Title
A molecular mechanism for thermal hypersensitivity

Author
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Publication Date
2003

Peer reviewed|Thesis/dissertation

A Molecular Mechanism for Thermal Hypersensitivity

by

Elizabeth D. Prescott

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Biochemistry and Molecular Biology

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

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Portions of chapter 2 were performed primarily in collaboration with Huai-hu Chuang, who carried out all experiments involving mammalian cell electrophysiology. Specifically, Figures 2, 5, 6, and 8 are a product of Dr. Chuang's experiments. Shannon Shields performed behavioral experiments described by Figure 1. Resiniferatoxin binding experiments, as described by Figure 9, were carried out by Dr. Sven Jordt. Coimmunoprecipitation experiments described in Figure 7 were done in collaboration with Dr. Haeyoung Kong.

The remaining experiments were conceived of and carried out by Elizabeth Prescott, in partial satisfaction of the Doctor of Philosophy requirements for the Department of Biochemistry and Biophysics, University of California, San Francisco.

Acknowledgements

I would like first and foremost to thank my parents, Allen and Cassandra Prescott for giving me the emotional, financial, and educational support that is required to even get to a wonderful institution like UCSF in the first place. I only hope that I can embody the selflessness, determination and generosity that they possess when it comes to my own children.

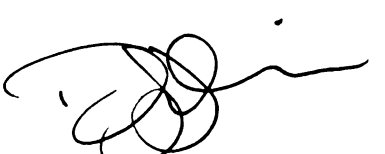
I am next indebted to the advice and mentorship of my advisor, David Julius. One resounding piece of "wisdom" imparted by countless colleagues and peers was that I would have days that made me question my decision to go to graduate school. Most also predicted that no matter my choice in labs, I would question that decision too. On both counts they were wrong. I continue to be grateful and satisfied with the decisions I made to both come to UCSF and in particular to join David's lab. I have grown not only as a scientist, but also as a person under his guidance, and I owe him a debt of gratitude that is almost beyond description.

My colleagues in the lab have also been instrumental in my personal and professional development over these last five years. While their critical insights were at first overwhelming and indeed disheartening, I grew to realize how lucky I have been to be surrounded by such competent scientists and friends. I am perhaps even more grateful, however, for the non-scientific characteristics of my lab mates. They made coming to lab (and sometimes leaving lab) enjoyable, and I am privileged to call them my friends.

I am also truly grateful for the friendship and camaraderie of my classmates and fellow graduate students at UCSF. In particular, I must thank Doris Fortin, Kanade Shinkai, and Neal Freedman for their continuous advice, support and friendship.

Lastly, I thank Daniel Stetson. In him I have found the respect, insight, and advice that is most valued in a colleague. In him I have found the humor, strength, and wisdom that is most treasured in a friend. And in him I have found the love, patience, understanding, and support that is cherished in a spouse. The relationship we have is the greatest discovery I made in graduate school.

Tissue injury generates endogenous factors that heighten our sense of pain by increasing the response of sensory nerve endings to noxious stimuli. Bradykinin and nerve growth factor (NGF) are two such pro-algesic agents that activate G protein-coupled (BK2) and tyrosine kinase (TrkA) receptors, respectively, to stimulate phospholipase C signaling pathways in primary afferent neurons. How these actions produce sensitization to physical or chemical stimuli has not been elucidated at the molecular level. Here we show that bradykinin- or NGF-mediated potentiation of thermal sensitivity in vivo requires expression of TRPV1, a heat-activated ion channel on sensory neurons. Diminution of plasma membrane PIP_2 levels through antibody sequestration or PLC-mediated hydrolysis mimics the potentiating effects of bradykinin or NGF at the cellular level. Moreover, recruitment of phospholipase C-γ to TrkA is essential for NGF-mediated potentiation of channel activity, and biochemical studies suggest that TRPV1 associates with this complex. Finally, we identify a site within the carboxy-terminal cytoplasmic domain of TRPV1 that is required for PIP_2–mediated inhibition of channel gating. Mutations that weaken PIP_2-TRPV1 interaction result in markedly decreased thresholds for chemical or thermal stimuli. TRPV1 channels in which this region has been replaced with a lipid binding domain from PIP_2-activated potassium channels remain PIP_2 inhibited, indicating that the directionality of modulation is determined not by the PIP_2 binding site, per se, but by other aspects of channel structure. Our results identify a modular PIP_2 interaction domain on TRPV1 that serves as a critical determinant of thermal threshold and dynamic sensitivity range, tuning the channel (and thus the sensory neuron) to appropriately detect heat under normal or pathophysiological conditions.



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CHAPTER 1: General Introduction

Pain and pain perception

The sensation we describe as pain can be simply thought of as a sensation that is elicited by real or impending tissue damage. From early in our lives, the sensation of pain serves as a warning sign or a cue to avoid potentially injurious situations. Indeed, rare genetic or medical conditions exist in which the ability to detect pain is absent. Patients with such disorders are unaware when injurious situations arise, and are observed to suffer severe injuries (such as digit or limb amputation) with no signs of discomfort (Rosemberg et al., 1994). Thus, the pain pathway is essential to our well being because it helps us to avoid situations that might cause tissue damage. Pain, is therefore unique among other sensory modalities, in that the only purpose it serves is protective. Unlike vision, hearing, or the sense of smell, which can give useful information about our environment, lead us to seek shelter, food, water, or provide information about predators and dangerous situations, pain is purely protective. It makes sense, then, that the processes that underlies pain sensation should be under tight regulation. Extraneous or chronic pain sensations serve no advantageous purpose, and in fact are only deleterious to our health and well-being.

In the visual and auditory systems of animals, specialized cells exist to detect sensory stimuli. In the mammalian visual system, light is detected by rod and cone photoreceptors in the retina. These specialized neurons then trigger a cascade of information exchange that ultimately leads to our sensation of light and movement (Hardie, 2001). Likewise, hair cells in the inner ear respond to changes in fluid pressure

within the cochlea and secrete neurotransmitters that ultimately activate the auditory nerve, resulting in our perception of sound (Kandel et al., 1991). Thus, it stands to reason that similar specialized "receptors" exist in the pain pathway (Sherrington, 1906). Until recently, however, the existence of such receptors was controversial (Melzack and Wall, 1965). The "pattern theory" held that the same neurons that detect innocuous stimuli, such as light touch and warmth, also conveyed noxious stimuli. This model predicts that the amount of output from such a neuron would occur in a graded fashion, increasing with the stimulus intensity. An important feature of this model is that the decoding of a stimulus and the discrimination between noxious and innocuous stimuli must occur centrally, i.e. in the spinal cord dorsal horn. On the other hand, the "specificity theory" held that a specific subset of neurons exists whose threshold is set such that they are only activated when stimuli capable of causing tissue damage are encountered. In other words, innocuous stimuli are incapable of activating these pain sensing cells, also called nociceptors or nociceptive fibers.

Work from the lab of Ed Perl in the 1960s first provided evidence in support of the specificity theory (Bessou and Perl, 1969; Burgess and Perl, 1967). These studies utilized electrophysiological approaches to demonstrate that a specific classes of neurons was only activated when exposed to stimuli that are considered noxious in animal models. On the other hand, a separate set of neurons was active at stimulus intensities not considered noxious, suggesting that in fact, specialized cells in the pain pathway (i.e. nociceptors) do exist.

Pain physiology

The sensation of pain is mediated by a network of neurons in both the peripheral and central nervous systems. The initial transduction of a pain-producing stimulus is mediated by primary afferent nociceptive neurons, or nociceptors. As initially characterized by Perl's lab and others, these neurons are specialized to detect stimuli that are capable of causing tissue injury. Nociceptive neurons project to the dorsal horn of the spinal cord and synapse on second order neurons that then project to the thalamus. Here, thalamocortical connections relay the signal to the cortex for interpretation. Primary afferent nerve fibers can be subdivided into categories based largely upon size, extent of myelination, and conduction velocity. Aβ fibers are slightly smaller and also myelinated with a somewhat slower conduction velocity. The majority of pain sensing myelinated fibers are Aβ fibers. These neurons respond to noxious mechanical stimuli, such as painful pressure, and some also respond to noxious temperatures. Unmyelinated, slow conducting C fibers make up the majority of nociceptive primary afferent neurons. These neurons have the smallest diameter of all the primary afferent neurons, and respond to noxious chemical, thermal, and mechanical stimuli; thus, they are often referred to as polymodal nociceptors.

C fibers are further subdivided by other biochemical and developmental criteria. Peptidergic C fibers produce neuropeptides such as CGRP and substance P, and cannot be labeled by the lectin IB4. Furthermore, in the adult animal, these neurons express the growth factor receptor TrkA. As such, they are often referred to as peptidergic, TrkA-positive, or IB4-negative C fibers. Non peptidergic C fibers, on the other hand, do not

produce neuropeptides but can be identified by labeling with the lectin IB4. As such, they are referred to as IB4-positive C fibers. It should be noted, however, that this categorization is not strict, and in fact can vary among species. For instance, some rat nociceptive neurons are both peptidergic and IB4 positive, whereas in human nociceptors these properties are mutually exclusive. Moreover, the complexity of sensory neuron subpopulations is expanding as more markers become available.

Nociceptor development: The neurotrophic hypothesis and neurotrophin receptors

Primary afferent neurons are derived from the embryonic ectoderm and somatic mesoderm. Following the development of the neural tube, neural crest cells migrate away from the neural tube and eventually give rise to peripheral sensory ganglia, as well as other cell types. (Burt, 1993; Kandel et al., 1991). Dorsal root ganglia cells, as such, do not actually appear until later in development, around E11 in the rat (Wall and Melzack, 1999). However, by birth, most polymodal C fibers are capable of activation by their full complement of stimuli, whereas functional refinement of the thresholds of activation for Aβ and Aδ fibers are not yet complete (Wall and Melzack, 1999).

The migration of neural crest cells and their subsequent maturation and specialization is dependent on target-derived trophic factors. These factors are postulated to promote neuronal survival, thereby ensuring that cells expressing receptors for these factors are selectively maintained at the site of the factor. In the absence of neurotrophin support, the neuron undergoes apoptosis, or programmed cell death (Patel et al., 2000). This so

called "neurotrophic hypothesis" has been supported by recent findings that selective subsets of neurons are lost upon genetic ablation or antibody mediated disruption of neurotrophin signaling. For instance, mice that lack the neurotrophin nerve growth factor (NGF) display a 70-80% reduction in DRG neuron numbers. These animals lack nociceptive neurons, and have reduced numbers of other sensory neurons, such as warm receptive neurons, and low threshold mechanosensitive neurons, as well as a reduction in some sympathetic neurons (Farinas, 1999). Mice lacking the neurotrophins BDNF and NT3 also show significant losses in DRG neurons. NT-3 knockout mice have reduced numbers of proprioceptive neurons, and BDNF knockout mice have a generalized loss of DRG derived neurons that is more modest than NGF knockout mice (Farinas, 1999). Importantly, detailed analysis of these genetically targeted mice indicates that the survival of many DRG neurons requires neurotrophic input even before the neurons reach their targets. Furthermore, a reduced number of BDNF requiring neurons is not observed until several days after birth, long after target innervation has been completed. Finally, only about 50% of DRG neurons remain responsive to NGF following birth, suggesting that their neurotrophin sensitivity switches postnatally. Taken together, these observations indicate that the role of neurotrophins is wider than originally proposed by the neurotrophic hypothesis and includes modulating function in adult life.

The receptors for neurotrophins are the Trk receptors. These receptor tyrosine kinases are more or less specifically activated by one neurotrophin each: TrkA is the NGF receptor, TrkB is activated BDNF, and TrkC is activated by NT-3. High concentrations of NT3 have been shown to activate TrkA in vitro, and this may account for the more

profound loss of DRG neurons in NT3 knockout mice compared to TrkC knockout mice. Approximately 80% of DRG neurons express TrkA during development, in agreement with a requirement for NGF. All nociceptive neurons initially express TrkA, and hence genetic ablation of either NGF or TrkA leads to a total loss of nociceptive neurons. In humans, several mutations in TrkA have been found to underlie a condition known as CIPA (congenital insensitivity to pain with anhidrosis). Patients with the defect display fevers, mental retardation, self-mutilating behavior, and the absence of responsiveness to noxious stimuli (Indo et al., 1996; Miura et al., 2000; Nagasako et al., 2003). Consistent with these symptoms, patients lack unmyelinated neurons and small diameter myelinated neurons, in other words nociceptors that express TrkA during embryonic development.

The low affinity NGF receptor, p75, was in fact the first neurotrophin receptor cloned and characterized. It is a member of the TNFR (tumor necrosis factor receptor) superfamily, and binds all neurotrophins with equal affinities (Dechant and Barde, 1997; Dechant and Barde, 2002). While a role of p75 alone has yet to be well described, it is thought to act as a neurotrophin co-receptor, forming complexes with either TrkA, B or C that have higher neurotrophin affinity than either receptor alone. p75 has also been implicated in mediating cell death in the absence of TrkA activation, making its ultimate role in cell death and survival enigmatic (Dechant and Barde, 1997).

Signaling downstream of Trk receptors is well characterized. Binding of the neurotrophin leads to dimerization of Trk monomers. Dimerization leads to activation of the Trk kinase domain and subsequent autophosphorylation of several tyrosine residues

on the cytoplasmic tail of the dimerized receptor. These phosphotyrosines comprise binding sites for adaptor proteins, leading to the activation of a variety of signaling cascades. For instance, phosphorylation of tyrosine 499 of rat TrkA leads to recruitment of the adaptor protein Shc. Binding of Shc to TrkA activates Shc, and recruits other adaptor molecules, ultimately resulting in the activation of MAP kinase and phosphorylation of transcription factors that regulate cell survival and differentiation (Friedman and Greene, 1999; Kaplan and Miller, 2000). Activation of TrkA also leads to the autophosphorylation of tyrosine 794, which recruits phospholipase C (PLC). Binding or PLC leads to its activation, and the cleavage of the membrane lipid phosphatidylinositol-4,5-bisphosphate (PIP_2). The downstream consequences of this signaling pathway are less well characterized and are only beginning to be elucidated (Choi et al., 2001; Yamada et al., 2002).

TRPV1: The Capsaicin Receptor

An important consideration of the specificity theory is that it predicts that there are molecular determinants of nociceptive neurons. In other words, specific proteins must endow the nociceptive fiber with the ability to detect stimuli whose magnitude exceeds a given threshold (i.e. pain producing). Historically, a characteristic feature of some nociceptive neurons is their sensitivity to the vanilloid compound capsaicin. Capsaicin is the pungent ingredient in chili peppers that produces a sensation of burning heat and irritation. Its application to cultured sensory neurons leads to the increase of intracellular cations (Oh et al., 1996; Wood et al., 1988) and prolonged application can lead to death

of some nociceptive neurons (Fusco and Giacovazzo, 1997). In 1997, the capsaicin receptor was cloned in the Julius lab using a novel expression cloning strategy (Caterina et al., 1997). The capsaicin receptor, also referred to as VR1, or vanilloid receptor-1, is a nonselective cation channel that is a member of the TRP superfamily of cation channels. VR1 is expressed in small to medium diameter sensory neurons that require NGF for survival during development. Postnatally, VR1 is found in both TrkA positive and TrkA negative, IB4 positive cells, reflecting the downregulation of TrkA. Although some reports indicate that resiniferatoxin, a high affinity VR1 ligand, can bind to VR1 expressing neurons in the brain (Mezey et al., 2000; Szallasi and Blumberg, 1999), and that VR1 nucleotide probes hybridize to brain sections, no biochemical evidence of VR1 protein expression outside of sensory neurons has yet been provided.

In addition to being activated in heterologous expression systems by capsaicin, VR1 is also activated by acidic pH as well as noxious temperatures, thus explaining why eating a spicy pepper elicits a sensation that is best described as "hot." Further work in the Julius lab revealed that not only is VR1 (now renamed TRPV1) activated by multiple noxious stimuli, but exposure to one modality sensitizes, or increases the sensitivity of the channel to other modalities (Tominaga et al., 1998). For instance, exposure of the channel to subthreshold levels of protons, i.e. pH levels incapable of directly eliciting channel activation, leads to an increased sensitivity to capsaicin or heat. These studies led to the idea that TRPV1 is a polymodal signal integrator, and suggested that TRPV1 is a critical determinant of nociceptor sensitivity (Caterina and Julius, 2001; Tominaga et al., 1998) under both normal and pathophysiological conditions.

The role of TRPV1 as a signal integrator was confirmed with the generation of genetically targeted mice that lack TRPV1 expression (Caterina et al., 2000; Davis et al., 2000). TRPV1 homozygous null mice failed to exhibit capsaicin sensitivity, and showed a profound decrease in hypersensitivity to noxious temperatures following inflammation, although sensitivity to mechanical stimuli was normal. Taken together these observations indicate that TRPV1 is a molecular determinant of a class of nociceptors, and is critical in mediating sensation of noxious chemical and thermal stimuli.

Pain thresholds and the inflammatory soup

For nociception to properly occur, neurons in the pain pathway must be activated only when situations are encountered that are capable of causing tissue damage. However, from our own experiences we know that pain sensation is not always a constant. For instance, we have all encountered pain in the absence of apparent injury – headaches are a common type of pain that do not appear to serve as a warning of tissue injury. Furthermore, the quality of pain can be variable from person to person, or even variable to one person in different circumstances. As an example, Howard Fields writes that the low back pain suffered by an otherwise healthy person following a day of moving furniture can be perceived very differently than that same stimulus intensity suffered by that same person if he is dying of cancer. In other words, the emotional or motivational aspect of pain can greatly influence the perception of pain. In this case "it is essential for the understanding of pain patients that the desire to escape from or terminate the

sensation be considered" (Fields, 1987). In the above example, the pain suffered after moving furniture can be tolerated since a clear expectation of an endpoint of the pain exists; whereas the same pain suffered as a consequence of a terminal illness can be perceived as much more intense, since the expectation is likely that the pain will not cease and might in fact, intensify. In this example, it is the emotional state of the person that can lead to alterations in pain perception.

A simpler situation in which a person has an altered pain threshold is that of injury. After burning your hand on a hot kettle, for instance, a normally mildly painful or even non-painful stimulus, such as a warm shower or mild pin prick, is perceived as much more painful. In this situation, the pain threshold, or the level at which a stimulus becomes perceived as painful, has shifted to lower levels. This shift in nociceptor activation threshold is thought to mediate a guarding reflex to protect against further injury to already damaged tissue.

Following tissue injury, a variety of inflammatory mediators are recruited to the site of damage. This so called "inflammatory soup" is comprised of neuropeptides, growth factors, ATP, and lipid metabolites produced by sensory or sympathetic neurons, as well as surrounding mast cells and neutrophils (Julius and Basbaum, 2001). Some of these factors then act on cognate receptors on nociceptors, leading to depolarization of the nerve fiber. For instance, ATP is released from damaged cells upon injury and acts on P2X receptors on sensory neurons, leading to nociceptor depolarization (Wall and Melzack, 1999). Similarly, the lipid metabolite anandamide, which is structurally related

to capsaicin, can activate the capsaicin receptor (Zygmunt et al., 1999), as can protons generated upon tissue acidosis following injury (Caterina et al., 1997; Tominaga et al., 1998). Other components of the inflammatory soup are not thought to act directly on ion channels, but rather on cognate metabatropic receptors that then sensitize nociceptor ion channels. Bradykinin is perhaps the most well characterized agent that acts on nociceptors during inflammation. It is a nine amino acid peptide that is produced at the site of injury and itself induces pain (Wall and Melzack, 1999). In addition, it has been well described that bradykinin application to cultured nociceptors sensitizes their responses to heat (Cesare and McNaughton, 1996). NGF has also been implicated in the sensitization of nociceptors to heat and capsaicin (Shu and Mendell, 1999a; Shu and Mendell, 1999b). While bradykinin acts on the Gq- coupled seven transmembrane receptor BK2R, NGF activates the receptor-tyrosine kinase TrkA. Although at the outset these receptor subtypes seem unlike each other, activation of each leads to the activation of PLC; BK2R couples to PLC-β while TrkA couples to PLC-γ. Following activation, PLC cleaves the membrane lipid PIP_2 to inositol trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 then binds IP_3 receptors on the endoplasmic reticulum, leading to release of calcium from intracellular stores. In conjunction with DAG, calcium activates protein kinase C (PKC), leading to phosphorylation of downstream targets (Alberts, 1994). Intriguingly, pharmacological activators of PKC, namely phorbol esters, mimic the sensitization of cultured neurons achieved by NGF or BK application (Premkumar and Ahern, 2000; Sugiura et al., 2002; Vellani et al., 2001b). These observation have thus led to speculation that TRPV1 is a target of PKC phosphorylation, and that this phosphorylation leads to channel sensitization and increased heat-evoked responses

following inflammation (Bonnington and McNaughton, 2003; Numazaki et al., 2002; Olah et al., 2002).

