## Title

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# TRPV1 channels are intrinsically heat sensitive and negatively regulated by phosphoinositide lipids

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## Summary

The capsaicin receptor, TRPV1, is regulated by phosphatidylinositol-4,5-bisphosphate ($PIP_2$), although the precise nature of this effect (i.e., positive or negative) remains controversial. Here, we reconstitute purified TRPV1 into artificial liposomes, where it is gated robustly by capsaicin, protons, spider toxins and, notably, heat, demonstrating intrinsic sensitivity of the channel to both chemical and thermal stimuli. TRPV1 is fully functional in the absence of phosphoinositides, arguing against their proposed obligatory role in channel activation. Rather, introduction of various phosphoinositides, including $PIP_2$, PI4P and PI, inhibits TRPV1, supporting a model whereby phosphoinositide turnover contributes to thermal hyperalgesia by disinhibiting the channel. Using an orthogonal chemical strategy, we show that association of the TRPV1 C-terminus with the bilayer modulates channel gating, consistent with phylogenetic data implicating this domain as a key regulatory site for tuning stimulus sensitivity. Beyond TRPV1, these findings are relevant to understanding how membrane lipids modulate other “receptor-operated” TRP channels.

## Introduction

Phosphoinositide lipids function as important regulatory factors for many types of membrane ion channels and transporters, including members of the Transient Receptor Potential (TRP) channel superfamily (Falkenburger et al., 2010; Gamper and Rohacs, 2012; Hilgemann et al., 2001; Qin, 2007). TRP channels represent a large and unusually diverse family of non-selective cation channels (>30 subtypes in mammals) that respond to an astoundingly wide range of environmental and endogenous stimuli of a chemical and/or physical nature (Montell et al., 2002; Ramsey et al., 2006). Although quite divergent in both sequence and function, many TRP channels share the ability to be regulated by one or more consequences of phospholipase C (PLC) activation, including hydrolysis of phosphatidylinositol-4,5-bisphosphate ($PIP_2$), production of polyunsaturated fatty acids and other metabolites, or release of calcium from intracellular stores (Hardie, 2007). In this way, neurotransmitters or hormones that activate PLC-coupled receptors can activate TRP channels or enhance their sensitivity to other physiologic stimuli. Indeed, some TRP channels respond to multiple physiological inputs, enabling them to function as polymodal

signal integrators capable of assessing complex changes in the cell or tissue environment (Nilius, 2007; Tominaga et al., 1998).

TRP channels were initially discovered in the *Drosophila* eye, where they are activated downstream of PLC-coupled rhodopsin (Hardie, 2007; Minke, 2001; Montell, 2012). While it is widely agreed that PLC-mediated hydrolysis of membrane $PIP_2$ is an obligatory step in this signaling pathway, it remains unclear how turnover of phosphoinositides promotes TRP channel gating (Montell, 2012). Genetic and electrophysiologic studies suggest that channel activation involves a combinatorial process requiring both depletion of phosphoinositides and consequent generation of a phosphoinositide-derived second messenger(s), such as polyunsaturated fatty acids and/or intracellular protons (Estacion et al., 2001; Huang et al., 2010). According to this model, TRP channels in the fly eye are negatively regulated by $PIP_2$ (and possibly other phosphoinositides), such that PLC-mediated hydrolysis removes this inhibitory constraint and allows the channel to then be fully activated by simultaneously produced metabolites or other physiologic stimuli (Raghu and Hardie, 2009). However, recent studies using engineered, membrane anchored phosphatases suggest that $PIP_2$ depletion (through conversion to other phosphoinositide species) neither activates nor potentiates fly TRP, arguing against a role for $PIP_2$ as a TRP channel inhibitor (Lev et al., 2012). While the *Drosophila* system has served as a powerful genetic model for understanding TRP channel function *in vivo*, difficulties in heterologous expression and purification of the fly TRP complex have hampered a more direct biochemical and mechanistic analysis of physiological gating mechanisms (Rosenbaum et al., 2011).

This controversy of $PIP_2$ modulation has also beset the study of vertebrate TRP channels, where both stimulatory and inhibitory actions have been reported for different family members, or even for a given channel subtype (Gamper and Rohacs, 2012). Some vertebrate TRPs, such as TRPM7 and the cold/menthol receptor, TRPM8, show clear functional hallmarks of $PIP_2$-dependency, including loss of activity (i.e. run-down) in excised membrane patches and restoration of activity by exogenous phosphoinositides (Rohacs et al., 2005; Runnels et al., 2002). But for most other TRP channels, conclusions have relied primarily on indirect analyses in which phosphoinositide levels were manipulated pharmacologically through application of antibodies, chelating agents, or exogenous lipids, or by heterologous expression of lipid kinases or phosphatases (Albert et al., 2008; Chuang et al., 2001; Lemonnier et al., 2008; Lev et al., 2012; Stein et al., 2006; Ufret-Vincenty et al., 2011). Thus, controversies and inconsistencies likely reflect the complexity of these systems and uncertainties regarding the identities of lipids, lipid-modifying enzymes, and other proteins in whole cells or excised membrane patches that may alter channel gating, desensitization, or other functional properties (Lemonnier et al., 2008).

A more definitive analysis requires the examination of purified channel protein in a defined membrane environment. This has recently been achieved for TRPM8 following reconstitution of heterologously expressed channels in artificial bilayers, where biophysical analysis confirms the role of $PIP_2$ as a positively acting, obligatory co-factor (Zakharian et al., 2010; Zakharian et al., 2009). In contrast, no such analysis has yet been carried out for a TRP channel proposed to be $PIP_2$-inhibitable, or known to be activated or potentiated downstream of a PLC-coupled receptor. This remains an important unresolved problem because many vertebrate TRP channels, like the prototypic *Drosophila* ancestor, likely function as ‘receptor-operated’ channels that are activated or potentiated downstream of PLC-coupled receptors.

One such example is the heat- and capsaicin-activated channel, TRPV1, arguably the best-characterized member of the vertebrate TRP channel family (Albert et al., 2008; Caterina et al., 1997). Its widely validated role in pain physiology, together with the availability of well

characterized pharmacological agents (natural and synthetic), make it a 'poster child' for elucidating basic principles underlying TRP channel pharmacology, structure, and regulation. TRPV1 is an essential component of the cellular signaling mechanisms through which injury produces thermal hyperalgesia (Basbaum et al., 2009). Activation of PLC-coupled receptors by a variety of pro-algesic agents sensitizes TRPV1 to produce pain hypersensitivity. Thus, TRPV1 serves as a vertebrate model for understanding how canonical 'receptor-operated' TRP channels are activated or potentiated downstream of PLC-coupled receptors.

On the basis of electrophysiological, pharmacological, and structure-function experiments, we proposed that phosphoinositide lipids inhibit TRPV1 by interacting with positively charged amino acid residues located within the C-terminal cytoplasmic tail, and that disruption of this interaction through PLC-mediated $PIP_2$ hydrolysis contributes to channel (and thus neuronal) sensitization (Chuang et al., 2001; Prescott and Julius, 2003). This model, however, has been challenged by studies that support a role for $PIP_2$ as an obligate co-factor of TRPV1, or a complex regulatory factor that exerts negative or positive effects on thermal and chemical sensitivity, depending on stimulus intensity (Klein et al., 2008; Lukacs et al., 2007; Qin, 2007; Ufret-Vincenty et al., 2011). However, a role for $PIP_2$ as an obligatory or stimulatory co-factor for TRPV1 is difficult to reconcile with the widely accepted observation that activation of PLC leads to robust enhancement of the channel's sensitivity to chemical and thermal stimuli (Ferreira et al., 2004). In addition to this important regulatory issue, other key aspects of TRPV1 function remain controversial, such as whether pro-algesic bioactive lipids function as direct allosteric modulators of the channel, whether heat sensitivity is intrinsic to the channel protein, and whether or to what extent thermal response properties (e.g. temperature activation threshold, activation kinetics, and other energetic parameters) are influenced by channel density, the cellular environment, or other extrinsic factors (Clapham and Miller, 2011; Patwardhan et al., 2010; Zhang et al., 2005).

