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INVITED REVIEW

Phosphoinositide regulation of TRPV1 revisited

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Abstract The heat- and capsaicin-sensitive transient receptor potential vanilloid 1 ion channel (TRPV1) is regulated by plasma membrane phosphoinositides. The effects of these lipids on this channel have been controversial. Recent articles re-ignited the debate and also offered resolution to place some of the data in a coherent picture. This review summarizes the literature on this topic and provides a detailed and critical discussion on the experimental evidence for the various effects of phosphatidylinositol 4,5-bisphosphayte [$PI(4,5)P_2$ or PIP_2] on TRPV1. We conclude that $PI(4,5)P_2$ and potentially its precursor PI(4)P are positive cofactors for TRPV1, acting via direct interaction with the channel, and their depletion by Ca^{2+}-induced activation of phospholipase Cδ isoforms (PLCδ) limits channel activity during capsaicin-induced desensitization. Other negatively charged lipids at higher concentrations can also support channel activity, which may explain some controversies in the literature. $PI(4,5)P_2$ also partially inhibits channel activity in some experimental settings, and relief from this inhibition upon PLCβ activation may contribute to sensitization. The negative effect of $PI(4,5)P_2$ is more controversial and its mechanism is less well understood. Other TRP channels from the TRPV and TRPC families may also undergo similar dual regulation by phosphoinositides, thus the complexity of TRPV1 regulation is not unique to this channel.

Keywords TRP channels · PIP2 · TRPV1 · Phosphoinositides

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Introduction

We published a focused review in 2008 about regulation of transient receptor potential vanilloid 1 ion channel (TRPV1) by the phospholipase C (PLC) pathway, with special emphasis on the role of phosphoinositides [94]. Since then, several reviews on more general topics discussed phosphoinositide regulation of TRPV1 [29, 40, 78, 95, 96]. Due to the unceasing interest and recent developments in this controversial topic [8, 65, 66, 84, 105, 114], however, I felt that a comprehensive and specific discussion is warranted on phosphoinositide regulation of TRPV1.

The capsaicin receptor in sensory neurons of the dorsal root ganglia (DRG) had been a topic of intensive research for several decades [115]. Its molecular identity as the TRPV1 channel was uncovered in the seminal 1997 paper by David Julius' laboratory [10]. Soon after its cloning, genetic deletion of TRPV1 in mice confirmed its role in thermal hyperalgesia, the increased sensitivity to heat, upon inflammation [11, 18]. Thermal hyperalgesia is mediated by pro-inflammatory agents, such as bradykinin, which sensitize the channel to its major activators: heat, capsaicin, and low extracellular pH, acting mainly via the phospholipase C (PLC) pathway. Protein kinase C (PKC), which is downstream of PLC, plays an important role in bradykinin-induced sensitization [12] and was shown to sensitize TRPV1 via direct phosphorylation of the channel protein [4, 79, 126].

$PI(4,5)P_2$ is a quantitatively minor phospholipid in the inner leaflet of the plasma membrane; its metabolism is depicted in Fig. 1. $PI(4,5)P_2$ is best known as the precursor for two classical second messengers inositol 1,4,5-trisphosphate (IP_3), which releases Ca^{2+} from the endoplasmic reticulum, and diacyl-glycerol (DAG), which activates PKC. Its immediate precursor, PI(4)P, is also a substrate for PLC, and its cleavage in cellular systems have been demonstrated [25], but probably all PLC isoforms are more active on $PI(4,5)P_2$

[89]. PI(4,5)P_2 also regulates many cellular processes such as cytoskeletal organization and vesicular traffic [2, 19, 101]. Phosphoinositides often act as membrane anchors for cytoplasmic proteins, and many of their biological effects are exerted via various lipid-binding domains such as the Pleckstrin homology (PH) domain [56].

PI(4,5)P_2 is also a general regulator of ion channels [29, 38, 40, 62, 112]. Shortly after the initial reports on the effects of PI(4,5)P_2 on inwardly rectifying K^+ (Kir) channels [3, 37, 41, 107, 113], TRPV1 was shown to be regulated by PI(4,5)P_2 [13]. As opposed to Kir channels, however, for which PI(4,5)P_2 is a positive co-factor, it was proposed that PI(4,5)P_2 inhibits TRPV1. Relief from this tonic inhibition upon activation of PLC by pro-inflammatory agents, such as bradykinin, was suggested to be responsible for sensitization of these channels [13], offering an alternative to the PKC hypothesis. In the following years, several papers reported a seemingly contradictory result: PI(4,5)P_2 in excised inside-out patches activated, rather than inhibited TRPV1 [49, 64, 109],

