 1994), the positively charged lysine Kir3.2(32K) may be permissive for a tyrosine phosphorylation event on Kir3.2. In confirmation of this hypothesis, the conductance of Kir 3.2(S146T)(P32K)(D41Y) was reduced by BDNF 40 $\pm$ 7% ($n= 33$) ($p<0.05$) (Fig. 13).

Figure 13. The effect of addition of tyrosine and alteration of the surrounding charge in the amino terminus of Kir 3.2(S146T). A. Oocytes were injected with 0.004 ng TrkB, and either 1 ng Kir3.2(S146T), 1 ng Kir3.2(S146T)(D41Y) or 1 ng Kir3.2(S146T)(P32K)(D41Y). Oocytes were pretreated for 10-15 min with BDNF (200 ng/ml). Bar graph summarizing the effects of BDNF compared to untreated, matched controls from the same batch. ($p<0.05$). As a control for the BDNF, experiments with the insensitive Kir 3.2 (S146T) were performed on the same day as experiments with BDNF-sensitive Kir3.1/3.2 channels.


Kir 3.2(S143T) + BDNF
Normalized Current
120
100
80
60
40
20
0
*
Kir 3.2
D41Y
P32K
D41Y

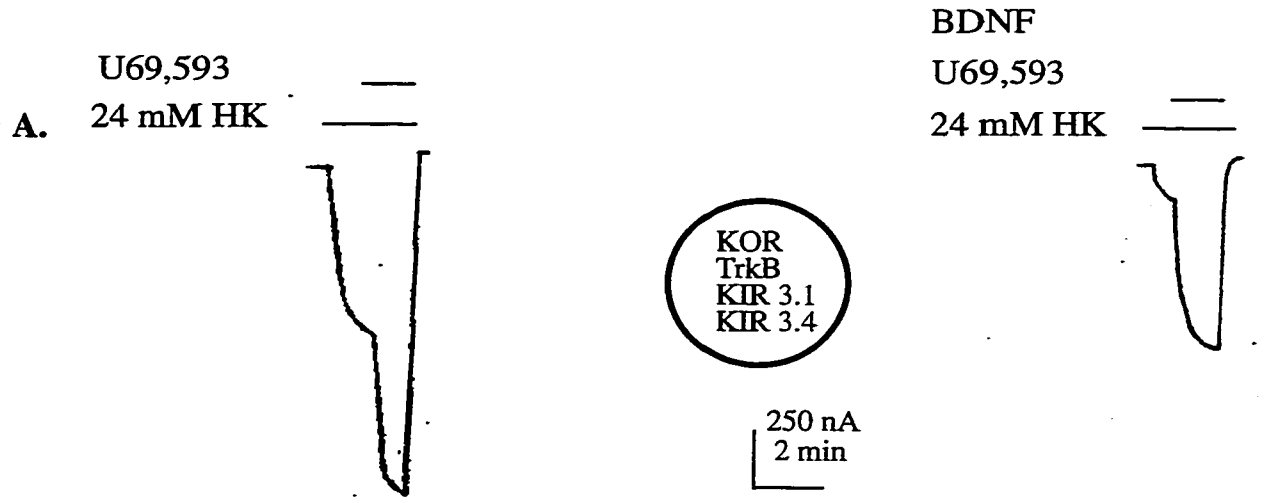
To further test that the charged lysine residue conferred sensitivity to Kir3.2 (D41Y), I mutated the proline to alanine. Kir 3.2(P32A)(D41Y) was not sensitive to BDNF (data not shown). The results thus help to define the motif required for BDNF sensitivity: two tyrosine residues in the amino terminus combined with a nearby cluster of positively charged residues seem to be required.

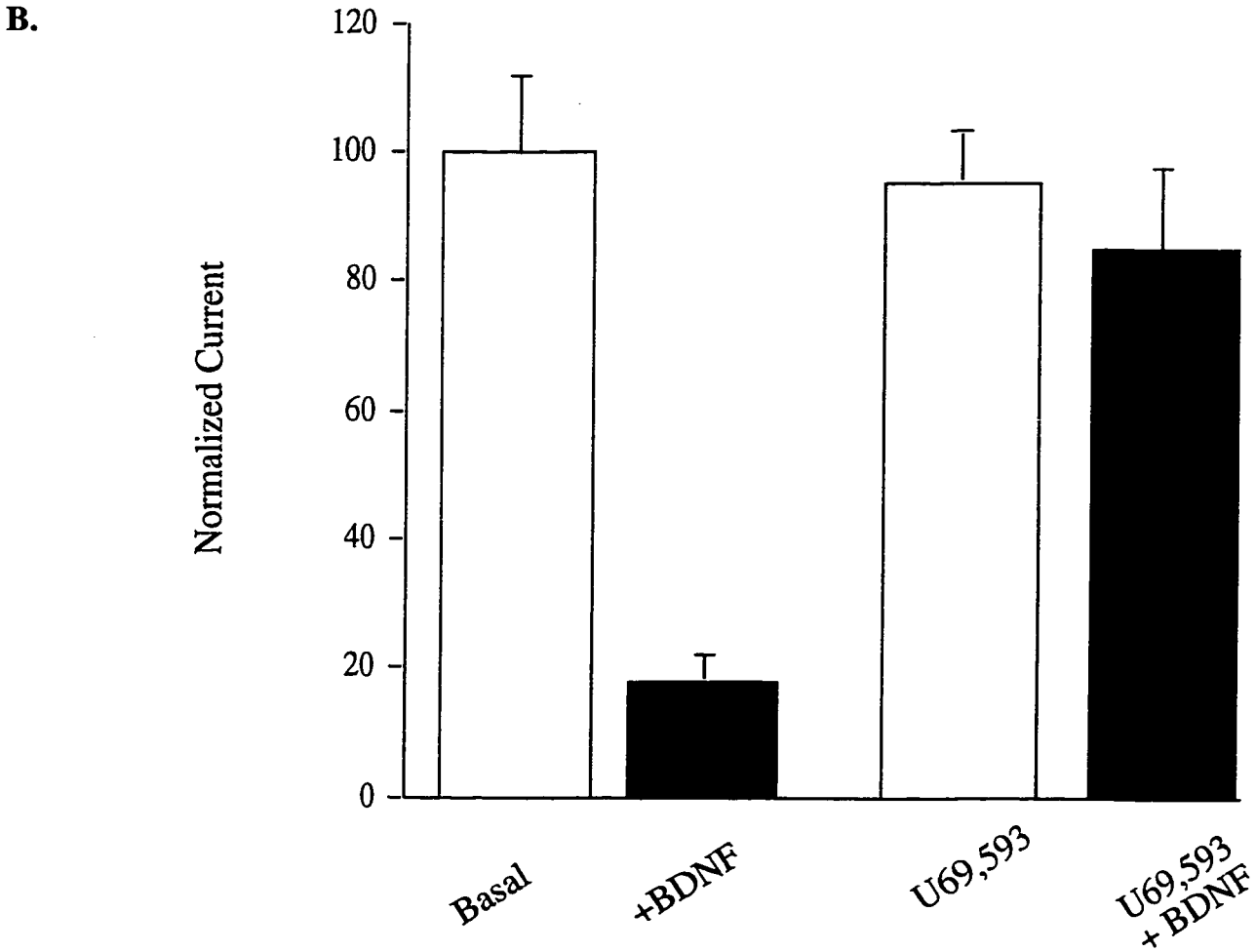
BDNF effects on Gβγ activation

A reduction in channel conductance may be caused either by reduced current through existing channels or by loss of channel from the plasma membrane. To distinguish between these two alternatives, I tested the effects of channel activation by G protein coupled receptor stimulation. I expressed the rat kappa opioid receptor (KOR), TrkB, Kir3.1 and 3.4 heteromultimers (Henry et al 1995; Appleyard et al 1999). Pretreatment with BDNF inhibited the channel conductance as shown previously (Fig 7). In contrast, the stimulation of Kir3 current by a maximally effective concentration of the kappa receptor by U69,593 was not inhibited by BDNF pretreatment (Fig 14). The results suggest that BDNF-induced phosphorylation shifts the channel from a basally active to a basally inactive state but does not prevent Gβγ activation of the channel.

Figure 14. The effect of BDNF on Gβγ activation of the kappa opioid receptor

A. The effects of BDNF treatment were measured in oocytes injected with 1.0 ng of the Kappa opioid receptor, 0.004 ng TrkB mRNA, 0.05 ng Kir3.1 and 0.05 ng Kir3.4. Representative current traces from oocytes expressing KOR, Kir3.1/3.4 and TrkB during perfusion with 24 mM KCl followed by KOR activation by the selective agonist U69,593 (500 nM). B. Bar graph summarizing the effects of BDNF on the channel response to 24mM KCl and the additional response caused by KOR activation with the selective agonist U69,593 (500 nM) compared to matched controls not treated with BDNF. Responses were then normalized as a percentage of the mean response to either KCl or U69,593 (500 nM) produced in untreated oocytes from the same batch and injection day. Data are from 5-6 oocytes and two independent experiments.


A.
U69,593
24 mM HK
BDNF
U69,593
24 mM HK
KOR
TrkB
KIR 3.1
KIR 3.4
250 nA
2 min


B.
Normalized Current
120
100
80
60
40
20
0
Basal
+BDNF
U69,593
U69,593
+ BDNF

DISCUSSION

The principal finding of these studies is that activation of TrkB by BDNF produces a robust inhibition of the G-protein gated inwardly-rectifying potassium channel (Kir3). These results support the conclusion that Kir3.1 and Kir3.4 require phosphorylation at two sites in the amino terminus. Removal of either blocked BDNF-inhibition. The data suggest that the related channel, Kir3.2 was insensitive to BDNF-induced inhibition because Kir3.2 has only a single tyrosine site in the amino terminus. As shown by immunoblot and channel mutagenesis results, channel inhibition likely was caused by direct channel phosphorylation on conserved tyrosine residues in the amino terminus of Kir3.1 and Kir3.4. These novel findings suggest that a component of BDNF action on neuronal excitability may include the regulation of G-protein receptor signaling. The resulting increase in excitability could contribute to the increases in synaptic plasticity and neurotransmitter release observed in intact neuronal preparations (Berninger and Poo 1996; Lessmann et al 1994).

In the mutagenesis studies described, I found that two tyrosine residues in the Kir3.4 sequence (Y32 and Y53) and two tyrosine residues in the Kir3.1 sequence (Y12 and Y67) were required for sensitivity to BDNF. Tyrosine phosphorylation of other tyrosine residues in Kir3 channels were not responsible for BDNF-induced inhibition. The lack of sensitivity of Kir3.2 to BDNF-inhibition was a striking finding that helped to define a potential consensus site. Because Kir3.2 contains a tyrosine residue surrounded by nearly the same motif as evident

in Kir3.1 and 3.4 (Fig 11 site B), I predicted that it should be phosphorylated following TrkB activation. By introducing a second tyrosine phosphorylation site in the amino terminus of Kir3.2 and adding an essential basic charge near the phosphorylation site, Kir3.2 became sensitive to BDNF-inhibition. I suggest that the change in charge created by the consensus site mutant enhanced the ability of tyrosine kinase to phosphorylate the Kir 3.2 (D41Y) tyrosine mutation. This reinforces the idea that tyrosine kinase phosphorylation is dependent upon charge interactions surrounding the phosphorylation site (Songyang et al 1994; Songyang and Cantley 1998). Alternatively, Kir3.2 may be insensitive because the phosphorylation site (Fig. 11B) does not interact with a complementary domain required for channel inhibition. A clearer understanding of the biophysical basis for the phosphotyrosine inhibition of the channel conductance is required before I can understand why Kir3.2 is insensitive to BDNF-inhibition.

As is evident from the surrounding sequences, all three BDNF-sensitive Kir3 channels have charged amino acids (aspartic acid or arginine) on the amino-side of the tyrosine and a hydrophobic residue on the carboxy-side. This general motif is characteristic of other sites phosphorylated by protein tyrosine kinases (Songyang et al 1994; Songyang and Cantley 1998); however, the motif is not sufficiently specific to allow the identification of the kinase involved. Although the specific kinase mediating the Kir3 inhibition was not defined in this study, the Src tyrosine kinase, Fyn, which is present in oocytes, associates with TrkB and

mediates some of the tyrosine phosphorylation events following TrkB activation (Iwasaki et al 1998).