Here, we resolve these controversies by examining the properties of heterologously expressed and purified TRPV1 protein in a reconstituted liposome system that supports channel analysis at the macroscopic level. In this robust system, reconstituted channels are fully functional and display both intrinsic heat and chemical sensitivity in the absence of any phosphoinositide species, arguing against a requirement for $PIP_2$ as an obligate cofactor. Addition of $PIP_2$ to this defined system produces a substantial rightward shift in the channel's thermal or chemical activation threshold, supporting a negative regulatory effect of $PIP_2$ and other phosphoinositides on TRPV1 function. Whereas $PIP_2$ and other phosphoinositides inhibit TRPV1, $PIP_3$ does not, suggesting that pro-algesic agents, such as nerve growth factor, that activate receptor tyrosine kinases, can sensitize TRPV1 through either PLC- or PI3 kinase-mediated turnover of $PIP_2$. Thus, our findings solidify a role for $PIP_2$ as a negative regulatory factor for this important class of 'receptor-operated/modulated' ion channels; they also implicate a wider range of phosphoinositide species than previously appreciated, including PI and PI4P, whose abundance in the plasma membrane rivals that of $PIP_2$. Using an orthogonal biochemical strategy, we further show that interaction between the TRPV1 C-terminal cytoplasmic tail and membrane lipids alters channel sensitivity to thermal and chemical stimuli, reminiscent of the interplay between inwardly rectifying potassium channels and $PIP_2$ (Hansen et al., 2011; Hilgemann et al., 2001; Suh and Hille, 2005).

## Results

### Functional reconstitution of TRPV1 in artificial membranes

To study the capsaicin receptor in a defined reconstituted system, we expressed TRPV1 in insect (Sf9) cells as a fully functional maltose binding protein (MBP) fusion, as determined by live-cell calcium imaging and whole-cell patch clamp recording (Figure 1A-C). Using this system, we could obtain biochemical quantities of detergent solubilized, homogeneous protein in a single step using amylose affinity chromatography (Figure 1D, inset). This material migrated as a stable and predominantly mono-dispersed species when analyzed by size exclusion chromatography (Figure 1D).

To assess functionality of the purified channel, we reconstituted MBP-TRPV1 protein into soybean lipid extracts (the MBP tag was retained to enhance protein stability and avoid a prolonged proteolysis step between purification and reconstitution). We characterized the reconstituted protein by carrying out electrophysiological recordings from excised 'inside-out' proteoliposome patches. Capsaicin elicited large, outwardly rectifying currents that were blocked by co-application of capsazepine, a prototypical TRPV1 antagonist (Figure 2A, B, S1A). In contract, capsaicin failed to evoke ionic current in liposomes containing a control channel, KcsA (Figure S1B). Similar results were obtained with other structurally distinct agonists, including resiniferatoxin (rtx) and 2-aminophenylborate (2-APB) (Figure 2C). Protons also activate TRPV1, but unlike capsaicin and other lipophilic ligands, do so only when applied to the extracellular face of the channel, where putative proton binding sites reside (Jordt et al., 2000; Ryu et al., 2007). Interestingly, we found that acidification of the perfusate failed to elicit responses in excised proteoliposome patches, whereas acidification of the pipette solution produced large, capsazepine inhibitable currents (Figure 2C, D). Similar results were obtained with DkTx spider toxin peptide (Figure 2E), which is also known to activate TRPV1 only from the extracellular side of the plasma membrane (Bohlen et al., 2010). The outwardly rectifying current evoked by various TRPV1 agonists suggests that this property is intrinsic to the channel protein, not involving other cellular factors. Taken together, these results demonstrate that purified TRPV1 channels retain pharmacological properties of native vanilloid receptors, and reconstitute into liposomes with a strong directional bias akin to the native cellular orientation (Figure 2F), and consistent with the observed outwardly rectifying current-voltage relation.

### TRPV1 is intrinsically heat sensitive

We next asked whether reconstituted TRPV1 channels could be activated by heat. Indeed, excised proteoliposome patches showed large, outwardly rectifying heat-evoked currents that were characterized by a thermal activation threshold of 41.5 ± 1.1°C and a steep temperature dependence ($Q_{10}$ = 23.5 ± 8.0)(n = 9)(Figure 3A–C), consistent with known properties of cellular TRPV1 channels (Caterina et al., 1997; Voets, 2012). Furthermore, heat-evoked responses were abolished by capsazepine (Figure 3D and S2A), as observed in whole cells. Interestingly, these values did not vary with the size of the evoked current (Figure S2B), indicating that thermal threshold is not dependent on the number of channels present in the system.

Temperature-evoked gating of TRPV1 likely involves a concerted and quick transition to the open state involving substantial conformational movement, as suggested by rather large changes in both enthalpy and entropy within a rapid (millisecond) time course of activation (Yao et al., 2010). To ask whether TRPV1 channels show similar rates and energetics of thermal activation in liposomes versus cell membranes, we used an infrared laser diode system to deliver fast (sub-millisecond) temperature jumps to excised proteoliposome patches while measuring evoked currents (Figure 3E, F). The resulting heat-evoked

responses showed the same thermal activation threshold (42.5 ± 0.16°C), rapid activation kinetics ($\tau$ = 3.5 ± 0.3 ms), and large enthalpic and entropic changes ($\Delta H$ = 86.2 ± 3.9 kcal/mol and $\Delta S$ = 249.2 ± 12.2 cal/mol·K, respectively)(n = 11) as previously determined in TRPV1-expressing mammalian (HEK293) cells (Yao et al., 2010). Taken together, these results show that purified TRPV1 protein is intrinsically heat sensitive and accounts for the basic thermal response properties associated with cellular capsaicin receptors.

## Modulation by lipid metabolites

A variety of inflammatory agents enhance TRPV1 sensitivity to heat, thereby producing thermal hyperalgesia in response to tissue injury. For some agents (such as extracellular protons) sensitization is clearly mediated by direct allosteric modulation of the channel, whereas others (such as bradykinin) exert their effects indirectly through activation of metabotropic receptors and downstream second messenger signaling pathways (Caterina and Julius, 2001; Rosenbaum and Simon, 2007). The reconstituted proteoliposome system allows us to ask whether specific factors activate or sensitize TRPV1 directly, in the absence of other cellular proteins or second messengers. As observed in whole cells (Tominaga et al., 1998), threshold doses of protons (pH 6, insufficient to activate the channel at room temperature) produced marked potentiation of heat sensitivity in proteoliposomes (Figure 4A, B and S3). Several bioactive lipids, such as anandamide (AEA), arachidonic acid (AA), and its metabolite 12-HPETE (Figure S3F), also produced marked thermal sensitization, even at concentrations well below that required for *de novo* channel activation at room temperature (Figure 4C–F and S1C, S3). In contrast, neither diacylglycerol (DAG) nor leukotriene B4 activated or sensitized TRPV1 in liposomes (Figure S3). As previously reported (Nieto-Posadas et al., 2012), lysophosphatidic acid (LPA) activated TRPV1 at room temperature, producing currents of equivalent magnitude to those elicited by capsaicin (Figure 4G and S1D). However, as opposed to anandamide, arachidonate, or HPETE, sub-threshold doses of LPA did not sensitize responses of TRPV1 to heat (Figure 4H), suggesting that LPA functions as an efficacious channel agonist, but a weak allosteric modulator, possibly reflecting distinct sites of action on TRPV1 for different lipid second messengers (Jordt and Julius, 2002; Nieto-Posadas et al., 2012). Thus, our findings demonstrate that these pro-algesic bioactive lipids act as direct agonists and/or allosteric modulators of the channel.

## Negative regulation of TRPV1 by phosphoinositide lipids

An ongoing controversy concerns the nature of TRPV1 interactions with membrane phosphoinositides, and whether such interactions facilitate or inhibit channel gating. Our ability to reconstitute purified TRPV1 protein into liposomes now provides a biochemically-defined system with which to resolve such controversies. Approximately 7% of the phospholipid content of soybean polar lipid extract is of unknown composition. Thus, we first pared our liposome system down to a set of fully defined components consisting only of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine, -phosphoethanolamine, and –phosphor-(1′-rac-glycerol) (POPC, POPE, and POPG), sphingomyelin, and cholesterol. When purified TRPV1 was reconstituted into this minimal system lacking phosphoinositides, robust outwardly rectifying currents were evoked by capsaicin or heat (Figure 5A, B), demonstrating that stimulus-evoked channel gating *per se* does not require $PIP_2$ or other phosphoinositides.