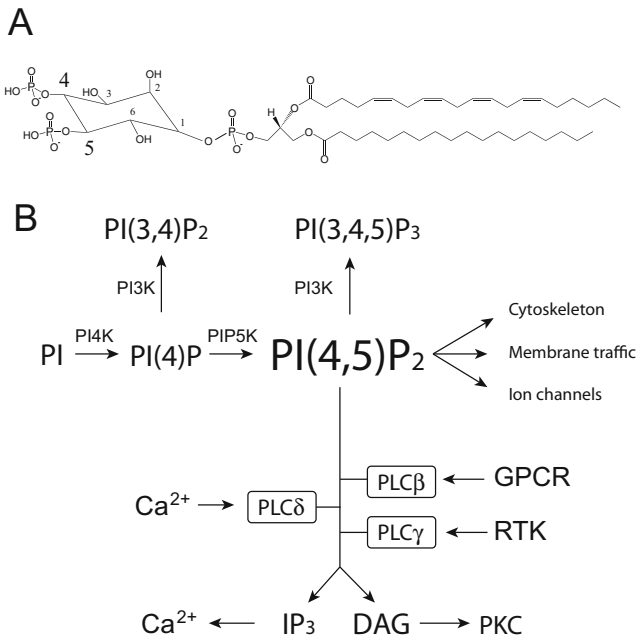

Fig. 1 Phosphoinositide metabolism in the plasma membrane. **a** Chemical formula of PI(4,5)P_2. **b** The metabolism of phosphoinositides: PI(4,5)P_2 is generated via phosphorylation of phosphatidylinositol (*PI*) by phosphatidylinositol 4-kinases (*PI4K*) to generate PI(4)P, which is further phosphorylated by phosphatidylinositol 4-phosphate 5′-kinases (*PIP5K*). PI(4,5)P_2 is hydrolyzed by various PLC enzymes to generate inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (*DAG*). PLCβ-s are stimulated by G-protein coupled receptors (GPCR), PLCγ-s are by Receptor tyrosine kinases (RTK). Not shown in the cartoon, but most PLC isoforms also hydrolyze PI(4)P even though less efficiently than PI(4,5)P_2. Phosphoinositide 3-kinases (*PI3K*) phosphorylate PI(4,5)P_2 and PI(4)P on the 3′-phosphate, and the resulting PI(3,4,5)P_3 and PI(3,4)P_2 are important second messengers for growth factor signaling. PI is relatively abundant in the plasma membrane; it is found up to 10 %; PI(4,5)P_2 and PI(4)P are found at ~1 %, whereas PI(3,4)P_2 and PI(3,4,5)P_3 are barely detectable in resting cells, and even in stimulated cells, they do not reach higher than 0.1 % [2]

raising doubts about PI(4,5)P_2 being inhibitory. Several other reports however were compatible with the existence of a partial inhibitory effect of PI(4,5)P_2, present in intact cells [45, 64, 75, 83]. Most of the data in the literature had been consistent with a general dependence of TRPV1 activity on phosphoinositides, most likely via direct interactions of these lipids with the channel, and the possible presence of an indirect partial inhibition by PI(4,5)P_2 [29, 95].

A recent paper shook up this relatively settled controversy by showing that the purified TRPV1 protein reconstituted in artificial lipid vesicles was fully active in the absence of phosphoinositides, arguing against dependence of channel activity on these lipids [8]. Furthermore, the sensitivity of the purified channel to capsaicin and heat decreased when phosphoinositides were included in the lipid mixture, suggesting a direct inhibition by these lipids [8].

Here, I will summarize the literature, critically discuss the experiments supporting the dependence of TRPV1 activity on phosphoinositides, and also review data in support of inhibition by PI(4,5)P_2, including recent articles that addressed several aspects of this controversy [65, 84, 105, 114]. In addition, I will also discuss implications of the recent side-chain resolution structures of TRPV1 [9, 57] and show examples on other TRP channels where similar dual effects of PI(4,5)P_2 have been reported.

Dependence of TRPV1 activity on phosphoinositides and its role in desensitization

Excised inside-out patch clamp measurements

Application to the "cytoplasmic" surface of excised inside-out patches has been the gold standard of demonstrating direct effects of phosphoinositides on ion channels. There is a universal agreement in the literature that phosphoinositides are positive regulators of TRPV1 in excised inside-out patches, see examples in Fig. 2. First, it was shown that TRPV1 heterologously expressed in F11 cells was inhibited by poly-lysine, an agent often used to chelate endogenous phosphoinositides [109]. The same article showed that both synthetic dipalmitoyl (diC_{16}) and dioctanoyl (diC_8) PI(4,5)P_2 increased the activity of TRPV1. Shortly after this report, Lukacs et al. demonstrated that TRPV1 channels expressed in *Xenopus* ooctyes were inhibited by poly-lysine and activated by both long acyl chain natural (arachidonyl-stearyl (AASt)) PI(4,5)P_2 (Fig. 2a) and synthetic diC_8 PI(4,5)P_2 (Fig. 2b). The same article also showed that many other diC_8 phosphoinositides, including PI(4)P, PI(3,4,5)P_3 and PI(3,4)P_2, also activate TRPV1 in excised inside-out patches (Fig. 2b) [64]. Klein et al. confirmed the activation of TRPV1 by PI(4,5)P_2, PI(4)P and PI(3,4,5)P_3 on TRPV1 channels expressed in F11 cells, and showed that the channel in excised patches was inhibited by the purified PH

