Regulation of the Temperature-dependent Activation of Transient Receptor Potential Vanilloid 1 (TRPV1) by Phospholipids in Planar Lipid Bilayers*

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Background: Regulation of the temperature-dependent activity of TRPV1 channels with phosphoinositides is not well understood.

Results: Phosphoinositides directly facilitate heat activation of TRPV1 reconstituted in neutral planar lipid bilayers. Different anionic lipids evoke TRPV1 channel activity with distinct open probability and conductance states.

Conclusion: Phosphoinositides are required for heat-induced activation of TRPV1.

Significance: Phosphoinositides are essential regulators of TRPV1 gating.

TRPV1 (transient receptor potential vanilloid 1) proteins are heat-activated nonselective cation channels. TRPV1 channels are polymodal in their function and exhibit multifaceted regulation with various molecular compounds. In this regard, phosphoinositides, particularly phosphatidylinositol 4,5-bisphosphate and phosphatidylinositol 4-phosphate, are important channel regulators. However, their effects on TRPV1 channel activity have not been conclusively determined. To characterize temperature-induced activation of TRPV1 in the presence of different phospholipids, we purified the TRPV1 protein from HEK-293 cells and incorporated it into planar lipid bilayers. In the presence of 2.5 μM phosphatidylinositol 4,5-bisphosphate, TRPV1 channels demonstrated rapid activation at 33–39 °C and achieved full channel opening at 42 °C. At this temperature range, TRPV1 heat activation exhibited steep temperature dependence (temperature coefficient (Q10) of 18), and the channel openings were accompanied by large changes in entropy and enthalpy, suggesting a substantial conformation change. At a similar temperature range, another phosphoinositide, phosphatidylglycerol, also potentiated heat activation of TRPV1, but with much lower efficiency. Negatively charged phosphatidylglycerol could also induce heat activation of TRPV1 channels, although with a small-conductance state. Our data demonstrate that phospholipids, specifically phosphoinositides, are important regulators of TRPV1 and are required for heat-induced channel activity.

The TRPV1 (transient receptor potential vanilloid 1) channel is the heat and capsaicin receptor (1). TRPV1 channel activity can be induced by heat, low pH, pungent chemicals, and a variety of endogenous mediators of nociception. Phosphoinositides, particularly phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2), are also important regulators of TRPV1 channels. However, the role of PI(4,5)P2 in TRPV1 potentiation or inhibition remains controversial. In the cellular system, depletion of PI(4,5)P2 by phospholipase C was initially proposed to sensitize TRPV1 channels (2). Later, TRPV1 trafficking to the plasma membrane by NGF was reported to underlie NGF-mediated increases in TRPV1 function, rather than PI(4,5)P2 cleavage by phospholipase C (3). Resynthesis of PI(4,5)P2 was found to sensitize TRPV1 (4), and also direct application of PI(4,5)P2 to inside-out patches strongly activated the channel (3, 5), supporting the positive effect of PI(4,5)P2 on TRPV1 gating. Together, these results indicate that due to the existence of complex signaling pathways, it is difficult to differentiate direct effects that PI(4,5)P2 exerts on TRPV1 channels in the cellular systems.

Recently, Cao et al. (6) reconstituted purified TRPV1 into artificial liposomes composed of both neutral and negatively charged lipids and characterized channel gating in this system. Their results show that 4% PI(4,5)P2 negatively regulates both capsaicin- and heat-mediated TRPV1 gating, where the rightward shifts in the conductance-voltage curve indicate an inhibition exerted by PI(4,5)P2. Another recent work by Senning et al. (7) demonstrates that membrane asymmetry is an important mediator of the regulatory effects exerted by phosphoinositides, where 4% PI(4,5)P2 inhibits TRPV1 activation when applied extracellularly, whereas 1% PI(4,5)P2 activates the channel when applied intracellularly. To distill effects of PI(4,5)P2 on TRPV1 channels, in our recent work, we incorporated the purified TRPV1 protein into planar lipid bilayers consisting of neutral lipids and demonstrated that low concentrations of PI(4,5)P2 (≤5 μM) facilitate TRPV1 activation by capsaicin (8).