While these minimal liposomes clearly support TRPV1 function, both thermal and chemical sensitivity was noticeably increased when compared with soybean proteoliposomes. Because soybean lipid extracts contain substantial levels (>15%) of phosphatidylinositol and other phospholipid species (Avanti Polar Lipids), we next asked whether addition of specific phospholipids to minimal proteoliposomes would alter chemical or thermal thresholds.

Indeed, we found that addition of PI, $PIP_2$, or PI4P decreased sensitivity to capsaicin as evidenced by a pronounced rightward shift in dose response curves (Figure 5C, Table S1), with PI and PI4P having the largest effects. Interestingly, equivalent levels of $PIP_3$ did not alter capsaicin sensitivity, indicating specificity for a subset of phosphoinositides. Similar results pertained to thermal sensitivity (Figure 5D, E and S4), where a variety of phosphoinosities produced substantial rightward shifts in thermal response profiles compared to minimal liposomes or those containing $PIP_3$. Thus, in minimal proteoliposomes lacking phosphoinostides, TRPV1-evoked responses resembled channels sensitized by protons (Figure 4B). Introduction of phosphoinositides (with the exception of $PIP_3$) markedly altered thermal response curves to more closely resemble those observed in soybean liposomes or whole cells. In contrast, phosphatidic acid (PA) had no effect (Figure S4H), further attesting to specificity for a particular class of anionic phospholipids. Taken together, these findings demonstrate that several phosphoinositide species, including $PIP_2$, inhibit TRPV1 by decreasing sensitivity to chemical and thermal stimuli.

It has been suggested that gating of some TRP channel subtypes is modulated by the mechanical properties of the membrane (Hardie and Franze, 2012; Rosenbaum et al., 2011). To ask whether changes in fluidity or curvature of minimal liposomes (containing 4% PI) affect TRPV1 thermosensitivity, we altered these properties of the membrane by incorporating different percentages of cholesterol or by perfusing liposome patches with lysophosphatidyl choline (LPA), respectively (Ferreira et al., 2004; Runnels et al., 2002). Neither of these manipulations affected the temperature response profile of reconstituted TRPV1 (Figure S5), indicating that the mechanical properties of the membrane do not significantly alter the intrinsic heat sensitivity of the channel protein.

## C-terminus-lipid interaction tunes channel sensitivity

Analysis of TRPV1 mutants and species orthologues have previously led us to propose that a basic, lysine/arginine-rich C-terminal region of TRPV1 interacts with negatively charged phospholipid head groups to inhibit channel activation; PLC-mediated $PIP_2$ hydrolysis would sensitize the channel by releasing the C-terminus from this inhibitory interaction (Chuang et al., 2001; Prescott and Julius, 2003). The reconstituted proteoliposome system provides an opportunity to further test this model.

First, we asked whether truncation of our TRPV1 construct at the beginning of a putative C-terminal $PIP_2$ interaction domain (Prescott and Julius, 2003) would affect phosphoinositide regulation. Indeed, liposomes containing such a protein did not show the PI-mediated shift in thermal sensitivity exhibited by the full-length channel (Figure 6A). We next asked whether PI modulation could be restored when a C-terminal-membrane interaction was established through an orthogonal (non-native) chemical strategy. This was achieved by adding a poly (8X)-histidine tag to the truncated C-terminus. The resulting protein (C-8xHis) was expressed, purified, and reconstituted into minimal lipsosomes that also contained PI plus a nitrilotriacetic acid (NTA) modified lipid (DGS-NTA), whose head group is capable of interacting with the histidine tag in a nickel-dependent manner. With liposomes containing C-8xHis, capsaicin dose-response curves showed a marked rightward shift in a nickel-dependent manner. No change in capsaicin sensitivity was observed with a control construct in which the 8xHis was placed at the N-terminus, a site distant to the putative $PIP_2$-binding site (Figure 6B and 6C). Thermal sensitivity in the C-terminally tagged construct also showed pronounced nickel-dependent shifts (Figure 6D-F). Taken together, these results support a direct functional interaction between the TRPV1 C-terminus and membrane lipids that regulates channel sensitivity to both chemical and thermal stimuli.

## Discussion

### $PIP_2$ as an inhibitor of 'receptor-operated' TRP channels

The canonical TRP channel was first identified in the fly eye, where it is activated downstream of PLC-coupled rhodopsin (Hardie, 2007; Minke, 2001; Montell, 2012). Although vertebrate TRP channels are widely divergent in their structures and physiologic functions, many, if not most, retain the ability to be activated or modulated by PLC-coupled receptors. For some TRP channels, this is likely mediated through the IP3/$Ca^{2+}$ release branch of the PLC signaling pathway (Liu and Liman, 2003), but for many others, lipid metabolism *per se* controls channel gating. For those in the latter category, including TRP channels in the fly eye, mechanisms linking PLC activation to channel gating remain controversial (Montell, 2012). One prevailing model suggests that two downstream events are essential: the depletion of $PIP_2$ and consequent production of second messenger metabolites (e.g. DAG, polyunsaturated fatty acids, or intracellular protons) (Estacion et al., 2001; Huang et al., 2010). Our findings support a similar model for TRPV1 modulation in which depletion of $PIP_2$ enhances channel sensitivity to other stimuli, including heat, simultaneously generated second messengers (e.g. AA, HPETE), or other inflammatory agents (e.g. extracellular protons, AEA). Indeed, our results using the reconstituted liposome system confirm the notion that numerous bioactive lipids capable of activating their own metabotropic receptors (e.g. AEA and LPA) also function as direct TRPV1 agonists (Nieto-Posadas et al., 2012; Zygmunt et al., 1999). Thus, pro-algesic agents such as bradykinin, that activate PLC-coupled receptors, likely elicit thermal hypersensitivity through a cascade of downstream mechanisms that include phosphoinositide lipid hydrolysis, production of second messengers (e.g. arachadonic acid metabolites) (Hwang et al., 2000), and PKC-mediated phosphorylation of TRPV1 (Bhave et al., 2003; Numazaki et al., 2002; Premkumar and Ahern, 2000) (Figure 7).

Our findings also identify a broad range of phosphoinositide species (PI, PI3P, PI4P, PI5P, $PIP_2$) that can act as negative regulators of TRPV1. PI3P and PI5P appear to be rare and reside mostly in intracellular membranes, and are therefore unlikely to modulate TRPV1 activity, although we cannot rule out the possibility that they may play a role in TRPV1 trafficking. On the other hand, PI, PI4P, and $PIP_2$ are present in the plasma membrane (Hammond et al., 2012), where they may serve as negative regulators to prevent uncontrolled activation of TRPV1 in resting cells. The ability of phosphoinosides other than $PIP_2$ to inhibit TRPV1 is in agreement with recent studies describing fly TRP modulation by PI and PI4P in excised membrane patches (Huang et al., 2010). This broad scale modulation of TRP channels by numerous phosphoinositide species raises an interesting question as to whether PLC-mediated hydrolysis of $PIP_2$ can account for sensitization of TRPV1. Interestingly, measurement of $PIP_2$ hydrolysis in microvilli of the fly eye indicates that photon absorption not only depletes $PIP_2$, but also diminishes the upstream phosphoinositide reserve pool (including PI and PI4P) (Hardie et al., 2004; Hardie et al., 2001). Similarly, PLC activation downstream of M1 receptors in mammalian cells leads to nearly complete depletion of PIP and $PIP_2$, as well as significant reduction in PI (Horowitz et al., 2005; Li et al., 2005). Moreover, $PIP_2$ metabolism and signaling may be spatially confined (Hilgemann, 2007; van den Bogaart et al., 2011), which should allow for efficient localized depletion of $PIP_2$ and other phosphoinositide pools within the vicinity of TRPV1 channels. Taken together, we believe that depletion of $PIP_2$ and other plasma membrane phosphoinositides contributes to sensitization of TRPV1 by proalgesic agents that activates PLC coupled receptors (Figure 7). Studies by others have shown that perfusion of $PIP_2$ onto plasma membrane patches excised from TRPV1-expressing cells enhanced capsaicin-evoked responses, indicative of a positive, rather than negative regulatory role (Lukacs et al., 2007; Ufret-Vincenty et al., 2011). The reason underlying this discrepancy with our results is unclear, but may reflect differences associated with alterations in bilayer $PIP_2$ as produced

by perfusion versus direct incorporation into liposomes. Indeed, the physiologic relevance of such manipulations is a topic of debate (Lemonnier et al., 2008).