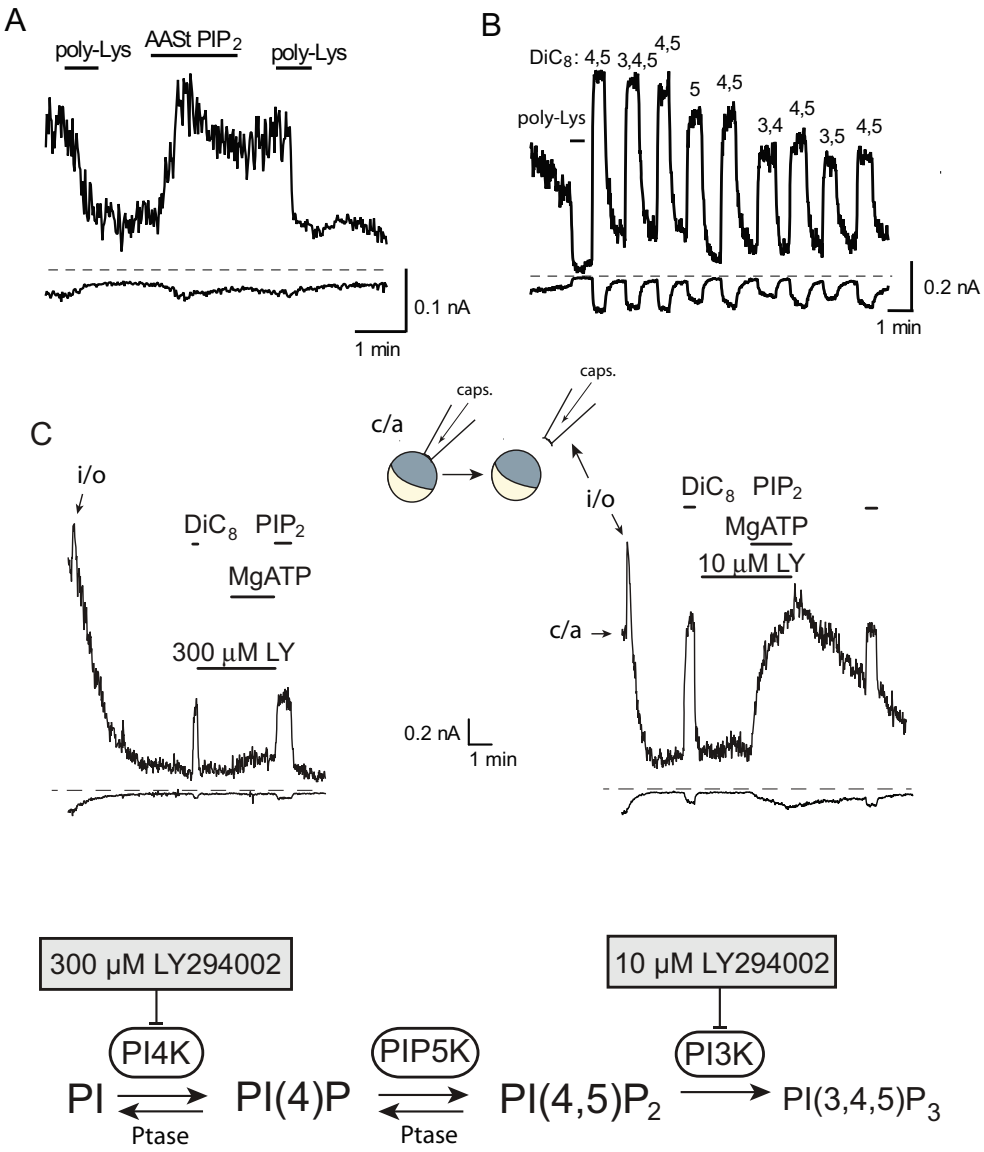

Fig. 2 The effects of exogenous and endogenous phosphoinositides in excised inside-out patches on TRPV1 from Refs. [64, 65] with permission. **a**, **b** Representative traces for measurements in excised inside-out patches in the presence of 1 μM capsaicin in the patch pipette at +100 and −100 mV. Application of 1 and 30 μg/ml poly-lysine and 5 μM AASt $PI(4,5)P_2$ (**a**) and 50 μM diC_8 $PI(4,5)P_2$, $PI(3,4,5)P_3$, $PI(3,4)P_2$, $PI(3,5)P_2$, and PI(5)P (**b**) are shown [64]. **c** Excised inside-out patch measurements in the presence of 0.2 μM capsaicin in the patch pipette, the applications of 2 mM MgATP, 300 and 10 μM LY294002, and 25 μM diC_8 $PI(4,5)P_2$ are shown [65]

domain of PLCδ1 which binds $PI(4,5)P_2$ [49]. A follow-up article from the same lab confirmed the positive effects of $PI(4,5)P_2$ in excised patches in DRG neurons and showed that a proximal C-terminal region of TRPV1 binds to $PI(4,5)P_2$ [123].

All experiments discussed so far were from two laboratories and were performed on capsaicin-stimulated TRPV1 channels. Data from a third lab showed that TRPV1 activity in the absence of capsaicin was also stimulated by $PI(4,5)P_2$ in excised patches in HeLa cells, even though the effect was much smaller than that evoked by capsaicin [47]. A fourth laboratory confirmed these data and showed that $PI(4,5)P_2$-stimulated TRPV1 in excised patches in HEK cells at positive voltages, and the effect was further enhanced by the application of capsaicin [84]. These data confirm the effects of $PI(4,5)P_2$ as a positive regulator of TRPV1 and show that the effect of this phosphoinositide does not per se depend on capsaicin. These data are also in line with $PI(4,5)P_2$ being a co-factor, rather than a bona fide agonist of the channel, because the effect of $PI(4,5)P_2$ at physiological voltages were very small [47], and $PI(4,5)P_2$ only induced substantial currents in the absence of capsaicin at extreme positive voltages [84], and as will be discussed later, voltage can also be considered an agonist of TRPV1 [127].