TRP channel properties

TRPV1 is a member of the TRP channel family, the founding member of which is named for the *Drosophila* mutant *trp*, which displays a diminished light response. Although the *trp* phenotype was first characterized in 1970s (Cosens and Manning, 1969; Minke, 1977) it was more than 10 years before the gene encoding the TRP protein was cloned (Montell and Rubin, 1989) and found to encode a putative integral membrane protein with 6 transmembrane domains. The TRP protein is in fact a calcium permeable cation channel and is responsible for calcium influx after light activates the G-protein coupled receptor rhodopsin (Hardie and Minke, 1992) in the rhabdomere of the fly eye. Subsequent work from a number of labs revealed the existence of other *Drosophila* TRP channels as well as TRP homologues from C. *elegans*, mice, and humans (reviewed in (Hardie, 2001; Harteneck et al., 2000; Hofmann et al., 2000). A number of these channels have been found to be activated by sensory stimuli including temperature (TRPV1, TRPV3, TRPV4, TRPM8, ANKTM1 (reviewed in (Jordt, 2003; Patapoutian et al., 2003)), taste (TRPM5 (Perez et al., 2003)), vision (TRP, TRPL) and hearing or mechanotransduction (nompC (Sidi et al., 2003) and Nanchung (Kim et al., 2003)).

The mechanism of gating of the canonical TRP channel, *Drosophila* TRP, is still of significant debate. It is now well accepted that TRP is activated downstream of

rhodopsin and that gating requires PLC activation, but the relative roles of PKC, PLC and calcium release are still uncertain (Chyb et al., 1999; Minke and Agam, 2003; Raghu et al., 2000). However, some consensus exists that cleavage of the membrane lipid PIP_2 might play a role in relieving inhibition of a Drosophila TRP, or producing a lipid activator of that channel (Hardie, 2003; Hardie et al., 2001). These and other observations indicate that TRP channels might be members of a larger family of lipid regulated ion channels.

Lipid regulation of channels & TRPV1 as a model

The regulation of ion channel by interaction with phospholipids is only beginning to be explored (reviewed in (Hilgemann et al., 2001). Perhaps the best characterized examples are the inward rectifier potassium channels (IRKs), all of which are thought to require interaction with the membrane lipid PIP_2 for full activation (Hilgemann et al., 2001; Huang et al., 1998; Kobrinsky et al., 2000; Lopes et al., 2002; Zhang et al., 1999). While direct evidence for a physical interaction between IRKs and PIP_2 has been difficult to obtain, significant physiological and genetic evidence supports the notion that channel-lipid interaction is mediated by multiple positive charges in the cytoplasmic portions of the channel. Mutation of these residues leads to channels that are unable to remain active, or whose activity rapidly runs down following excision of a channel containing patch from an intact cell (Huang et al., 1998; Zhang et al., 1999). Recently, a TRP channel was identified that also appears to be positively regulated by PIP_2 interaction (Runnels et al., 2002). TRPM7 is a ubiquitously expressed mammalian TRP channel that

interacts with PLC. Channel activity can be abrogated by cleavage of PIP_2 resulting from activation of PLC through either Gq-coupled receptors or receptor tyrosine kinases, and channel activity can be restored upon application of exogenous PIP_2. A putative PIP_2 binding domain has not yet been identified for these channels, so a direct comparison of their binding sites to that of IRKs is not yet possible.

On the other hand, some channels appear to be negatively regulated by PIP_2 interaction. TRPL and cyclic nucleotide gated potassium channels are examples of channels that are activated following receptor mediated PIP2 hydrolysis (Hilgemann et al., 2001; Womack et al., 2000).

Because TRPV1 can be activated by a variety of stimuli, and because it can be easily expressed in a variety of heterologous systems, it is an attractive member of the TRP channel family with which to pursue further understanding of this new and exciting class of channels as well as mechanisms of lipid regulation. Furthermore, due to the clear and important role of TRPV1 in transducing thermal and chemical stimuli, understanding its regulation will provide key insights into normal and pathophysiological pain states.

Chapter 2: Bradykinin and Nerve Growth Factor Release the Capsaicin Receptor from PIP_2-Mediated Inhibition

Tissue injury generates endogenous factors that heighten our sense of pain by increasing the response of sensory nerve endings to noxious stimuli (Bevan, 1999; McMahon, 1999). Bradykinin and nerve growth factor (NGF) are two such pro-algesic agents that activate G protein-coupled (BK2) and tyrosine kinase (TrkA) receptors, respectively, to stimulate phospholipase C signaling pathways in primary afferent neurons (Burgess et al., 1989; Ganju et al., 1998). How these actions produce sensitization to physical or chemical stimuli has not been elucidated at the molecular level. Here we show that bradykinin- or NGF-mediated potentiation of thermal sensitivity in vivo requires expression of VR1, a heat-activated ion channel on sensory neurons. Diminution of plasma membrane PIP2 levels through antibody sequestration or PLC-mediated hydrolysis mimics the potentiating effects of bradykinin or NGF at the cellular level. Moreover, recruitment of phospholipase C-γ to TrkA is essential for NGF-mediated potentiation of channel activity, and biochemical studies suggest that VR1 associates with this complex. These studies delineate a biochemical mechanism through which bradykinin and NGF produce hypersensitivity, and may explain how activation of phospholipase C signaling systems regulates other members of the TRP channel family.

Introduction

Nociceptors are specialized sensory neurons that detect chemical, physical, or mechanical stimuli capable of causing tissue damage. Inflammation increases levels of extracellular protons, neurotransmitters, and growth factors within the local tissue environment, thereby decreasing activation thresholds and enhancing excitability of the nociceptor (Bevan, 1999; Woolf and Salter, 2000). Included among these agents are the nonapeptide, bradykinin, and the neurotrophin, NGF (Bevan, 1999; Koltzenburg et al., 1999; Nicholas et al., 1999; Shu and Mendell, 1999b) Sensitization by bradykinin or NGF may involve long-term changes in gene expression (Ganju et al., 1998; Koltzenburg, 1999; Woolf, 1996) but the ability of these factors to promote nociceptor sensitization within several minutes suggests that post-translational mechanisms also come into play. The downstream effector molecules that mediate sensitization by bradykinin or NGF have not been fully characterized (Ganju et al., 1998). One candidate target on nociceptors is VR1, a cation channel that is activated by pungent vanilloid compounds (such as capsaicin), extracellular protons, or noxious heat (Caterina et al., 1997). This ability to integrate thermal and chemical stimuli makes VR1 potentially well suited to modulate sensitivity of the nociceptor following tissue injury (Tominaga et al., 1998). Indeed, VR1-deficient mice do not develop thermal hyperalgesia in response to inflammation (Caterina et al., 2000; Davis et al., 2000).

Results

Because injury generates an array of chemical mediators, we asked whether bradykinin and NGF, specifically, contribute to VR1-dependent thermal hypersensitivity. We injected wild type and VR1-deficient mice with bradykinin or NGF and measured paw withdrawal latencies from a radiant heat source before and after treatment. Each agent produced substantial sensitization in wild-type animals, but not in VR1-null mice (Fig. 1), demonstrating that VR1 is essential for the development of bradykinin- or NGF-induced thermal hypersensitivity *in vivo*.

Whereas extracellular protons interact directly with VR1 to potentiate its sensitivity to capsaicin or heat (Jordt et al., 2000; Tominaga et al., 1998), NGF or bradykinin may modulate VR1 by binding to their own receptors on sensory neurons and activating second messenger signaling cascades. Indeed, VR1 belongs to the TRP channel family, some members of which are activated downstream of phospholipase C-coupled receptors through as yet undefined mechanisms (Harteneck et al., 2000). To examine this possibility, we expressed VR1 and BK_2 receptors together in human embryonic kidney (HEK293) cells and examined their sensitivity to VR1 agonists before and after exposure to bradykinin. Moderately acidic solutions (pH 6.4) evoked small, but discernable inward currents at negative holding potentials. Subsequent treatment with bradykinin (20 nM) dramatically increased these responses by ~25-fold (Fig. 2a). Even at physiological pH, bradykinin produced a slight increase in holding current that was attributable to VR1 based on characteristic outward rectification and inhibition by the vanilloid receptor antagonist, capsazepine (Fig. 8). At a low dose of capsaicin (10 nM), bradykinin also produced a substantial (~5-fold) and prolonged potentiation of evoked currents (Fig. 2b).

In all cases, modulation was observed only in cells co-expressing VR1 and BK_2 receptors.

To determine whether potentiation involves a change in channel gating, we analyzed the kinetic properties of capsaicin-evoked currents before and after bradykinin exposure. In the absence of any stimulus, VR1 displayed slight basal channel activity characterized by strong outward rectification; inward current at negative holding potential was negligible (Fig. $2c_1$). Application of capsaicin produced a greater proportional increase in currents at negative membrane potentials, resulting in weaker outward rectification (Fig. $2c_2$). Upon activation of BK_2 receptors, capsazepine-inhibitable basal currents grew slowly over 2 - 3 minutes and rectification gradually weakened (Fig. $2c_3$). A second challenge with capsaicin further weakened the outward rectification, preferentially augmenting the inward component (Fig. $2c_4$). A similar phenomenon was observed when protons were used as agonist (not shown). These observations suggest that potentiation of VR1 by bradykinin primarily involves an alteration in channel gating.

Previous studies have shown that exposure of cultured dorsal root ganglion (DRG) neurons to NGF for 10 minutes produces acute sensitization of capsaicin-evoked responses (Shu and Mendell, 1999a). Similarly, we found that NGF produced robust (~30-fold) increases in proton (pH 5.5)-evoked currents in oocytes co-expressing TrkA and VR1 (Fig. 3a). Potentiation was specific for VR1 since we found no change in the activity of co-expressed $5HT_3$ serotonin-gated channels. Moreover, NGF had no effect unless TrkA was present (Fig. 3a). We also compared temperature response profiles for NGF-treated versus untreated cells and found that NGF produced a substantial decrease in the thermal threshold for VR1 activation, such that significant currents were observed even at room temperature (Fig. 3b). This was accompanied by an increase in the magnitude of responses at temperatures both below and above the threshold (~43°C)

normally required for VR1 activation. These effects, as well as those described above for bradykinin, are reminiscent of the signature changes in nociceptor excitability produced by inflammation, including development of ongoing activity and sensitization to thermal stimuli (Koltzenburg, 1999). Although the neurotrophin receptor, p75, was usually co-expressed with TrkA in these experiments, no difference was observed in its absence (not shown), consistent with recent evidence that p75 is not required for NGF-evoked thermal hypersensitivity *in vivo* (Bergmann et al., 1998).

PLC stimulation is common to both NGF and bradykinin receptor activation, and we therefore asked whether this is an obligate step in the VR1 potentiation process. A TrkA mutant (Y794F) that specifically abrogates recruitment of PLC-γ to the receptor complex (Stephens et al., 1994) showed greatly diminished NGF-mediated potentiation of proton-evoked currents (14±7% of the wild type receptor), even though Western blot analysis showed that mutant and wild type receptors were expressed at comparable levels (Fig. 4a). By contrast, another TrkA mutant (Y499F) that disrupts coupling to the MAP kinase pathway (Stephens et al., 1994) was fully capable of potentiating VR1-evoked responses (Fig. 4a). These results demonstrate that PLC activation is the predominant pathway involved in VR1 potentiation by NGF. To determine whether PLC activity alone can potentiate VR1 responses, we applied a recombinant inositide phospholipid-specific PLC (PI-PLC) directly to patches excised from VR1-expressing HEK293 cells. PI-PLC enhanced both basal channel activity and capsaicin-evoked responses, and these effects were significantly reduced by a neutralizing monoclonal antibody to the enzyme (Fig. 4b).

PLC catalyzes the hydrolysis of membrane phosphatidylinositol-4,5-bisphosphate (PIP_2) to yield inositol trisphosphate and diacylglycerol (DAG), one consequence of which is activation of protein kinase C (PKC). Pharmacological studies suggest that bradykinin

potentiates heat-evoked currents through activation of PKC, and that PKC potentiates capsaicin-evoked responses in VR1-expressing oocytes.(Cesare et al., 1999; Premkumar and Ahern, 2000). However, we found that neither staurosporine nor Ro-31-8425, two PKC inhibitors, blocked NGF-elicited potentiation of VR1 in oocytes (not shown). In addition, attempts to mimic potentiation with the PKC activator phorbol 12-myristate 13-acetate (PMA or TPA) were confounded by our observation that this agent antagonizes binding of 3-resiniferatoxin (a high affinity capsaicin receptor agonist) to VR1, suggesting that PMA interacts directly with the channel (Fig. 9). Importantly, we also found that PI-PLC potentiated VR1 currents in the absence of ATP.

Taken together, these results suggest that modulation of VR1 involves both PKC-dependent and -independent mechanisms, with the latter possibly occurring at a more proximal point in the pathway, namely, as a direct consequence of PIP_2 hydrolysis. A number of ion channels are regulated in this manner, as exemplified by PIP_2 inhibition of cyclic nucleotide-gated channels (Womack et al., 2000), or PIP_2 potentiation of G protein-gated inwardly rectifying potassium channels (Huang et al., 1998; Zhang et al., 1999). Anti-PIP_2 monoclonal antibodies sequester membrane PIP_2 and thus shift the sensitivity of these channels to cyclic GMP or $G_{\beta\gamma}$, respectively. When we applied this antibody to the cytoplasmic side of excised inside-out patches from VR1-expressing oocytes we observed a gradual stimulation of basal channel activity (Fig. 6a left) that displayed characteristic outward rectification. PIP_2 antibody also potentiated capsaicin-evoked currents, which normally display tachyphylaxis. Additionally, when the pipette solution was made more acidic (pH 6.4), PIP_2 antibody induced large inward currents (not shown). A similar induction was observed at neutral pH in cells expressing VR1 mutants E600Q or E600K (not shown) which, under normal physiological conditions, behave like proton-potentiated wild type channels (Jordt et al., 2000). PIP_2 antibody did not evoke responses in membrane patches from control oocytes (not shown). Taken

together, these results demonstrate that PIP_2 antibody-activated currents are carried by VR1.

Cell types differ in membrane lipid composition and we asked whether PIP_2 sequestration would also sensitize VR1 channels in mammalian cells, including sensory neurons. Very small inward currents were observed when 20 nM capsaicin was applied to inside-out membrane patches excised from VR1-transfected HEK293 cells. Application of PIP_2 antibody enhanced basal and capsaicin-induced currents. Both of these currents were suppressed by capsazepine (Fig. 8) and antibody effects were partially reversed upon washout (Fig. 6a middle). This action was observed only when antibody was applied to the cytoplasmic side of the membrane (where PIP_2 resides), and a monoclonal antibody against progesterone had no effect (not shown). PIP_2 antibody also shifted the heat sensitivity of VR1 to a lower temperature range. When excised patches from these cells were exposed to a temperature ramp plateauing at 37°C, a small VR1-mediated basal current developed. Brief application of antibody to this membrane patch then triggered a large inward current, indicating that the thermal sensitivity of VR1 can be modulated by PIP_2 in a membrane-delimited fashion (Fig. 6a right). PIP_2 antibody also stimulated basal currents and sensitized capsaicin-evoked responses in rat DRG neurons (Fig. 6b). These results suggest that endogenous PIP_2 inhibits VR1 and that repression can be alleviated by agents that activate PLC.

Mammalian TRPC3 channels associate with TrkB receptors in the brain, and these channels can be stimulated by brain-derived neurotrophic factor through a TrkB/PLC-dependent signaling pathway (Li et al., 1999). We therefore asked whether VR1 and TrkA associate to form an analogous signaling complex. Immunoprecipitates prepared from HEK293 cells co-expressing VR1 and TrkA contained both proteins, irrespective of whether VR1 or TrkA antiserum was used to form the precipitate (Fig. 7a). Moreover,

endogenous PLC-γ was detected in these samples, demonstrating that VR1, TrkA, and PLC-γ can associate to form a ternary complex. Significantly less PLC-γ was recovered in immunoprecipitates from cells expressing TrkA (Y794F) mutant receptors (Fig. 7a), consistent with our electrophysiological results showing reduced NGF potentiation of VR1 currents in oocytes expressing this construct. In addition, VR1 associated with a catalytically inactive TrkA (K547A) mutant (Fig. 10), demonstrating that receptor kinase activity is not a prerequisite for interaction. Although we have not shown that TrkA and VR1 associate in sensory neurons, the interaction that we observe in HEK293 cells is specific since the epidermal growth factor (EGF) receptor, a closely related receptor tyrosine kinase, does not co-immunoprecipitate with VR1 when expressed in these cells (Fig. 7b).

Discussion

A subset of vertebrate and invertebrate TRP channels are activated by neurotransmitter, hormone, or growth factor receptors that couple to PLC signaling pathways. How PLC activation leads to TRP channel opening is still a question of significant debate, but a number of mechanisms have been proposed that involve calcium store depletion, direct interaction with IP_3 receptors, or activation by lipid metabolites produced downstream of PLC (Harteneck et al., 2000). Our data suggest that VR1 can be activated or potentiated at a very proximal point in the pathway as an immediate consequence of PLC activation and PIP_2 hydrolysis. Indeed, application of $G_{\alpha 11}$ to inside-out membrane patches activates *Drosophila* TRPL channels through a PLC-mediated mechanism (Harteneck et al., 2000), and application of PIP_2 partially inhibits TRPL opening (Estacion et al., 2001). The ability of exogenously applied anandamide or DAG to activate VR1 or other TRP

channels in a PKC-independent manner (Hofmann et al., 1999; Zygmunt et al., 1999) raises the possibility that these lipid messengers mediate their effects by displacing PIP_2 or other membrane lipids from an inhibitory site on the channel complex.

In the *Drosophila* eye, TRP channels exist in a complex with other components of the phototransduction machinery, an arrangement that influences sensitivity and kinetics of the signaling process (Chevesich et al., 1997; Scott and Zuker, 1998). Whether a similar signaling scaffold exists in nociceptors remains to be determined. Interestingly, we have found that a variety of PLC-coupled receptors modulate VR1 function in heterologous systems (not shown). Moreover, preliminary experiments suggest that EGF receptor activation also sensitizes VR1, albeit to a lesser extent (~ 5-fold) than that typically observed with NGF (Fig. 11). Thus while formation of a specific protein complex is not a prerequisite for modulation, complex formation may facilitate the process. Activation of different PLC-coupled receptors by individual components of the inflammatory milieu may allow for spatially and quantitatively distinct mechanisms of nociceptor sensitization.