Some pro-algesic agents, such as nerve growth factor (NGF), activate PLC as well as PI3 kinase, raising questions as to which pathway is most responsible for TRPV1 sensitization (Chuang et al., 2001; Stein et al., 2006; Zhang et al., 2005; Zhuang et al., 2004). Our finding that $PIP_3$ has no inhibitory effect on TRPV1 suggests that both pathways may contribute to this process through a common mechanism involving depletion of $PIP_2$ through hydrolysis or phosphorylation (Figure 7). Moreover, our finding that PI4P inhibits TRPV1 to the same or greater extent as $PIP_2$ likely explains why $PIP_2$ depletion with an inducible 5′ lipid phosphatase diminishes, rather than enhances mammalian TRPV1, or has no effect on fly TRP function (Klein et al., 2008; Lev et al., 2012). These results have been used to argue against a role for $PIP_2$ as a negative regulatory factor, but our data now suggest how these observations may, indeed, be consistent with a mechanism of phosphoinositide-mediated inhibition.

Our findings in the reconstituted system also show that the thermal activation profile for TRPV1 is independent of channel number. This is relevant because some pro-algesic agents, such as nerve growth factor, have been suggested to enhance TRPV1-mediated currents and produce thermal hypersensitivity through a PI3 kinase-dependent pathway that increases insertion of channels at the plasma membrane by ~ 2-fold (Stein et al., 2006; Zhang et al., 2005). Our findings suggest that increased cell surface expression may account for a component of thermal hypersensitivity, but not for aspects that involve a decrease in thermal threshold.

### TRPV1 is intrinsically heat sensitive

TRPV1 channels support heat-evoked currents whether examined in whole cell or excised membrane configurations, consistent with the idea that the channel is intrinsically thermosensitive. However, neither preparation is completely free of native cytoplasmic or membrane-associated factors, making it impossible to formally rule out involvement of other cellular proteins or second messengers. Indeed, it has been suggested that TRPV1 responds to thermal stimuli through a mechanism involving heat-evoked generation and release of oxidized linoleic acid derivatives that then serve as TRPV1 agonists (Patwardhan et al., 2010). Our reconstitution data argue against such indirect mechanisms and support intrinsic sensitivity of the channel to heat. Moreover, the thermal response properties of reconstituted TRPV1 protein in soybean lipids (including activation rate and threshold, $Q_{10}$, $\Delta H$ and $\Delta S$) recapitulate those observed in cells (Yao et al., 2010), demonstrating that TRPV1, itself, underlies heat-evoked currents measured in biological membranes, and accounts for the energetic profile of these responses.

A recent study of TRP channels in the fly eye suggests that light-evoked hydrolysis of $PIP_2$ leads to contraction of photoreceptor cells as a consequence of changes in membrane curvature. This raises the possibility that PLC enhances the activity of some ‘receptor-operated’ TRP channels through mechanical changes to the plasma membrane (Hardie and Franze, 2012). Our results suggest that alteration of membrane curvature has no significant effect on the thermal activation profile of TRPV1, consistent with thermal sensitivity being determined by the channel *per se*, rather than the biophysical properties of the membrane.

### C-terminal domain as a determinant of channel sensitivity

Several studies suggest that cellular proteins such as calmodulin, AKAP150, or Pert contribute to $PIP_2$ regulation of TRP channels (Jeske et al., 2011; Kim et al., 2008; Kwon et al., 2007), although this too remains controversial (Ufret-Vincenty et al., 2011). While other

cellular proteins may, indeed, contribute to this process, our findings strongly suggest that, as in the case of inwardly rectifying potassium channels (Hansen et al., 2011), phospholipids are capable of engaging in direct interactions with TRPV1 *per se*. Furthermore, our truncation and poly-(8xHis) tag-based tethering experiments support the idea that interaction between the TRPV1 C-terminus and membrane lipids represents a key mechanism for regulating channel sensitivity. Although the precise location of a phosphoinositide interaction domain within the C-terminus remains controversial (Brauchi et al., 2007; Prescott and Julius, 2003; Ufret-Vincenty et al., 2011), our findings are generally consistent with the observation that natural variation within this region is associated with species-specific tuning of TRPV1 thermal activation profiles (Gracheva et al., 2011). It has also been reported that the TRPC5 channel can be activated by calpain-mediated proteolytic cleavage of its C-terminal domain (Kaczmarek et al., 2012), but whether phosphoinositides contribute to this mechanism is not known. The studies presented here provide biochemical and physiological tools to facilitate structural and functional studies aimed at delineating the precise nature of these and other physiologically important TRP channel regulatory mechanisms.

## METHODS

### Protein expression and purification

An expression cassette containing 8xHis tag-MBP (maltose binding protein) and rTRPV1 (2–839) was cloned into the pFastbac1 vector and recombinant baculovirus obtained following the manufacturer's protocol (Bac-to-Bac expression system, Invitrogen). For protein expression, sf9 cells were infected with recombinant baculovirus and harvested by centrifugation 72 hours post infection. Cells were broken by passing through an emulsifier twice in hypotonic buffer (36.5mM sucrose, 50mM Tris, 2mM TCEP, pH8.0) supplemented with 1mM phenylmethanesulphonylfluoride, 3μg/ml aprotinin, 3μg/ml leupeptin, 1μg/ml pepstatin (all from Roche). Cell debris was cleared by low-speed centrifugation (8000g x 20 min). Membranes were collected by ultracentrigation (200,000g x 1 hr) and solublized in Buffer A (200mM NaCl, 2mM TCEP, 10% glycerol, 50mM HEPES, pH8.0) supplemented with 20mM DDM (Anatrace). Detergent insoluble material was removed by centrifugation (30,000g x 20 min), and the supernatant fluid incubated with amylose resin (New England Biolabs). The resin was washed with Buffer A containing either 0.5mM DDM with 10μg/ml soybean lipids, or 1mM DDM with 10μg/ml synthetic lipid mixture. rTRPV1 was eluted with same buffer supplemented with 20mM maltose. Immediately after elution, rTRPV1 protein was mixed with lipids for reconstitution. To analyze size-homogeneity of the sample, rTRPV1 protein was separated on a Sepharose 6 gel filtration column (GE Healthcare) in Buffer A supplemented with 0.5mM DDM and 10μg/ml soybean lipids.

### Liposome reconstitution

Reconstitution experiments were performed as previously described (Delcour et al., 1989; Knol et al., 1998) with modifications. All lipids were purchased from Avanti Polar Lipids. 2.5mg soybean polar lipid extract in chloroform was dried under argon stream and residual chloroform further evaporated by vacuum desiccation (~2 hours). Lipids were rehydrated in 0.5ml Buffer B (5mM MOPS, 200mM KCl, pH7.0) containing 2mM TCEP for 30 minutes, then subjected to 10 freeze-thaw cycles by alternately placing the sample in liquid nitrogen and warm water prior to bath sonication for 10 min. Liposomes were destabilized by adding DDM to a final concentration of 4mM and incubating for 30 min at room temperature with gentle agitation. For reconstitution, purified rTRPV1 protein and destabilized liposomes were mixed at 1:5–10 (wt/wt) protein-to-lipid ratios and incubated at room temperature for 1 hr with gentle agitation. Detergents were subsequently removed by adding four aliquots of Bio-Beads SM-2 (30mg, 30mg, 50mg, and 100mg, respectively) at 1 hr intervals with gentle

agitation at room temperature. After adding the last aliquot of Bio-Beads SM2, the mixture was incubated overnight at 4°C with gentle agitation. Bio-Beads were removed by filtration over a disposable polyprep column, and the eluate centrifuged at 100,000g x 1 hr to collect proteoliposomes, which were resuspended in 48 μl Buffer B, flash-frozen in aliquots, and stored at −80°C until use. Chemically defined liposomes were composed of 5 basic lipids: 2.5mg lipids-2:1:1 POPE:POPC:POPG, 62.5μg cholesterol, 62.5 μg sphingomyelin (porcine brain). Various phosphoinositides (0.1mg)-$PIP_2$ (porcine brain), PI (porcine liver), $PIP_3$, PI3P, PI4P (porcine brain), PI5P- were added to the basic lipid mixture to examine their effects on TRPV1 function. To investigate the effect of channel-lipid interaction on TRPV1 function using defined chemistry, the basic lipid cocktail plus 0.1 mg PI and 62.5 μg head group modified lipids (i.e. DGS-NTA+Ni or DGS-NTA-Ni) was mixed with 8xHis-MBP-rTRPV1 (2–783) or MBP-rTRPV1 (2–783)-8xHis protein for reconstitution. The same protocol was used to reconstitute rTRPV1 into these chemically defined liposomes.