A hallmark of $PI(4,5)P_2$-activated ion channels is the decrease of activity (rundown) in excised patches, which is caused by the gradual dephosphorylation of $PI(4,5)P_2$ and PI(4)P by phosphatases in the patch membrane [41, 113, 137]. TRPV1 activity in the presence of saturating capsaicin concentrations shows only moderate and slow rundown [64], which can be explained by the very high apparent affinity of these channels to $PI(4,5)P_2$ [49]. We recently showed that the activity of TRPV1 in the presence of low capsaicin concentrations ran down to ~10 % of initial activity after excision of the patch into an ATP-free medium (Fig. 2c) [65]. Channel

activity was slowly restored by supplying MgATP to excised patches, and this effect was prevented either by inhibiting type 3 phosphatidylinositol 4-kinases (PI4K) by high concentrations of LY294002 (Fig. 2c) or by removing phosphatidylinositol (PI) the substrate of PI4K by a PI-specific bacterial phospholipase C enzyme (PI-PLC) [65]. These data show that endogenous $PI(4,5)P_2$ and PI(4)P, generated by lipid kinases, support TRPV1 activity.

The data briefly described here from four different laboratories, using different expression systems as well as native DRG neurons show that phosphoinositides reproducibly activate TRPV1 channels in excised patches. This effect was demonstrated using both synthetic and natural lipids applied exogenously and stimulating endogenous lipid kinases with MgATP. Importantly, to my knowledge, there is no report claiming no effect or inhibition by phosphoinositides in excised inside-out patches on TRPV1.

Inducible lipid phosphatases

The data described so far unanimously support positive regulation of TRPV1 by $PI(4,5)P_2$; iterations of one experimental technique however may lead to erroneous conclusions. On several voltage-gated K^+ channels, for example, excised patch data suggested various effects of $PI(4,5)P_2$, which could not be confirmed by subsequent experiments in intact cells using inducible lipid phosphatases [52], for detailed discussion see Ref. [39]. Chemically inducible and voltage-sensitive phosphoinositide phosphatases dephosphorylate $PI(4,5)P_2$ and in some cases PI(4)P, and thus decrease phosphoinositide levels without generating second messengers, offering a "cleaner" way than PLC activation to reduce $PI(4,5)P_2$ levels [74, 111, 125].

The first one of these tools, based on the rapamycin-induced heterodimerization of FKBP12 and the FRB fragment of the mammalian target of rapamycin was developed independently by Meyer's [111] and Balla's [125] laboratories. In these systems, application of rapamycin leads to the translocation of a 5′-phosphatase to the plasma membrane, where it dephosphorylates $PI(4,5)P_2$ [111, 125]. The first article using the inducible phosphatase developed by the Balla lab showed that capsaicin-induced TRPV1 activity was not inhibited by rapamycin [64]. This was explained by the fact that channel activity is also supported by PI(4)P, which is not degraded by the 5′-phosphatase. Two subsequent publications confirmed this interpretation using a newly developed rapamycin-inducible dual 4′- and 5′-phosphatase termed pseudojanin. Both articles found that only combined depletion of both $PI(4,5)P_2$ and PI(4)P inhibited TRPV1 stimulated by capsaicin [33] or by low pH [66]. Two articles however found that the rapamycin-inducible 5′-phosphatase developed by the Meyer lab [111] slowly but significantly inhibited TRPV1 [49, 134].

Using voltage-dependent phosphatases (VSP), cloned from various organisms, including *Ciona intestinalis* (ciVSP) and *Danio rerio* (drVSP), is an alternative approach to selectively deplete $PI(4,5)P_2$ [74, 82]. The phosphatase domain of VSPs is a homologue of PTEN, which removes the 5′ phosphate from either $PI(3,4,5)P_3$ or $PI(4,5)P_2$. Because $PI(3,4,5)P_3$ in resting cells is essentially undetectable [2], the main effect of activating VSPs by depolarization is conversion of $PI(4,5)P_2$ into PI(4)P. TRPV1 activity was shown to be slowly inhibited upon repeated depolarizations in excised inside-out patches expressing ciVSP, arguing for an essential role for $PI(4,5)P_2$ [49]. In excised inside-out patches however, TRPV1 activity also shows spontaneous rundown (Fig. 2c) [65] thus it is difficult to tell how much of the decrease in activity was due to VSP activity and spontaneous dephosphorylation by endogenous lipid phosphatases in the patch. In another publication, drVSP exerted no inhibition upon a 3-s depolarizing pulse in whole cell patch clamp experiments. This maneuver fully inhibited Kir2.1 channels that show high specificity and high affinity for $PI(4,5)P_2$ [66]. This result with drVSP is in line with the earlier proposal that PI(4)P is also capable of supporting TRPV1 activity.

Overall, all experimental data described so far support, or compatible with, the dependence of TRPV1 activity on phosphoinositides, but there is a debate on the relative contributions of $PI(4,5)P_2$ and PI(4)P. One group of papers concluded that $PI(4,5)P_2$ is the key co-factor for TRPV1 activity, and depletion of this lipid inhibits the channel [49, 134]. Other articles concluded that $PI(4,5)P_2$ and PI(4)P are similarly important, and only their combined depletion inhibits channel activity [33, 64, 66].

What could explain the discrepancy between the experimental results from different groups? To attempt this, we need to revisit the effects of PI(4)P and $PI(4,5)P_2$.