The conductance of the G protein gated inwardly rectifying potassium channel is controlled by a wide range of regulatory factors as would be expected for a ubiquitous channel having a key role in the regulation of neuronal excitability. Kir3 is activated by Gβγ, phosphotidylinositol-4,5-bisphosphate (PIP_2) (Huang et al 1998) and sodium binding and inhibited by protein kinase phosphorylation (Sui et al 1999). Logothetis and colleagues suggest that PIP_2 binding generally regulates the inwardly rectifying potassium channels. The Kir3 channel interaction with PIP_2 additionally requires both sodium and Gβγ binding to the carboxy terminus of the channel (Zhang et al 1999). My results show that U69, 593-activation of the kappa opioid receptor that acts by increasing Gβγ concentration, overcomes the basal inhibition of the conductance caused by BDNF treatment. This result shows that the tyrosine phosphorylated Kir3 channel still can be activated by PIP_2, sodium, and Gβγ binding. I suggest that BDNF induced phosphorylation of Kir 3 causes a conformational change in the channel that reduces the ability of intrinsic activators to basally activate the channel. The observation that Gβγ, increased by kappa receptor activation, can overcome the BDNF effect suggests that the inhibition by phosphorylation is independent of the activation by endogenous regulators PIP_2, sodium, and Gβγ.

Evidence suggests that growth factors have diverse effects on ion channels. For example, the growth factor NGF regulates both the number and distribution of delayed rectifying K channels in PC 12 cells (Sharma et al 1993). However, growth factors can also have extremely selective effects on ion channels. The growth factors FGF1 and FGF2 have opposing effects in the regulation of Kir in cardiac myocytes via distinct second messenger pathways (Chauhan-Patel and Spruce 1998). My data demonstrate that the growth factor BDNF selectively modulates Kir3 through a unique pathway. That growth factors can control both ion channel distribution and regulation suggests that BDNF modulation of Kir3 may be a determinant in synaptic transmission. The results in this study suggest an additional mechanism for BDNF regulation of ion channel conductance, an important mechanism regulating neuronal excitability. My analysis utilized a malleable expression system to define a novel regulatory mechanism and predict a physiological relationship between neurotrophin action and potassium channel function.

CHAPTER IV

EICOSANOIDS INHIBIT THE G-PROTEIN GATED INWARDLY RECTIFYING POTASSIUM CHANNEL (KIR3) AT THE NA/PIP2 GATING SITE

INTRODUCTION

I previously showed that activation of the human endothelin A receptor (HETAR) by endothelin-1 (Et-1) produced products of PLA2 metabolism to inhibit Kir3 heteromultimers. This chapter presents evidence that Kir3 inhibition by eicosanoids is channel subunit dependent and I identify the site within the channel required for arachidonic acid sensitivity. Kir3 subunits share common gating components. A principal difference in gating components between subunits is the presence of a Na activation site in Kir3.2 and Kir3.4. In this chapter, I show that the critical aspartate residue required for eicosanoid sensitivity is the same residue required for Na regulation of PIP2 gating.

The G-protein gated inwardly rectifying potassium channels (Kir3) provide essential regulation of neuronal and cardiac excitability (Jan and Jan 1997). By mediating the effects of cholinergic, monoamine and peptide receptor activation, Kir3 channels respond to a wide range of transmitters. The Kir3 family of G-protein gated inwardly rectifying potassium channels consists of subunits (Kir3.1-Kir3.5) that can assemble to form functional heteromultimers. Kir3 channels share a common design characterized by cytoplasmic N and C termini and two

transmembrane domains M1 and M2 that surround a potassium selective pore region (P or H5) (Dascal 1997)(Fig.15). The activation of Kir3 is complex and not completely understood. Kir3 channels require the interaction of multiple components to produce channel gating including Gβγ, Na, the phospholipid PIP2 and ATP (Sui et al 1996; Krapivinsky et al 1998; Logothetis and Zhang 1999; Zhang et al 1999; Kim and Pleumsamran 2000). Huang and colleagues proposed that Gβγ activates Kir3 by stabilizing interactions between PIP2 and the potassium channel. They showed that PIP2 depletion blocks activation of Kir3 by both Gβγ and Na (Huang et al 1998). ATP is another regulatory element in Kir3 gating that may act to maintain phosphorylation of PIP2. The response to activation of Kir3.1 and Kir3.4 heteromultimers is rapidly inactivated (run down) when internal ATP is depleted. Sui et al (1998) show that Kir3 activity is ATP dependent and is mediated by PIP2; moreover, ATP hydrolysis enables both Na and Gβγ activation (Sui et al 1998). These studies support the hypothesis that Kir3 gating components are interdependent.

Kir3 normally exists as a heterotetramer, but the gating properties of individual subunits may be studied by using channel mutants able to form functional homomeric channels: Kir3.1(F137S) (Chan et al 1997), Kir3.2(S146T) (Rogalski et al 2000) and Kir3.4(S143T) (Vivaudou et al 1997). Although members of Kir3 are similar in structure, one primary difference in the subunits is the presence of a Na activation site in Kir3.2 and Kir3.4, that is not found in

Kir3.1. Ho and Murrell-Lagnado used chimeras of the Na-insensitive Kir3.1 and the Na sensitive-Kir3.2 to define the site of the channel that was sensitive to Na activation. Substitution of asparagine for aspartate 226 in Kir3.2(D226N) abolished Na-dependent activation for both the Kir3.2 homomer and Kir3.1/Kir3.2 heteromultimers without altering the amplitude of receptor-activation (Ho and Murrell-Lagnado 1999; Ho and Murrell-Lagnado 1999). Ho and Murrell-Lagnado proposed that Na binding to an aspartic acid residue masks the charge and permits PIP2 binding.

In contrast to the process of channel activation by PIP2, Na, and Gβγ, the mechanism of arachidonic acid inhibition of Kir3 is less well defined. Arachidonic acid and its metabolites modulate several ion channels including Kir3.1 and Kir3.4 heteromultimers in cardiac myocytes (Kurachi et al 1989). Unsaturated free fatty acids such as oleic, linoleic and arachidonic acid inhibit Kir3.1 and Kir3.4 heteromultimers channels by blocking ATP-dependent gating in atrial cells (Kim and Pleumsamran 2000). I previously showed that arachidonic acid inhibits the DAMGO activation of the MOR in *Xenopus* oocytes expressing MOR and Kir3 heteromultimers. Channel sensitivity to arachidonic acid was shown to be specific to the channel subtype: heteromultimers consisting of Kir3.1 and Kir3.2 or Kir3.1 and Kir3.4 were more sensitive to arachidonic acid than heteromultimers consisting of Kir3.1 and Kir3.5 (Rogalski et al 1999). These results suggest that eicosanoids have direct effects on G-protein gated inwardly rectifying potassium channels by modification of the channel conformation, however the molecular basis

for eicosanoid inhibition of Kir3 was not defined. In the present study, I explored the hypothesis that eicosanoids generated by HETA-activation may directly modulate Kir3 gating. Using site-directed mutagenesis to identify the eicosanoid-sensitive regulatory site on the Kir3 channel, I provide evidence that eicosanoid induced-inhibition of Kir3 requires the Na-dependent gating site.

MATERIALS AND METHODS

Complementary DNA clones and mRNA synthesis

The rat mu opioid receptor clone was obtained from Dr. Lei Yu (GenBank accession number L13069). cDNA for the human endothelin A (HETA) receptor (Genbank accession no: S67127) was obtained from Dr. Richard Kris. cDNAs for the Kir 3.1 (GIRK1) (Genbank accession no: U01071) and Kir 3.2 (GIRK 2) (Genbank accession no: U11859) were obtained from Drs. Cesar Lebarca and Henry Lester. Dr. John Adelman provided the Kir 3.4 (GIRK4) clone (Genbank accession no: X83584). Kir3 chimeras were the kind gift of Dr. Diomedes Logothetis. Point mutations to produce functional homomeric channels; Kir3.1(F137S) (Chan et al 1997), Kir3.2(S146T) (Rogalski et al 2000) and Kir3.4(S143T) (Vivaudou et al 1997) were produced. Mutations were introduced by polymerase chain reaction amplification using PFU turbo DNA polymerase with complementary oligonucleotide primers incorporating the desired mutation. Positive clones were confirmed by automated sequencing. Plasmid templates for

constructs were linearized prior to *in vitro* mRNA synthesis using mMessage Machine (Ambion Inc, TX).

Oocyte maintenance and injection

Healthy stage V and VI oocytes were harvested from mature anesthetized *Xenopus laevis* (Nasco, Ft. Atkinson, WI) and defolliculated enzymatically as described previously (Snutch 1988). The oocytes were maintained at 18°C in standard oocyte buffer, ND96 (96 mM NaCl, 2 mM KCl, 1 mM CaCl2, 1 mM MgCl2, 5 mM HEPES, pH 7.5) supplemented with 2.5 mM sodium pyruvate and 50 μg/ml gentamicin (Sigma Chemical Co.). One day after harvest, each oocyte was injected with 50 nl of cRNA for the mu opioid receptor (MOR) (1ng) and human endothelin A receptor (HETA)(1ng) and G-protein inwardly rectifying potassium channels. Concentrations of cRNA for different Kir3 channels generated in this study were injected into oocytes: G-protein inwardly rectifying potassium channel heteromultimers Kir3.1 (0.025 ng) and Kir3.2 (0.025ng), Kir3.1 (0.025ng) and Kir3.4 (0.025 ng), or homomultimers Kir3.1(F137S) (1 ng), or Kir3.2(S146T) (1 ng) or Kir3.4(S143T) (1ng) into the vegetal pole. Recordings were made at least 48 hr after cRNA injection.

Electrophysiological studies

A Geneclamp 500 amplifier was used for standard two-electrode voltage-clamp experiments. The pCLAMP program (Axon Instruments) was used for data acquisition and analysis. Oocytes were removed from incubation medium, placed

in the recording chamber containing ND 96 medium, and clamped at –80 mV. Recordings were made in hK buffer (2 mM NaCl, 96 mM KCl, 1 mM CaCl2, 1 mM MgCl.2, 5 mM HEPES, pH 7.5). Microelectrodes were filled with 3 M KCl and had resistances of 0.5 - 1.0 MΩ. Currents were measured without leak subtraction. Individual comparisons of drug effects on Kir 3 were conducted using oocytes from the same harvest and injection batch. Pharmacologic agents were perfused or placed directly into the bath from freshly made stock solutions.

Materials

Stock solutions of AACOCF3, arachidonic acid, bacalein and PD98059 were dissolved in dimethylsulfoxide (DMSO); the final concentration of DMSO applied to the oocytes was ≤ 0.02%. Arachidonic acid was stored at -70°C until use. Nitrogen was bubbled through water prior to dissolving endothelin. Endothelin-1 and DAMGO were obtained from Peninsula Laboratories, Belmont, CA. Arachidonic Acid, AACOCF3, PD98059 were from Calbiochem, La Jolla, CA. U73122 was from BioMol, Plymouth Meeting, PA. DMSO was from Sigma Chemical Co, St. Louis, MO.

Statistical analysis

Data are presented as means ± SEM. The statistical significance of differences between results was calculated using a paired t test. A probability of $p < 0.05$ was considered statistically significant.

Figure 15. Diagram of Kir3.