### Sf9 cell imaging and electrophysiology

48 hours post-infection, Sf9 cells were loaded with Fura-2 AM in MBS buffer (10mM $CaCl_2$, 60mM KCl, 17mM $MgCl_2$, 10mM NaCl, 4mM glucose, 110mM sucrose, 0.1% BSA, 10mM MES, pH6.2) for ratiometric calcium imaging. Currents were recorded at room temperature in the whole-cell patch-clamp configuration. The intracellular solution contained 140 mM Cs-methanesulfonate, 2.5mM NaCl, 10mM EGTA, 10mM HEPES, pH7.2. The extracellular (bath) solution contained 150mM Na-gluconate, 10mM glucose, 2mM $MgCl_2$, 10mM HEPES, pH7.2.

### Proteoliposome electrophysiology

4–8 μl of frozen proteoliposomes were thawed and supplemented with sucrose to a final concentration of 20 mM before being placed on a clean glass slide for dehydration in a vacuum desicator (30 min at room temperature). Proteoliposomes were then rehydrated by adding 10 μl Buffer B to the surface of dried lipid film and incubating overnight at 4°C in a sealed, humidified chamber. For patch clamp recording from giant soybean liposomes, a small amount of rehydrated proteoliposomes was dislodged and dropped on a glass coverslip, which was then placed in a petri dish containing Buffer B. Proteoliposomes were allowed to settle onto the cover slip for 5–10 min and floating liposomes carefully removed by suction. Liposomes formed from chemically defined lipids required incubation at room temperature for ~2 hr to facilitate fusion prior to recording. Patch pipettes typically displayed open resistances of 1.5–3 MΩ and were filled with Ringer's solution (140mM NaCl, 5mM KCl, 2mM $MgCl_2$, 2mM $CaCl_2$, 10mM HEPES, pH7.3). Capsaicin, resiniferatoxin, anandamide, and capsazepine were all purchased from Tocris; Arachidonic acid, 2-Aminoethyldiphenylborinate, and di-oleoyl-glycerol were purchased from Sigma; Lysophosphatic choline (LPC) was purchased from Anvanti; D (+)-sn-1-O-oleoyl-glycerol-3-phosphate was purchased from echelon bioscience; Leukotriene B4 and 12(S)-hydroperoxyeicosa-5Z,8Z,10E,14Z-tetraenoic acid (HPETE) were purchased from Enzo Life Science. All drugs were diluted into Ringer's buffer immediately before use and applied via an in-line perfusion system (Automate Scientific). For electrophysiological recording of proteoliposomes containing DGS-NTA-Ni or DGS-NTA+ Ni, both pipette solution and perfusate contained 200 mM NaCl, 10 mM HEPES, pH 7.3. Ringer's solution was not used to avoid complications from divalent $Ca^{2+}$ and $Mg^{2+}$ ions, both of which could be potentially chelated by the NTA head group. Temperature ramps were generated with a Peltier device (Reid Dan Electronics), and temperature measured using an IT-24P microprobe (Physitemp Instrument, Inc.). Currents were recorded with an Axopatch 200B amplifier (Molecular Devices) using a 500 ms ramp from −80mV to +80mV delivered once per second.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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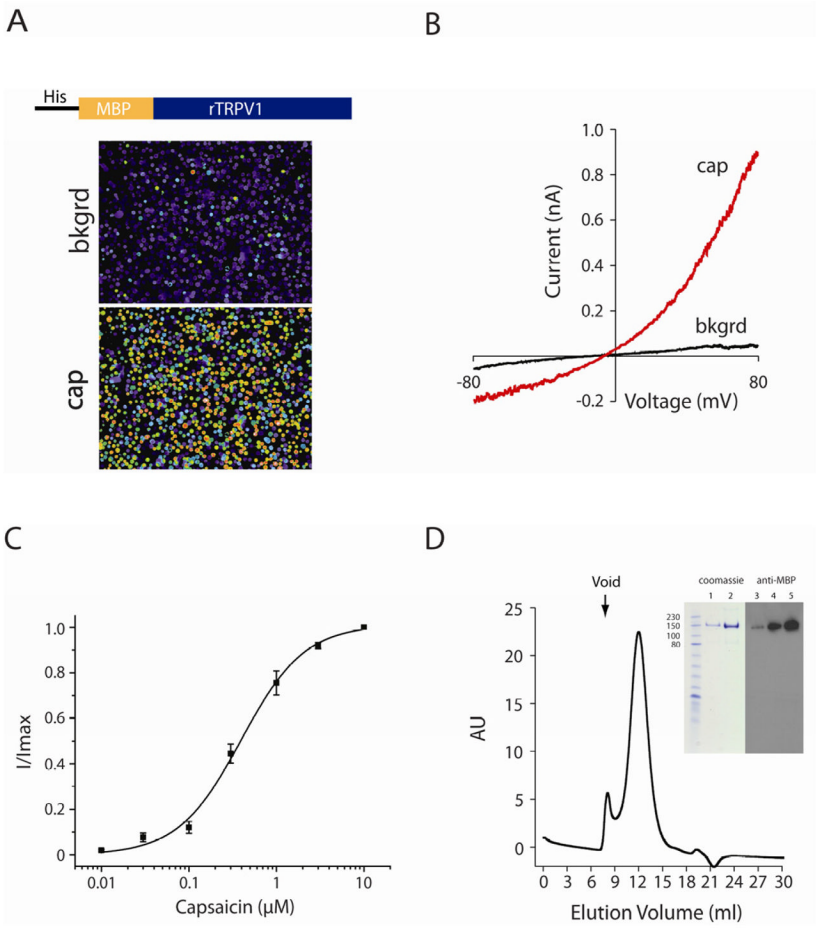


**Figure 1. Purification of functional TRPV1 protein**

(**A**) Rat TRPV1 protein was expressed as an amino (N)-terminal 8xHis-MBP fusion (referred to as rTRPV1 hereafter) in Sf9 cells, where capsaicin (10 μM) elicited robust channel activation as assessed by ratiometric calcium imaging.

(**B**) Representative whole cell recording from Sf9 cells expressing rTRPV1. Capsaicin (10 μM) evoked large, outwardly rectifying current.

(**C**) Capsaicin dose-response curve for MBP-TRPV1 expressing Sf9 cells reveals an $EC_{50}$ = 0.4 μM; n = 6 independent whole cell recordings.

(**D**) rTRPV1 protein elutes predominantly as a symmetric peak within the included volume of a Sepharose 6 size exclusion column. (Inset) Affinity purified rTRPV1 protein was analyzed by SDS-PAGE (4–12% gel) and visualized by coomassie staining (~1 and 5 μg; lanes 1 and 2) or western blotting (~2 ng, 6 ng, or 18 ng; lanes 3–5) using anti-MBP antibody.