The relative roles of $PI(4,5)P_2$ and PI(4)P and interpretation of the inducible phosphatase data

There are two published dose responses of $PI(4,5)P_2$ and PI(4)P on TRPV1. Lukacs et al. [64] found that the channel expressed in *Xenopus* oocytes, had an EC_{50} of 4.9 μM for $PI(4,5)P_2$ and 32.4 μM for PI(4)P in the presence of 1 μM capsaicin at +100 mV. At −100 mV, the apparent affinities for both $PI(4,5)P_2$ and PI(4)P were somewhat lower. Klein et al. [49] found that TRPV1 expressed in F11 cells had EC_{50} values of 1.3 μM for $PI(4,5)P_2$ and 16.7 μM for PI(4)P in the presence of 0.3 μM capsaicin. Even though the numbers differ somewhat, there is an agreement between the two papers that in excised inside-out patches the apparent affinity of TRPV1 for $PI(4,5)P_2$ is higher than that for PI(4)P, but the maximal effects of the two lipids are similar [49, 64].

Using the data obtained in F11 cells [49], Collins et al. [15] calculated the mole fraction of diC_8 $PI(4,5)P_2$ and diC_8 PI(4)P

that is reached in the plasma membrane during dose–response measurements in excised inside-out patches, taking into consideration the different extent of incorporation of $PI(4,5)P_2$ and PI(4)P into biological membranes (Fig. 3). Here, we briefly discuss quantitative considerations, based on their calculations, to understand the effects of conversion of $PI(4,5)P_2$ to PI(4)P on TRPV1 activity, assuming that the resting concentrations of both lipids in the plasma membrane are 1 mole %.

Based on the data of Collins et al., resting cellular $PI(4,5)P_2$ levels would fully saturate TRPV1; a 10-fold decrease would not inhibit the channel substantially and full inhibition would require a 100-fold drop in the concentration of $PI(4,5)P_2$ (Fig. 3). Resting PI(4)P on the other hand would support a ~50 % activity, in other words, it is around EC_{50} where the channel is most sensitive to changes in the concentration of the lipid. These affinities have significant implications for interpreting the inducible 5′-phopshatase data, and predict that even small differences in experimental conditions may give different results. Rapid conversion of $PI(4,5)P_2$ to PI(4)P theoretically would double the concentration of PI(4)P, which would maintain an ~80 % activity of TRPV1, even if $PI(4,5)P_2$ is completely eliminated. Thus theoretically, using these numbers, acute activation of a 5′-phosphatase should induce a ~20 inhibition. Reduction of $PI(4,5)P_2$ to zero however is unlikely, because PIP5K enzymes continuously resynthesize $PI(4,5)P_2$. PIP5K enzymes are much faster than PI4Ks [24], thus it is possible that they can maintain some minimal levels of $PI(4,5)P_2$ even when a phosphatase is turned on. Even a small amount of residual $PI(4,5)P_2$, or a minor deviation from these numbers between different cell preparations and experimental conditions, could easily shift this balance to no detectable inhibition. On the other hand, the cell may decrease PI(4)P

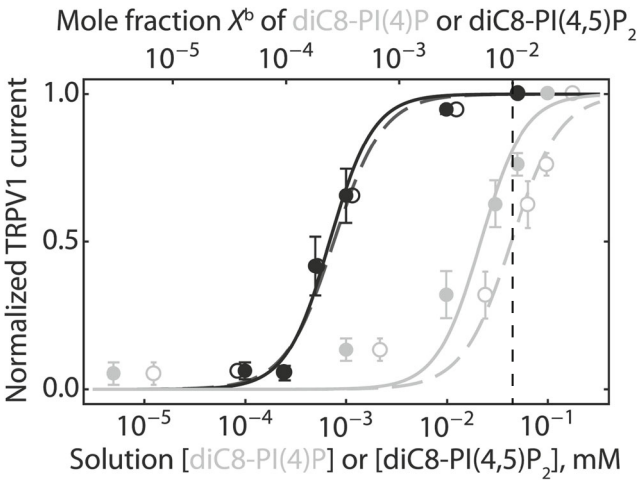

Fig. 3 Quantification of the effects of $PI(4,5)P_2$ and PI(4)P in excised inside-out patches, from Ref. [15] with permission. The *vertical dashed line* shows 1 % which we assume corresponds to cellular levels of both $PI(4,5)P_2$ and PI(4)P. The *dashed curve* fit to the PI(4)P data shows theoretical PI(4)P dose response corrected for the more efficient incorporation of PI(4)P into biological membranes due to its higher lipophilicity compared with $PI(4,5)P_2$, see [15] for details

over time back to its original level by feedback regulations, where it would exert a ~50 % activity, again, where the channel is the most sensitive to changes of the concentration of the lipid. Even a small activity of the 5′-phosphatase towards the 4′-phosphate, or differences in the experimental conditions, such as cell type, ATP concentration could easily shift this balance towards a significant inhibition.

There have been many differences in experimental conditions between the publications reporting seemingly opposing results using the inducible 5′-phosphatases. These include differences in cell types, composition of intracellular solutions, drug concentrations used, and treatment protocols; detailed discussion of these would probably not lead any closer to a resolution. One simple and consistent experimental difference however will be briefly discussed here. The two articles showing inhibition of TRPV1 by the rapamycin-inducible 5′-phosphatase [49, 134] both used the construct developed by Meyer's laboratory [111], which contains the Inp54p from yeast. The articles not finding inhibition by the rapamycin-inducible 5′ phosphatase [33, 64] used a different construct that had the human type IV phosphatase INPP5E. Both these phosphatases were shown to be specific for the 5′ phosphate in vitro, but there is no guarantee that in the given system their specificities and efficiencies are exactly the same. As discussed before, either a small difference in the level of selectivity of the phosphatases between the 5′- and 4′-phosphates, or differences in their ability to fully eliminate $PI(4,5)P_2$ may contribute to the different results obtained with them.