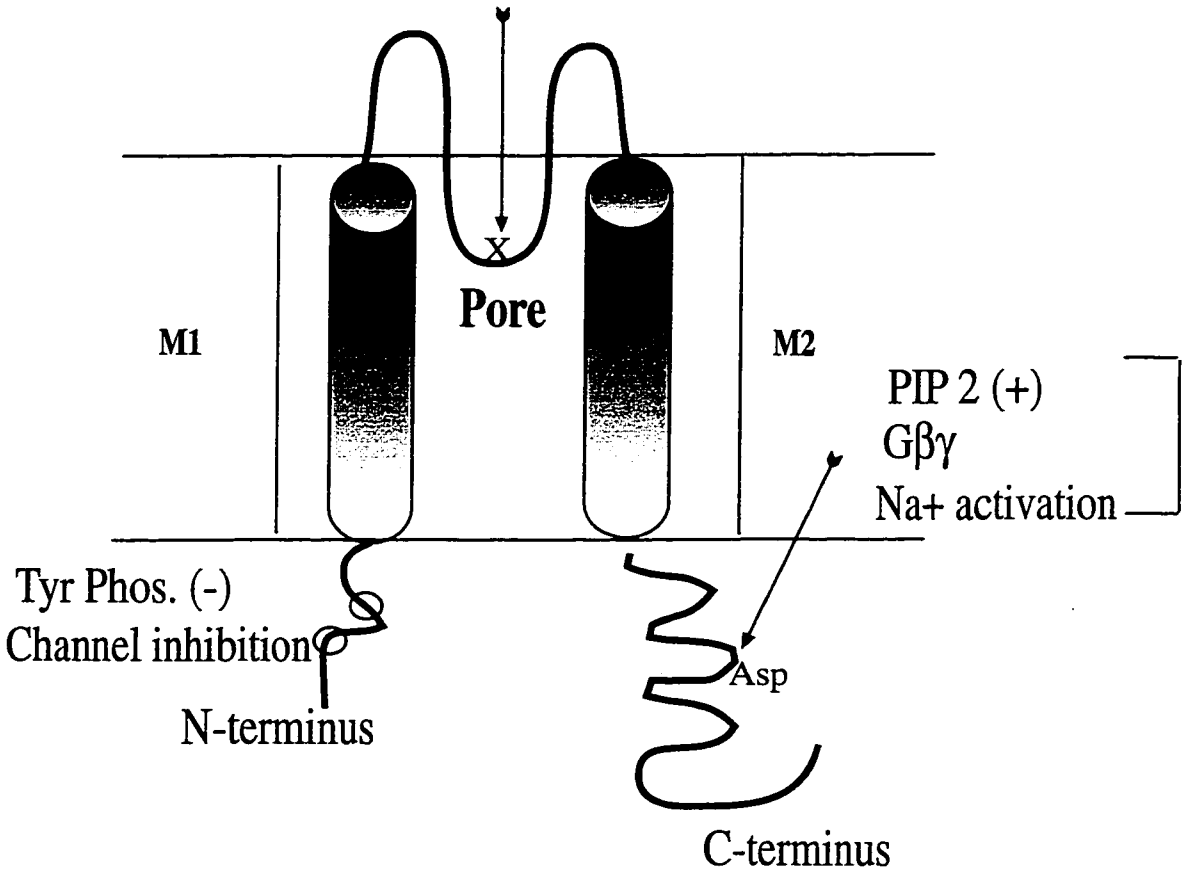
Kir3 channels share a common design characterized by a cytoplasmic N terminus (90 amino acids) and C terminus with two transmembrane domains M1 and M2, that surround the ion selective pore region (P or H5). Kir3 channels function as active heteromultimers (Kir3.1 pairs with other subtypes). Mutations in the P-region enhance the activity of homomers. This diagram indicates the P region with the site of specific point mutations that produce functional homomeric channels; Kir3.1(F137S), Kir3.2(S146T), and Kir3.4(S143T). The presumed PIP2-Na-G$\beta\gamma$ interaction site in the C-terminus is indicated. Kir3 is also inhibited by tyrosine phosphorylation (Rogalski et al 2000) with phosphorylation sites in the amino-terminus noted by the 'o'.

Homomultimers

Kir 3.1 (F137S)

Kir 3.2 (S146T)

Kir 3.4 (S143T)



RESULTS

Effects of endothelin-1 on Kir3 subunits and heteromultimers after DAMGO activation of the MOR.

Heterologous expression of MOR and Kir3 in *Xenopus* oocytes generated a system for the study of channel activation (Fig. 16). Activation of the Gi/Go coupled opioid receptors by the agonist DAMGO (1 μM) released Gβγ that activated Kir3 and produces a robust inward current (Fig. 16A). Activation of the heterologously expressed Gq-coupled endothelin receptor produced a cascade of signals that resulted in the reduction of the DAMGO-evoked response

To identify the Kir3 subunit conferring sensitivity to endothelin I used the strategy developed by Logothetis and colleagues to generate functional homomeric Kir3 channels. Mutation in the pore of these channels, Kir3.1(F137S) and Kir3.4(S143T) greatly increased expression and the current evoked by the channel homomers. In agreement with Vivaudau and colleagues, I noted that the wild type channel Kir3.4 does not produce functional currents when expressed alone in *Xenopus* oocytes. In contrast, the homomer Kir3.4(S143T) (1 ng RNA/oocyte) produced a robust current with an average receptor-activated current of 1-2 μA in response to agonist. Although the wild-type channel Kir3.1 forms functional channels with the intrinsic *Xenopus* subunit Kir3.5, the current response produced was small (50-100 nA) compared to the large currents (1-2 μA) produced by the functional homomer Kir3.1(F137S). This observation is consistent with the report of Chan et al. Similarly, Kir3.2(S146T) produced larger currents (500 nA to 1 μA) compared to wild-type Kir3.2 (100 nA), which normally forms a homomeric

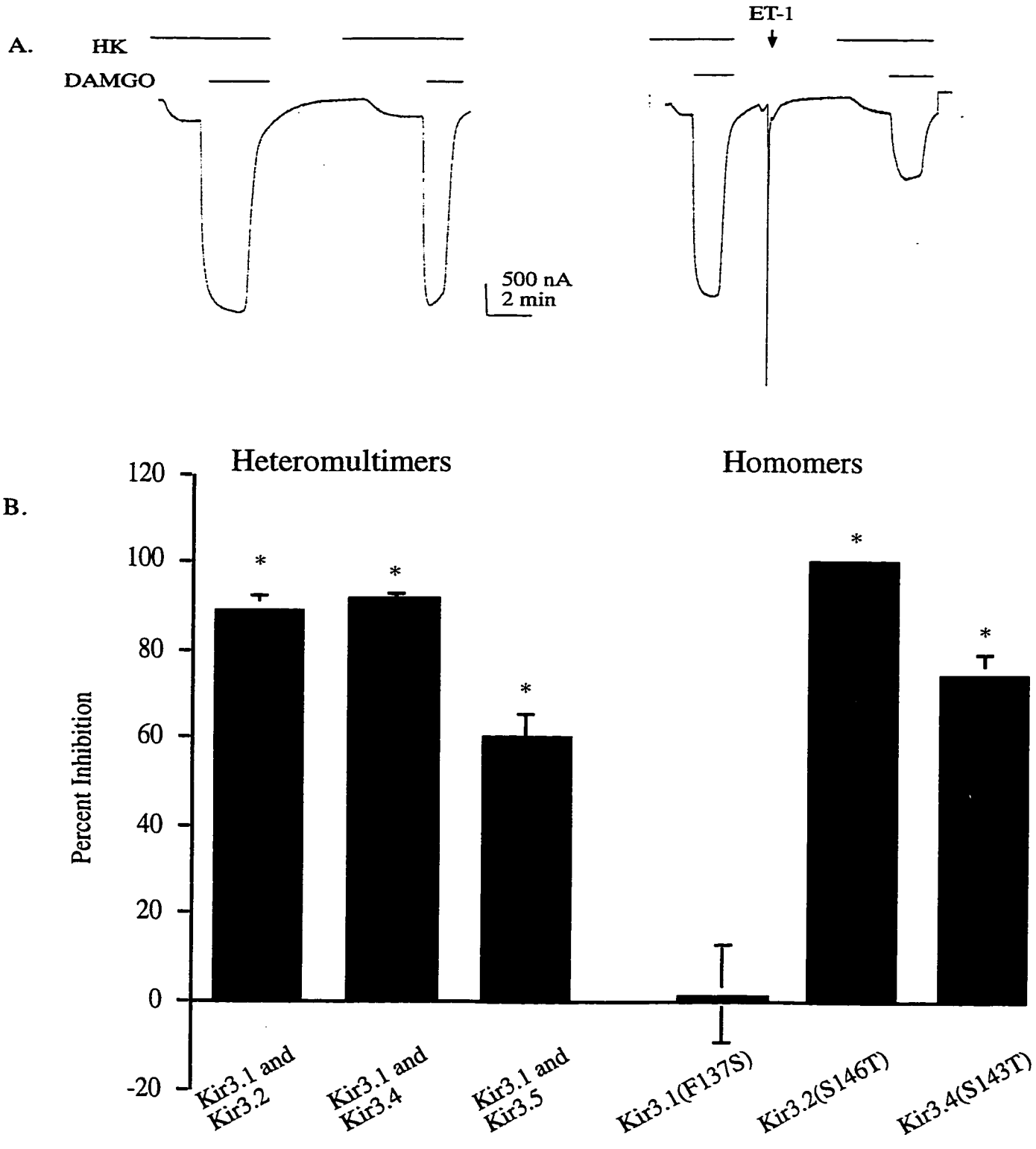
channel. Moreover, the point mutation of Kir3.2(S146T) served as a control as this mutation in the pore was similar to Kir3.1(F137S) and Kir3.4(S143T). Thus, oocytes expressing homomeric forms of Kir3.1, 3.2, and 3.4 were functional and were robustly activated by Gβγ released by mu opioid receptor activation.

Oocytes expressing either Kir3.2(S146T) or Kir3.4(S143T) treated with Et-1 prior to the second DAMGO challenge showed a marked inhibition of the second opioid response. The amplitude of the second opioid response in Kir3.2(S146T) homomers after Et-1 treatment was inhibited by 100 ± 0% (n=9). The amplitude of the second opioid response in Kir3.4(S143T) homomers after Et-1 treatment was inhibited by 74 ± 5% (n=18) ($p < 0.01$) (Fig 16B). In contrast, the second opioid response in oocytes injected with Kir3.1(F137S) treated with Et-1 prior to the second DAMGO challenge was not inhibited, and the second opioid response after Et-1 treatment was not significantly different from untreated oocytes. The amplitude of the second opioid response in Kir3.1(F137S) homomers after Et-1 treatment was 1 ± 11% (n=16). The channel type selectivity evident from this experiment further supports the conclusion that the endothelin-induced suppression of the DAMGO-activated response was caused by a direct modification of the channel. I previously showed that the PLA2 inhibitor AACOCF3 selectively blocked the endothelin receptor mediated effect (Rogalski et al 1999). These results suggested that an eicosanoid such as arachidonic acid produced by PLA2 activation following HETA receptor activation was responsible for channel inhibition. Another interpretation of my data was that the endothelin effect was caused by PLC mediated PIP2 depletion. To test the latter hypothesis, oocytes were pretreated with the PLC inhibitor, U73122 (5 μM) (Vickers, 1993) for ten minutes and tested for endothelin sensitivity. The amplitude of the second

opioid response in Kir3.4(S143T) homomers after Et-1 treatment was inhibited by 61 ± 5% (n=8). Pretreatment with the PLC inhibitor U73122 (5 μM) did not block the inhibition of the second opioid response to Et-1. The amplitude of the second opioid response of Kir3.4(S143T) homomers after Et-1 treatment was inhibited by 66 ± 5% (n=8) (not significantly different than in the absence of U73122). Thus, PLA2 activation and subsequent eicosanoid production likely caused the endothelin induced inhibition.

Figure 16. Effects of endothelin-1 on the DAMGO elicited mu opioid response (MOR) mediated by functional heteromultimers and channel homomers expressed in *Xenopus* oocytes. Oocytes were injected with a mixture of the following cRNAs: 1 ng MOR, 1 ng HETAR and either Kir 3.1 and Kir 3.2 (0.05 ng), or Kir3.1 and Kir3.4 (0.05 ng) or Kir3.1 and Kir3.5(0.05 ng) or 1 ng Kir 3.1(F137S), or 1 ng Kir 3.2(S146T), or 1 ng Kir3.4(S143T). A. Representative current traces from oocytes stimulated by perfusion with the mu opioid agonist (1 μM) DAMGO in oocyte buffer containing 96 mM KCl in two applications. The first panel shows the response without Et-1. The second panel includes a pulse of Et-1 in ND96 applied directly to the oocyte in the recording chamber. Application of vehicle had no effect. For these traces, oocytes were clamped at -80 mV in normal saline buffer. Horizontal bars indicate the duration of drug perfusion. The opioid response was only observed in oocytes injected with both receptor and channel cRNA. The endothelin-induced activation of the endogenous chloride current was only observed in oocytes injected with the endothelin A receptor cRNA.