Methods

Behavior. Bradykinin (20 µl of a 10 nM solution in 0.9% saline) was injected i.pl. into one hind paw of wild type or VR1 -/- mice (adult males, ~25 g body weight) and response latencies to a radiant heat source were measured as described (Caterina et al., 2000). NGF (2.5S, generous gift of Dr. William Mobley) was administered subcutaneously (1 mg/kg in 0.9% saline). Significance was determined using repeated measures ANOVA with Fisher's PLSD post-hoc analysis.

Oocyte electrophysiology. Defolliculated stage VI *Xenopus laevis* oocytes were injected with 0.1-1.0 ng rat VR1, rat TrkA, rat p75, human EGFR or mouse $5HT_3R$-A cRNA. Oocytes were maintained in Modified Barth's saline solution for 4 - 6 days prior to analysis. Two-electrode voltage-clamp analysis was performed using a Geneclamp 500 amplifier (Axon Inst.) and MacLab A/D converter (E_{hold} = -45 mV). ND-96 (with Ba^{2+}) recording solution was buffered with 10 mM Na-MES (pH 5.5) or 10 mM Na-HEPES (pH 7.5), and bath chamber perfused at ~1ml/min. Temperature ramps were generated as described (Tominaga et al., 1998). For patch-clamp experiments, 2 - 10 ng of VR1 cRNA was injected into oocytes and responses measured 2 - 5 weeks after injection. Recombinant rat NGF-β, human EGF (Sigma) and bradykinin (Bachem) were diluted into perfusate.

Mammalian cell electrophysiology. HEK293 cells were cultured in DMEM/F12 with 10% FBS and transfected using Lipofectamine, Superfect, or Fugene 6 according to manufacturers' protocols. Cells were transfected with 400 ng plasmid DNA encoding rat VR1, with or without 400 ng human BK_2 receptor cDNA. To identify transfected cells, an enhanced green fluorescence protein reporter plasmid was co-transfected at one-tenth the concentration of receptor cDNAs. Cells were plated onto coverslips 1 - 3 days prior to recording, and examined 4 - 7 days after transfection. Dissociated neurons from rat or mouse DRG were prepared as described (Caterina et al., 2000). Voltage-clamp experiments were performed at -60 mV holding potential with 320 ms voltage ramp from -120 to +80 mV at 1 Hz. Data were acquired using pClamp (Axon Instruments) or Pulse-Pulsefit (HEKA, GmBH) software. Recordings were filtered at 5 kHz and sampled at 1kHz. Standard bath solution for patch-clamp experiments contained (in mM): 10 Tris/HCl, 1 EGTA, 1 $MgCl_2$, 150 CsCl at pH 7.4. The pH 6.4 solution contained (in mM): 20 citric acid, 1 $MgCl_2$, titrated with CsOH to pH 6.4, and supplemented with CsCl to make the final Cs^+ concentration 150 mM. For excised patch experiments, standard bath solution

was used in both the pipette and perfusion solution. No ATP was added to the solutions. Diameters of patch pipettes were 20 - 50 μm for oocyte recordings and 12 - 15 μm for HEK293 cells or neurons. For whole cell recording, a 0.9x standard bath solution supplemented with 0.24 mM $CaCl_2$, 0.2 mM Na_2GTP and 0.3 mM Na_2ATP was used in the pipette, and the final pH adjusted with Tris-base to 7.3. Pipette resistance in this solution was typically 2 - 3 MΩ. PI-PLC and its neutralizing antibody were from Molecular Probes, and PIP_2 and progesterone antibodies from Perseptive Biosystem. Titers of PIP_2 Ab used in individual experiments were 1:200 (Fig 6a left and right), 1:2000 (Fig 6a center) and 1:500 (Fig 6b). A perfusion stage with heating resistors was used to generate a heat ramp plateauing at 37°Cand bath temperature was monitored with probes (Warner Instruments).

Biochemistry. HEK293 cells were maintained in DMEM containing 10% FBS. Cells were plated at 70-80% confluency and subjected to calcium phosphate or lipofectamine transfection with approximately equal amounts of plasmid DNAs. 24 - 48 hours post-transfection, cells were harvested and lysed in 1 ml TNE buffer (10 mM Tris, pH 8.0; 150 mM NaCl; 1 mM EDTA; 1% NP40) containing 0.12 mg/ml PMSF, 2 μg /ml leupeptin, 1 μg /ml aprotinin, 10 mM NaF, and 1 mM Na_3VO_4 on ice. Lysates of equivalent protein content were incubated overnight at 4°C with anti-pan-Trk polyclonal antibody C14 (Santa Cruz Biotech) (1.5 μg) or anti-VR1 antiserum (Tominaga et al., 1998) (1:1000). Immune complexes were immobilized on protein A-Sepharose beads, washed with ice-cold TNE, boiled in SDS-sample buffer, separated by SDS-PAGE, and transferred onto PVDF membrane. Membranes were blocked in TBST buffer (20 mM Tris, pH 7.5; 500 mM NaCl; 0.1% Tween-20) containing 5% nonfat milk, then incubated for 2 hrs at room temperature or overnight at 4°C in TBST with 1% milk and one of the following primary antibodies: anti-Trk 45 antiserum (1:2000); anti-VR1 antiserum (1:2000); or anti-PLCγ antibody (Upstate Biotech) (0.7 μg /ml). Membranes were washed with TBST, then

incubated with HRP-conjugated goat anti-rabbit or goat anti-mouse secondary antibodies. Immunoreactive protein bands were detected by enhanced chemiluminescence using ECL reagents. For western blot analysis of oocyte proteins, 10 cells were lysed in 100 µl 100mM NaCl, 1% Triton X-100, 20 mM Tris-Cl pH 7.6 and complete protease inhibitor cocktail (Roche) by drawing 5 - 10 times each through 15g and 26g needles. Lysates were centrifuged 10 min. x 12,000 rpm and supernatant fractions passed twice over 0.22 µm Spin-X filters (Costar). Approximately 20 µl of final flow through was denatured in SDS sample buffer and analyzed as above.

RTX Binding. HEK293 cells were transfected with rat VR1 cDNA using Lipofectamine Plus reagent (Life Technologies). Membranes were prepared as described (Szallasi et al., 1999; Wahl et al., 2001) 12-O-tetradecanoylphorbol-13-acetate (TPA) and phorbol-12,13-dibutyrate (PDBu) were purchased from Sigma; [^{3}H]-resiniferatoxin (RTX) from NEN. EC_{50} for [^{3}H]-RTX = 200 ± 30 pM from three independent saturation binding experiments; Hill coefficient = 1.6 ± 0.2. These data are consistent with previous studies using membranes from rat DRG (Szallasi and Blumberg, 1990) or transfected HEK293 cells (Wahl et al., 2001) . Binding assays were set up on ice and contained 1µg membrane protein, 200 pM [^{3}H]-RTX, non-radioactive ligand and 0.25 mg/ml BSA in assay buffer (5mM KCl, 5.8mM NaCl, 0.75 mM $CaCl_2$, 2mM $MgCl_2$ 10mM HEPES pH 7.4). After incubation for 1 hour at 37 C, reactions were terminated on ice, collected on Whatman GF/B filters, and washed 3 x 10 ml ice-cold assay buffer. The retained radioactivity was determined by liquid scintillation counting. Non-specific binding was 32 ± 5 % of total, as defined by binding in the presence of 200 nM unlabeled RTX.

Acknowledgments

We are grateful to W. Neuhausser for providing dissociated DRG neurons, W. Mobley for 2.5S NGF, D. Shelton for advice regarding NGF administration, and J. Poblete and K. Simpson for expert technical assistance. We thank members of our laboratories for many helpful suggestions and constructive criticism. This work was supported by a NSF predoctoral fellowship (E.P.), NIH predoctoral neuroscience training grant (S.S.), a German Academy of Natural Scientists Leopoldina postdoctoral fellowship (S-E.J.) and by the NIH (M.V.C., A.I.B. and D.J.).

Figure 1

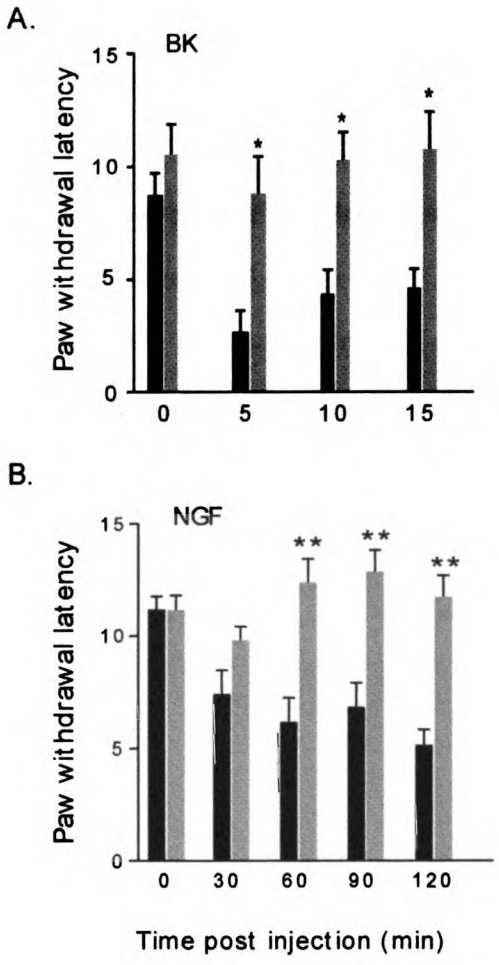

A.
BK
Paw withdrawal latency
15
10
5
0
0
5
10
15
*
*
*
B.
NGF
Paw withdrawal latency
15
10
5
0
0
30
60
90
120
**
**
**
Time post injection (min)

Figure 1 VR1 is essential for development of bradykinin- or NGF-evoked thermal hypersensitivity in vivo. **A.** Wild type (black bars) or VR1 -/- (gray bars) mice were injected intraplantar with bradykinin and response latency to radiant heating of the hind paw was measured at various times after injection (zero min. shows pre-injection response latency). *$p < 0.05$ for VR1 +/+ versus VR1 -/-; $p = 0.02$ for entire time course; $n = 7$ mice per genotype. **B.** NGF-evoked thermal hypersensitivity was measured as above. **$p < 0.02$ for VR1 +/+ versus VR1 -/-; $p = 0.01$ for the entire time course; $n = 7$ mice per genotype. Note that NGF was administered subcutaneously and delivery to the receptive field (i.e. the paw) may account for the slower onset of hyperalgesia relative to bradykinin. Neither group showed significant change in response latencies following saline injection (not shown).

Figure 2

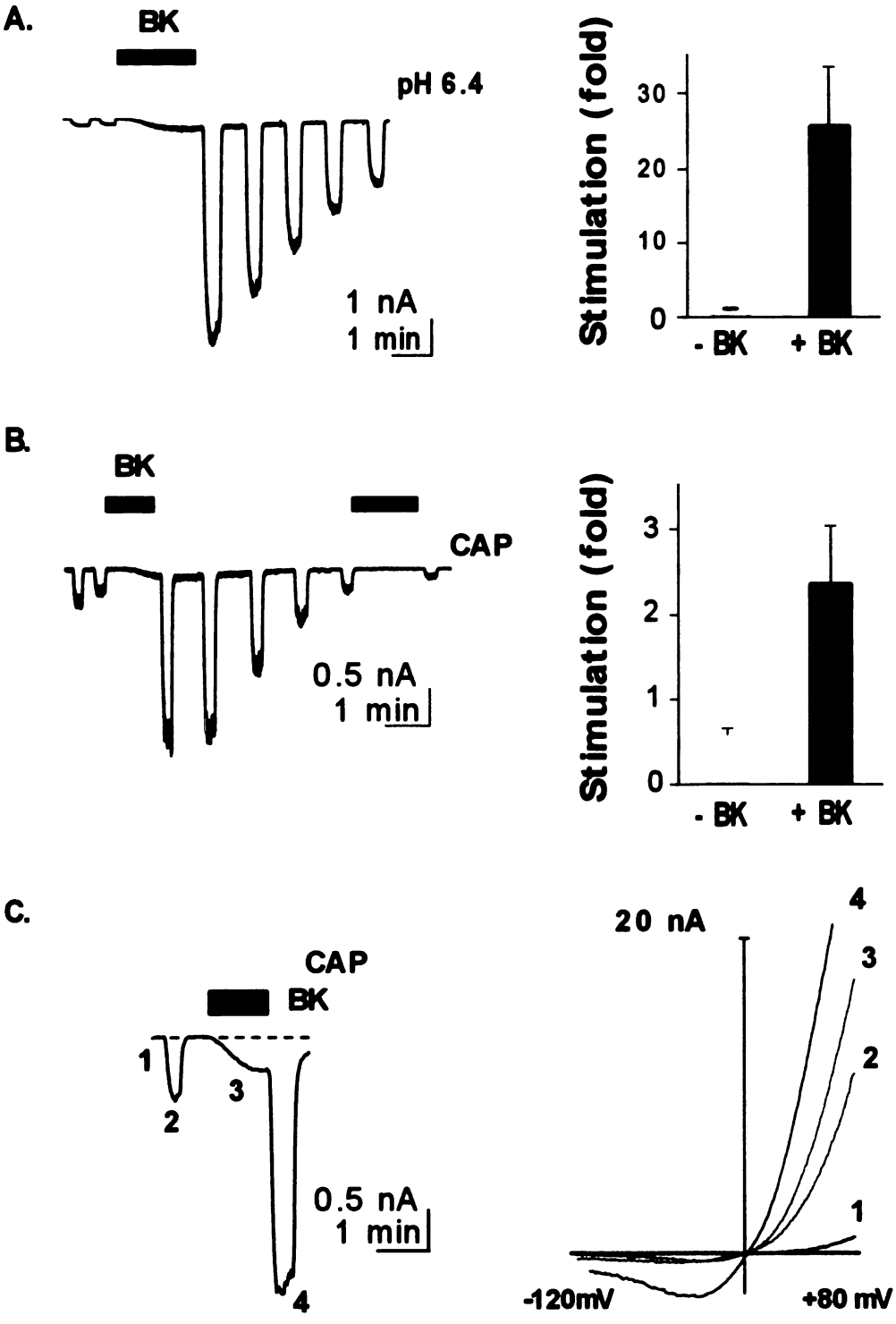

A.
BK
pH 6.4
1 nA
1 min
Stimulation (fold)
30
20
10
0
- BK
+ BK
B.
BK
CAP
0.5 nA
1 min
Stimulation (fold)
3
2
1
0
- BK
+ BK
C.
CAP
BK
1
2
3
4
0.5 nA
1 min
20 nA
4
3
2
1
-120mV
+80 mV

Figure 2 Bradykinin (BK) sensitizes VR1 in heterologous expression systems. **A**, Left, BK (20 nM) potentiated proton (pH 6.4)-evoked responses in a voltage-clamped VR1-expressing HEK293 cell. Right, normalized proton response before (gray bar) and after (black bar) BK application ($n = 8$, $p < 0.05$). **B**, Left, BK potentiated capsaicin (CAP; 10 nM)-evoked responses in a voltage-clamped VR1-expressing HEK293 cell. Right, capsaicin response normalized to a previous application before (gray bar) and after (black bar) BK treatment ($n = 16$, $p < 0.05$). **C**, Current-voltage relationships of VR1 responses to capsaicin (CAP; 10 nM) before and after BK application reveal a change in gating. Numbers indicate points at which voltage-ramp traces (right) were obtained from current trace (left).

Figure 3

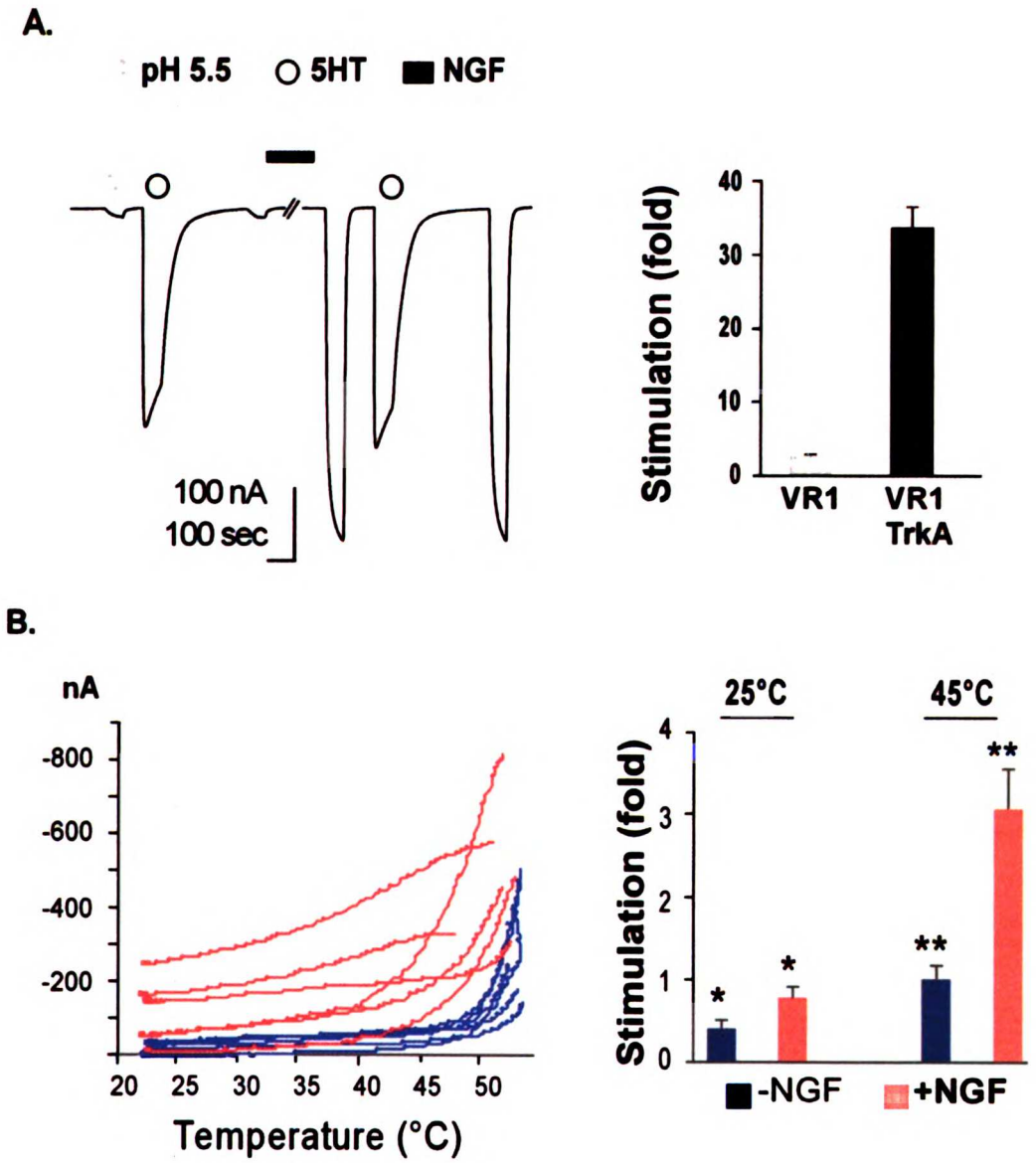
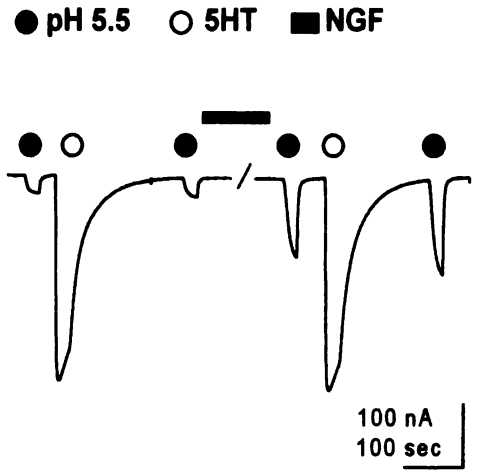

A.
pH 5.5
5HT
NGF
100 nA
100 sec
Stimulation (fold)
40
30
20
10
0
VR1
VR1
TrkA
B.
nA
-800
-600
-400
-200
20
25
30
35
40
45
50
Temperature (°C)
25°C
45°C
Stimulation (fold)
4
3
2
1
0
*
*
**
**
-NGF
+NGF

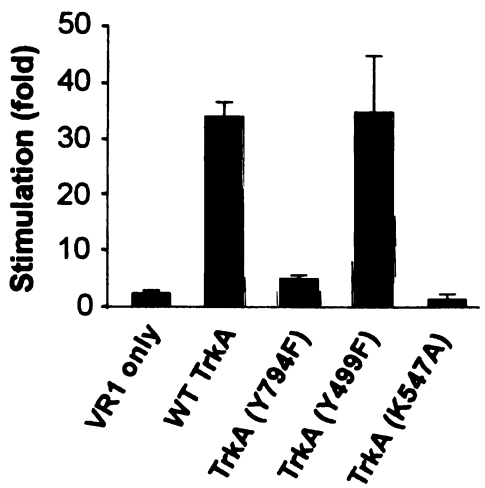
Figure 3 Nerve growth factor (NGF) sensitizes VR1 in heterologous expression systems. **A**, Left, NGF sensitizes proton-evoked currents in oocytes expressing VR1, TrkA, p75 and $5HT_3R$-A. Break indicates 10 min NGF (0.75 nM) application. Protons (pH 5.5) and serotonin (5HT, 10 μM) were perfused for 30 seconds. Right, averaged potentiation by NGF in these cells (black bar) versus oocytes expressing VR1 alone (gray bar; $n = 10$, $p < 0.001$). **B**, Heat-evoked currents in oocytes co-expressing TrkA. Cells were incubated with NGF (red bar, 0.75 nM) or vehicle (blue bar) for 10 min at room temperature prior to analysis ($n = 14 - 16$, $*p < 0.05$, $**p < 0.001$). Water injected oocytes did not respond to heat (not shown). Averaged values (right) were normalized to current responses of vehicle-treated cells at 45°C.