These calculations contain a number of uncertainties, including for example local concentrations of phosphoinositides, and the potentially different effectiveness of diC_8 and natural $PI(4,5)P_2$. Also, as mentioned earlier, the two reported that phosphoinositide dose responses were not identical; in Lukacs et al. [64], the affinity of TRPV1 for $PI(4,5)P_2$ was somewhat lower, but also the channel was less selective for $PI(4,5)P_2$ over PI(4)P than that reported by Klein et al. [49]. The sole purpose of this exercise was to demonstrate the theoretical possibility that PI(4)P contributes to channel activity in a cellular environment and that selective conversion of $PI(4,5)P_2$ to PI(4)P does not inhibit TRPV1 [33, 66], despite the lower affinity of the channel for PI(4)P.

Overall, the role of $PI(4,5)P_2$ in supporting TRPV1 activity in cellular systems is very well established by both excised patch data and inducible lipid phosphatases, how much PI(4)P contributes to channel activity is debated and potentially depends on the experimental conditions.

The functional role of phosphoinositide dependence in desensitization

TRPV1 activity decreases during prolonged stimulation by saturating concentrations of capsaicin in the presence of extracellular Ca^{2+} (Fig. 4), similar to many other Ca^{2+} permeable

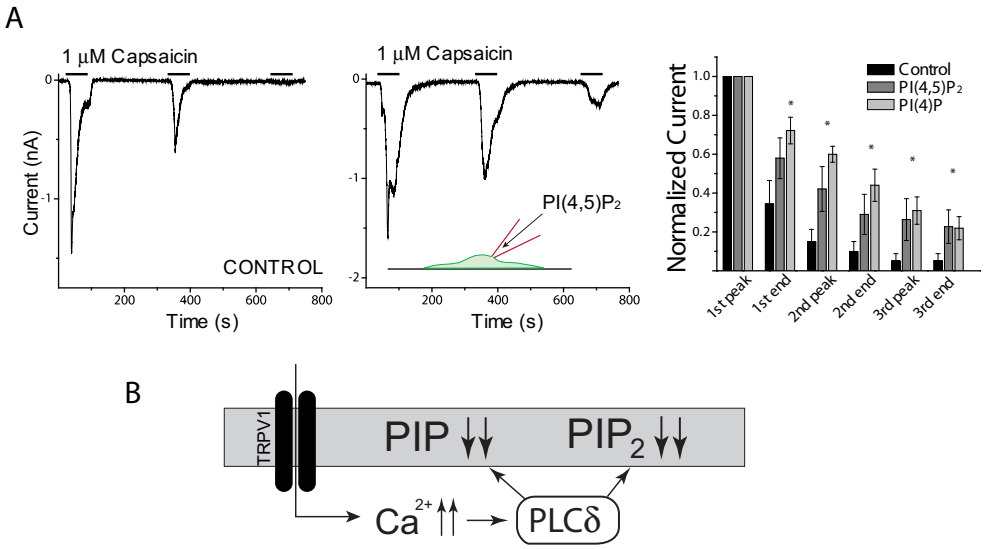

Fig. 4 Desensitization of TRPV1 in DRG neurons, from reference [66] with permission. **a** Whole cell patch clamp measurements in DRG neurons at −60 mV with and without 100 μM diC_8 $PI(4,5)P_2$ in the patch pipette. *Right*, quantification of the data normalized to the peak current evoked by capsaicin. **b** Cartoon showing the model for desensitization, in which Ca^{2+} influx though TRPV1 activates a highly Ca^{2+}-sensitive PLCδ isoform and the robust decrease in both $PI(4,5)P_2$ and PI(4)P inhibits channel activity. See text for more details

ion channels. This phenomenon is termed desensitization [50] or adaptation [134], and it may contribute to the paradoxical local analgesic effect of this compound [115]. The first indication for the involvement of phosphoinositides came from Liu et al. who showed that recovery from desensitization required intracellular MgATP and was abolished by inhibiting PI4K with high concentrations of wortmannin [60]. They also showed that capsaicin application inhibited the $PI(4,5)P_2$ dependent Kir2.1 channels, suggesting $PI(4,5)P_2$ depletion upon TRPV1 activation. This paper was published when the prevailing view was that $PI(4,5)P_2$ inhibited TRPV1 channels, and the effects of phosphoinositides were not tested in excised patches. As discussed earlier, shortly after this publication, Klein et al. [49] and Lukacs et al. [64] showed that indeed $PI(4,5)P_2$ activated TRPV1 in excised patches. Lukacs et al. also showed that PLC inhibitors as well as inclusion of $PI(4,5)P_2$ and PI(4)P in the whole cell patch pipette inhibited desensitization of recombinant TRPV1 channels. Furthermore, activation of PLC upon capsaicin application was confirmed by both fluorescence based $PI(4,5)P_2$ and PI(4)P measurements and by demonstrating IP_3 formation in TRPV1 expressing cells [64]. Subsequent publications confirmed that $PI(4,5)P_2$ dialysis through the patch pipette inhibited desensitization of TRPV1 expressed in SF9 insect cells [58], and in the HEK293 derivative tsA201 cells [135]. Finally, it was shown that activation of native TRPV1 by capsaicin in DRG neurons leads to depletion of $PI(4,5)P_2$ and PI(4)P, and that inclusion of $PI(4,5)P_2$ or PI(4)P in the whole cell patch pipette inhibited desensitization (Fig. 4a) [66]. TRPV1 desensitization was also diminished in DRG neurons isolated from mice lacking the highly Ca^{2+}-sensitive PLCδ4 isoform [66].