Initially, oocytes were bathed in normal oocyte saline buffer containing 2 mM KCl and clamped at –80mV. Oocytes were then perfused with a saline buffer containing 96 mM KCl. Elevation in potassium concentration facilitates current measurement by increasing the basal current through the inwardly rectifying potassium channels. Application of the mu opioid agonist DAMGO (1 μM) in oocyte buffer containing 96 mM KCl increased the current. After the response reached steady state, normal oocyte saline buffer was washed on for 2 min. A


A.
HK
DAMGO
ET-1
500 nA
2 min
B.
Heteromultimers
Homomers
Percent Inhibition
120
100
80
60
40
20
0
-20
*
Kir3.1 and Kir3.2
Kir3.1 and Kir3.4
Kir3.1 and Kir3.5
Kir3.1(F137S)
Kir3.2(S146T)
Kir3.4(S143T)

second application of DAMGO produced a similar response (left trace). The amplitude of the second opioid response was 99 ± 2.9% (n=34) of the first response. When endothelin (50 nM) was applied after the first DAMGO application, there was a robust inhibition of the second DAMGO-evoked response (right trace). The percent inhibition of the second mu opioid response after Et-1 pretreatment is shown. B. Bars represent mean ± SEM of 18-20 oocytes from three batches. Recordings were performed 2-3 days post injection. Oocytes used were injected with MOR, HETAR and the Kir3 cRNA listed on the x-axis.

Endothelin effects on Kir3 channel truncations and chimeras.

To determine the basis for the insensitivity of Kir3.1(F137S) to endothelin receptor activation, the Et-1 insensitive Kir3.1 and Et-1 sensitive Kir3.4 sequences were aligned and compared. These Kir3 subunits share the most homology in the pore region and the greatest heterogeneity in the tail regions. Macica and colleagues identified a serine residue in the distal amino terminus in the inward rectifier ROMK1 that confers sensitivity to arachidonic acid (Macica et al 1996; Macica et al 1998). I wanted to test the hypothesis that an amino-terminus residue was responsible for eicosanoid-sensitivity in the inward rectifier Kir3. Sequential truncations of the amino terminus were made and the effect of Et-1 on Kir3 activation was tested. Oocytes expressing a truncated Kir3.4(S143T) lacking amino acids (Δ1-57) produced strong potassium currents and a robust response following DAMGO activation of MOR. The amplitude of the second opioid response in the channel with the amino terminus truncation Kir3.4(S143T)(Δ1-57) after Et-1 treatment was inhibited, a result not significantly different than the parent (p>0.05) (Fig 17B). Truncation of the first 23 amino acids in Kir3.2 also did not block either the DAMGO activation or endothelin sensitivity (data not shown).

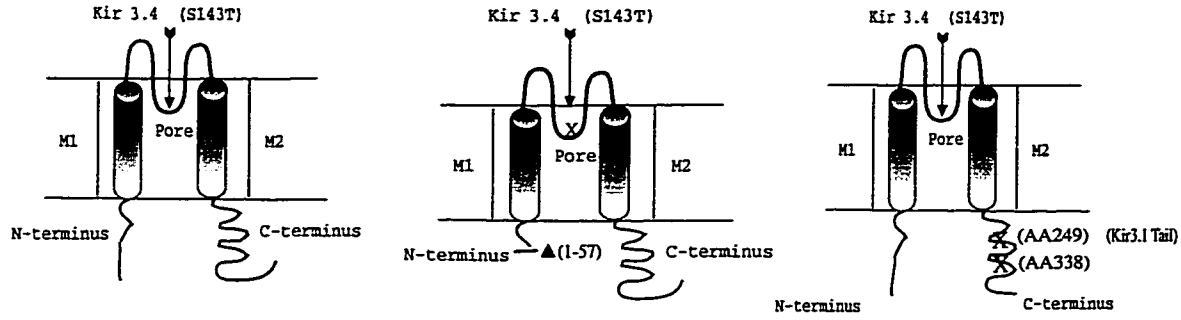
In contrast, the C-terminus of Kir3.1 and Kir3.4 differed significantly in both amino acid length and amino acid sequence. Chimeras composed of the Et-1 sensitive Kir3.4(S143T) amino terminus and pore region with the Et-1-insensitive Kir3.1 C-terminal tail Kir3.4(S143T)(1-338)/Kir3.1(333-501) and Kir3.4(S143T)(1-249)/Kir3.1(244-501) were used to define the region of endothelin-sensitivity. The MOR response to DAMGO following Et-1 treatment in the channel chimera Kir3.4(S143T)(1-338)/Kir3.1(333-501) with Kir3.1 C-terminal tail was inhibited, a result not significantly different than Kir3.4(S143T)

($p > 0.05$) (Fig 17B). Similarly, the amplitude of the second opioid response in the chimera Kir3.4(S143T)(1-249)/Kir3.1(244-501) with Kir3.1 C-terminal tail after Et-1 treatment was blocked, a result not significantly different than Kir3.4(S143T) ($p > 0.05$) (Fig 17B). Thus, neither the amino terminus nor the distal carboxyl terminus of Kir3 was responsible for the Et-1 induced inhibition. The chimera and truncation data suggest that the channel domain responsible for endothelin-sensitivity was within the proximal C-terminal region near the membrane.

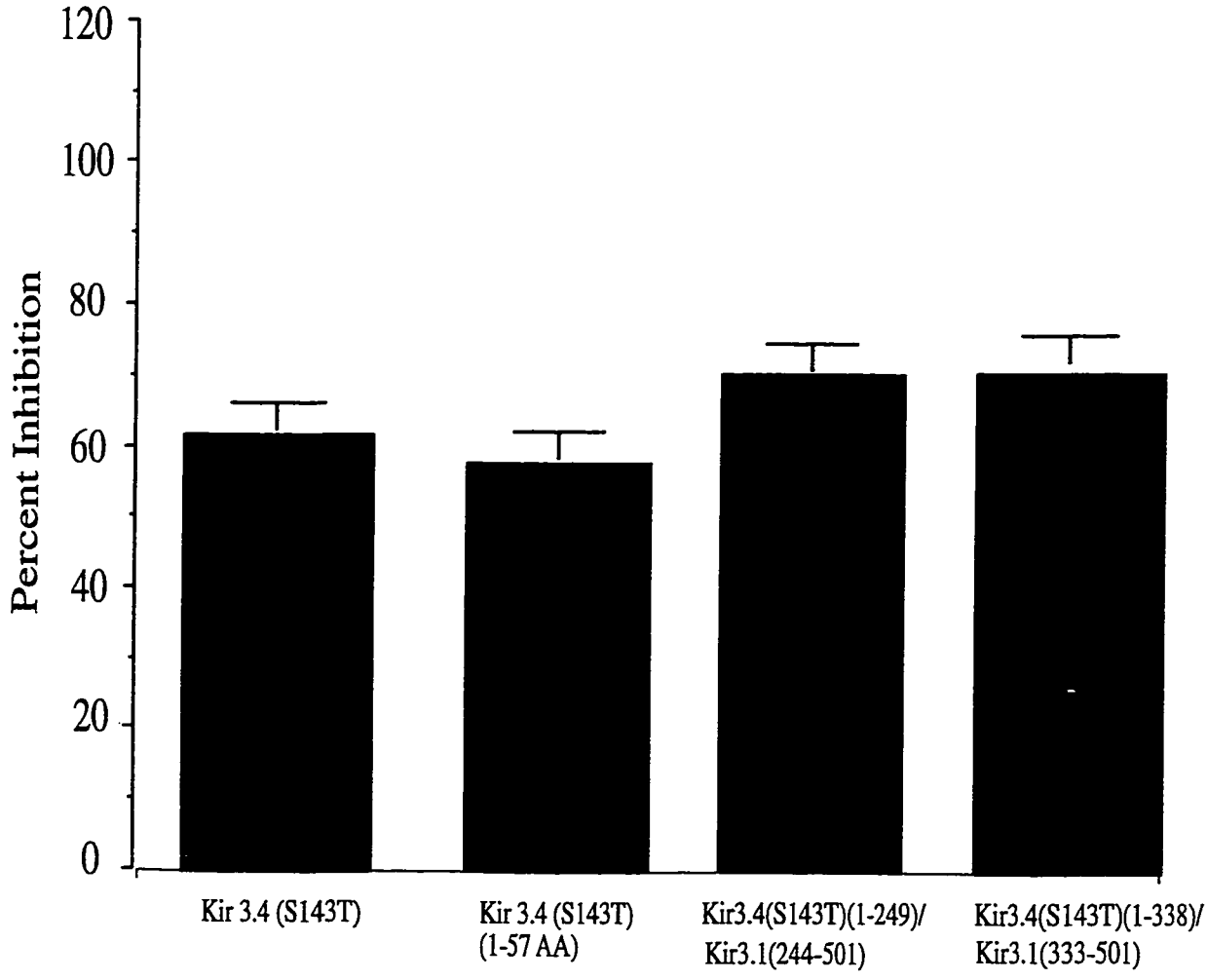
Figure 17. Endothelin effects on channel mutants. A. Diagrams depict channel mutants: Kir3.4 (S143T) parent, amino terminus truncation and chimeras. I produced the Kir3.4(S143T) using a cDNA template for cRNA coding a Kir3.4(S143T) having a truncated (1-57) amino-terminus. I used Kir3 chimeras: Kir3.4(S143T)(1-338)/Kir3.1(333-501) and Kir3.4(S143T)(1-249)/Kir3.1(244-501). B. Oocytes were injected with either 1 ng MOR and 1 ng HETAR, and either 1 ng Kir3.4(S143T), 1 ng Kir3.4(S143T)(Δ1-57), 1 ng Kir3.4(S143T)(1-338)/Kir3.1(333-501), or 1 ng Kir3.4(S143T)(1-249)/Kir3.1(244-501). The percent inhibition of the second mu opioid response after Et-1 pretreatment is shown. B. Bar graph summarizing the effects of Et-1 on the DAMGO activation of MOR compared to untreated controls from the same batch. Data are means ± SEM from 4-7 oocytes and 2-3 independent experiments.

The proximal domain of the channel contains sites responsible for PIP2 and Na gating (Sui et al 1996, Ho and Murrell-Lagnado 1999; Ho and Murrell-Lagnado 1999). I noted a correlation between endothelin sensitivity and Na gating sensitivity. Kir3.1 was not sensitive to endothelin, whereas Kir3.2 and Kir3.4 were sensitive (Fig 16B). Moreover, Kir3.1 is not gated by Na, whereas, Kir3.2 and Kir3.4 are gated by Na (Zhang et al 1999; Ho and Murrell-Lagnado 1999; Ho and Murrell-Lagnado 1999). Based on this correlation, I explored the hypothesis that the Kir3 gating domain was important in the observed eicosanoid-inhibition of Kir3. Na sensitivity was reported to depend on the presence of a critical aspartate

A.


Kir 3.4 (S143T)
M1
Pore
M2
N-terminus
C-terminus
Kir 3.4 (S143T)
M1
Pore
M2
N-terminus
▲(1-57)
C-terminus
Kir 3.4 (S143T)
M1
Pore
M2
(AA249)
(Kir3.1 Tail)
(AA338)
N-terminus
C-terminus

B.


Percent Inhibition
120
100
80
60
40
20
0
Kir 3.4 (S143T)
Kir 3.4 (S143T) (1-57 AA)
Kir3.4(S143T)(1-249)/ Kir3.1(244-501)
Kir3.4(S143T)(1-338)/ Kir3.1(333-501)