Figure 4

A.


pH 5.5
5HT
NGF
100 nA
100 sec

B.


Stimulation (fold)
50
40
30
20
10
0
VR1 only
WT TrkA
TrkA (Y794F)
TrkA (Y499F)
TrkA (K547A)

C.

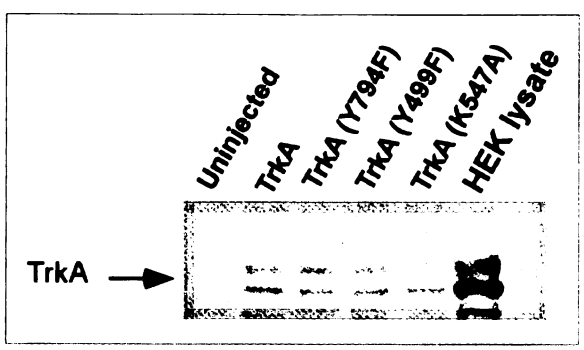

Uninjected
TrkA
TrkA (Y794F)
TrkA (Y499F)
TrkA (K547A)
HEK lysate
TrkA

Figure 4 Phospholipase C is involved in NGF modulation of VR1 function. **A.** Oocytes injected with VR1, TrkA(Y794F), p75, and $5HT_3R$-A, were analyzed as described in Fig. 3. ($n = 10$, $p < 0.001$ versus cells expressing wild type TrkA). **B.** Histogram shows average potentiation of proton-evoked responses in cells co-expressing VR1 and wild type or mutant TrkA receptors. **C.** Western blot of oocyte extracts indicates similar levels of wild type and mutant TrkA receptor expression. Lysate from TrkA-transfected HEK293 cells provides a control for TrkA immunoreactivity.

Figure 5

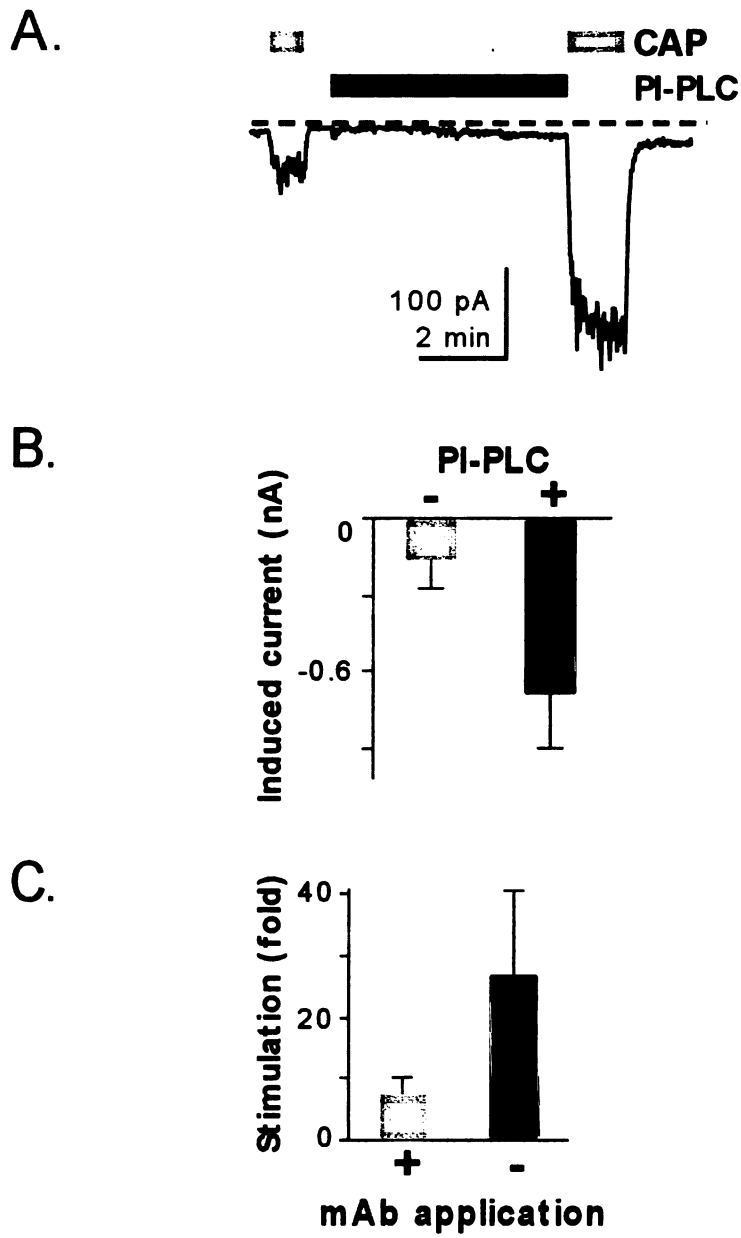

A.
CAP
PI-PLC
100 pA
2 min
B.
PI-PLC
-
+
0
-0.6
Induced current (nA)
C.
Stimulation (fold)
40
20
0
+
-
mAb application

Figure 5 **A**. Representative trace from an inside-out membrane patch excised from and HEK293 cell expressing VR1 (-60 mV holding potential.) **B.** Capsaicin (20 nM)-evoked currents before (gray bar) and after (black bar) application of PI-PLC (0.5 u/ml) to inside-out membrane patches excised from HEK293 cells expressing VR1 (-60 mV holding potential; $n = 12$, $p < 0.01$). **C.** PI-PLC potentiation (black bar) was reduced upon pretreatment of PI-PLC with anti-PLC Ab 72-24 (gray bar). Current was normalized to first capsaicin application ($n = 7$, $p < 0.05$).

Figure 6

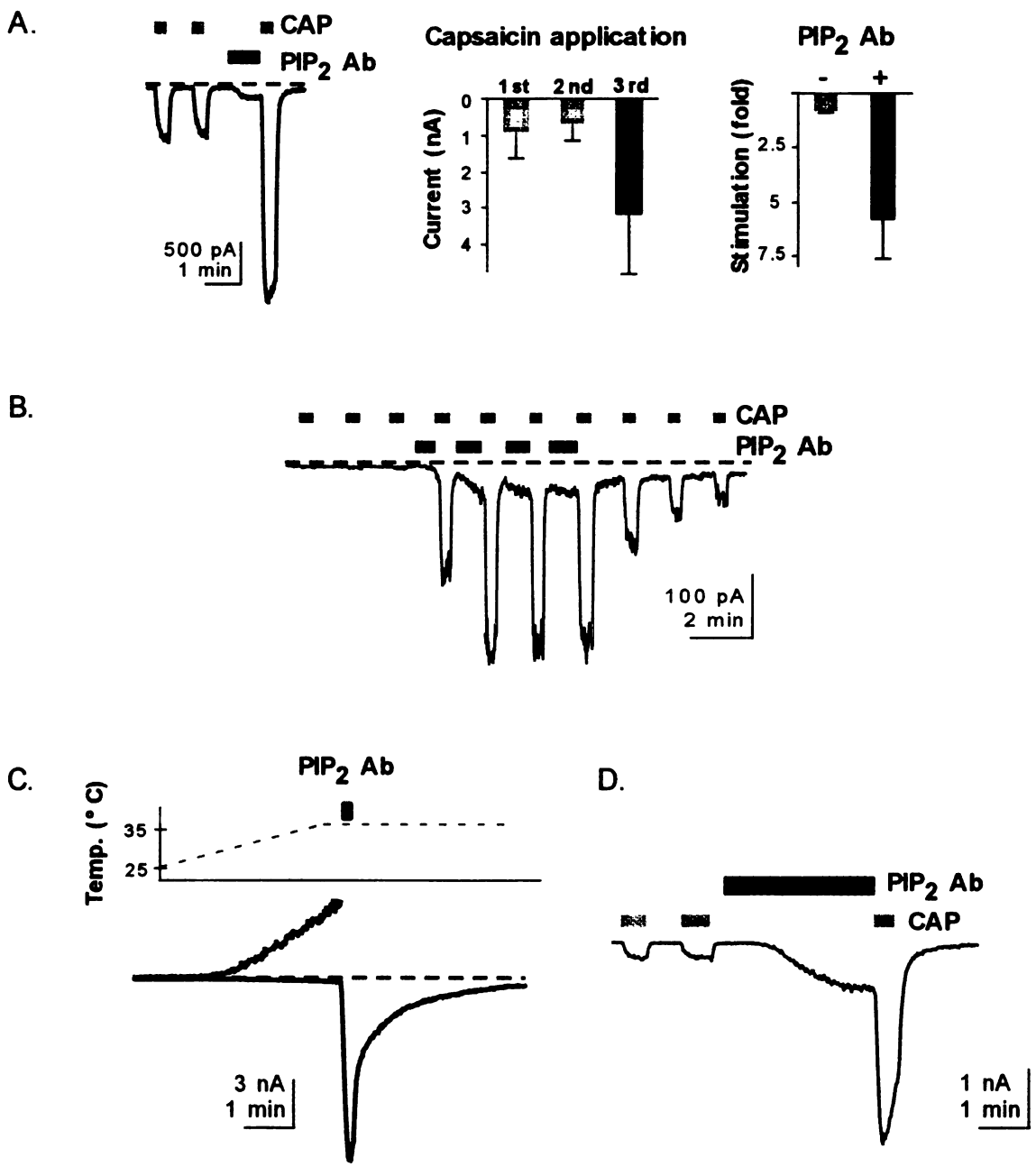
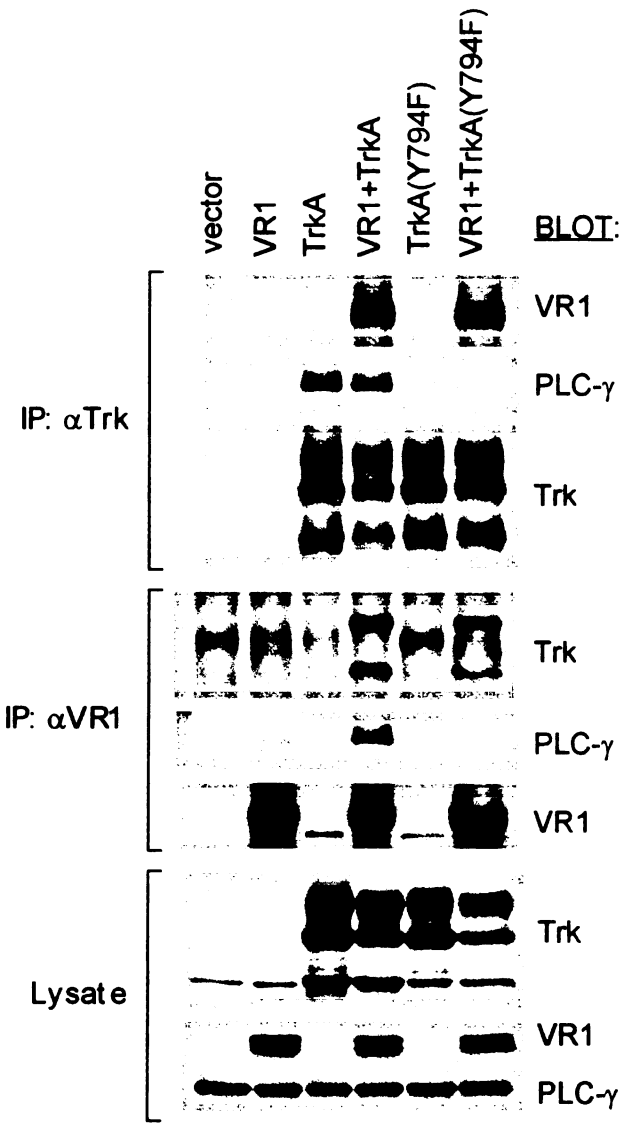

A.
CAP
PIP2 Ab
500 pA
1 min
Capsaicin application
1st
2nd
3rd
Current (nA)
0
1
2
3
4
PIP2 Ab
-
+
Stimulation (fold)
2.5
5
7.5
B.
CAP
PIP2 Ab
100 pA
2 min
C.
PIP2 Ab
Temp. (° C)
35
25
3 nA
1 min
D.
PIP2 Ab
CAP
1 nA
1 min

Figure 6 PIP_2 antibody application mimics modulation of VR1 by BK or NGF. **A**, Left, application of PIP_2 monoclonal antibody (PIP_2 Ab) to an inside-out membrane patch excised from VR1-expressing oocytes induced basal current and potentiated capsaicin (CAP, 20 nM)-evoked currents. Middle, averaged response to capsaicin challenge in the absence (1st and 2nd, grey bars) and presence (3rd, black bar) of PIP_2 Ab. Right, current responses induced by capsaicin (normalized to previous application) showed >6-fold potentiation by PIP_2 Ab ($n = 12$, $p < 0.001$). **B**, Modulation by PIP_2 Ab was also observed in membrane patches excised from VR1-expressing HEK293 cells. **C**, Heat application to an excised membrane patch from a VR1-expressing HEK293 cell elicited a strongly rectifying basal current below 37°C (upward deflection shows outward current at +70 mV holding potential before Ab application). Brief application of PIP_2 Ab then provoked a large inward current (-60 mV holding potential) at 37°C. **D**, Potentiation of capsaicin (20 nM)-evoked responses by PIP_2 Ab was also observed in membrane patches excised from rat DRG neurons.

Figure 7

A.


vector
VR1
TrkA
VR1+TrkA
TrkA(Y794F)
VR1+TrkA(Y794F)
BLOT:
VR1
PLC-γ
Trk
IP: αTrk
Trk
PLC-γ
VR1
IP: αVR1
Trk
VR1
PLC-γ
Lysate

B.

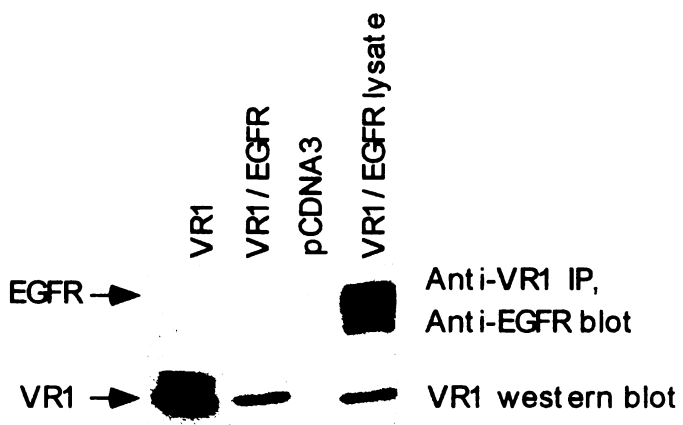

VR1
VR1 / EGFR
pCDNA3
VR1 / EGFR lysate
EGFR
Anti-VR1 IP,
Anti-EGFR blot
VR1
VR1 western blot

Figure 7 VR1, TrkA, and PLC-γ form a signaling complex. **A.** HEK293 cells were transfected with the indicated cDNAs (top). Immunoprecipitates were formed with antibodies indicated at right and visualized on Western blots with antibodies indicated at left. Total lysates were also analyzed to demonstrate equivalent protein expression among samples. Note that TrkA is typically expressed as three differentially glycosylated isoforms migrating as 140, 110, and 80 kD species. **B.** VR1 does not form a complex with EGFR. HEK293 cells were transfected with indicated cDNAs and immune complexes formed using VR1 antiserum. Blots were probed with EGFR antibody. Rightmost lane contains lysate from cells co-expressing VR1 and EGFR. Lower panel shows VR1 expression in total cell lysates.

Figure 8

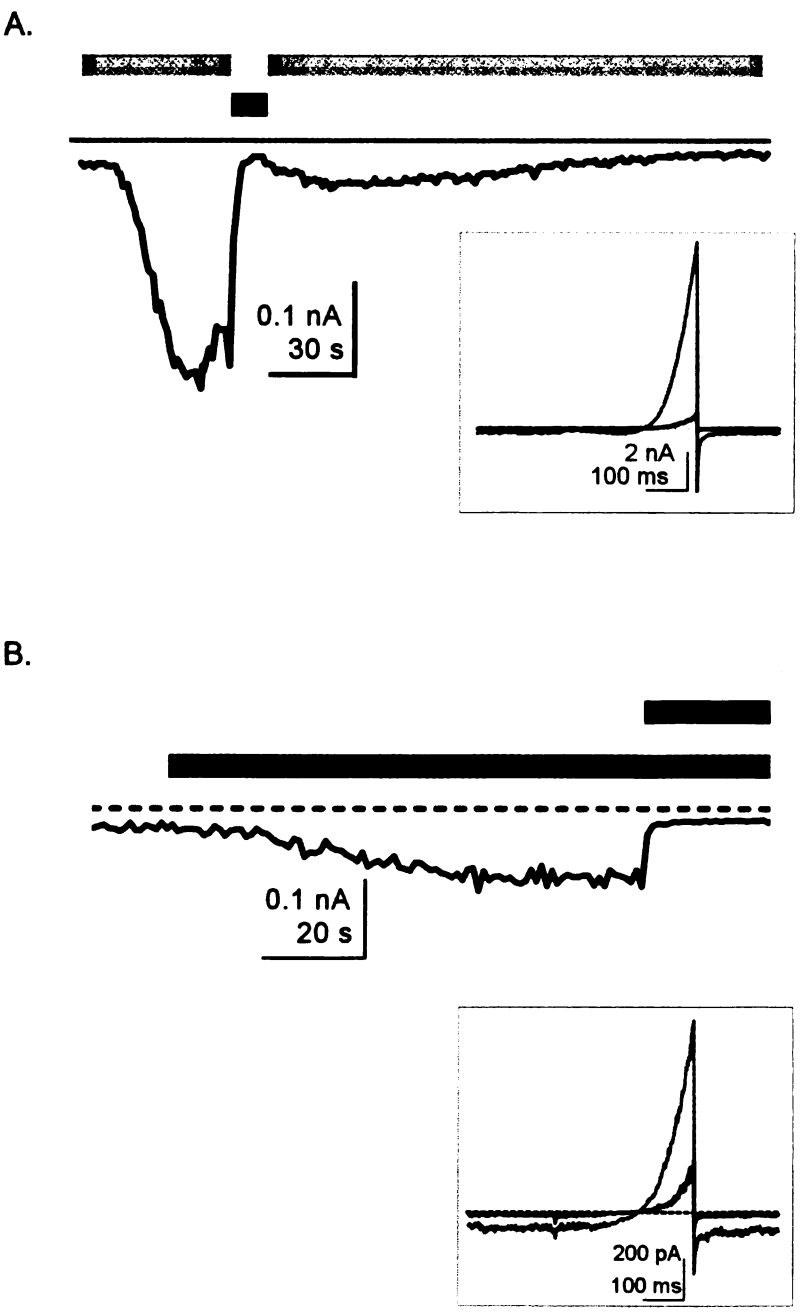


Figure 8 A. In transfected HEK cells, BK (20 nM, grey bar) elicits a strongly rectifying VR1 basal current that is inhibited by 5 mM capsazepine (black bar), as shown by the holding current trace and the ramp traces (inset, black trace represents current blocked by capsazepine) **B.** PIP2 Ab-induced VR1 basal current (-60 mV, gray bar) is suppressed by 3 mM capsazepine (black bar); representative ramp traces at inset (black trace represents current blocked by capsazepine.)