In this work, we continued our study on the phosphoinositide-dependent regulation of TRPV1 channel activity. Here, we aimed to explore direct effects of PI(4,5)P2 on the temperature dependence of TRPV1 in planar lipid bilayers in comparison...
with the effects elicited by other negatively charged lipids. The purified TRPV1 protein was incorporated into neutral planar lipid bilayers. The effects of complex cell signaling pathways and negatively charged lipids were thus excluded, enabling exploration of direct effects of PI(4,5)P2. Our data show that (1) phosphoinositides potentiate heat activation of TRPV1; (2) the activation temperature range in the presence of 2.5 \( \mu \text{M} \) PI(4,5)P2 is \( \sim 33-39 \) °C; and (3) apart from phosphoinositides, negatively charged 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-1-rac-glycerol (POPG) lipids are also able to induce heat activation of TRPV1, although with a small single-channel conductance state. Overall, these results indicate that the negative charge on the lipids is required for TRPV1 channel activity.

**EXPERIMENTAL PROCEDURES**

**Expression and Purification of TRPV1 Protein**—HEK-293 cells stably expressing TRPV1 or TRPM8 (transient receptor potential cation channel, subfamily M, member 8) tagged with the Myc epitope at the N terminus were grown to 80–90% confluency, washed, and collected with cold PBS. Cells from eight 100-mm dishes were harvested and resuspended in sodium chloride-based buffer containing 500 mM NaCl, 50 mM NaH2PO4, 20 mM HEPES, and 10% glycerol (pH 7.5) and supplemented with protease inhibitor PMSF (1 mM) and \( \beta \)-mercaptoethanol (5 mM). The cells were then lysed by freeze-thawing and centrifuged at 40,000 \( \times g \) for 2.5 h, and the pellet was resuspended in sodium chloride-based buffer supplemented with protease inhibitor mixture (Roche Applied Science), 0.1% Nonidet P-40 (Roche Applied Science), and 0.5% dodecyl maltoside (Calbiochem). The suspension was incubated overnight at 4 °C on a shaker with gentle agitation and then centrifuged for 1 h at 40,000 \( \times g \). The TRPV1 or TRPM8 protein was purified by immunoprecipitation using Protein A/G magnetic beads (Thermo Fisher Scientific) conjugated with anti-Myc antibody as described previously (8). Protein was eluted from the beads with Myc peptide (150 \( \mu \text{g/ml} \); Sigma-Aldrich) in the presence of 0.5% dodecyl maltoside or 0.03% lauryl maltose neopentyl glycol (Anatrace, Maumee, OH). All purification steps were performed at 4 °C.

Protein purity was examined by silver staining. Proteins were electrophoretically separated on 10% Tris-HCl gel (Bio-Rad) using Tris/glycine/SDS buffer (Bio-Rad) at a constant voltage of 180 V. Protein bands were visualized by silver staining according to the manufacturer’s protocol.

**Planar Lipid Bilayer Experiments**—Planar lipid bilayer experiments were performed as described previously (8). Bilayers were formed from a solution of synthetic 1-palmitoyl-2-oleoylglycerol-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoylglycerol-3-phosphoethanolamine (POPE) (Avanti Polar Lipids, Alabaster, AL) at a 3:1 ratio in \( n \)-decane (Sigma-Aldrich). For some experiments, POPG (Avanti Polar Lipids) was included in the bilayer system at a ratio to POPC/POPE of 3:3:1. The solution was used to paint bilayers in an aperture of \( \sim 150 \) \( \mu \text{m} \) in diameter on a Delrin cup (Warner Instruments, Hamden, CT) between symmetric aqueous bathing solutions of 150 mM KCl, 0.02 mM MgCl2, and 20 mM HEPES (pH 7.2) at 22 °C. Bilayer capacitances were in the range of 50–75 picofarads. The purified TRPV1 or TRPM8 protein was incorporated into lipid micelles consisting of a mixture of POPC and POPE (3:1, \( v/v \)). After the bilayers had formed, 0.2 \( \mu \text{l} \) of the TRPV1 or TRPM8 micellar solution (0.001 \( \mu \text{g/ml} \)) was applied to the \( cis \)-chamber with stirring or additional painting to facilitate protein incorporation. Air bubble glass capillaries were used for painting. Unitary currents were recorded with an Axopatch 200B amplifier. Currents through the voltage-clamped bilayers (background conductance of <3 picosiemens (pS)) were filtered at the amplifier output (low pass, \( \sim 3 \text{ dB at } 10 \text{ kHz, 8-pole Bessel response} \)). Data were secondarily filtered at 100 Hz through an 8-pole Bessel filter (Model 950, tunable filter, Frequency Devices, Ottawa, IL) and digitized at 1 kHz using an analog-to-digital converter (Digitdata 1322A, Molecular Devices, Sunnyvale, CA) controlled by pCLAMP 10.3 (Molecular Devices). Data were analyzed using ClampFit 10.3 (Molecular Devices). Dioctanoylphosphoinositides were purchased from Cayman Chemical (Ann Arbor, MI). The solvent in all bilayer experiments was \( n \)-decane.