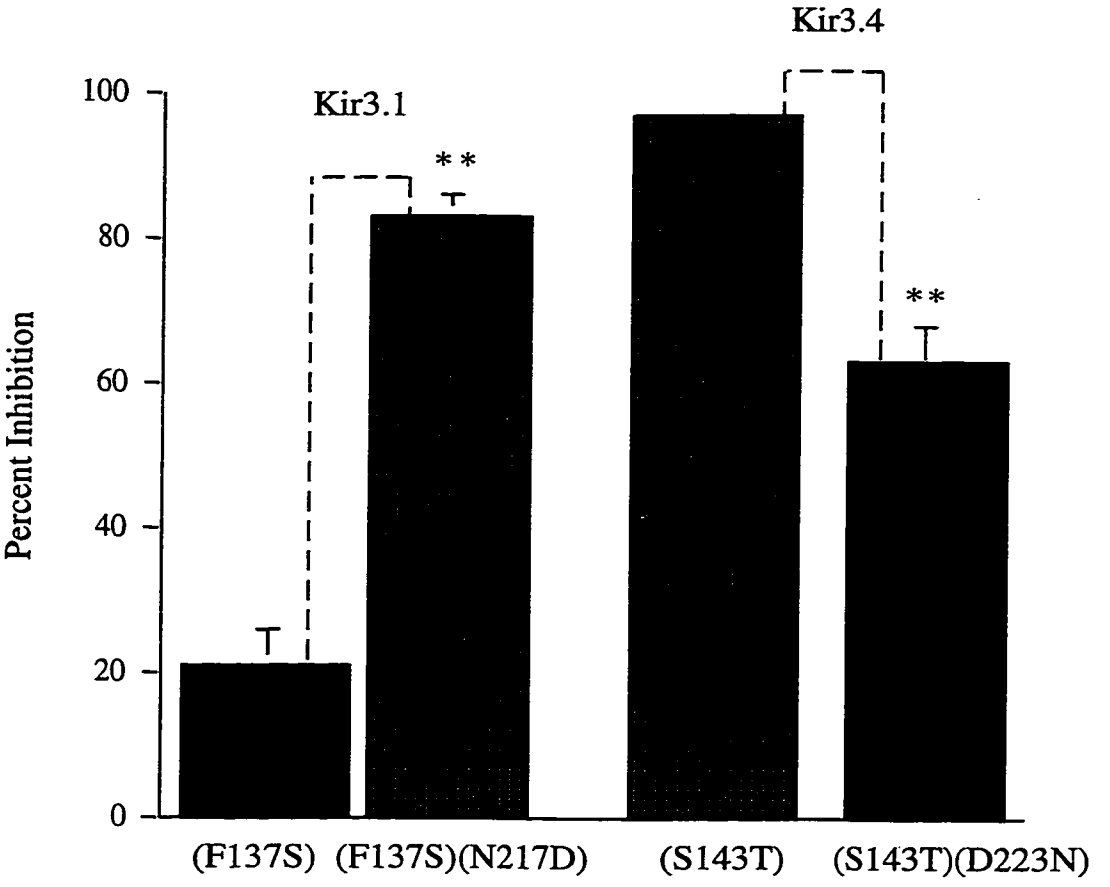
residue in the gating domain Na (Zhang et al 1999; Ho and Murrell-Lagnado 1999; Ho and Murrell-Lagnado 1999). Kir3.1(F137S) and Kir3.4(S143T)(D223N) lack the aspartate thought to be responsible for Na-gating and the corresponding channels Kir3.4(S143T) and Kir3.1(F137S)(N217D) contain this residue. These channel variants were produced and expressed in oocytes (Fig. 18).

The effect of the sodium binding site on the endothelin-1 inhibition of Kir3.

I coexpressed either Na-sensitive or Na-insensitive channel mutants of Kir3.1 or Kir3.4 with cRNA for the MOR and cRNA for the human endothelin A receptor. The amplitude of the second opioid response in Kir3.1(F137S) (Na-insensitive) homomers after Et-1 treatment was inhibited by 22 ± 5% (n=18) (Fig. 18). Introduction of the aspartate residue that confers Na sensitivity also conferred endothelin sensitivity. Oocytes injected with Kir3.1(F137S)(N217D) (Na-sensitive) and then treated with Et-1 prior to the second DAMGO challenge showed a marked inhibition of the second opioid response. The second opioid response after Et-1 treatment was inhibited by 82 ± 5% (n=16) ($p< 0.01$). For Kir3.4, removal of the critical aspartate significantly reduced endothelin sensitivity (Fig. 18). The second mu opioid response after Et-1 activation in oocytes expressing the HETA receptor, the MOR and Kir3.4(S143T) (Na-sensitive) was inhibited by 97± 1% (n=7). Oocytes expressing Kir3.4(S143T)(D223N) (Na-insensitive) treated with Et-1 prior to the second DAMGO challenge showed significantly less inhibition of the second opioid response; endothelin reduced the

response to DAMGO by 63± 6% (n=12). Kir3.4(S143T)(D223N) remains partially sensitive to endothelin treatment, but the sensitivity was significantly reduced compared to Kir3.4(S143T). These data indicate that endothelin-sensitivity of Kir3 depended on the presence of the aspartate residue also responsible for Na gating.

Figure 18. The effect of the sodium binding site on the endothelin-1 inhibition of Kir3 homomers. Oocytes were injected with a mixture of the following mRNAs: 1 ng MOR, 1 ng human endothelin A receptor and either 1 ng Kir 3.1(F137S), or 1 ng Kir 3.1(F137S)(N217D), or 1 ng Kir3.4(S143T) or 1 ng Kir3.4(S143T)(D223N). Recordings were performed 2-3 days post injection. The experiment was performed as above. Bar graph compares Kir3.1(F137S) versus Kir3.1(F137S)(N217D) with addition of a site for Na-dependent gating. Second set of bars are Kir3.4(S143T) compared to Kir3.4(143T)(D223N); a mutant with a deletion of the Na-activation site.

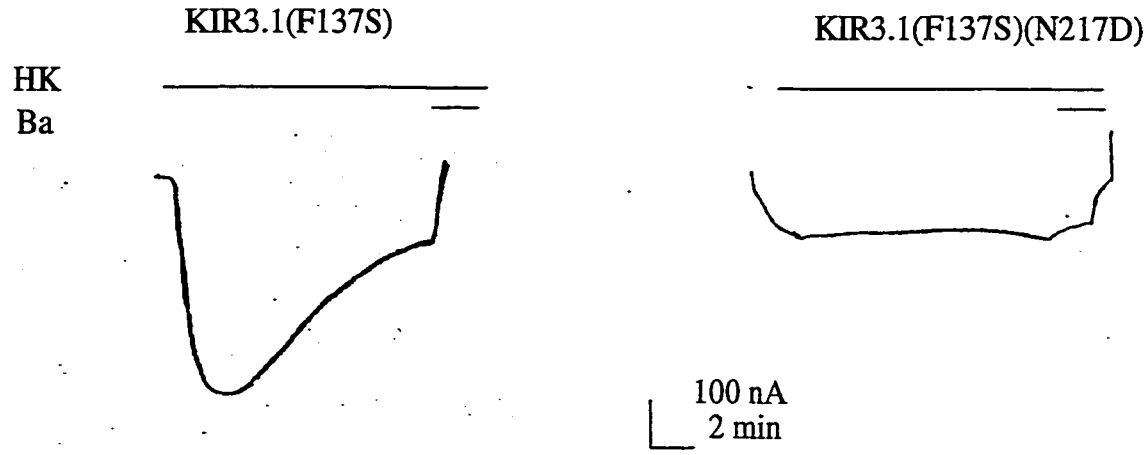

Kir3.4
Kir3.1
100
80
60
40
20
0
Percent Inhibition
**
**
(F137S)
(F137S)(N217D)
(S143T)
(S143T)(D223N)

Desensitization of Kir3 is dependent on the Na gating site.

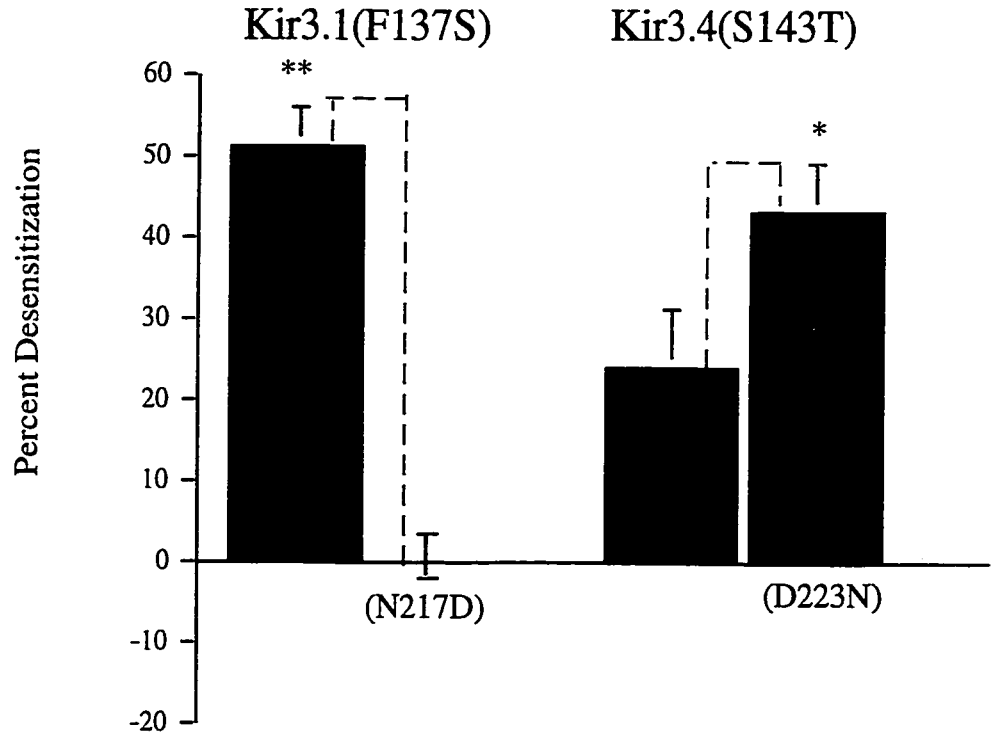
These four channel mutants provided additional insight to the mechanisms of Kir3 gating. Kir3.1(F137S) lacking a Na-gating site rapidly desensitized during prolonged hK application (Fig. 19A left trace). Adding a putative Na-gating site to produce Kir3.1(F137S)(N217D) resulted in a channel having smaller currents that did not desensitize (Fig. 19A right trace). For Kir3.4, removal of the putative Na-gating site to produce Kir3.4(S143T)(D223N) enhanced desensitization in hK buffers (Fig. 19B). The results suggest that this critical aspartate residue required for Na-gating controls channel desensitization during prolonged exposure to an extracellular buffer containing 96 mM K and 2 mM Na concentrations.

Figure 19. Desensitization of the current response to hK buffer is dependent on Na gating. I expressed cRNA for channel mutants: Na-insensitive Kir 3.1(F137S), the Na-sensitive mutant: Kir 3.1(F137S)(N217D) the Na-sensitive Kir3.4(S143T) and Na-site deletion mutant Kir3.4(S143T)(D223N). Recordings were performed 2-3 days post injection. A. Representative traces show the potassium current response measured in oocytes expressing either Kir3.1(F137S) or Kir3.1(F137S(N217D) during high potassium buffer perfusion. High potassium buffer was applied until the peak response was reached and continued for a ten-minute interval. At ten minutes, 300 μM barium in high potassium buffer was applied. B. Bar graph compares the desensitization rates of Kir3.1(F137S) versus Kir3.1(F137S)(N217D) with the addition of a site for Na dependent gating. Second set of bars are Kir3.4(S143T) compared to Kir3.4(S143T)(D223N); a mutant with deletion of the Na activation site. Using *Xenopus* oocytes expressing the Kir3.1(F137S) I noted a rundown of current during perfusion with high potassium buffer (51 ± 5% residual/peak). In oocytes expressing the Na-sensitive Kir3.1(F137S)(N217D) the channel response to high potassium buffer did not run down (0 ± 2%) ($p<0.01$). I noted that the peak current in the Na-sensitive Kir3.1(F137S)(N217D (F137S) homomer (220 ± 78 nA, n=12) was significantly smaller than the Kir3.1 (994 ± 119 nA, n=16) ($p<0.01$). Similarly, in oocytes expressing the Na-sensitive Kir3.4(S143T) the current response to high potassium buffer did not run down as significantly (20 ± 7%) than the Na-insensitive

A.


KIR3.1(F137S)
KIR3.1(F137S)(N217D)
HK
Ba
100 nA
2 min

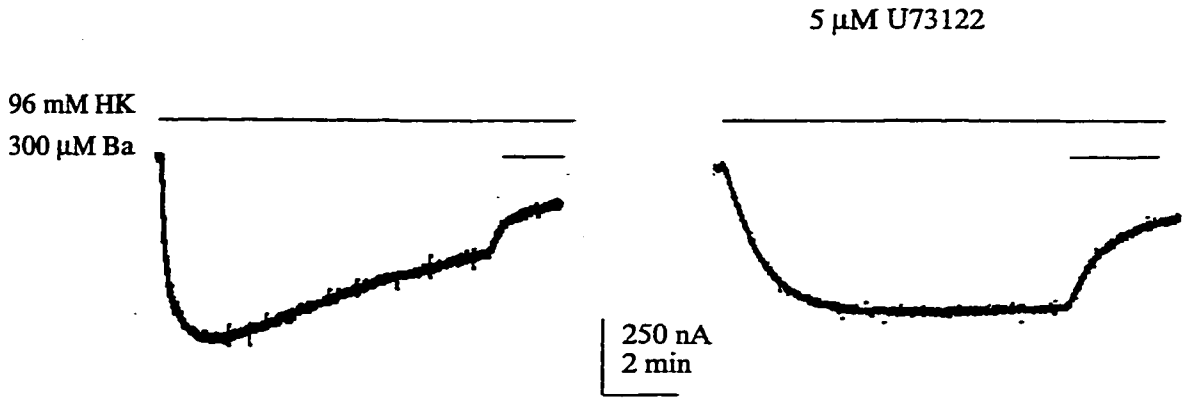
B.