Figure 9

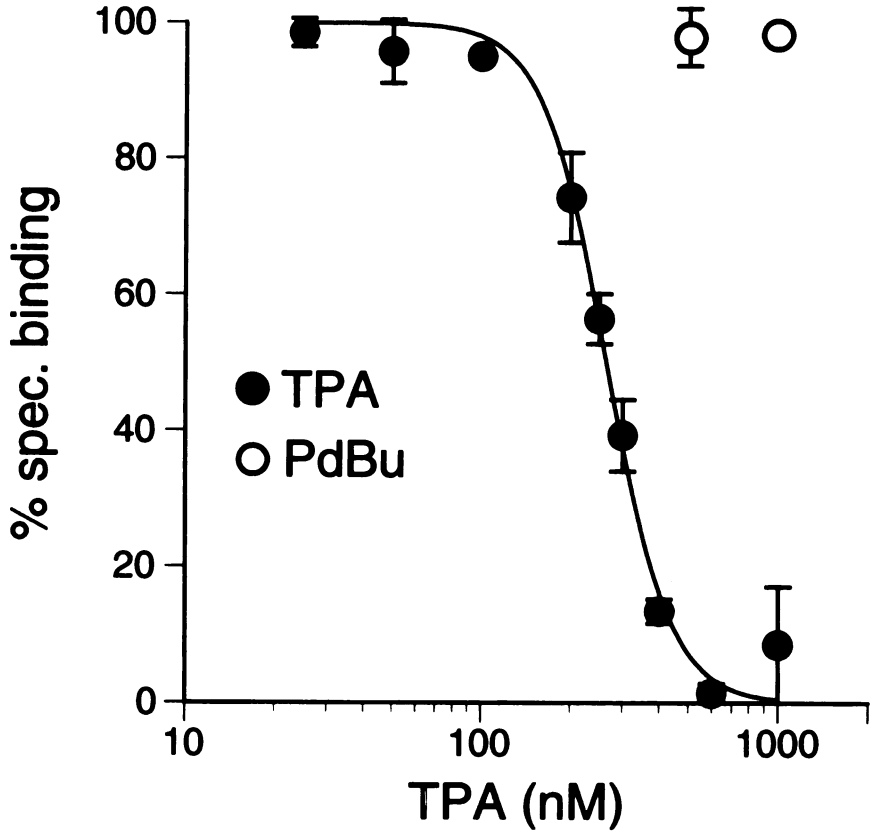


Figure 9 [^{3}H]-RTX binding to VR1 and displacement by TPA. Data are plotted as percentage of specific [^{3}H]-RTX binding. IC_{50} for TPA = 256 ± 8 nM (average of four independent experiments; each point in duplicate). Note that phorbol-12,13-dibutyrate (PDBu) did not affect [^{3}H]-RTX binding. Data were fit to the Hill equation

Figure 10

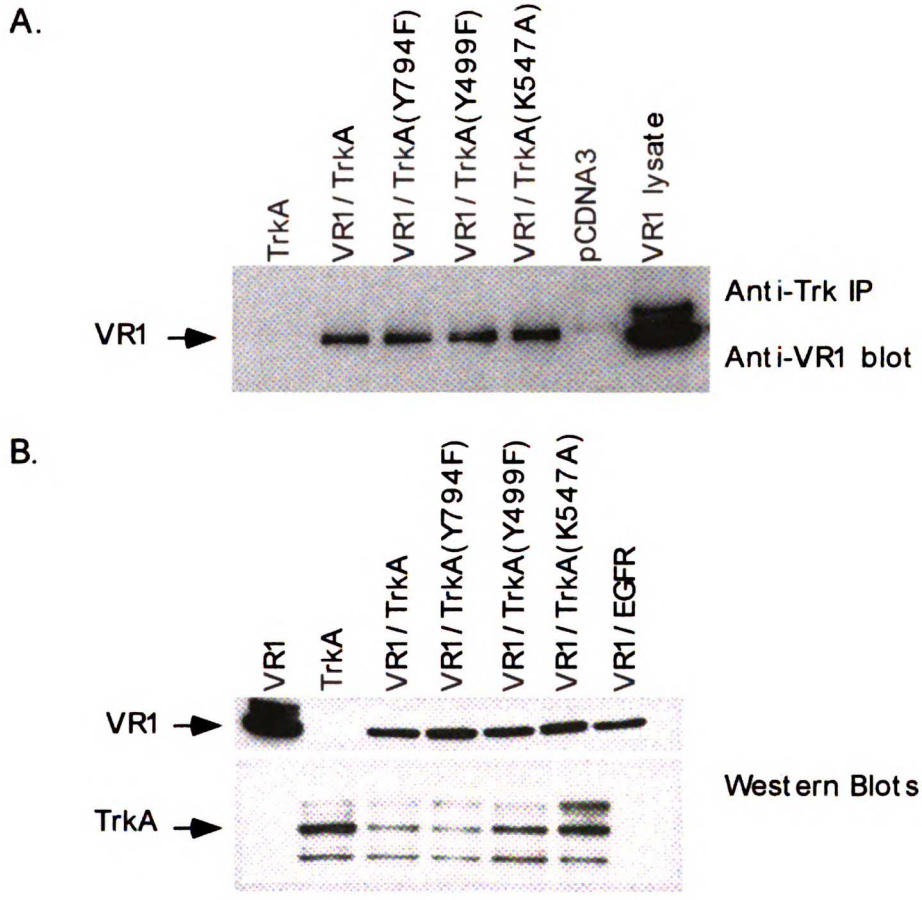

A.
TrkA
VR1 / TrkA
VR1 / TrkA(Y794F)
VR1 / TrkA(Y499F)
VR1 / TrkA(K547A)
pCDNA3
VR1 lysate
VR1
Anti-Trk IP
Anti-VR1 blot
B.
VR1
TrkA
VR1 / TrkA
VR1 / TrkA(Y794F)
VR1 / TrkA(Y499F)
VR1 / TrkA(K547A)
VR1 / EGFR
VR1
TrkA
Western Blots

Figure 10 VR1 associates with wild type TrkA and TrkA mutants. HEK cells were transfected with equal amounts of VR1 and TrkA or TrkA mutant cDNAs. **A.** Immune complexes were formed and analyzed as described in text. **B.** Western blots of 40 ug total soluble HEK protein indicate equal expression levels of VR1 and Trks among samples.

Figure 11

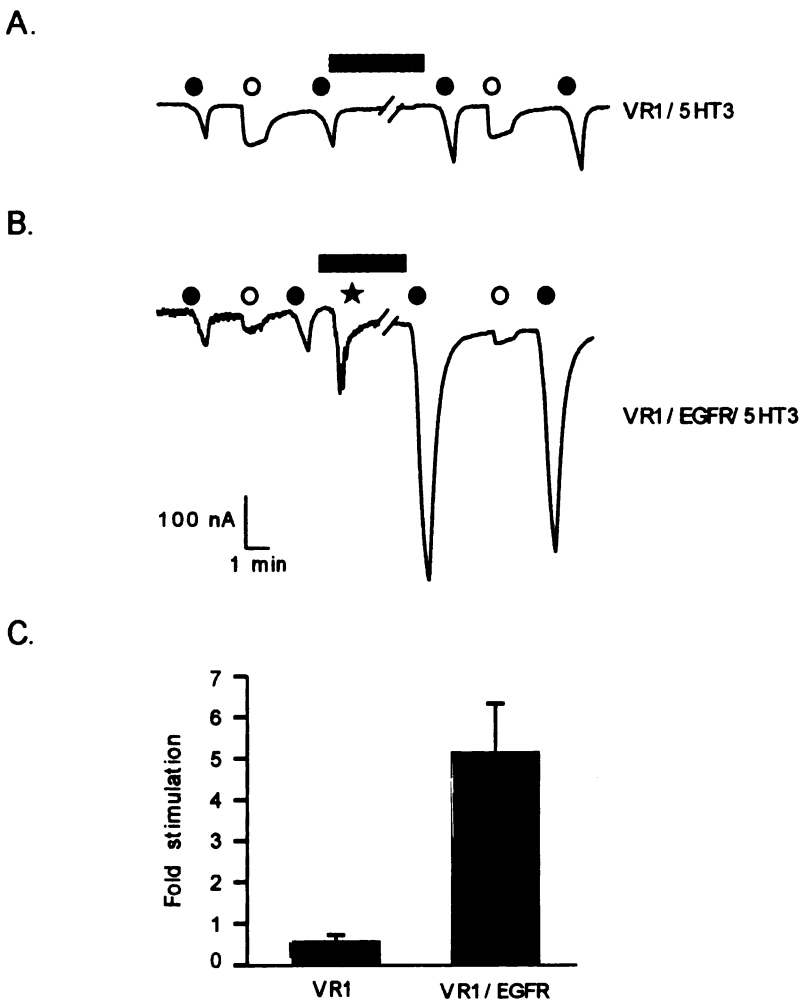

A.
VR1 / 5HT3
B.
VR1 / EGFR/ 5HT3
100 nA
1 min
C.
Fold stimulation
0
1
2
3
4
5
6
7
VR1
VR1 / EGFR

Figure 11 Effects of EGFR activation on VR1 currents. Two-electrode voltage clamp analysis was performed as described in text. Representative traces are shown in **A** and **B**, and quantitation of 5 VR1 injected and 8 VR1/EGFR injected oocytes (shown in **C** as SEM), $p < 0.005$. Note calcium activated chloride current (denoted with star) indicative of EGFR stimulation and consequent PLC activation.

Chapter 3: A Modular PIP_2 Binding Site as a Determinant of Capsaicin Receptor Sensitivity

The capsaicin receptor (TRPV1), a heat-activated ion channel of the pain pathway, is sensitized by phosphatidylinositol-4,5-bisphosphate (PIP2) hydrolysis after phospholipase C activation. We identify a site within the C-terminal domain of TRPV1 that is required for PIP2-mediated inhibition of channel gating. Mutations that weaken PIP2-TRPV1 interaction reduce thresholds for chemical or thermal stimuli, whereas TRPV1 channels in which this region is replaced with a lipid-binding domain from PIP2-activated potassium channels remain inhibited by PIP2. The PIP2-interaction domain therefore serves as a critical determinant of thermal threshold and dynamic sensitivity range, tuning TRPV1, and thus the sensory neuron, to appropriately detect heat under normal or pathophysiological conditions.

Introduction

Charged membrane phospholipids are thought to regulate a variety of ion channels and transporters (Hilgemann et al., 2001). For example, recent electrophysiological studies suggest a role for the membrane phospholipid PIP_2 as a modulator of transient receptor potential (TRP) channels, many of which contribute to the detection of sensory stimuli. TRP channels in the *Drosophila* eye (Hardie, 2003) and TRPV1 channels in the mammalian pain pathway (Chuang et al., 2001; Tominaga et al., 2001) are activated or potentiated when PIP_2 is hydrolyzed, whereas the ubiquitously expressed mammalian TRPM7 channel is inhibited by PIP_2 cleavage (Runnels et al., 2002). Phospholipase C catalyzes the hydrolysis of PIP_2 to inositol trisphosphate (IP_3) and diacylglycerol (DAG) and has been implicated in the release of TRPV1 from PIP_2-mediated inhibition (Chuang et al., 2001), although the underlying mechanism for such regulation has not been elucidated. Despite functional evidence that TRP channels are directly regulated by PIP_2, there is now no structural basis to account for this effect. TRPV1 is an especially tractable model for addressing this question because it can be directly gated by a number of stimuli, including the pungent vanilloid compound capsaicin, extracellular protons (pH<6.0), or noxious heat (>43 C) (Caterina et al., 1997; Tominaga et al., 1998). Moreover, genetic studies have shown that TRPV1 is an essential component of the signaling pathway through which PLC-coupled receptors increase behavioral sensitivity to heat (Caterina et al., 2000; Chuang et al., 2001; Davis et al., 2000), making elucidation of this regulatory pathway of physiological interest.

Results

To investigate the molecular basis of PIP_2-dependent regulation, we determined which regions of TRPV1 were required for PLC-mediated potentiation. We reasoned that mutations affecting such domains should render TRPV1 insensitive to PIP_2 inhibition, and therefore, we identified mutants exhibiting increased responses to capsaicin or extracellular protons. A TRPV1 mutant lacking a segment of the C-terminal cytoplasmic domain (TRPV1 Δ777-820) produced much larger currents compared to wild type channels in response to low doses of capsaicin (250nM) or protons (pH 5.5) when expressed in Xenopus oocytes (Fig. 12). These enhanced currents could not be attributed to increased cell surface expression because biotinylation experiments revealed equivalent levels of the mutant and wild type TRPV1 at the plasma membrane (Fig. 15B). To determine whether amino acids 777-820 were also required for PLC-mediated potentiation, we exposed oocytes expressing TRPV1 channels and the nerve growth factor (NGF) receptor TrkA/p75 to NGF, a treatment that normally elicits a robust potentiation wild type TRPV (~20-fold) of responses through activation of PLC-γ (Chuang et al., 2001). Currents from TRPV1Δ777-820 were unchanged, which suggests that these residues are critical for mediating potentiation downstream of PLC-coupled receptor stimulation. Moreover, potentiation of TRPV1Δ777-820 was not observed when other PLC-coupled receptors were activated, including the epidermal growth factor receptor (EGFR) and the G protein-coupled m1 muscarinic acetylcholine receptor . Loss of potentiation was not simply due to increased agonist sensitivity of the mutant channel because the response to either a low dose of capsaicin (100nM) or protons (pH 6.1) was unchanged after PLC activation (data not shown). In addition, the

thermal threshold of TRPV1Δ777-820 was markedly shifted to lower temperatures, and the overall currents were larger than those of wild type channels (Fig. 12D), a phenotype reminiscent of wild type channels that have been potentiated by PLC coupled receptor activation (Chuang et al., 2001).

If potentiation of TRPV1 involves release from PIP_2-mediated inhibition, then regions required for potentiation are potential sites of PIP_2 interaction. PIP_2 binding domains of ion channels are loosely characterized by clusters of basic residues interspersed with hydrophobic amino acids, an arrangement that is believed to facilitate interactions with the negatively charged head group of the phospholipid (Hilgemann et al., 2001). In agreement with this, the region 777-820 of TRPV1 has a predicted isoelectric point (pI) of 11 and contains 8 positively charged residues. To determine which amino acids are most important for mediating potentiation, we generated deletion and point mutations within this region. Removal of as few as 16 residues (777-792) completely inhibited potentiation (Fig. 13A, B). Furthermore, neutralization of as few as two positive charges reduced potentiation by 94±1.1% (Fig. 13B), which suggests that clusters of basic residues are important for mediating TRPV1 potentiation. These mutants not only exhibited increased sensitivity to chemical stimuli, but were also sensitized to heat, as indicated by a shift of activation thresholds to substantially lower temperatures and production of larger currents at supra-threshold temperatures (Fig. 14A, B). Moreover, as observed for capsaicin- and proton-evoked responses, larger deletions within the 777-820 domain had a more profound phenotype (as indicated by a greater shift in thermal

threshold) than small deletions or point mutations, which suggests that residues affecting activation threshold and potentiation are distributed throughout this region.

The most well characterized PIP_2-binding domains in channels are those of the inward rectifier potassium (IRK) channels. All members of this family are thought to be positively regulated by PIP_2 interaction (Hilgemann et al., 2001), although binding affinities differ among family members (Huang et al., 1998; Zhang et al., 1999). Even though PIP_2 activates IRKs and inhibits TRPV1, are regulated in opposite directions by PIP_2, we asked whether potentiation could be restored to TRPV1Δ777-820 by inserting a region from an IRK family member. Chimeras of TRPV1 were therefore generated in which amino acids 777-820 were replaced with residues 207-244 from the cytoplasmic C-terminal tail of IRK1 (Fig. 15A), a region shown to be important for PIP_2-dependent activity of this channel (Zhang et al., 1999). The resultant chimera (TRPV1.IRK) was responsive to capsaicin, protons, and heat, but showed relatively small currents compared with those of wild-type TRPV1 (29.4±3.5% for pH 5.5) (data not shown), even though both channels were expressed at comparable levels as determined by cell surface biotinylation (Fig. 15B). This apparent decrease in activity was accompanied by a marked rightward shift in half-maximal effective concentration for capsaicin (EC_{50}= 3.0±0.2 μM for chimera and 0.42±0.03 μM for wild-type TRPV1, Fig. 15C), which suggests that the PIP_2-binding domain from IRK1 enhances interaction of the channel with phospholipids, and thus renders it more avidly inhibited than wild-type TRPV1.

PLC-mediated modulation of IRK1 is nearly undetectable in Xenopus oocytes (Kobrinsky et al., 2000), presumably reflecting the relatively high affinity of this channel for PIP_2, and our initial attempts to potentiate the TRPV1.IRK chimera were similarly unsuccessful. We therefore asked whether the presumed high affinity of this chimera for PIP_2 could be overcome, either by lowering phospholipid availability or weakening PIP_2 affinity. Phenylarsine oxide (PAO), is a cell permeable, reversible inhibitor of phosphatidylinositol 4-kinase (PI4-K) that limits PIP_2 resynthesis (Wiedemann et al., 1996). Treatment of TRPV1-expressing oocytes with PAO produced potentiation of proton-evoked currents and subsequent activation of TrkA receptors had minimal additional effect on channel activity (Fig. 16A). To further decrease PIP_2 levels, we combined PAO treatment with activation of EGFR. Unlike TrkA, this PLC-coupled receptor can be expressed at high levels in oocytes without toxicity, allowing for reliably robust ligand-dependent cleavage of PIP_2 (data not shown). Cells expressing wild type or TRPV1.IRK channels showed significant potentiation (11.3±2.5 and 4.2±0.8 fold, respectively) when treated with EGF and PAO for 5 minutes, whereas TRPV1Δ777-820 showed no potentiation (0.75±0.02 fold) (Fig. 16B). These results demonstrate that the PIP_2 binding domain from IRK1 can substitute for residues 777-820 of TRPV1 and support potentiation, so long as PIP_2 levels are sufficiently diminished.