**Temperature Studies**—For temperature studies, the bilayer recording chamber was fitted on a conductive stage containing a pyroelectric heater/cooler, which was driven by a temperature controller (CL-100, Warner Instruments). Deionized water was circulated through this stage, pumped into the system to remove the heat generated. The temperature of the bath was continuously monitored with a thermoelectric device in the \( cis \)-chamber (the ground side), and it could be reliably controlled within \( \pm 0.5 \) °C. The \( Q_{10} \) for open probability (\( P_o \)) or single-channel conductance was obtained from Equation 1.

\[
Q_{10} = \left( \frac{X_2}{X_1} \right) \frac{10}{T_2 - T_1} \tag{Eq. 1}
\]

Activation energies (\( E_a \)) were calculated from the Arrhenius equation or directly from the slope of the Arrhenius plot (Equation 2),

\[
E_a = \frac{R T_1 T_2}{T_2 - T_1} \ln \frac{X_2}{X_1} \tag{Eq. 2}
\]

where \( R \) is the gas constant (8.314 J/mol), \( T_1 \) and \( T_2 \) are temperatures in Kelvin, and \( X_1 \) and \( X_2 \) are \( P_o \) or single-channel conductance at relative temperatures.

Data are presented as means ± S.E. Student’s unpaired \( t \) test was used to test significance for some experiments.

**RESULTS**

**Preparation of TRPV1 Protein**—The TRPV1 protein purification procedure was performed as described previously (8), except for using a different detergent. TRPV1 was purified from HEK-293 cells stably expressing the protein using the immunoprecipitation method. Originally, when dodecyl maltoside was used as the primary detergent in the elution buffer, the purified TRPV1 proteins were easily aggregated, which diminished the success rates of incorporation of single channels into the bilayers. Here, we substituted dodecyl maltoside with lauryl maltose neopentyl glycol in the elution buffer. The purified TRPV1 protein eluted in the presence of lauryl maltose neopentyl glycol was then tested in the bilayer experiments and demonstrated an
increased success rate of single-channel incorporation. This protein was further used in all our bilayer experiments.

**TRPV1 Gating Properties upon Heat Activation in the Presence of PI(4,5)P2**—We previously demonstrated that capsaicin activation of TRPV1 channels requires PI(4,5)P2 in planar lipid bilayers (8). In this study, we performed a detailed analysis of heat activation of TRPV1 in the presence of PI(4,5)P2. Fig. 1 shows representative current traces of TRPV1 obtained at 24, 33, and 43 °C and 100 mV and the corresponding all-points histograms. At 33–39 °C, the channel $P_o$ at 100 mV shifted dramatically from $0.21$ to $0.99$. The increase in $P_o$ was sigmoid, with a steep slope, negligible activity below 33 °C, and a fully open channel above 39 °C (Fig. 2A). Thermodynamic analysis provided a value for the temperature coefficient of TRPV1 activation. We plotted the log of $P_o$ values versus temperature and obtained the temperature-dependent phase of activation (Fig. 2B). Although it deviated from the linearity of the Arrhenius plot, the transition phase exhibited high-temperature sensitivity with $Q_{10} = 18.3$ at 33–39 °C. The $E_a$ for this temperature range was $−155$ kcal/mol. In Fig. 2C, the data with $P_o$ are fitted in a van’t Hoff plot, which represents the relationship of $\ln(K_{eq})$ versus $1/T$, where the equilibrium constant ($K_{eq}$) was determined as $P_o/(1 − P_o)$. Using $\ln(K_{eq}) = −(\Delta H/RT) + (\Delta S/R)$, the values for change in enthalpy and entropy were obtained from the linear fit of the plot, which demonstrated a strong temperature dependence of TRPV1. The best linear fit for the main shift in the openings of TRPV1 was observed between 33 and 39 °C. It exhibited large transitional changes in enthalpy and entropy with $\Delta H = 155$ kcal/mol and $\Delta S = 502$ cal/mol·K. The strong temperature dependence of TRPV1 activation and large values for enthalpy and entropy changes but the small free-energy change obtained for TRPV1 (Fig. 2D) are consistent with previous reports in live cells (1, 9–11).