As mentioned earlier, TRPV1 has very high affinity for $PI(4,5)P_2$ (Fig. 3), and theoretically, a 100-fold decrease may be required for its full inhibition. This is in line with the finding that most PLC-stimulating G-protein coupled receptor (GPCR) agonists do not inhibit its activity. Capsaicin is not primarily known as a PLC activator; how can it induce phosphoinositide depletion to an extent that significantly limits channel activity? While the mechanism is not fully elucidated, the extreme increase in cytoplasmic Ca^{2+} in response to TRPV1 activation by capsaicin is likely to be an important factor. All PLC isoforms require Ca^{2+} for activity, but Ca^{2+} alone probably activates predominantly PLCδ isoforms [89]. In our hands, capsaicin-induced activation of TRPV1 was a more efficient way to reduce not only PI(4)P but also $PI(4,5)P_2$ levels than PLCβ activation via GPCRs by bradykinin in native DRG neurons [66] and even in recombinant systems over-expressing Gq-coupled M1 muscarinic receptors [5]. In DRG neurons, capsaicin evoked a larger Ca^{2+} signal and a larger decrease in $PI(4,5)P_2$ and PI(4)P levels than activation of voltage gated Ca^{2+} channels by 30 mM KCl, when measured with a low affinity Ca^{2+}-sensitive dye [66]. TRPV1 is a non-selective cation channel with relatively high Ca^{2+} permeability; when activated by capsaicin, ~10 % of the current is carried by Ca^{2+} [102]. TRPV1 is highly expressed in DRG neurons, its activation results in currents well over 1 nA, thus it probably induces very large elevations of Ca^{2+} in the cytoplasm. Capsaicin however also induced a small but statistically significant reduction in $PI(4,5)P_2$ in the absence of extracellular Ca^{2+} in DRG neurons [66] thus it is possible that the efficiency of TRPV1 activation as a PLC stimulus has components additional to the large increase of cytoplasmic Ca^{2+}.

Overall these data provide strong support to the model in which Ca^{2+} influx through TRPV1 activates Ca^{2+}-sensitive PLC isoforms (mainly PLCδs) leading to depletion of $PI(4,5)P_2$ and PI(4)P, which limits channel activity (Fig. 4b). This model does not exclude the contribution of other mechanisms to desensitization, such as calmodulin [58, 80,

98], calcineurin [20, 71], ATP [58], or internalization [103], since neither PLC inhibitors nor phosphoinositides inhibited desensitization completely in most publications.

As it will be discussed later, activation PLCβ via GPCRs usually potentiate TRPV1; one notable exception is discussed here. It was proposed that prostatic acid phosphatase (PAP) inhibits TRPV1 and reduces pain signaling via generating adenosine, which activates PLC and decreases $PI(4,5)P_2$ acting on A_1R adenosine receptors [108]. Intrathecal injection of $PI(4,5)P_2$ together with a carrier molecule that allows its intracellular entry, inhibited hyperalgesia. The authors explained the inhibitory effect of adenosine with earlier findings showing that A_1R does not desensitize, which predict more robust decrease in $PI(4,5)P_2$ levels than activation of other GPCRs that sensitize rather than inhibit TRPV1 [108].

Does TRPV1 really need phosphoinositides for activity?

Apart from the debate on the relative roles of $PI(4,5)P_2$ and PI(4)P, all data presented so far overwhelmingly support the idea that phosphoinositides serve as cofactors, required for TRPV1 activity, and that their depletion inhibits the channel and contributes to desensitization. How do we reconcile this seemingly clear picture with the full functionality of the purified TRPV1 in liposomes in the absence of phosphoinositides [8]?

To address this question, we need to revisit lipid specificity. As mentioned earlier, phosphoinositide activation of TRPV1 is not specific to $PI(4,5)P_2$ and PI(4)P, but many other phosphoinositides, such as $PI(3,4,5)P_3$ [49, 64], $PI(3,4)P_2$, $PI(3,5)P_2$, and PI(5)P also activated these channels [64] (Fig. 2b). Can this low specificity explain the apparent lack of dependence of the activity of the reconstituted channel on phosphoinositides [8]? The purified channels were reconstituted in two different lipid mixtures, one was a soybean polar lipid extract that contains ~18 % phosphatidylinositol (PI) and ~7 % unknown lipids. The other fully defined lipid combination contained various neutral phospholipids and ~25 % phosphatidylglycerol (PG), which has a negative charge [8]. The promiscuity of TRPV1 raised the possibility that the high concentrations of negatively charged lipids such as PG (or PI) can support channel activity. We tested this hypothesis and found that high concentrations of PG indeed stimulated TRPV1 channels after rundown in excised inside-out patches (Fig. 5a, c) [65]. Furthermore, two other negatively charged phospholipids PI and phosphatidylserine (PS) also activated the channels (Fig. 5c), but the neutral phosphatidylcholine (PC) had no effect (Fig. 5b, c). PG, PS, and PI have only one phosphate that couples the head group to the glycerol backbone, thus their charge is much less than that of the three phosphates of $PI(4,5)P_2$ or the two phosphates of PI(4)P. The net charge of $PI(4,5)P_2$ is generally considered to be −4, but depending on its environment, it can range from −3 to −5 [68]. Consistent with their low charge densities, PS, PG, or PI only activated TRPV1 partially, and at extremely high concentrations 250–500 μM in excised patches, close to their limit of solubility after extensive sonication [65]. Other negatively charged lipids not related to phosphoinositides, such as DGS-NTA and long acyl chain CoA (LC-CoA) also activated TRPV1 in excised patches; in line with their higher charge densities, they had larger effects and required lower concentrations than PG, PS, or PI [65, 135].