To structurally weaken PIP_2-channel interactions, we generated a TRPV1.IRK1 chimera harboring a mutation (R218Q) that in the context of IRK1 produces a channel with faster kinetics of inhibition, suggesting a decreased affinity for PIP_2 (Zhang et al., 1999). This chimera, (TRPV1.IRK R218Q) showed significantly greater potentiation than

TRPV1.IRK, and was indistinguishable from wild type TRPV1 in this regard (Fig. 16C). Furthermore, the capsaicin dose response curve for TRPV1.IRK R218Q was closer to that obtained with wild-type TRPV1 channels (Fig. 15C), consistent with a return to a milder form of PIP_2-mediated inhibition compared to TRPV1.IRK. Furthermore, consistent with their relative EC_{50} values for capsaicin, TRPV1.IRK-mediated currents were activated only at very high temperatures (48±0.4°C), whereas the chimera with weakened PIP_2 binding affinity (TRPV1.IRK R218Q) had a thermal threshold (44±0.3°C) closer to that of wild type TRPV1 channels (44°C) (Fig. 17A,B).

Several reports have recently suggested that potentiation of TRPV1 is achieved through PKC-dependent channel phosphorylation (Premkumar and Ahern, 2000; Vellani et al., 2001a). Moreover, two putative phosphorylation sites on TRPV1 have been identified and mutations at these residues abolish phorbol ester-mediated potentiation of capsaicin-evoked currents (Numazaki et al., 2002). One of these sites, S800, is contained within the region we implicate in PIP_2-mediated inhibition and we therefore asked whether phosphorylation at this site is required for receptor tyrosine kinase-mediated potentiation. Accordingly, we mutated S800 and found that this mutant (TRPV1 S800A) displayed normal capsaicin-evoked currents and normal EGFR-mediated potentiation (6.6±1.2 fold-for wild-type TRPV1 and 5.1±0.5-fold for S800A, Fig. 18A). TrkA activation also produced robust potentiation of both wild type and S800A channels, although a statistically significant difference was observed in the absolute magnitude of the effect (17.1±6.1 fold for wild-type and 8.6±0.5 for S800A, Fig. 18B). Thus, phosphorylation at S800 is not absolutely required for EGFR- or TrkA-mediated potentiation. Additionally,

phosphorylation by PKC probably does not contribute to potentiation of the TRPV1.IRK chimeras, because the PIP_2 binding domain from IRK1 contains no PKC consensus sites, yet supports significant receptor-mediated potentiation in the context of TRPV1. Indeed, robust potentiation can also be achieved simply by inhibition of PIP_2 synthesis, independent of either PLC or PKC activation (Fig. 16A). However, the reduced level of TrkA-mediated sensitization observed with the S800A mutant raises the possibility that phosphorylation can modulate potentiation efficacy, perhaps by increasing negative charge density at this site and weakening electrostatic interactions between PIP_2 and the channel. Alternatively, we have shown that TrkA (but not EGFR) forms a complex with TRPV1 and PLC-γ (Chuang et al., 2001), and thus the reduced sensitization of the S800A mutant by NGF could also reflect a decreased physical interaction and efficiency of local PIP_2 hydrolysis.

Discussion

Our findings provide a structural basis to support the hypothesis that PLC-mediated regulation of TRP channels involves the removal of PIP_2 from a binding site on the channel protein. Although channel-PIP_2 binding domains exhibit little linear sequence homology, our studies suggest that they function as modular regulatory domains, mediating inhibition or activation by phospholipids depending on their channel context. Several TRP channels can be directly activated in vitro by DAG or other polyunsaturated lipids, such as anandamide in the case of TRPV1(Hardie, 2003; Hofmann et al., 1999; Zygmunt et al., 1999). These lipid second messengers could activate or modulate TRP channels by displacing PIP_2 from an inhibitory binding site on the channel complex.

However, capsaicin and anandamide sensitivity map to a domain which is distinct from the C-terminal domain that we identify here as being important for PLC modulation (Jordt and Julius, 2002). Furthermore, TRPV1 mutants that loose the ability to be potentiated retain capsaicin sensitivity, which suggests that PIP_2 and unsaturated lipid agonists interact with TRP channels at separable sites, at least in the case of TRPV1.

The capsaicin receptor TRPV1 is normally activated with a threshold that corresponds to the onset of tissue damage and pain (~43 C) (Caterina et al., 1997). Our studies suggest that this physiologically crucial set point is determined, at least in part, by the strength of TRPV1-PIP_2 interactions. If this interaction is too strong, then the activation threshold would be too high to serve as an effective warning mechanism, as is illustrated by the VR1.IRK chimera. The more tempered affinity of TRPV1 for PIP2 not only establishes a physiologically relevant activation threshold, but also enables the channel to be dynamically modulated by inflammatory products that activate PLC. Finally, it is interesting to note that the C-terminal domain of TRPV3, a warm-sensitive channel with an activation threshold of ~35°C (Peier et al., 2002; Smith et al., 2002; Xu et al., 2002), conspicuously lacks a region corresponding to the 777-792 domain of TRPV1, the minimal essential core of the predicted PIP_2 binding site. Thus, modification of this PIP_2 regulatory domain by genetic, biochemical, or pharmacological mechanisms may have profound effects on sensitivity of primary afferent nerve fibers to chemical and thermal stimuli under normal or pathological conditions.

Methods

Molecular biology. Mutations of TRPV1 were made using oligonucleotide directed mutagenesis according to the methods described by Kunkel (Kunkel et al., 1991). Chimeras with rTRPV1 and mIRKs were made using standard polymerase chain reaction based methods.

Oocyte electrophysiology. Defolliculated stage VI *Xenopus laevis* oocytes were injected with 0.1–1.0 ng rat TRPV1 or TRPV1 mutants, rat TrkA, rat p75, or human EGFR cRNA. Oocytes were maintained in Modified Barth's saline solution for 4 to 6 days before analysis. Two-electrode voltage-clamp analysis was performed using a Geneclamp 500 amplifier (Axon Institute) and MacLab A/D converter (E_{hold} = –45 mV). ND-96 (with Ba^{2+}) recording solution was buffered with 10 mM Na-MES (pH 5.5) or 10 mM Na-Hepes (pH 7.5), and bath chamber perfused at ~1 ml/min. Temperature ramps were generated as described (Tominaga et al., 1998) or using continuous temperature feedback Peltier system (Reid et al., 2002). Recombinant rat NGF-®, human EGF (Sigma) and phenylarsine oxide (Calbiochem) were diluted into perfusate to concentrations indicated. Temperature response thresholds (Fig. 17) were determined as follows: a single line representing the steady basal current for each oocyte was determined. A second line representing a 20° shift in the baseline current was next determined, and point at which the second line was tangential to the heat curve was defined as the temperature threshold. Using this protocol, the temperature threshold for each oocyte was determined and averaged. Statistical analysis was performed using the students T-test and compared chimeras to wild-type VR1. No statistical difference was seen between TRPV1 and TRPV1.IRK R218Q.

Cell surface biotinylation. Biotinylation was performed as described (Ennion and Evans, 2002). Briefly, oocytes expressing the indicated constructs (15 each construct) were incubated with 0.5 mg/ml sulfo- NHS-LC-biotin (Pierce) in ND96 recording buffer for 30 min at 17°C. Oocytes were rinsed in 5 changes of ND96 then homogenized through a p200 pipette tip in Buffer H (100 mM NaCl, 20 mM Tris-Cl pH 7.4, 1% Triton X-100, and protease inhibitor cocktail (COmplete Protease Inhibitors, Boehringer Manheim) in a volume of 40 ∝l per oocyte. Lysates were centrifuged at 16,000*g*, 2 min. Cleared lysate (10 ∝l) was reserved for whole-cell lysate sample. The remaining lysate was probed with 45 ∝l 50% streptavidin agarose slurry (Sigma) for 3 hours at 4°C. Beads were washed 5 times in Buffer H and separated along with whole-cell lysates on a 10% acrylamide gel using SDS-PAGE. After transfer of the proteins to PVDF membranes, western blotting using antibodies to the C-terminal and extracellular domains of TRPV1 was performed to visualize TRPV1 constructs. Immunoreactive protein bands were detected by enhanced chemiluminescence using ECL reagents (Amersham).

Acknowledgements

We thank R. Nicoll and D. Minor for many helpful suggestions and encouragement throughout this project, G. Reid for help with the Peltier system, and members of our lab for advice. This work was supported by pre-doctoral fellowships from the NSF and UCSF Chancellor's Fund (E.D.P.) and by the NIH (D.J.)

Figure 12

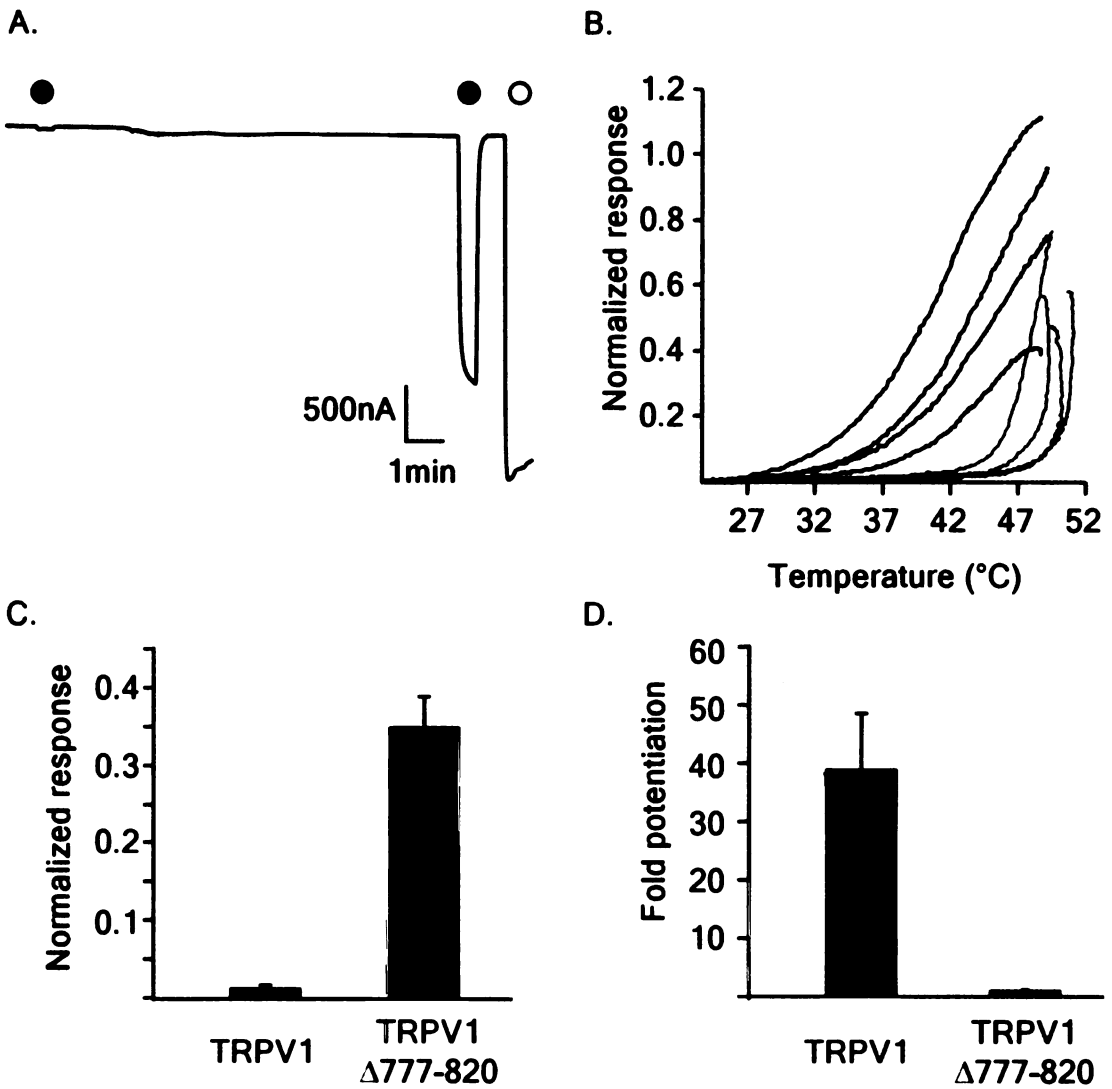
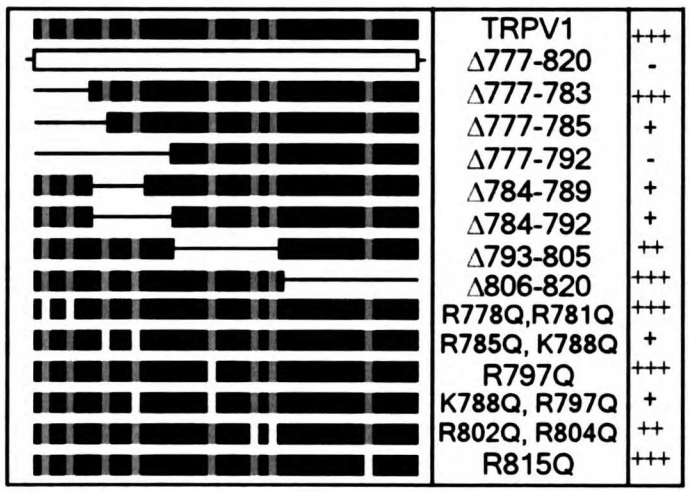

A.
500nA
1min
B.
Normalized response
1.2
1.0
0.8
0.6
0.4
0.2
27 32 37 42 47 52
Temperature (°C)
C.
Normalized response
0.4
0.3
0.2
0.1
TRPV1
TRPV1
Δ777-820
D.
Fold potentiation
60
50
40
30
20
10
TRPV1
TRPV1
Δ777-820

Figure 12. A C-terminal region of TRPV1 is required for PLC-mediated potentiation. **A** Continuous current recording from oocyte expressing TRPV1 and the NGF receptor TrkA/p75 NGF (*24*). Oocyte was exposed to pH 5.5 buffer for 30 seconds (black circles), followed by 750pM NGF for 10 minutes (grey bar.) After NGF treatment, oocyte was exposed to pH 5.5 again, followed by a saturating (10 uM) dose of capsaicin (open circle.) **B.** Heat-evoked currents in oocytes expressing TRPV1 (gray traces) or TRPV1Δ777-820 (black traces) were measured in responses to temperature ramps and normalized to a saturating dose of capsaicin (10 μM). **C.** Oocytes expressing indicated channel and TrkA/p75 were challenged with pH 5.5 buffer. Responses were normalized to current evoked with saturating dose of capsaicin (10 uM) according to the protocol indicated in Fig. 12A. **D.** Oocytes expressing indicated channels and TrkA/p75 were assayed for PLC-mediated potentiation. Results are measured as the fold increase in response to 30 sec. challenge with pH 5.5 buffer following 10 min treatment with 750pM NGF.

Figure 13

A.


TRPV1 +++
Δ777-820 -
Δ777-783 +++
Δ777-785 +
Δ777-792 -
Δ784-789 +
Δ784-792 +
Δ793-805 ++
Δ806-820 +++
R778Q,R781Q +++
R785Q, K788Q +
R797Q +++
K788Q, R797Q +
R802Q, R804Q ++
R815Q +++

B.

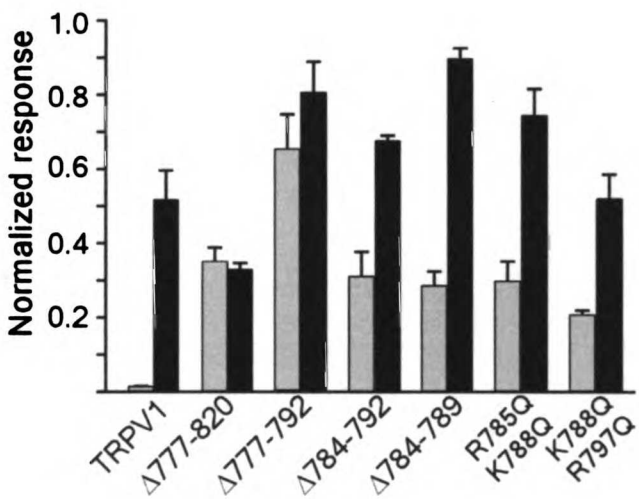

Normalized response
1.0
0.8
0.6
0.4
0.2
TRPV1
Δ777-820
Δ777-792
Δ784-792
Δ784-789
R785Q K788Q
K788Q R797Q

Figure 13. Delineation of a potentiation domain of TRPV1. **A** Representation of amino acid region 777-820 of rat TRPV1. Red boxes indicate basic residues; gray boxes indicate neutralized residues (K_Q or R_Q); lines indicate deletions. Relative degree of potentiation obtained with each mutant is indicated at right: (+++) maximal potentiation, (-) no potentiation. **B** Responses to a 30 second pulse of pH 5.5 buffer were measured before (grey bars) and after (black bars) exposure to 750 pM NGF for 10 minutes. Responses are normalized to maximal currents obtained with a saturating dose of capsaicin (10uM); n = 5 to 6 oocytes per construct. Potentiation is measured as the fold increase in response to 30 sec. challenge with pH 5.5 buffer following 10 min treatment with 750pM NGF: 39.0±9.5 (TRPV1), 0.94±0.9 (TRPV1Δ777-820), 1.24±0.1 (TRPV1Δ777-792), 2.45±0.3 (TRPV1Δ784-792), 3.17±0.5 (TRPV1Δ784-789), 2.51±0.4 (TRPV1 R785Q, K788Q), 2.61±0.5 (TRPV1 K788Q, R797Q)

Figure 14

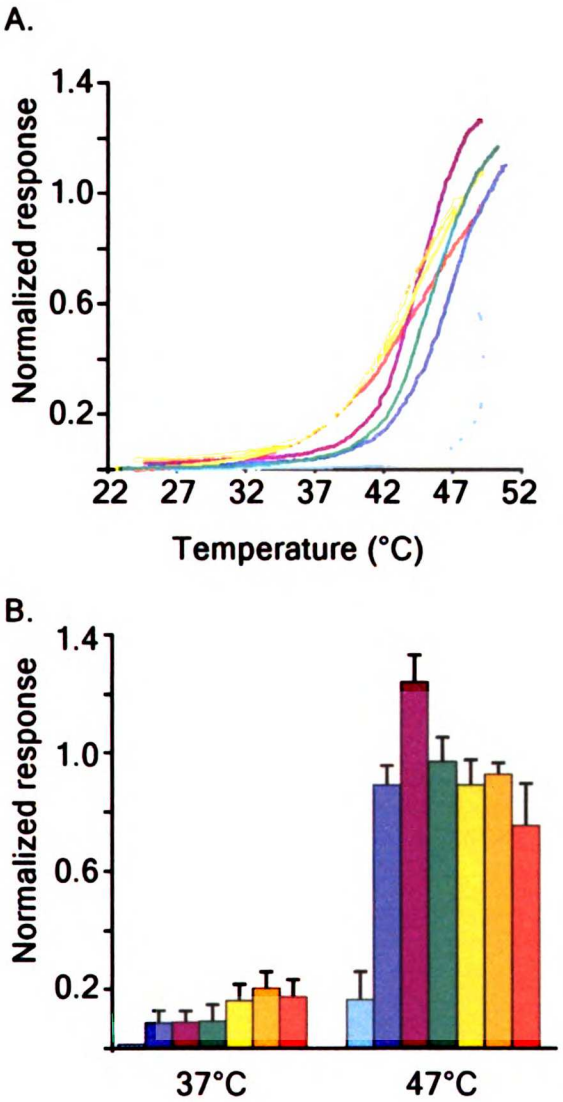
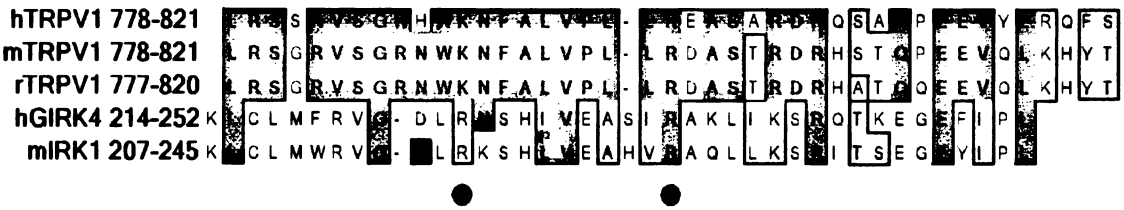

A.
Normalized response
1.4
1.0
0.6
0.2
22 27 32 37 42 47 52
Temperature (°C)
B.
Normalized response
1.4
1.0
0.6
0.2
37°C
47°C

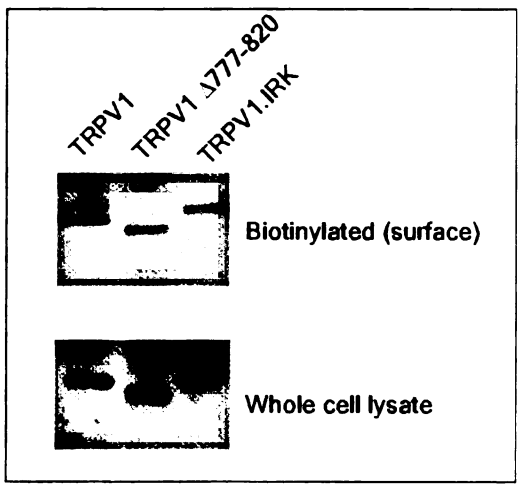
Figure 14. A Heat-evoked currents were measured in responses to temperature ramps and normalized to a saturating dose of capsaicin (10 uM). Traces are representative of responses from 4 to 5 oocytes per construct. Light blue (TRPV1), dark blue (TRPV1 R785Q, K788Q), purple (TRPV1Δ784-792), green (TRPV1 K788Q, R797Q), yellow (TRPV1 R785Q, K788Q), orange (TRPV1Δ777-792), and red (TRPV1Δ777-820). Average responses at subthreshold and suprathreshold temperatures are plotted in **B**. In all experiments, oocytes also expressed TrkA/p75.