Fig. 2E demonstrates changes in the conductance of the outward and inward currents under the heating conditions. The changes in conductance exhibited $Q_{10}$ of 2.4 and 2.9 (Fig. 2F) for outward and inward currents, respectively. The temperature analysis of TRPV1 was derived from seven independent experiments, with 73,445 events analyzed.

**Specific Profile of Heat Activation of TRPV1 Channels in Planar Lipid Bilayers by Different Lipids**—To test whether other negatively charged lipids also potentiate heat activation of TRPV1, we characterized TRPV1 activation at 42 °C in the presence of POPG, 2.5 μM dioctanoylphosphatidylinositol 4-phosphate (PI(4)P), and 2.5 μM dioctanoyl-PI(4,5)P2. With each particular lipid type, we found that the channel demonstrated very different behavior and alterations in both $P_o$ and single-channel conductance (Fig. 3).
The addition of POPG in the planar lipid bilayers upon temperature-induced activation of TRPV1 resulted in high channel activity with $P_o = 0.987 \pm 0.003$ obtained at 42 °C and 100 mV (4,277) (Fig. 3, A, upper traces, and C). However, TRPV1 was open only into a small-conductance state (36.2 ± 0.4 pS at 42 °C) (Fig. 3, A, upper traces, and B).

Unlike POPG, in the presence of PI(4)P at 42 °C, TRPV1 demonstrated low $P_o$ ($P_o = 0.131 \pm 0.027$ at 100 mV, 4,277) (Fig. 3, A, middle traces, and C). Further dissimilarity was evident in the conductance values of TRPV1, where the channel exhibited high-current amplitude with a large-conductance state of 108.2 ± 5.6 pS (Fig. 3, A middle traces, and B).

The highest TRPV1 activity at 42 °C was obtained in the presence of PI(4,5)P$_2$, where the channel was open in a large-conductance state of 98.1 ± 1.2 pS and also showed high $P_o$ of 0.926 ± 0.023 (100 mV, 4,277) (Fig. 3, A, lower traces, B, and C). These experiments demonstrate that negatively charged lipids alter TRPV1 channel gating and that the highest channel activity can be obtained in the presence of PI(4,5)P$_2$. The single-channel conductance and $P_o$ previously observed for TRPV1 in cellular systems are similar to the TRPV1 activity in artificial membranes obtained in the presence of PI(4,5)P$_2$ (Fig. 3).

Interestingly, upon temperature activation, phospholipids differentially affect not only channel gating behavior but also single-channel conductance. We detected that PI(4)P induced negligible channel activity starting at 24 °C (Fig. 4), which was not observed in the presence of other lipids (Fig. 1). At 24 °C, PI(4)P induced TRPV1 channel opening in a small-conductance state (12.4 ± 1.3 pS) and showed minor $P_o$ (0.00567, number of events analyzed = 97) (Fig. 4A). However, upon temperature increase, TRPV1 underwent strong rearrangements, where the channel sharply stepped up into a high-conductance state (108.5 ± 5.6 pS at 42 °C), indicating a pore conformational change. To further evaluate this interesting feature, we compared heat-induced TRPV1 activation and cold-induced TRPM8 activation in the presence of PI(4)P. Fig. 4 shows single-channel recordings of TRPV1 and TRPM8 activated under...
heating and cooling conditions, respectively. Similar to TRPV1, the temperature-induced activation of TRPM8 in the presence of PI(4)P (5 μM) resulted in a dramatic increase in $P_o$ (at 20 °C, $P_o = 0.214 \pm 0.032$, and at 18 °C, $P_o = 0.789 \pm 0.055$; $n = 12$). Moreover, at 18 °C, TRPM8 channels abruptly switched into a higher conductance state, where conductance ($G$) = 2.899 ± 0.063 pS obtained at 20 °C increased by ~10-fold with $G = 29.629 \pm 0.32$ pS (Fig. 4B). This unusual behavior was very different from that of cold-induced TRPM8 activation in the presence of PI(4,5)P$_2$ (13), where strong conductance changes do not take place upon cooling, but the channel is initially open in the low-conductance state. These results indicate that both TRPV1 and TRPM8 channels undergo strong conformational changes upon temperature activation, and this regulation is highly dependent on the type of phosphoinositide present in the membrane.