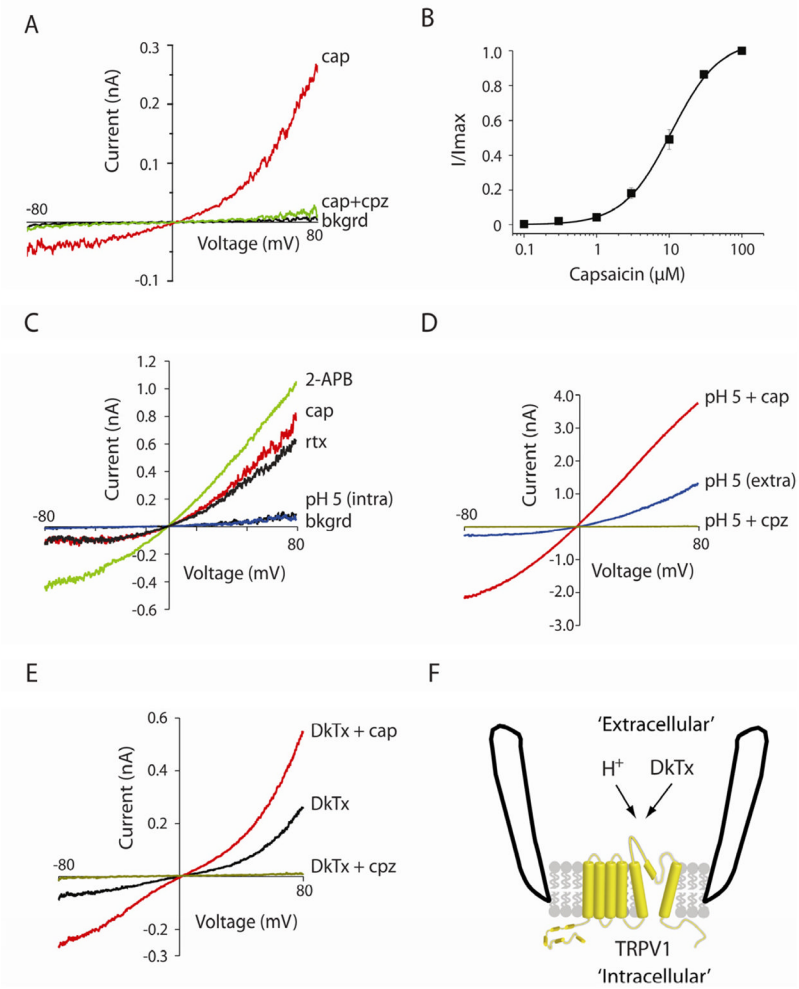


**Figure 2. Functional characterization of purified TRPV1 in reconstituted soybean liposomes**

(**A**) Patches excised from TRPV1 containing proteoliposomes showed capsaicin (cap, 10 μM)-evoked responses that were blockable by the antagonist, capsazepine (cpz, 20 μM). Current-voltage relationships showed outward rectification characteristic of TRPV1 channels.

(**B**) Capsaicin dose-response curve for TRPV1 proteoliposomes ($EC_{50}$ = 10.7 μM; n = 11 independent patches).

(**C**) Activation of TRPV1 proteoliposomes by various agonists, including 2-aminophenyl borate (2-APB, 1 mM), cap (10 μM), resiniferatoxin (RTX, 1.2 μM), and protons (pH 5 solution applied to 'intracellular' face of the liposome). Except for 'intracellular' protons, all other TRPV1 agonists produced robust responses.

(**D**) Application of protons to the 'extracellular' face of proteoliposomes evoked capsazepine-blockable currents that were enhanced by addition of capsaicin (10 μM).

(**E**) Application of spider toxin (DkTx, 2 μM) to the 'extracellular' face of proteoliposomes evoked capsazepine-blockable currents that were enhanced by addition of capsaicin (10 μM).

(**F**) Schematic representation of TRPV1 orientation in 'inside-out' proteoliposomes patches as determined by sensitivity to protons or DkTx.

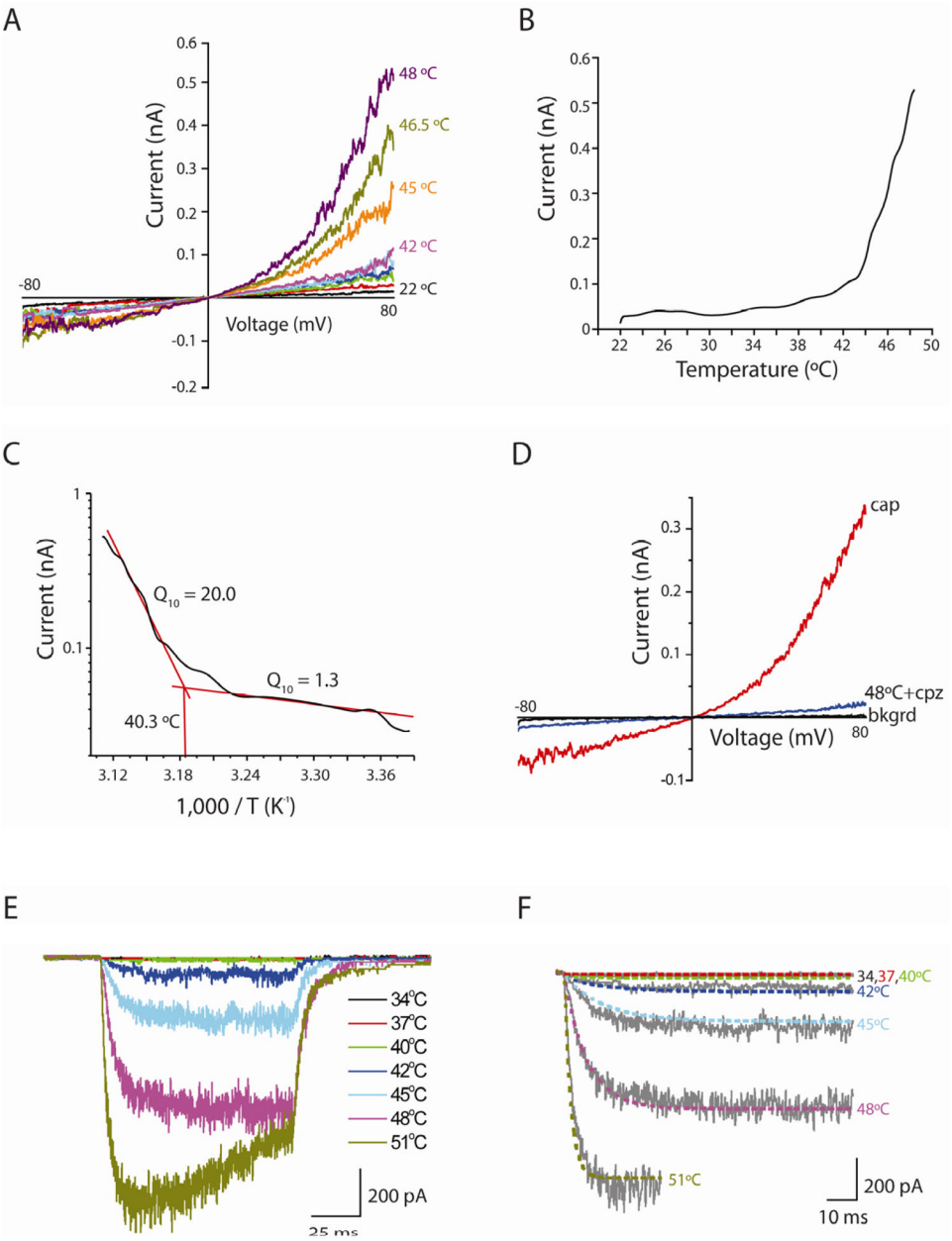


**Figure 3. TRPV1 is an intrinsically heat sensitive channel**

(**A**) Outwardly rectifying currents recorded from TRPV1-containing soybean proteoliposomes in response to a temperature ramp (22–48°C).

(**B–C**) Thermal response profile and Arrhenius plot derived from responses in (**A**) reveal activation threshold (40.3°C) and temperature coefficient ($Q_{10}$ = 20.0) resembling that of TRPV1 channels in native biological membrane.

(**D**) Capsaicin (5 μM)-evoked current demonstrates presence of functional TRPV1 channels in a proteoliposome patch, where subsequent response to heat (48°C) challenge was blocked by co-application of capsazepine (20 μM).

(**E**) TRPV1 responses evoked by rapid temperature jumps delivered to proteoliposome patches using an infrared (IR) laser ($V_h$ = −60 mV; duration of heat pulse = 100 msec).

(**F**) Activation time course for IR laser-evoked responses fit by a single exponential (stimulus temperatures shown at right).