Figure 15

A.



B.



C.

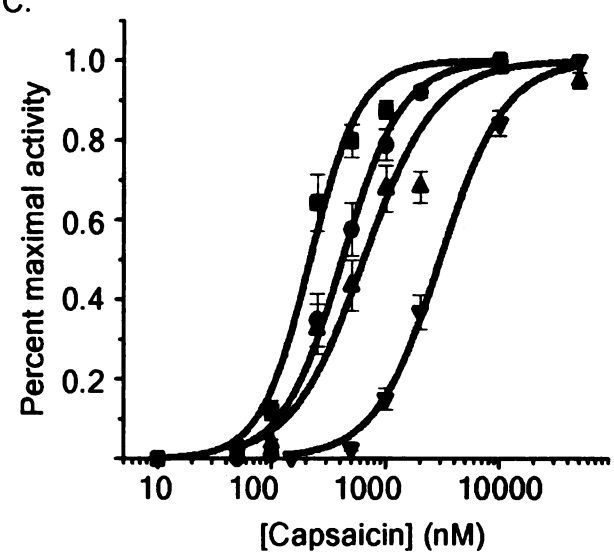
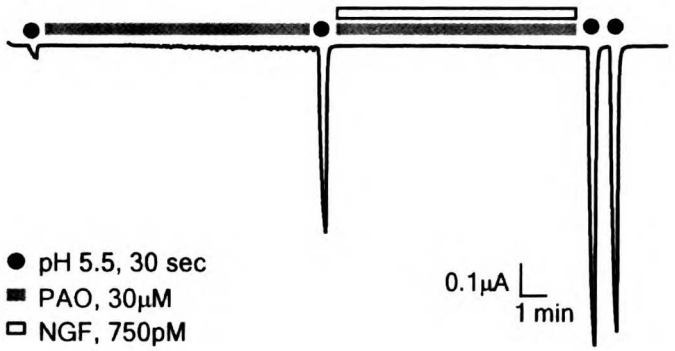


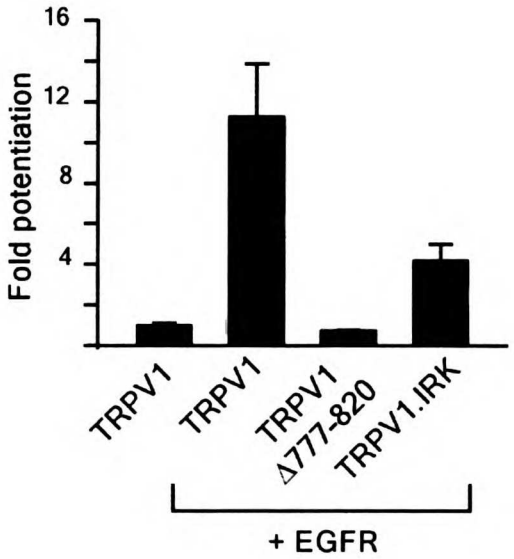
Figure 15. Functional conservation of a PIP_2 binding domain from distinct ion channel families. **A** Comparison of TRPV1 C-terminal regions with putative PIP_2 binding domains from IRK1 and GIRK4 (Huang et al., 1998; Zhang et al., 1999). Two basic residues required for PIP_2 interaction in IRK and GIRK are indicated with black circles. **B.** TRPV1, TRPV1Δ777-820, and TRPV1.IRK are equivalently expressed in oocytes. Oocytes were labeled with membrane impermeant sulpho-NHS-biotin Pierce for 30 min prior to lysis. Western blotting of whole cell lysates shows similar total expression levels for all three constructs in oocytes. Cell surface protein was immunoprecipitated with streptavidin-agarose and identified by Western blotting using anti-TRPV1 antiserum **C.** Agonist-response relationships for TRPV1 and mutants. Responses at each dose of capsaicin were normalized as percentage of maximal activity for each mutant. squares: TRPV1D777-820, circles: TRPV1, upright triangles: TRPV1.IRK R218Q, inverted triangles: TRPV1.IRK. Data were fit to the Hill equation, yielding the following hill coefficients: 1.68±0.18 (TRPV1, n = 8); 2.0±0.31 (TRPVΔ777-820, n = 11); 1.39±0.19 (TRPV1.IRK R218Q, n=8); 1.54±0.10 (TRPV1.IRK, n=5)

Figure 16

A.



B.



C.

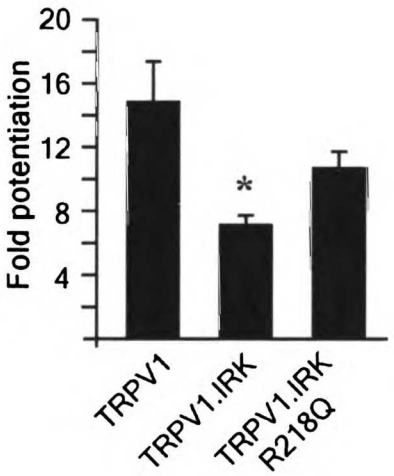
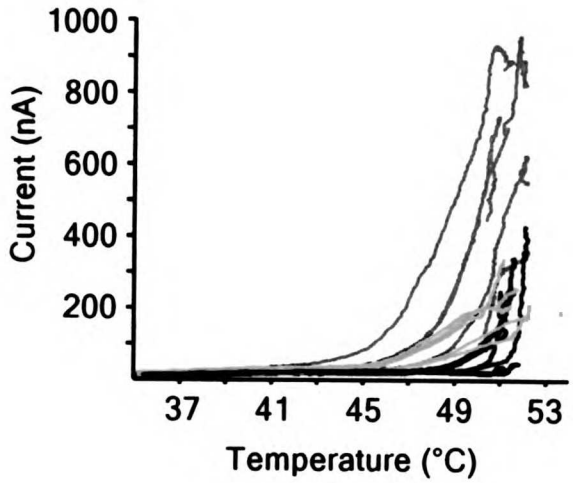


Figure 16 A. Inhibition of PI4-K enhances TRPV1-mediated currents. Representative voltage-clamp trace (E_{hold} = -45 mV) from a TRPV1-expressing oocyte challenged with pH 5.5 buffer (30 sec; black dots). Protons elicited a small current that was greatly enhanced following 10 min exposure to PAO (30uM, grey bars). Further treatment with NGF (750 pM, open bar) and PAO (30 uM, 10 min) produced only minimal further enhancement. **B**. Replacement of the C-terminal basic domain of TRPV1 with the PIP_2 binding site of IRK1 yields channels that can still be potentiated. Oocytes expressing constructs indicated at bottom (along with EGFR, unless indicated) were exposed to pH 5.5 buffer for 30 sec before and after 5 min treatment with EGF (50ng/ml) and PAO (15 uM). Potentiation represents the fold increase in current after treatment with EGF and PAO; n=5-8 oocytes per construct. **C**. PIP_2 binding affinity correlates with ability of chimeric channels to be potentiated. Oocytes expressing wild type or chimeric channels, together with EGFR, were exposed to pH 5.5 buffer for 30 sec before and after 5 min treatment with EGF (50 ng/ml) and PAO (15 uM). Potentiation represents the fold increase in current after treatment with EGF/PAO; n = 5 oocytes per construct. Asterisk indicates $p<0.02$ (student T-test). No statistical difference was seen between TRPV1 and TRPV1.IRK R218Q

Figure 17

A.


1000
800
600
400
200
Current (nA)
37
41
45
49
53
Temperature (°C)

B.

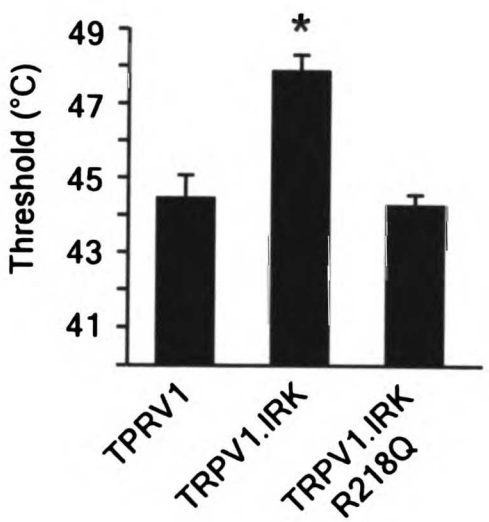

49
47
45
43
41
Threshold (°C)
*
TPRV1
TRPV1.IRK
TRPV1.IRK R218Q

Figure 17 PIP_2-binding affinity correlates with temperature sensitivity. **A.** Representative temperature-response profiles from oocytes expressing TRPV1 or TRPV1.IRK chimeras. Each trace represents a single oocyte expressing TRPV1 (blue), TRPV1.IRK (green), or TRPV1.IRK R218Q (yellow). Currents obtained from water-injected oocytes are indicated in black. .**B.** The average temperature response threshold for oocytes expressing TRPV1.IRK (47.9±0.4°C) was significantly higher than for those expressing wild-type TRPV1 (44.5±0.6°C, asterisk indicates $p<0.01$, students T-test, n = 5 oocytes per construct). No significant difference was seen between TRPV1 and TRPV1.IRK R218Q (44.3±0.3°C).

Figure 18

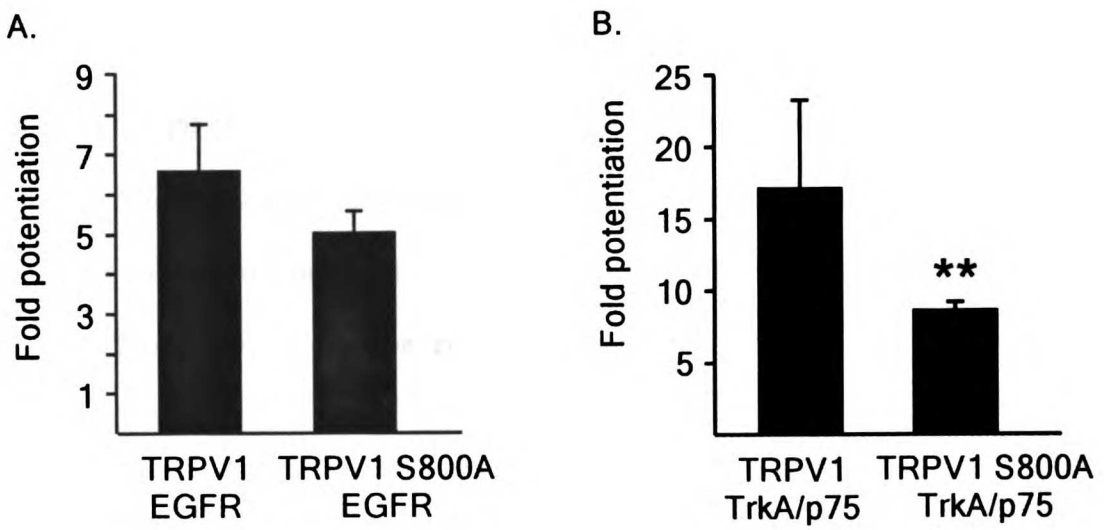

A.
Fold potentiation
9
7
5
3
1
TRPV1 EGFR
TRPV1 S800A EGFR
B.
Fold potentiation
25
20
15
10
5
**
TRPV1 TrkA/p75
TRPV1 S800A TrkA/p75

Figure 18 A. Mutation of a putative PKC phosphorylation site has no effect on EGFR-mediated potentiation. Oocytes expressing wild type or S800A mutant channels, together with EGFR, were exposed to capsaicin (250nM for 30 sec) before and after treatment with EGF (100ng/ml for 5 min). Potentiation represents the fold increase in current after treatment with EGF; n = 8 oocytes per construct. No significant difference was seen between TRPV1 and TRPV1S800A (student T-test). **B.** Oocytes expressing wild type or S800A mutant channels, together with TrkA/p75, were exposed to capsaicin (250nM for 30 sec) before and after treatment with NGF (750pM for 10 min). Potentiation represents the fold increase in current after treatment with NGF (double asterisk indicates $p<0.02$, student T-test, n = 9 oocytes per construct).

CHAPTER 4: Conclusions and Remaining Queries

Phospholipid gating of ion channels

TRP channels comprise a family of 6 transmembrane cation channels with wide distribution. Several TRP channels are thought to be integral to sensory perception and indeed have been shown to be of critical importance in the visual, auditory, gustatory, and nociceptive pathways. However, information regarding the gating of these interesting channels has been difficult to obtain. Drosophila TRP expresses poorly in heterologous systems, making standard structure function approaches difficult. Genetic and pharmacological evidence, however, suggests that TRP exists in a regulated complex that is scaffolded by the protein INAD. Other members of the phototransduction cascade are linked to TRP through interaction with INAD, including PLC and PKC. Genetic ablation of PKC does not lead to visual impairment, but rather to a modest defect in TRP channel desensitization. Moreover, TRP has been found to be activated in excised patches upon application of polyunsaturated lipids that mimic DAG. Taken together with the requirement of PLC activity for TRP channel activation, these observations have since led to a model of TRP channel gating that requires cleavage of PIP_2, which simultaneously relieves an inhibitor (PIP_2) and generates a lipid agonist (DAG) leading to full channel activation.

Regulation of channel activity by phospholipids is an emerging theme in channel biology. While the exact mechanism whereby PIP_2 interaction controls gating of IRK channels has yet to be determined, current work indicates that channel is not completely closed in the absence of PIP2, suggesting that at gating of the channel involves more than just lipid interaction (Xiao et al., 2003). Indeed, an interesting observation from our studies is that

the PIP_2 binding domain of IRK1 can foster channel inhibition in the context of TRPV1, as opposed to activation in the context of IRK1. Thus, channel gating determinants cannot be entirely comprised by this domain and depends on other aspects of channel structure. Certainly more extensive structural and biochemical analyses will shed light on the molecular determinants of channel gating by PIP_2 and its binding site(s).

Phospholipids and thermosensitive TRP channels

The putative TRPV1-PIP_2 interaction clearly plays an important role in setting the thermal threshold for activation. Given the similarity in the C-terminal region of TRPV3 to that of TRPV1, it is tempting to speculate that other thermosensitive TRP channels also rely on an interaction with phospholipids (or lack thereof) to modulate their thermal thresholds. For example, given its high thermal activation threshold and close similarity to TRPV1, it is reasonable to predict that TRPV2 harbors a particularly strong PIP_2 interaction domain. However, this channel is not regulated by PLC-coupled receptors (personal observations), and sequence alignment with TRPV1 reveals little similarity within their carboxy terminal domains. On the other hand, preliminary experiments with a chimeric TRPV3 bearing the "missing" PIP_2 binding domain suggests that insertion of this module yields a channel that can be modulated by PLC activation. The thermal activation threshold for this chimeric channel has not yet been examined, but it is predicted to be substantially higher than that of TRPV3. Further experiments to address the modular nature of the TRPV1 PIP2 binding domain are ongoing. It is also interesting to note that the recently described *Drosophila* protein Painless does appear to have a domain that could interact with PIP_2, insofar as it is comprised of a cluster of basic

residues interspersed with hydrophobics, in a configuration that resembles the PIP_2 binding domain of TRPV1 and IRK1. Whether or not this channel can be regulated downstream of PLC coupled receptors has not yet been tested. Finally, several experiments from this laboratory have indicated that the cold activated channel TRPM8 is not modulated downstream of PLC-coupled receptors, suggesting that PIP_2 regulation is likely not universal among TRP channels.