**DISCUSSION**

The role of phosphoinositides in TRPV1 has been studied extensively, yet it is heavily debated whether PI(4,5)P$_2$ facilitates or inhibits channel activity (3, 5, 6, 8, 14). The reasons for these contradictory findings could arise from the different experimental conditions, approaches, or complex regulatory mechanisms.

One of the recent studies performed on the purified TRPV1 channel reconstituted into artificial liposomes demonstrated an inhibitory role of PI(4,5)P$_2$ on TRPV1 upon heat- or capsaicin-induced activation (6). However, it has been demonstrated that purified TRPV1 incorporated into planar lipid bilayers requires the presence of PI(4,5)P$_2$ for activation with capsaicin (8).

A further complexity of this regulation could originate from side-specific effects of PI(4,5)P$_2$, where the phosphoinositide exerts an inhibitory action on TRPV1 from the extracellular side but an activating effect when present on the inner side of the membrane (7). In our study, we did not observe side-specific effects of PI(4,5)P$_2$, as we added the phosphoinositide to both the cis- and trans-compartments, and it is difficult to evaluate asymmetry in the bilayers due to the lack of preferred orientation of the channel in the membrane. These discrepancies in the results could be due to the different concentrations. The concentration of PI(4,5)P$_2$ used in this study (2.5 μM) is much lower than those used in the previous work that specified asymmetry (50 μM at the intracellular side and 200 μM at the extracellular side). It is possible that in addition to the asymmetry, high doses of PI(4,5)P$_2$ may also cause differential effects.

Another factor that should be considered is the regulation of TRPV1 activity by the other negatively charged lipids. As was shown recently, phospholipids with a single negative charge, including phosphatidylinositol, phosphatidylserine, and POPG, induced TRPV1 activity in excised patches (8). Here, we demonstrated that TRPV1 can be activated by heat in the presence of negatively charged POPG lipids (Fig. 3). Importantly, when TRPV1 channels are incorporated into neutral membranes, activation with capsaicin (8) or heat (Figs. 1, 4) requires the addition of phosphoinositides.

In planar lipid bilayers, the initial activation temperature of TRPV1 in the presence of 2.5 μM PI(4,5)P$_2$ is ~33 °C, which is 9 °C lower compared with the cell recordings (42 °C) (1, 9). The plasma membrane is composed of different lipids that could affect the function of the channels. For instance, higher cholesterol levels in the membrane were shown to increase the temperature threshold of TRPV1 activation in HEK-293 cells without changing the intrinsic gating properties of the channel (9). Our lipid bilayers consisted of only POPC/POPE and no cholesterol, which might be the reason for the lower temperature threshold of the reconstituted TRPV1 channels.

Intriguingly, in the presence of different phospholipids, the TRPV1 channel not only demonstrates a difference in gating
behavior but also maintains various conductance states (Figs. 3 and 4). Similar behavior was observed previously for the cold and menthol receptor TRPM8, where the channel exhibited significantly different gating modes and conductance states when activated with menthol in the presence of various phosphoinositides (13). Furthermore, we compared temperature-induced activation of TRPV1 and TRPM8 in the presence of PI(4)P. With this phosphoinositide, both channels initially open with a small-conductance state (Fig. 4). However, upon temperature change (increase in the case of TRPV1 and decrease in the case of TRPM8), both channels show a sharp increase in their conductance states (Fig. 4). These characteristics of the temperature-dependent TRP channels indicate that they undergo drastic conformational rearrangements with