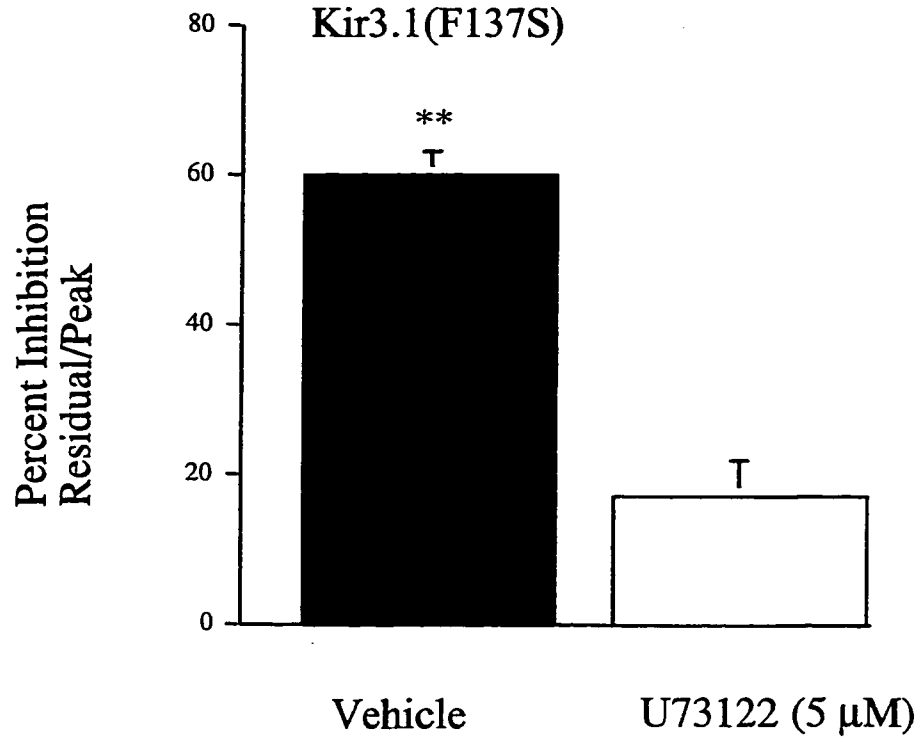

Kir3.1(F137S)
Kir3.4(S143T)
Percent Desensitization
60
50
40
30
20
10
0
-10
-20
**
*
(N217D)
(D223N)

Kir3.4(S143T)(D223N). The channel response to high potassium buffer was diminished residual/peak response by (43 ± 6%) ($p<0.05$).

In a recent report, Kobrinsky and colleagues reported that the rapid desensitization of the muscarinic M2 receptor activation of Kir3.1 and Kir3.4 heteromultimers expressed in *Xenopus* oocytes could be blocked by pretreatment with the PLC inhibitor U73122. They concluded that channel rundown was caused by PIP2 depletion (Kobrinsky et al 2000) To determine whether a similar mechanism was responsible for the channel rundown observed, I expressed Kir3.1(F137S) channel in Xenopus oocytes and pretreated with vehicle or the PLC inhibitor U73122 (5 μM). In the Na-site deficient Kir3.1(F137S) the current response exhibited rundown (60 ±3% residual/peak) (Fig. 20A). In contrast, the rundown of current during perfusion with high potassium buffer was significantly reduced by U73122 pretreatment to (17 ± 5% residual/peak) ($p<0.01$) (Fig. 20A). Inhibition of PLC by U73122 is expected to block PIP2 production that may reduce channel activation. Consistent with this, the initial current response to high potassium buffer was inhibited by 50% in oocytes pretreated with U73122, compared to untreated oocytes. The results suggest that PIP2-depletion produces channel desensitization in the Kir3.1(F137S) channel and the rundown in the acetylcholine evoked response reported by Kobrinsky and colleagues was likely caused by an effect on the channel and not the receptor. However, the mechanism by which the elevation of extracellular potassium concentration caused PIP2 depletion was not defined.

Figure 20. Rundown of the current response to hK buffer in Kir3.1(F137S) is blocked by inhibition of PLC. Oocytes were injected with 1 ng Kir 3.1(F137S) and recordings were performed 2-3 days post injection. High potassium buffer was applied until the peak response was reached and continued for a ten-minute interval. At ten minutes, 300 μM barium in high potassium buffer was applied. Oocytes were either pretreated in vehicle or 5 μM U73122. A. Representative traces of Kir3.1(F137S) and 5μM U73122-treated Kir3.1(F137S). B. Bar graph compares desensitization rates of Kir3.1(F137S) versus 5 μM U73122-treated Kir3.1(F137S).

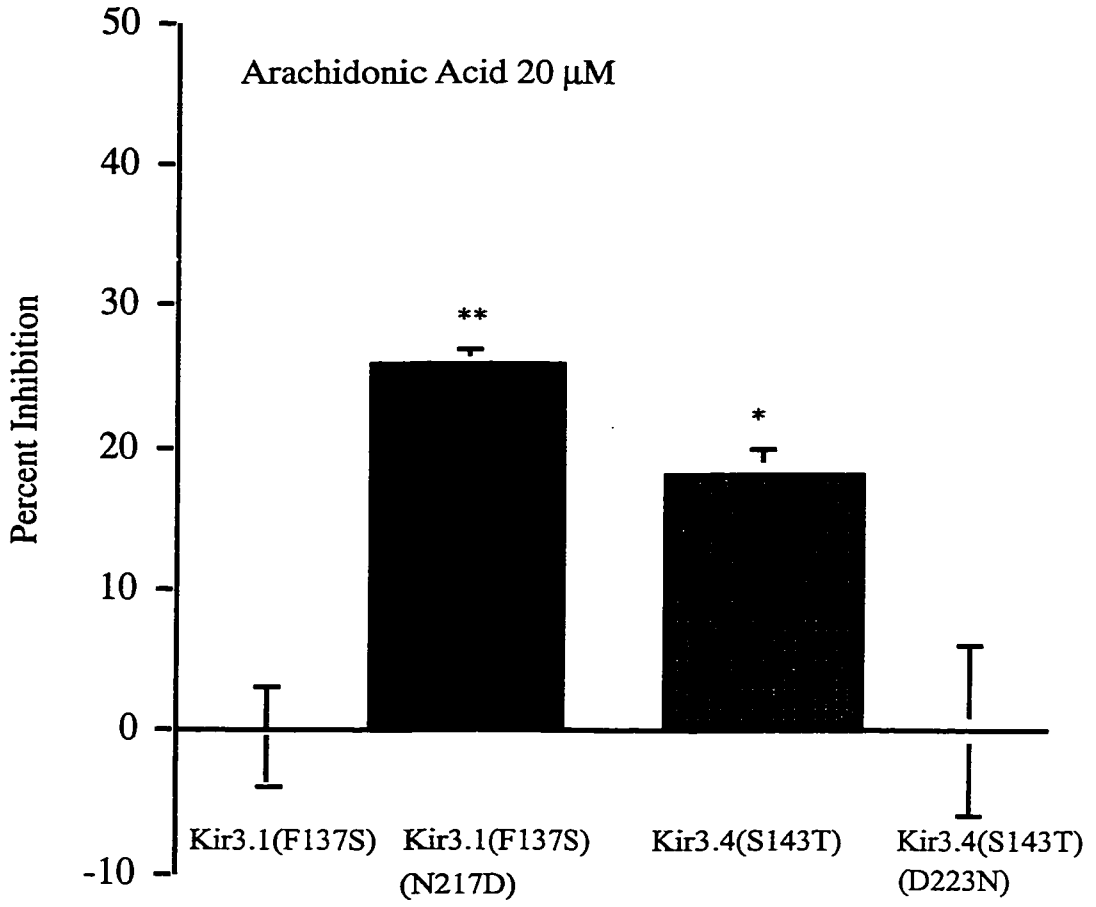

5 μM U73122
96 mM HK
300 μM Ba
250 nA
2 min


Kir3.1(F137S)
Percent Inhibition
Residual/Peak
80
60
40
20
0
**
Vehicle
U73122 (5 μM)

Arachidonic Acid effects on channel homomers.

The results suggested that endothelin activation of PLA2 produced an eicosanoid that inhibited Kir3 channels by interacting at the Na gating domain. Prior work showed that arachidonic acid was the most potent of the eicosanoids at inhibiting Kir3 in cardiac myocytes (Kim and Pleumsamran 2000). Based on this finding, we tested the effectiveness of arachidonic acid application on the Na-sensitive and insensitive Kir3 variants. In oocytes expressing the channel homomer Kir3.1(F137S), arachidonic acid did not inhibit the second DAMGO-elicited response of MOR ($0 \pm 3\%$). In oocytes expressing the channel with a site for Na activation Kir3.1 (F137S)(N217D) arachidonic acid produced an inhibition of the second opioid response; the second DAMGO response was inhibited by ($26 \pm 1\%$) ($p<0.05$) (Fig 21). Moreover, arachidonic acid inhibited the Na-sensitive Kir3.4(S143T) ($18 \pm 2\%$) compared to oocytes with the analogous Na-site removed Kir3.4(143T)(D223N) ($-2 \pm 6\%$).

Figure 21. The effects of arachidonic acid on Na-activation site mutants in homomers Kir 3.1(F137S) and Kir3.4(S143T). Oocytes were injected with a mixture of the following mRNAs: 1 ng MOR, and either 1 ng Kir 3.1(F137S), 1 ng Kir 3.1(F137S)(N217D), or 1 ng Kir3.4(S143T) or 1 ng Kir3.4(S143T)(D223N). Recordings were performed 2-3 days post injection. Oocytes expressing the channel variants were challenged with DAMGO then treated with 20 μM arachidonic acid placed directly into the bath for 6 min prior to activation of the second opioid response. Bar graph compares arachidonic acid effects on Kir3.1(F137S) versus Kir3.1(F137S)(N217D) with addition of a site for Na-dependent gating. Second set of bars are Kir3.4(S143T) compared to Kir3.4(143T)(D223N); a mutant with a deletion of the Na-activation site.


Arachidonic Acid 20 μM
Percent Inhibition
50
40
30
20
10
0
-10
**
*
Kir3.1(F137S)
Kir3.1(F137S) (N217D)
Kir3.4(S143T)
Kir3.4(S143T) (D223N)

DISCUSSION

The principal finding of my study is that arachidonic acid inhibits G-protein gated potassium channels at the Na/PIP2 gating domain. The identification of this mechanism helps clarify the effects of eicosanoids generated by phospholipase A2 activation. The results provide new insights to the gating process controlling Kir3 function. Previous studies established that the elevation of Gβγ concentration following Gi/o coupled receptor activation increases Kir3 current (Henry et al 1995). Gβγ was shown to work in concert with PIP2 to induce a conformational change in the channel and open the K selective pore. In addition to Gβγ, Kir3.2 and Kir3.4 channel subunits also require the presence of Na for activation (Ho and Murrell-Lagnado 1999; Ho and Murrell-Lagnado 1999). The results provide support for the hypothesis that arachidonic acid is an additional component regulating Kir3 gating.

The results obtained provide evidence that the production of an eicosanoid by endothelin receptor activation is responsible for the observed inhibition of Kir3. My prior study showed that endothelin effects were blocked by the PLA2 inhibitor AACOCF3 (Rogalski et al 1999). As PLA2 metabolizes PIP2 to arachidonic acid (Fig. 21), inhibition of Kir3 by depletion of PIP2 is an alternative mechanism of endothelin action. This alternative is excluded by the data presented. The HETAR is a Gq coupled receptor that activates PLC to produce the calcium-activated chloride conductance (Rubanyi and Polokoff 1994). Although PLC activation

might be expected to deplete PIP2 and thus cause Kir3 inactivation, I found that PLC inhibition by U73122 did not block endothelin action but did reverse channel rundown. The second finding is that endothelin receptor activation did not affect Kir3.1, a channel that is dependent on PIP2 for activation (Fig 16). The third observation is that the Kir3 subunit sensitivity to arachidonic acid matches endothelin sensitivity. Thus, results support the conclusion that endothelin activation of PLA2 produces an eicosanoid that inhibits Kir3. The specific eicosanoid generated by endothelin receptor activation in *Xenopus* oocytes was not identified; however, arachidonic acid is a reasonable candidate based on its high potency shown by Kim and Pleumsamran (Kim and Pleumsamran 2000). In addition, arachidonic acid exhibited an inhibition profile that was similar to endothelin receptor activation (Fig 21).