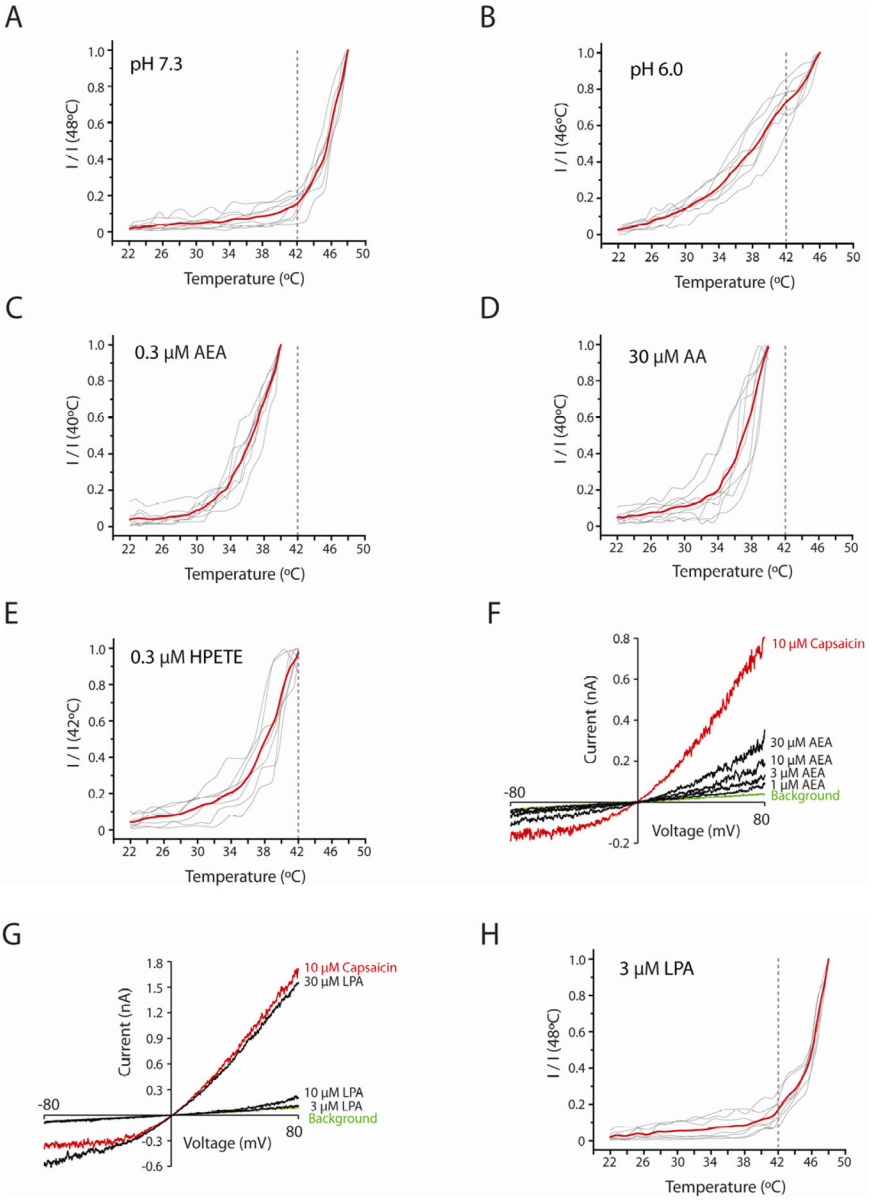


**Figure 4. Direct activation or modulation of TRPV1 by lipid metabolites**

(**A–E**) Thermal response profiles from TRPV1-containing proteoliposome patches in the absence or presence of inflammatory agents, including protons or lipid metabolites, as indicated. All agents potentiated TRPV1 heat sensitivity at doses below thresholds required for direct channel activation at room temperature. All agents were delivered by bath perfusion, except for protons, which were introduced in the electrode buffer (pH 6.0). Individual and averaged responses are plotted as light gray and red traces, respectively (n > 7 patches per condition).

(**F–G**) Both anandamide (AEA) and lysophosphatidic acid (LPA) directly activated TRPV1 in proteoliposome patches. Responses evoked by various concentrations of these agonists are shown relative to capsaicin.

(**H**) Thermal response profiles obtained from TRPV1-containing proteoliposome patches in the presence of 3 μM LPA. Individual and averaged responses are plotted as light gray and red traces, respectively (n = 7).

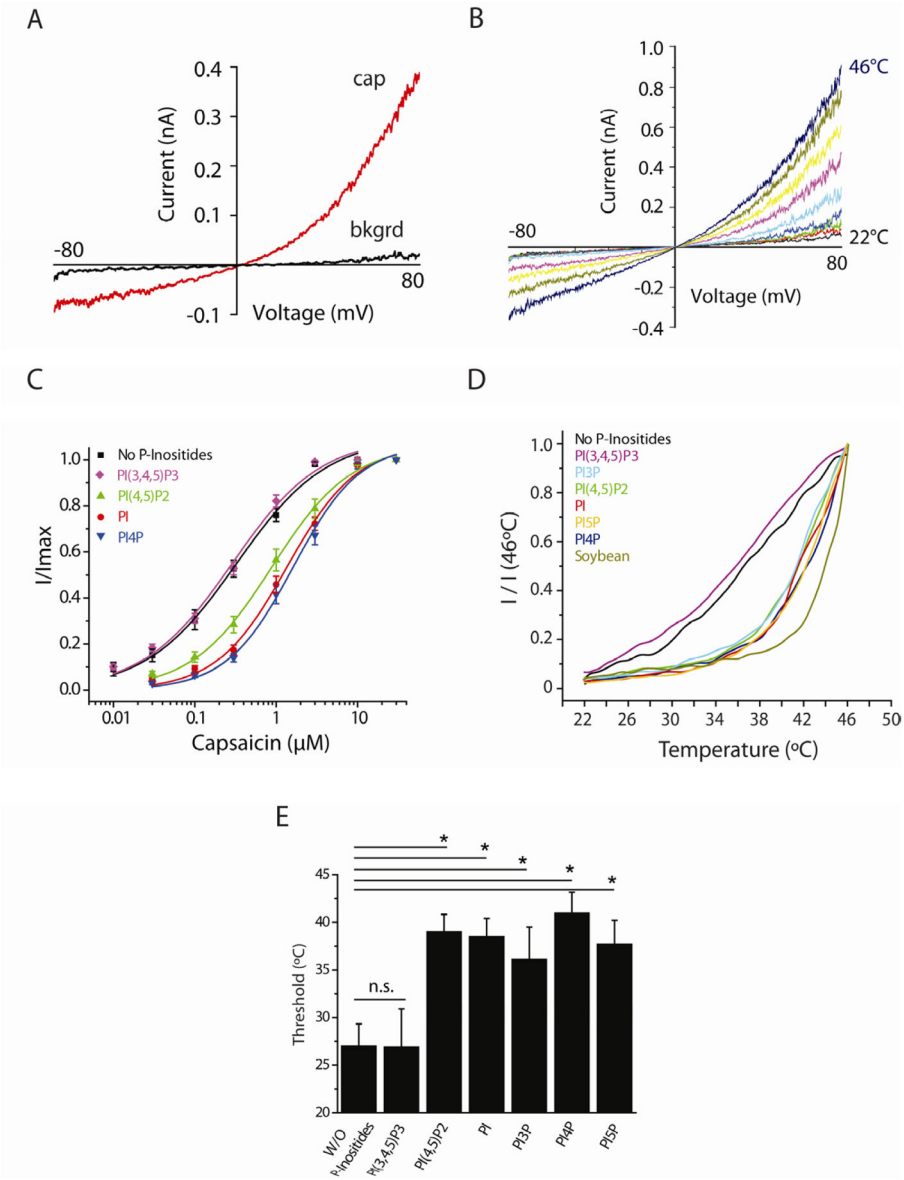


**Figure 5. Regulation of TRPV1 by phosphoinositides**

(**A–B**) Capsaicin (10 μM)- or heat-evoked currents were observed in minimal TRPV1-containing proteoliposomes lacking phosphoinostides.

(**C**) With the exception of $PIP_3$ (4%), phosphoinositides (4%) produced rightward shift in capsaicin dose-response curve relative to minimal proteoliposomes ($EC_{50}$ =0.33, 0.29, 0.88, 1.36, and 1.63 μM for no phosphoinositides, $PIP_3$, $PIP_2$, PI, and PI4P, respectively).