*Phospholipids Potentiate Heat Activation of TRPV1*

**FIGURE 4.** TRPV1 and TRPM8 undergo essential conformational rearrangements during temperature activation in the presence of PI(4)P. Planar lipid bilayer experiments were performed on temperature-activated TRPV1 and TRPM8 proteins in the presence of PI(4)P (PIP). Experimental conditions were as described in the legend to Fig. 1. The TRPM8 protein was purified from HEK-293 cells as described previously (13, 17–19). A, representative single-channel current trace recordings of TRPV1 channels obtained upon heating the bilayer system from 24 to 42 °C in the presence of 2.5 μM PI(4)P. Representative current trace recordings demonstrate different segments upon heating. The temperatures are indicated above the traces. Clamping voltages are shown. B, representative single-channel current trace recordings of TRPM8 channels obtained at 20, 18, and 16 °C in the presence of 5 μM PI(4)P. C and D, P<sub>v</sub> and conductance changes recorded for TRPV1 and TRPM8 channels, respectively, at the temperature ranges indicated. The values were obtained at 100 mV. For TRPV1, P<sub>v</sub> values differed significantly, with p = 0.0084 between 24 and 42 °C, and p = 0.0086 for 37 and 42 °C. Similarly, P<sub>v</sub> values were significantly different for TRPM8, with p = 0.01107 between 20 and 18 °C, and p = 0.00426 for 20 and 16 °C. Conductance values were significantly higher for both channels: for TRPV1, p = 0.00537 between 24 and 37 °C, and p = 0.00326 for 24 and 42 °C; for TRPM8, p = 1.46 × 10<sup>-4</sup> between 20 and 18 °C, and p = 1.24 × 10<sup>-4</sup> for 20 and 18 °C. *, p < 0.05; **, p < 0.01; ***, p < 0.005.
temperature change and that phosphoinositides are important regulators of both the gating transitions and conductance states. At first glance, this behavior might seem puzzling, as conductance is generally considered an intrinsic signature of ion channels, and this property should be constant for a particular channel under the defined ionic conditions. Understanding the phenomenon of the complex gating and permeation mechanisms of TRP channels has been only hypothetical. However, recent advances in the cryo-electron microscopy approach made it possible to obtain the molecular structure of TRPV1 at high resolution, which provided valuable and exciting insights into the structural arrangements of the channel with the ligands (15, 16). In particular, Cao et al. (15) demonstrated that the TRPV1 pore region adopts dual gating that confers distinct conformations in the presence of different agonists. Various structural adaptations of TRPV1-ligand complexes further indicate a possibility for the altered conformational arrangements of the channel in the presence of its critical gating cofactors, phosphoinositides.

Another interesting aspect is that transitions in temperature-induced TRPV1 and TRPM8 activation in the presence of PI(4)P demonstrate a sharp increase in conductance along with $P_o$ (Fig. 4). Although an increase in conductance would be expected for the heat-activated TRPV1 channel, this behavior is rather unusual for TRPM8. It is interesting that when TRPM8 is activated by cold in the presence of PI(4,5)P$_2$, the conductance, which is initially large (68 pS at 20 °C), gradually decreases along with the temperature decline (13), although in the case of PI(4)P, the conductance of TRPM8 first jumps from 2.9 pS at 20 °C to 29 pS at 18 °C and then starts to decrease gradually. It is noteworthy that a similar tendency is observed for TRPV1. Upon temperature-induced activation in the presence of PI(4,5)P$_2$, the TRPV1 channel also undergoes gradual changes in conductance (Fig. 2), whereas PI(4)P causes its sharp increase (Fig. 4). It is possible that with fewer phosphate groups in the case of PI(4)P, the protein/lipid-interacting domain could be unstable, which could result in sharp conformational changes upon temperature-induced activation. This could also indicate that PI(4,5)P$_2$ is a better fit for interaction with the channel with regard to the number of phosphate groups as (1) charge contributors and (2) occupants of the lipid-binding domain. Overall, our results demonstrate that phosphoinositides are essential regulators of TRP channels and that TRPV1, similar to TRPM8, requires their presence in the membrane for temperature-induced activity.

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