The basis for the eicosanoid effect on Kir3 was not defined, but the study provides suggestions. The correlation between Na sensitivity and arachidonic acid sensitivity suggests that the binding site of eicosanoids is part of the domain controlled by the Na binding site. Moreover, the Na binding site is also controls PIP2 binding. Because arachidonic acid shares structural features with PIP2, the PIP2 binding region of Kir3 may also bind arachidonic acid (Fig. 22). The inhibition of gating caused by arachidonic acid may result from a competition with PIP2 for binding at this site. In the absence of structural data, I suggest the physical basis for the interaction between these gating components and the ion channel. Additional work will be required to further define the interaction between

eicosanoids and PIP2 at Kir3. However, my data show that the mechanism of Kir3 sensitivity to arachidonic acid differs from that reported for ROMK channels by Macica and colleagues (Macica et al 1996; Macica et al 1998).

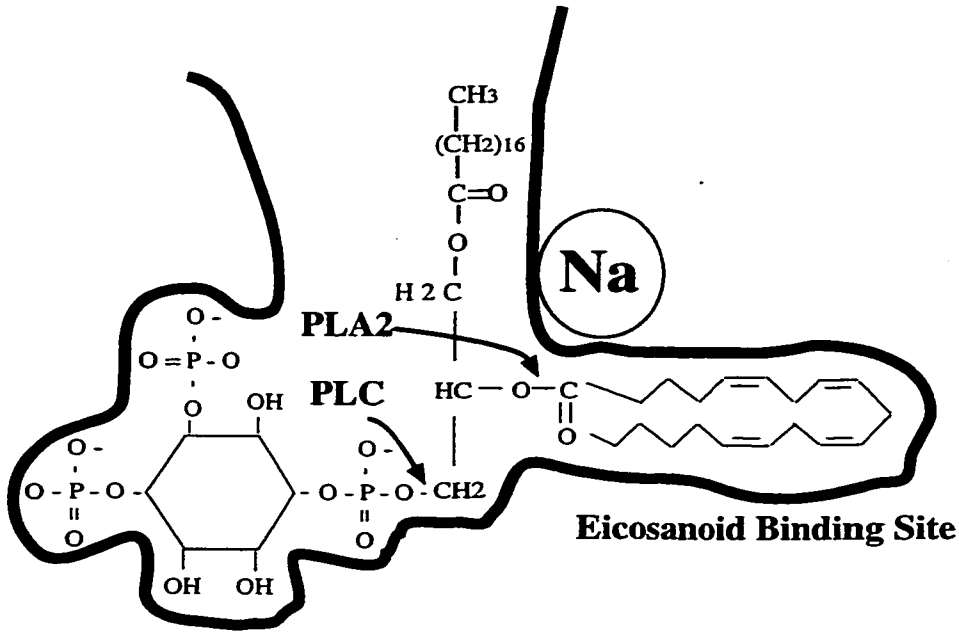
In my studies, I show that the Kir3.1 channel lacking the Na gating site exhibits a pronounced desensitization of the basal response in high potassium buffer. In contrast, Kir3.1(F137S)(N217D) (Na-sensitive) did not desensitize in the presence of high potassium buffer. The inverse correlation between the presence of the Na gating site and the rate of desensitization in hK buffer is intriguing; however, the basis for the relationship was not established in this study. The inhibitory effects of the PLC inhibitor on Kir3 desensitization suggest that PIP2 depletion results in channel rundown. One explanation for the lack of desensitization observed in Na-sensitive channels is that they may exist in a desensitized state. Consistent with this explanation is the observation that the currents are smaller after treatment with U73122 and that the Na-sensitive Kir3.1(F137S)(N217D) expresses smaller channel currents. Although the data suggest that Na-site deficient channels rapidly desensitize, I noted that the desensitization rate was faster for Kir3.1(F137S)(Na-insensitive) compared to Kir3.4(S143T)(N223D) (Na-insensitive). Vorobiov et al suggest that application of high K buffer to *Xenopus* oocytes expressing Kir3 heteromultimers causes intracellular Na depletion which leads to channel rundown (Vorobiov et al 1998). Although I do not dispute that alteration of internal Na levels may alter Kir3 desensitization, my data suggests that Kir3 subunits desensitize in the absence of a

specific Na-gating site. High K levels may alter the internal Na levels in oocytes in a manner that destabilizes binding, which subsequently allows rapid depletion of PIP2.

I explored the mechanism of endothelin receptor induced inhibition of mu opioid receptor activation of Kir3 *in vitro*. Although *Xenopus* oocytes are a complex expression system with interacting signaling molecules, the insights to Kir3 gating mechanisms are evident. The endothelin receptor is one member of the Gq-coupled seven-transmembrane superfamily of receptors (Rubanyi and Polokoff 1994). Other Gq-receptors that regulate Kir3 by eicosanoid production may also require specific gating components to exert effects on the channel. For example, activation of the Gq-coupled substance P receptors expressed in locus coeruleus neurons inhibits Kir3 currents (Koyano et al 1994; Velimirovic et al 1995). Although the mechanism of inhibition was not established in that study, the results shown here may be relevant. Eicosanoid regulation of Kir3 as described in this study may be relevant to the pathological situations that occur during inflammation. As Kir3 has in important role in cardiac excitability, cardiovascular disease may elicit an inflammatory response to produce eicosanoids that inhibit Kir3 (Van der Vusse et al 1997; Sugden and Clerk 1998; Yamazaki et al 1999). Nevertheless, the physiological significance of these findings needs to be directly established.

Figure 22. Proposed Mechanism of Eicosanoid modulation of Kir3. The figure shows a proposed binding site for PIP2 in the proximal domain of the carboxyl-terminus of Kir3.4. The site for Na-activation may shield adjacent charged residues, which is permissive for the PIP2/Arachidonic acid complex to bind the channel. The diagram also shows the sites of PLA2 and PLC cleavage of PIP2.

PIP2 in Kir3.4



Addendum

Effects of inhibition of map kinase activity

My data suggests that Kir3 channel inhibition by eicosanoids is related to channel gating. Pathophysiological conditions such as cardiac reperfusion injury or cardiac hypertrophy may provide the link between eicosanoids, Kir3 gating, and downstream signaling events in intact systems. Phosphorylation events that include PKC (Henrich and Simpson 1988; Dunnmon et al 1990; Exton 1996) and mitogen-activated protein kinases (MAPK) occur in disease states in cardiac cells. ERK activation produces cellular proliferation in myocytes (Sugden and Clerk 1997; Sugden and Clerk 1998)(Yamazaki et al 1999). Because MAPK is activated in models of hypertrophy in cardiac myocytes (Sugden and Clerk 1998; Yamazaki et al 1999), I wanted to determine if map kinase was a component of the pathway that between HETA and Kir3. Oocytes expressing the endothelin-sensitive Kir3.4(S143T) homomer were pretreated for 25 min with the map kinase inhibitor, PD98059 (Dudley et al 1995) prior to activation with Et-1. PD98059 (20 μM) treatment had no effect on resting membrane current (data not shown). The second DAMGO response after treatment with Et-1 was inhibited 89 ± 3% (n=9). The inhibitory effects of 100 nM Et-1 were significantly reduced; the Et-1 effect was only 51 ± 13% (n=9) following pretreatment with PD98059 (Fig. 23). The data suggest that map kinase pathway is involved in HETAR regulation of Kir3.

Figure 23. Effects of pretreatment with the map kinase inhibitor PD98059 on endothelin-1 inhibition of the MOR response in the Kir3.4(S143T) homomer. Bar graph summarizing the effect of PD98059 on the endothelin inhibition of the second opioid response.

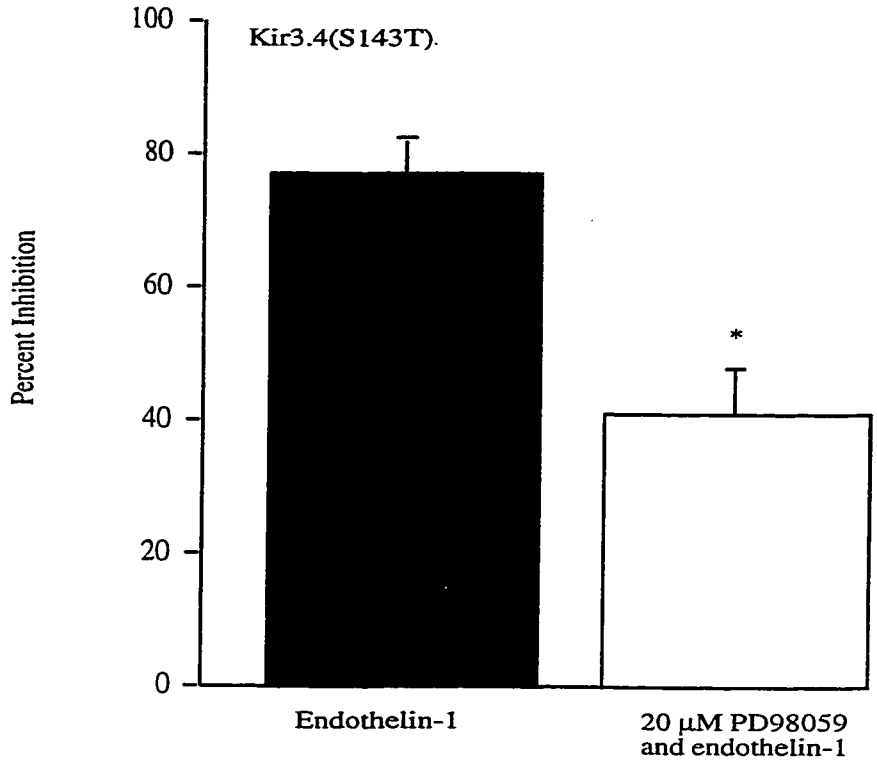
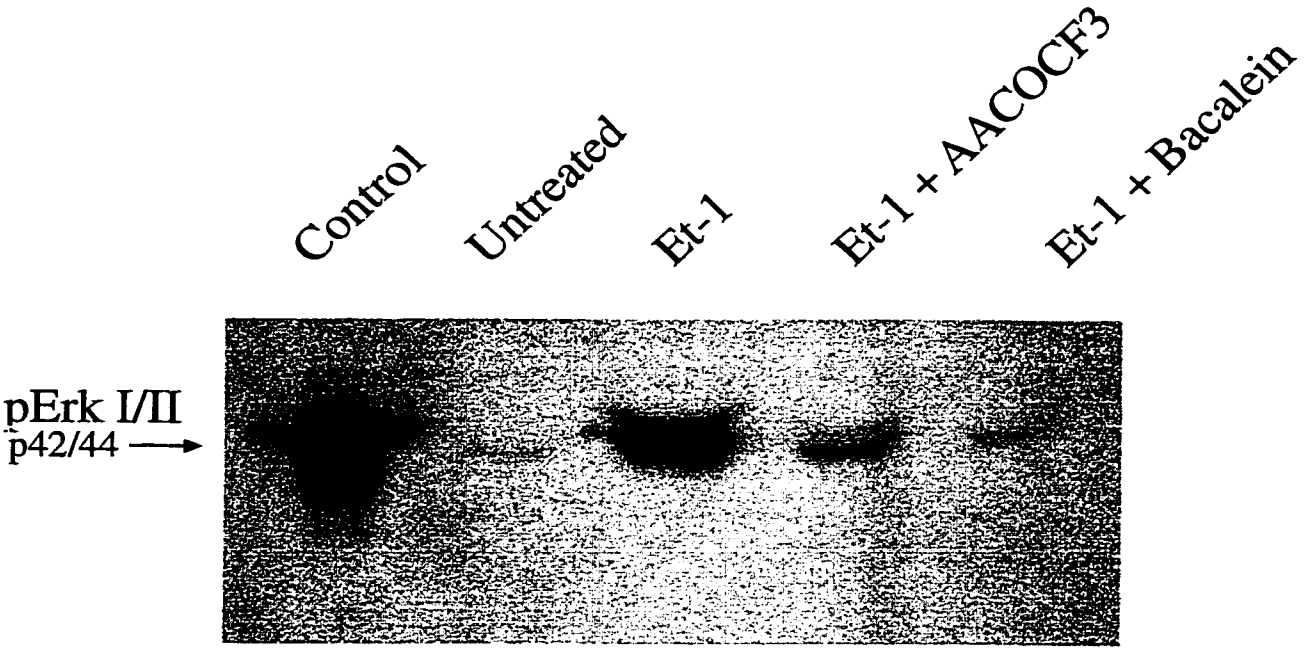

Kir3.4(S143T).
Percent Inhibition
100
80
60
40
20
0
*
Endothelin-1
20 μM PD98059
and endothelin-1

Figure 24. Immunodetection of map kinase ERK activity of the HETA receptor after treatment with ET-1, AACOCF3 and Bacalein. 42-44kD protein band indicating ERK immunoreactivity following Et-1 treatment. Membrane compares untreated cells, Et-1 treated cells, AACOCF3 + Et-1 treated cells and Bacalein+Et-1 treated cells.