The role of PKC

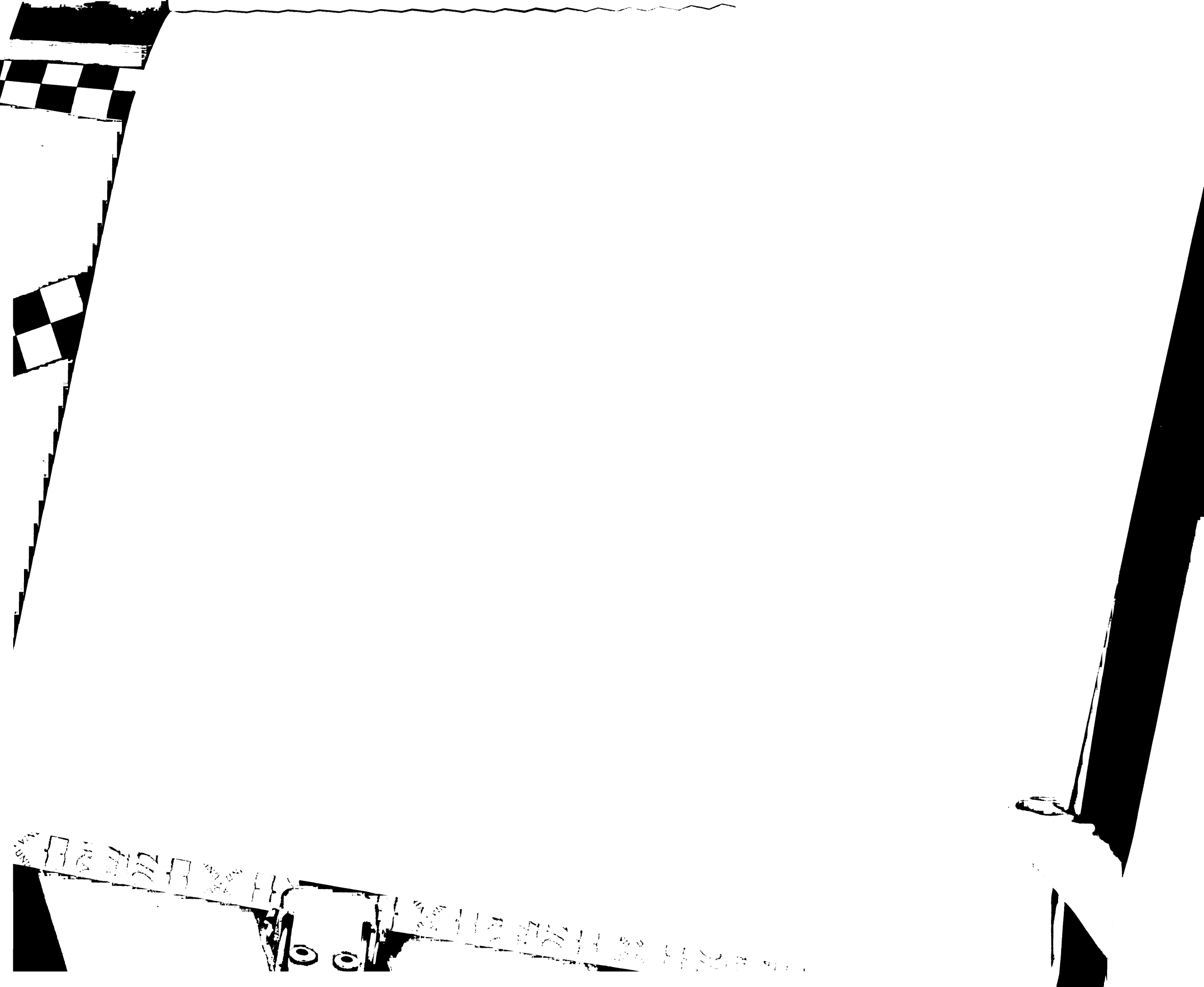
The use of phorbol esters has greatly enhanced the study of PKC dependent processes. These PKC activators are effective at very low concentrations, are cell permeable, and their effects can usually be inhibited with PKC inhibitors. The use of these compounds to study the effect of bradykinin and ATP on cultured neurons has led to the hypothesis that TRPV1 potentiation by inflammatory products relies on PKC activation and channel phosphorylation. In fact, two putative PKC phosphorylation sites on TRPV1 were recently identified in in vitro kinase assays. Mutation of these residues abolishes the ability of PMA to potentiate TRPV1 responses in cultured mammalian cells.

The PKC isoform that has been implicated in underlying thermal hypersensitivity following inflammation is PKCε. Mice that have been genetically targeted to not express this isoform of PKC show decreased thermal and mechanical hypersensitivity following epinephrine and acetic acid injection (Khasar et al., 1999). However, studies of bradykinin mediated hypersensitivity were not carried out by these investigators. Experiments with phorbol esters in this lab have yielded conflicting data. On the one

hand, PMA application to TRPV1 expressing oocytes leads to potentiation of capsaicin evoked responses at concentrations of PMA as low as 50nM (unpublished observations). Furthermore, a phorbol that is unable to activate PKC, 4α phorbol, does not potentiate capsaicin evoked responses. However, neither PMA mediated potentiation nor potentiation elicited by PLC activation could be blocked by PKC inhibitors, even at 200-fold molar excess. Furthermore, recent studies also suggest that PMA mediated activation of PKC leads to the depletion of PIP2 from cell membranes (Zeng et al., 2003). Taken with our observations that phorbol esters can interact with TRPV1-containing membranes (Chuang et al., 2001), these data indicate that the role of PKC in mediating TRPV1 potentiation is at the very least complex, and further experiments are required to determine whether PKC plays a role downstream of PLC coupled receptors in a physiological context, and not just upon maximal activation by pharmacological agents. It remains possible, for instance, that PKC effects modulation of channel activity by direct phosphorylation, and works in concert with PIP_2 cleavage following PLC coupled receptor activation. Phosphorylation of S800, which falls within the predicted PIP_2 binding domain of TRPV1, would alter the net charge of this region and weaken electrostatic interactions with the lipid head group of PIP_2. In this way, phosphorylation by PKC could shift the binding affinity of the channel for PIP_2, favoring disinhibition and channel potentiation. .

How is thermal threshold set?

Perhaps the most intriguing and difficult question remaining in TRPV1 biology is how the channel is gated by temperature. While work in this study has indicated that lipid



interaction is an important determinant of threshold, mutant channels that presumably do not interact with PIP_2 do not lack thermal sensitivity, as might be expected if PIP_2 binding were the only determinant of threshold. While these channels are activated at lower temperatures, they still are inactive at room temperature, and activate with a relatively steep slope at physiologically relevant temperatures. A logical hypothesis is that the channel is gated by fluctuations of membrane fluidity that occur as a result of temperature changes. Thus, conventional structure function analyses and even crystallographic analysis may not be sufficient to gain insight into this fascinating question. Of course, since there are several thermosensitive TRP channels, the use of chimeras is an appealing method to perhaps elucidate which part of the channel proper confers gating at a particular temperature. But these experiments are confounded by the propensity of chimeric channels to be completely inactive or not expressed at all. The answer to this question will likely take a combination of all of these techniques, and perhaps the development of novel techniques to assess the role of phospholipids and other membrane lipids in channel physiology.

CHAPTER 5: Methods

Molecular Biology

Mutagenesis

Point mutations, deletions, and insertions were made using the method of single stranded mutagenesis first described by Kunkel (Kunkel et al., 1991). Mutagenic oligonucleotides were allowed to anneal to the single stranded template, following second strand synthesis, heteroduplex plasmids were transformed into E. *coli* (DH5a). Restriction digest was employed to screen for mutation, and positive clones were confirmed by DNA sequencing. cDNAs were cloned into pCDNA3 or the combined oocyte/mammalian expression vector pFROG3 containing 5_- and 3_- untranslated regions of *Xenopus* _-globin (Jordt et al., 2000)

cRNA preparation

Copy RNA (cRNA) was prepared from linearized plasmid templates using in AmpliCap T7 kit (Epicentre). Approximately 1ug of linearized cRNA was used in each reaction. cRNA was quantified by agarose gel electrophoresis.

Electrophysiology

Xenopus laevis oocyte harvest

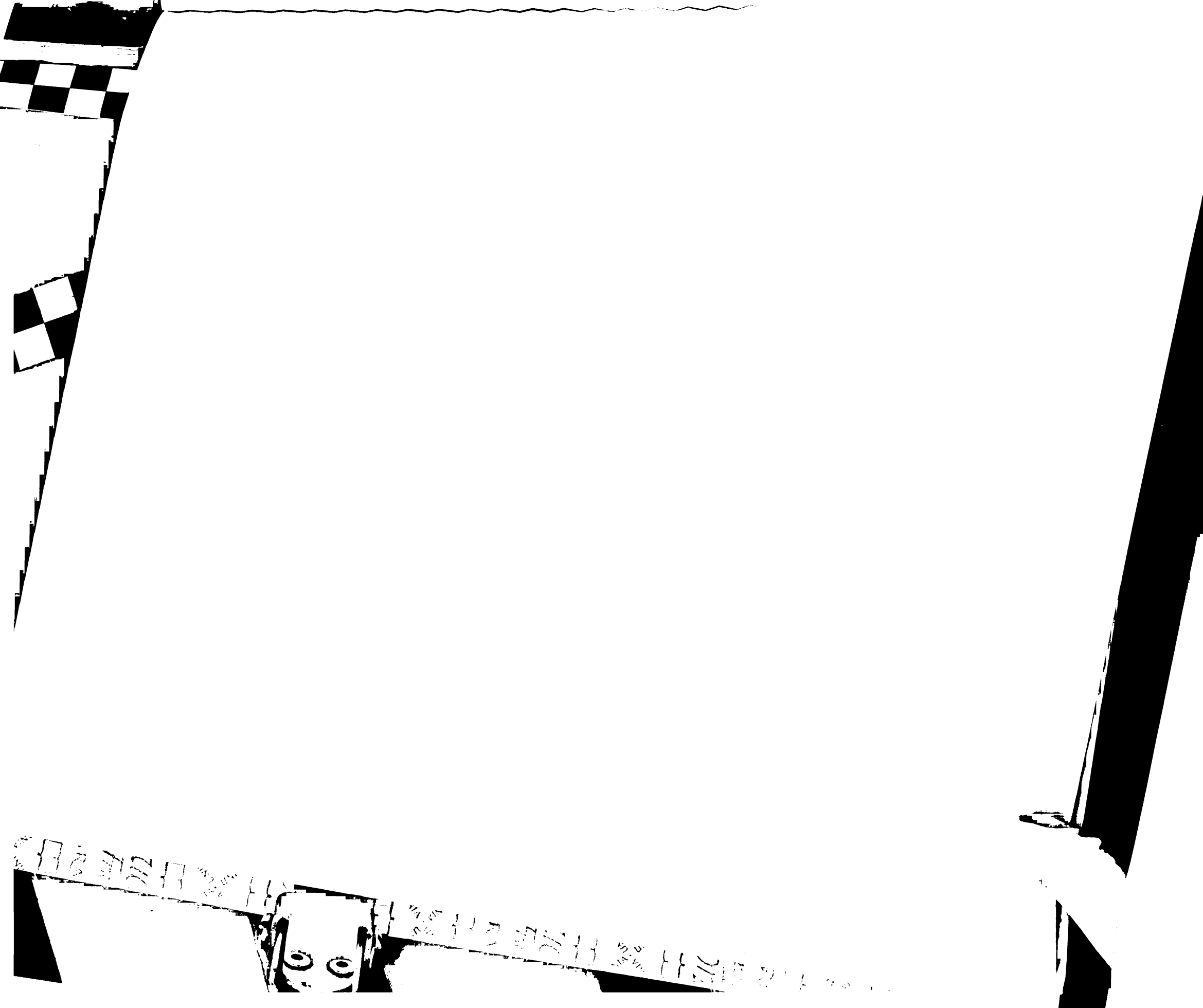
Female xenopus laevis frogs were obtained from either Nasco or Xenopus One suppliers. Animals were maintained in pond water. Prior to harvest, animals were anaesthetized using a solution of Tricaine (3-aminobenzoic acid ethyl ester methane sulfonate salt, 0.75g/L) and sodium bicarbonate (NaHCO3, 3.9 g/L) at room temperature. Anesthesia

was considered complete when animals failed to respond to toe pinch. Surgery was performed with the animal supine on ice. A small (2 cm) incision was made over the ovarian lobe. A similarly sized incision was made in the underlying muscle layer, exposing the ovarian lobe. Approximately one half of one lobe was removed per harvest. Non resorbable sutures were used to close the muscle and skin layer, and the animal allowed to recover in pond water. Upon full recovery from anesthesia, the animal was returned to the pond.

Oocyte preparation

Following harvest, oocytes were dissected into small clusters of approximately 10-20 oocytes each. Oocytes were washed one time in 5-10 volumes modified Barth's saline solution (MBSS: 88mM NaCl, 1mM KCl, 2.4mM NaHCO3, 0.82 mM MgSO4, 7.5mM Tris, 0.1% Ficoll, 2.5uM Na pyruvate, 100unit/mL penicillin, 100ug/mL streptomycin, 50ug/mL gentamycin). Oocytes were then digested with collagenase (Type 1, Worthington Biochemicals, 1-2mg/mL in MBSS) with rotation. Two one-hour digestions were typically necessary to separate oocytes from the ovarian lobe. Following collagenase treatment, oocytes were washed extensively (5x10 minutes) in MBSS with rotation. Oocytes were then defolliculated by gentle washing with MBSS+Ca^{2+} (MBSS supplemented with 330uM $Ca(NO_3)_2 \cdot 4H_2O$ and 410uM $CaCl_2 \cdot 2H_2O$) 3x30 min. Defolliculated oocytes were stored at 17°C in MBSS+ Ca^{2+} prior to injection.

Oocyte injection



Injection needles were pulled from Drummond glass capillary tubes (3.5", .53mm internal diameter) using a Narshige glass microelectrode puller and microforge. Needles were filled with sterile mineral oil, then backfilled using a Drummond nanoject microinjection device. Oocytes were injected with 40nL cRNA premix, consisting of cRNAs of interest diluted in RNAse free H_20. Oocytes were maintained in 6 well tissue culture dishes in MBSS+ Ca^{2+}.

Two-electrode voltage clamp recording

Two-electrode voltage-clamp analysis was performed using standard techniques 4-7 days following injection. Electrodes (Narshige glass capillaries, 90mm with filament) were pulled at 99°C and broken to achieve resistances of 0.5-1.0 MOhm. Oocytes membranes were clamped at –45mV and currents measured using a GeneClamp 500 amplifier (Axon Instruments). ND96 recording buffer (86 mM NaCl, 2mM KCl, 1mM MgCl2, 0.1mM BaCl2, 10mM Na HEPES (for buffer pH 7.5) or 10mM Na MES (for buffer pH 5.5)) was continuously perfused into the recording chamber using a ValveBank perfusion system (AutoMate Scientific). For heat experiments, solution temperature was controlled with an in-line SH-27A solution heater and TC-324B thermal controller (Warner Instruments). A thermistor was placed within 1 mm of the oocyte to record bath temperatures and regulate the heat controller. Data were collected using MacLab software (ADI instruments).

Biochemistry

Mammalian cell culture

Human embryonic kidney cells (HEK293) were maintained in DMEM cell culture medium, supplemented with 10% fetal bovine serum, penicillin/streptomycin, and glutamine. Cells were grown at 37°C in 5% CO2. One day prior to transfection, cells were split to 1-2x10^6 cells per 10cm/dish.

Transfection

Cells were transfected with the indicated constructs using Lipofectamine Plus reagent (LifeTechnologies) according to manufacturer's instructions. Cells were maintained 40-48 hours post transfection.

Lysate preparation

Prior to lysis, plates of transfected cells were washed once in cold phosphate buffered saline (PBS). 1mL cold lysis buffer (150mM NaCl, 10mM TrisCl pH 8.0, 1%NP-40, 1X protease inhibitor cocktail (CØmplete protease inhibitor cocktail, Boehringer Mannheim) was then added to each 10 cm plate and cells were scraped with a cell lifter. Cell slurry was then collected into an eppendorf tube and placed on ice for one hour. Cell lysate was then subjected to centrifugation at 20,000g for 10 minutes at 4°C. Cell supernatant was collected and the insoluble pellet was discarded. Protein quantity was determined using Commassie Plus reagent (Pierce) according to manufacturer's instructions.

Immunoprecipitations

Immunoprecipitation was performed using indicated antibodies. Antibody was added to cell supernatant at indicated concentrations and immune complexes were allowed to form at 4°C, 3h-overnight with gentle rocking. Approximately 20uL washed ProteinA-agarose beads (Sigma) were then added to each mg cell lysate/antibody mixture. Complexes were allowed to form for 3 hr at 4°C with gentle rocking. Beads were then pelleted by centrifugation at 1000g for 30 sec. Beads were washed with 500uL lysis buffer 5 times. Following the final wash step, beads were drained of buffer and an equal volume of 2x Laemli protein buffer and heated to 65°C for 10 minutes. Denatured supernatants were then subjected to SDS-PAGE and Western blotting.

Western blotting

Following electrophoresis, gels were transferred to PVDF membranes via semidry transfer. Blots were briefly stained with Ponceau S to identify protein standards and then blocked in Super Blotto buffer (500mM NaCl, 50mM Tris Cl pH 7.6, 1%NP-40, 3% nonfat dry milk) for 1 hour at room temperature with gentle agitation. Blots were typically incubated with primary antibody at the indicated concentrations in Super Blotto overnight at 4°C, although on some occasions primary antibody incubation was performed at room temperature for one hour with equivalent results. Following primary antibody incubation, blots were washed 3 times, 5 minutes each with Super Blotto. Secondary antibody incubation was also performed in Super Blotto for 1 hour at room temperature. Blots were washed again, 3x5 minutes. Immune complexes were detected upon incubation with enhanced chemiluminescence reagent (Amersham) according to the manufacturer's instructions.

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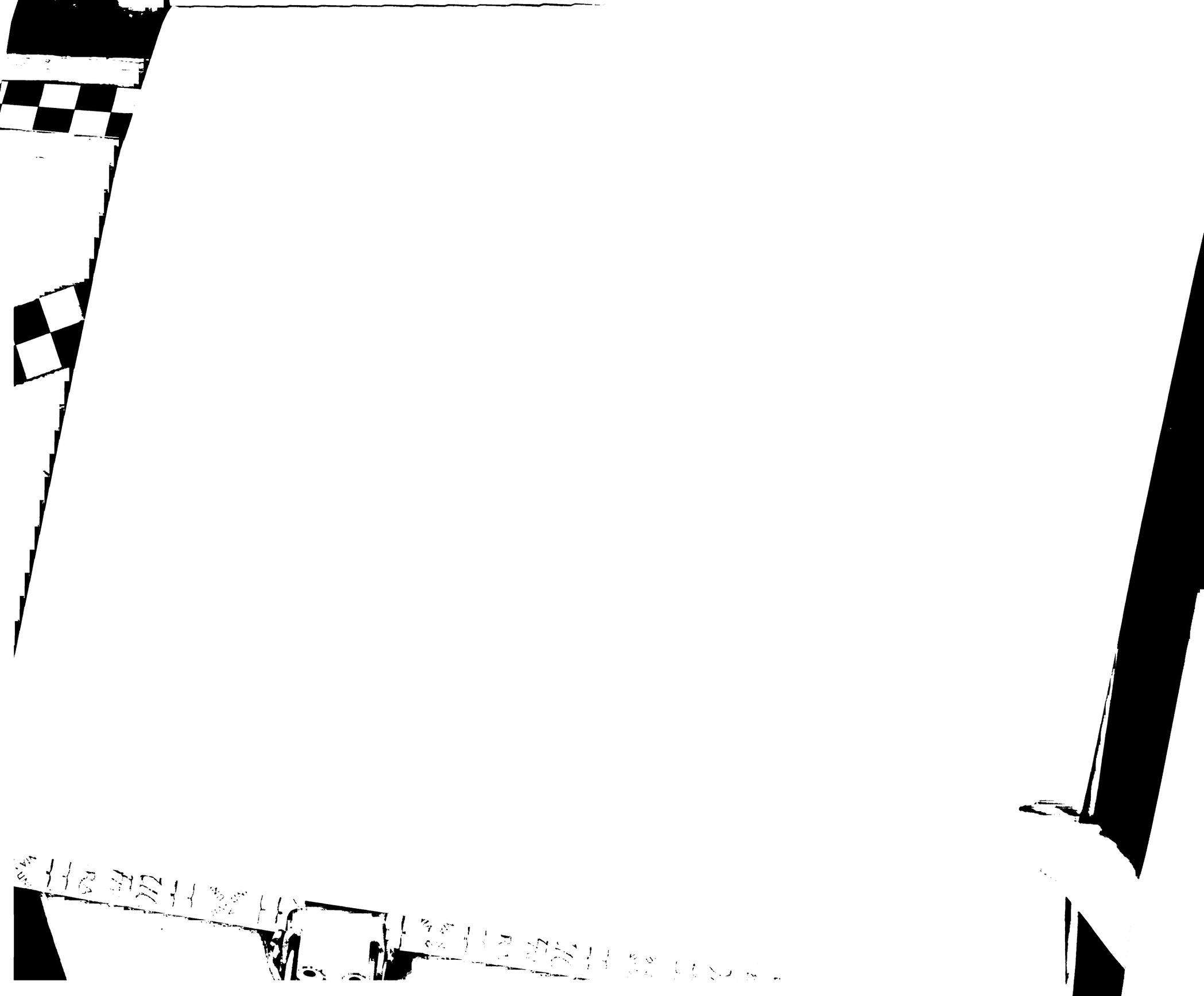

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