(**D**) Inclusion of various phosphoinositides (4%) produced marked rightward shift in thermal response profiles. TRPV1 proteoliposomes without phosphoinositides or with $PIP_3$ (4%) exhibit thermal response profiles characteristic of sensitized channels. Traces in (**D**) show averaged profiles (n > 7 patches per condition; individual traces shown in Figure S4A-G).

(**E**) Thermal activation thresholds for curves shown in (**D**) Error bar denotes mean ± s.d., *p<0.0001, Student's t-test, n > 7 per condition.

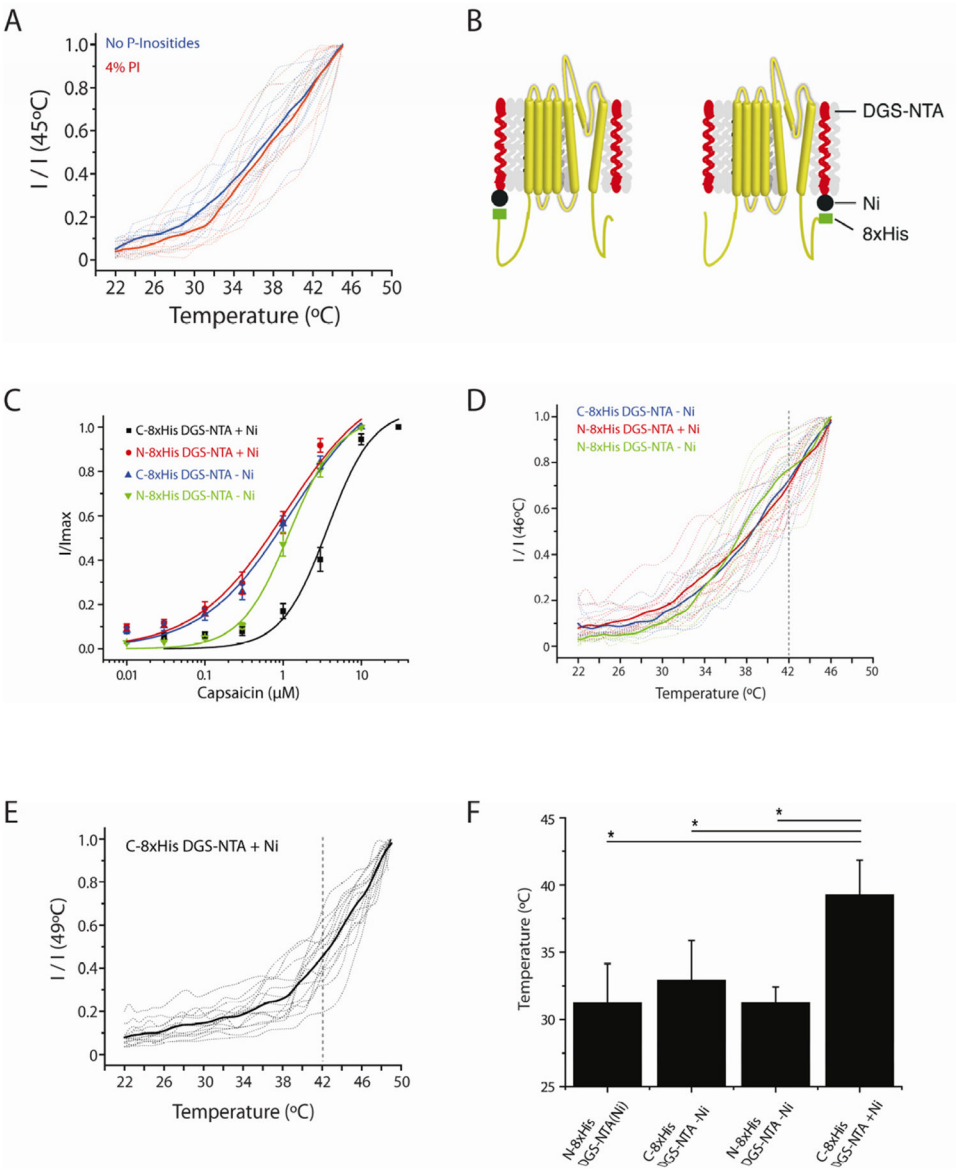


**Figure 6. TRPV1 sensitivity is tuned by C-terminal- membrane interaction**

(**A**) A C-terminally truncated TRPV1 is not inhibited in reconstituted minimal liposomes containing phosphatidyl inositol (PI, 4%).

(**B**) Schematic depicting orthogonal chemical strategy for modulating TRPV1-lipid interactions. TRPV1 (yellow) was tagged with 8xHis (green) at the N- or C-terminus to promote interaction with head group modified lipids (DGS-NTA) in a nickel (Ni)-dependent manner.

(**C**) Capsaicin dose-response curves for 8xHis-tagged rTRPV1 in liposomes containing DGS-NTA. C-terminal tagged channel in the presence of Ni (black) showed rightward shift ($EC_{50}$ = 3.69 μM; n = 8 independent patches) relative to proteoliposomes containing C-terminal fusion without Ni (blue; $EC_{50}$ = 1.25; n = 19 independent patches), or N-terminal fusion with Ni (red; $EC_{50}$ = 1.06; n = 14 independent patches) or without Ni (green; $EC_{50}$ = 1.20; n = 9 independent patches).

(**D–E**) Specific interaction between TRPV1 C-terminus and membrane lipids also inhibits thermal sensitivity. Averaged or individual responses are shown as solid or dotted traces, respectively.

(**F**) Corresponding averaged thermal activation thresholds. Error bar denotes mean ± s.d., *p<0.0001, Student's t-test, n > 8 per condition.

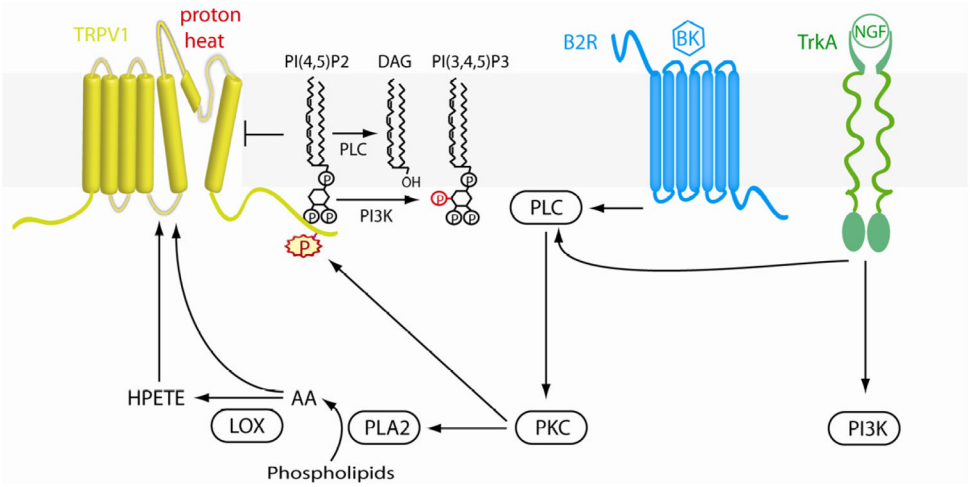


**Figure 7. Model depicting sensitization of TRPV1 by lipid metabolism**

TRPV1 channel is inhibited by association of its C-terminus with membrane phosphoinositides (i.e, PI, PI4P, and $PIP_2$). Activation of PLC downstream of bradykinin or TrkA receptors by their agonists (i.e, BK or NGF, respectively) may potentiate TRPV1 through three major subsequent events: i) PLC mediated depletion of $PIP_2$ (as well as PI and PI4P) releases TRPV1 from negative inhibition by these lipids; ii) activated PKC phosphorylates and sensitizes TRPV1; iii) lipid metabolites such as AA and HPETE generated by PLA2 sensitize TRPV1 by directly targeting the channel. In addition, given that $PIP_3$ has no effect on TRPV1, on site conversion of $PIP_2$ to $PIP_3$ by PI3K may also contribute to sensitization by NGF and other factors that activate PI3K.