Control
Untreated
Et-1
Et-1 + AACOCF3
Et-1 + Bacalein
pErk I/II
p42/44

CHAPTER V

SUMMARY AND FUTURE DIRECTIONS

In this thesis I have described what I have learned about how effectors regulate downstream signaling events to modulate Kir3. Using two-electrode voltage clamp techniques to test *in vitro* G-protein coupled receptor-ion channel paradigms, I have provided evidence for novel mechanisms of direct and indirect modulation of Kir3 by effector regulation. Using molecular biology techniques to mutate amino acid residues of interest on members of the Kir3 family, I show that Kir3 is phosphorylated by tyrosine kinases on specific conserved amino acids in the amino terminus of the channel. My experiments show that a tyrosine kinase modulates Kir3 by a direct phosphorylation event on the channel. In the mutagenesis studies described, I found that two tyrosine residues in the Kir3.4 sequence (Y32 and Y53) and two tyrosine residues in the Kir3.1 sequence (Y12 and Y67) were required for sensitivity to tyrosine kinase activation. Phosphorylation of other tyrosine residues in Kir3 channels were not responsible for BDNF-induced inhibition of Kir3. Taken together with evidence generated by others, these studies suggest that tyrosine phosphorylation has direct effects on many ion channels and that these phosphorylation events may be conserved across ion channel families.

Further experimentation to determine the identity of the tyrosine kinase is in Kir3 inhibition would provide further insight into channel regulation. These experiments would be performed by simple patch recordings on oocytes injected with the various channel mutants as described in Chapter 3. Direct application of commercially available or synthesized kinases to the excised patch area may help define which family of tyrosine kinases are responsible for the observed inhibition. Moreover, I may determine the time course of BDNF-action. It has been recently shown that BDNF acts as a neurotransmitter, activating the sodium channel (Kafitz et al 1999). Kaftiz et al show that brain-derived neurotrophic factor (BDNF) at low concentrations excited neurons in the hippocampus, cortex and cerebellum as rapidly as the neurotransmitter glutamate. The tyrosine kinase inhibitor K252A blocked the neurotrophin-induced depolarization that resulted from the activation of a sodium ion conductance. In my studies on BDNF inhibition of Kir3, I obtained reversal of the BDNF-induced Kir3 inhibition by K252A. By utilizing the same methodology of Kafitz, et al, the time course of BDNF action on TrkB and its actions on intact preparations may allow us to determine if TrkB acts as a neurotransmitter in addition to its role as a nerve growth factor.

BDNF-sensitive Kir3 channels have charged amino acids (aspartic acid or arginine) on the amino-side of the tyrosine and a hydrophobic residue on the carboxyl-side. This general motif is characteristic of other sites phosphorylated by protein tyrosine kinases (Songyang et al 1994; Songyang and Cantley 1998). In the BDNF-insensitive Kir3.2, I show that electrostatic interactions are required to

define a consensus site for tyrosine phosphorylation. My experiments define that tyrosine modulation of Kir3.2 requires conserved amino acid sequences upstream of the tyrosine phosphorylation site. Further mutagenesis studies on the conserved charged residues on the BDNF-sensitive Kir3.1 and Kir3.4 may provide new insight on the amino acid requirements for tyrosine kinase phosphorylation. In the BDNF studies outlined in Chapter 3, I used commercial bioinformatics programs to align Kir channels to determine homologous domains. Because tyrosine phosphorylation events require charged-consensus sites, I altered amino acids upstream of tyrosine to provide a recognition site for phosphorylation. Using more sophisticated bioinformatics programs combined with site-directed mutagenesis may provide further insight into what constitutes a tyrosine phosphorylation consensus site in the Kir3 family.

In another study, I showed that endothelin-1 activation of the Gq-coupled human endothelin A receptor reduces the response evoked by DAMGO activation of the Gi-coupled mu opioid receptor coexpressed with Kir3 heteromultimers. I provide evidence that arachidonic acid inhibits Kir3 heteromultimers in a channel subtype-specific manner. I used *Xenopus* oocytes injected with cRNA for the mu opioid receptor, the human endothelin A receptor and Kir3 heteromultimers, and two-electrode voltage clamp techniques to define indirect actions by lipids on Kir3. The data obtained suggest that PLA2 antagonists blocked the endothelin-induced inhibition of Kir3. Recently, Kim and Pleumsamran verified my finding that arachidonic acid inhibits Kir3 I_{KACH}. Kim used patch recordings and applied a large

variety of unsaturated fatty acids to cardiac cells. Kim and Pleumsamran determined that arachidonic acid inhibits Kir3 in primary cultures (Kim and Pleumsamran 2000). This result paralleled my results in the *Xenopus in vitro* system.

Dual regulation of Kir3 by converging receptors remains a controversial area of study. I believe that it cannot be generalized that all Gq-coupled receptors and Gi-coupled receptors activate standard pathways when they converge on Kir3. Although Gq-coupled receptors generally activate PLC, the downstream effectors that are activated are dependent upon the specific receptor. My data and that of other researchers define a pathway for the Gq-coupled endothelin receptor through PLA2. I do not generalize this finding to all Gq-coupled receptors. I suggest that the specificity of receptor signaling to downstream effectors provides insight into cellular regulation. This is supported by the PLC-inhibitor studies shown in Chapter IV. Although, endothelin-1 activates PLC; inhibition of PLC by U73122 does not reverse desensitization of the channel response.

Using Kir3 homomers, I show that inhibition of Kir3 by PLA2 is subunit specific and dependent upon gating by Na. Kir3.1, which does not have a site for Na activation is not inhibited by endothelin-1 production of PLA2. In contrast, the subunit of Kir3.4 has a site for Na-activation and is sensitive to endothelin-1 production of PLA2. The data suggest that Na is required for eicosanoid inhibition of Kir3. Application of high potassium buffer to oocytes expressing Kir3 homomers and Kir3 homomer channels with Na-mutations show distinct responses to high potassium buffer. The Kir3.1 channel does not have a Na site and it shows a pronounced desensitization of the basal response in high potassium buffer. In contrast, Kir3.4 and Kir3.1(N217D) (Na-sensitive) did not desensitize in the

presence of high potassium buffer. In a recent report, Kobrinsky and colleagues suggest that PIP2 downregulation produces desensitization of I_{KACH}. My data with the Na-site deficient Kir3.1(F137S) suggests that Kir3 desensitization occurs more rapidly in the absence of a Na-gating site. I suggest that a possible role for Na is to provide stability to the PIP2/ATP complex that activates the channel.

In my thesis work, I provide multiple forms of regulation of Kir3 by diverse stimuli. I would suggest that my *in vitro* studies parallel the actions of excitable tissue in the organism. In the nervous system and in cardiac physiology, opposing actions provide for homeostasis. That is, different receptors activating multiple signaling cascades converge on an ion channel to provide cellular regulation.

Channel behavior is a combination of both rapid responses and long-term cellular changes. Each type of response to a stimulus activates distinct signal transduction systems to define a cellular response. One cellular input may activate both a rapid response and produce cellular change. My data suggests that endothelin may produce PLA2 which rapidly modulates Kir3. Moreover, PLA2 activates the Map Kinase pathway; which may produce long-term cellular changes. Gating molecules such as Na or PIP2 may be the link between transient effects on Kir3 and long-term cellular changes. Kir3.1 functional homomers that lack a site for Na activation cannot be modulated by endothelin-production of PLA2 or arachidonic acid. This data provides a link between gating molecules and the downstream signals that regulate Kir3.

G-protein coupled receptors release $G\beta\gamma$ to modulate Kir3 to activate downstream effectors. Kir3 require multiple components to produce channel gating including $\beta\gamma$, Na, the phospholipid PIP2 and ATP. I know that the G-protein coupled receptor releases $G\beta\gamma$ to modulate Kir3 to activate downstream

effectors. Moreover, my data suggests that receptor tyrosine kinases phosphorylate Kir3. That both Trk receptors and multiple GPCR modulate Kir3 suggest that Kir3 must have the structural capability to define multiple inputs. That Kir3 may be directly phosphorylated by activation of TrkB as well as gated by a complex of factors may provide understanding of how Kir3 may be regulated by so many signal transduction pathways.

While my studies have focused on molecular mechanisms of Kir3 modulation and the relationship with downstream signaling events, the goal of this work was to gain insight into how this complex family of receptor activated ion channels regulate physiologic responses. Understanding the complexities of the relationship between channel gating and cellular responses is critical in determining the nuances of neuronal and cardiac function. My thesis provides a new link for the molecular basis of how Kir3 regulation activates downstream signal transduction pathways to modulate excitability.

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SHERRI L. ROGALSKI

Dept. of Pharmacology
University of Washington
PO Box 357280
Seattle, Washington 98195

24900 NE 52nd Place
Redmond, WA 98053
(425) 868-2656
RROGALSKI@aol.com

EDUCATION

University of Washington
Seattle, WA 98195
Graduate Student Dept. of Pharmacology 1996-2000
Ph.D. November, 2000

Dissertation Title: **Modulation of Kir3 by Lipids and Tyrosine Phosphorylation**

University of Washington
Seattle, Washington
Dept. of Laboratory Medicine
Certification in Hematology 1990
American Society of Clinical Pathologists Certification, 1990

Eckerd College
St. Petersburg, Florida
BS Interdisciplinary Sciences 1978

RESEARCH EXPERIENCE

Research Assistant, Dept. of Pharmacology. University of Washington, Seattle, Washington. Advisor: Charles Chavkin, Ph.D.

PROFESSIONAL EXPERIENCE

Tutor neuropharmacology - UW Medical Students
2000

Supervise and train undergraduate students
in the lab of Charles Chavkin, Ph.D.
1997-Present

Teaching Assistant
Dept. of Pharmacology, University of Washington, Seattle, Washington
1996-1997

Hematology specialist and student instructor
Perform clinical hematology tests, review and identify normal and abnormal blood morphology, clinical coagulation studies, Quality Assurance
Harborview Medical Center, Seattle, Washington
1990-1993

PROFESSIONAL AFFILIATIONS

Society for Neuroscience, student member since 1997
American Society of Clinical Pathologists 1990-1993

HONORS

Industrial Relations Grant to intern in a local biotechnology company – awarded spring, 2000
Molecular Pharmacology Training Grant/National Institute of Drug Abuse Training Grant1996-1998
Eckerd College Scholarship 1976-1978

PUBLICATIONS

Rogalski, SL and Chavkin, C (2000). Eicosanoids inhibit the G-protein gated inwardly rectifying potassium channel (Kir3) at the Na/PIP2 gating site. (Submitted)

Rogalski SL, Appleyard SA, Pattillo A, Terman GW, Chavkin C. (2000). TrkB Activation by BDNF Inhibits the G Protein Gated Inward Rectifier Kir3 by Tyrosine Phosphorylation of the Channel. JBC 275:25082-25088.

Rogalski SL, Cyr C, Chavkin C. (1999). Activation of the endothelin receptor inhibits the G-protein coupled inwardly rectifying potassium channel by a PLA2 mediated mechanism. J Neurochem 72:1409-16.

ABSTRACTS AND PRESENTATIONS

Rogalski SL, Appleyard SA, Pattillo A, Terman, GW, Chavkin C. (2000). TrkB Activation by BDNF Inhibits the G Protein Gated Inward Rectifier Kir3 by Tyrosine Phosphorylation of the Channel. *INRC Abstracts* (Vol. 31).

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Cyr C, Henry D, Chavkin C. (1997). Regulation of Inwardly Rectifying Potassium Channels (GIRKs) by the Endothelin-A Receptor. *Society for Neuroscience Abstracts.* (Vol. 27).

COMMUNITY RELATIONS

Lab tours for individual students and local high schools for the University of Washington Dept. of Community Relations
1998-Present .

Present 'Neuroscience for Kids' in elementary schools and for UW Brain Awareness Week
1997-Present