When the purified TRPV1 protein was incorporated into planar lipid bilayers consisting of neutral phospholipids (PC)/phosphatidyletanolamine (PE) 3:1, it showed no activity in response to capsaicin [65]. When $PI(4,5)P_2$ was added to the lipid mixture, it induced a robust activation of the channel in the presence of capsaicin (Fig. 5d) [65]. In a subsequent paper, it was shown that PG could also support heat-induced TRPV1 activity in planar lipid bilayers in a lipid mixture of PG/PC/PE=3:3:1 with high open probability but lower unitary conductance [114].

What is the role of various negatively charged plasma membrane phospholipids in intact cells?

The data just described indicate that not only phosphoinositides but many other negatively charged phospholipids may also support TRPV1 activity at sufficiently high concentrations. What happens in cellular membranes? The plasma membrane contains up to 1 % $PI(4,5)P_2$ and similar amounts of PI(4)P [2, 22, 27]. PI on the other hand is found at much higher concentrations, up to 10 %, whereas PS is up to 5 % and PG is usually less than 1 % [81, 124, 129]. Since PS and PI are found at higher concentrations than $PI(4,5)P_2$ and PI(4)P, but they are also much less potent, it poses a question whether they contribute to TRPV1 activity in a cellular context.

To answer this question, the stimulation level of the channel needs to be taken into consideration. It has been shown that TRPV1 channels desensitized by 1 μM capsaicin can be reactivated by supramaximal stimulations, such as 100 μM capsaicin [134]. Since $PI(4,5)P_2$ and PI(4)P are depleted during desensitization, the activation by stronger stimuli can be explained in two different ways. Either the channel's apparent affinity becomes so high that very low residual $PI(4,5)P_2$ levels can support activity, or the channel does not require phosphoinositides anymore, and other negatively charged lipids such as PI or PS can support its activity.

It has been shown that higher concentrations of capsaicin shift the dose–response relationship of diC_8 $PI(4,5)P_2$ to the left, i.e., increase its apparent affinity [64]. Accordingly, the velocity of rundown in excised patches decreases at higher capsaicin concentrations, and rundown becomes incomplete [64, 65]. Slow rundown is generally thought to indicate higher

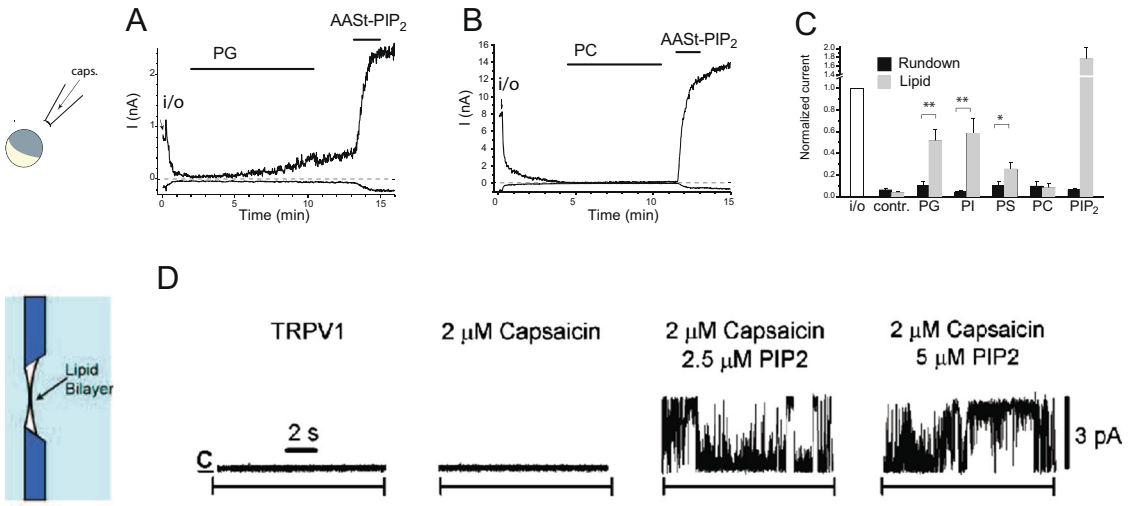

Fig. 5 The role of negatively charged phospholipids in supporting TRPV1 activity, from Ref. [65] with permission. **a**, **b** Excised inside-out patch clamp measurements in the presence of 0.5 μM capsaicin in the patch pipette, shown at +100 and −100 mV. The applications of 250 μM PG, 250 μM PC, and 20 μM $PI(4,5)P_2$ are indicated by the *horizontal lines*. **c** Quantification of the effects of various phospholipids. **d** Planar lipid bilayer measurements in a neutral phospholipid mix (POPC/POPE 3:1), in the absence and presence of $PI(4,5)P_2$ from Ref. [65]

apparent affinity for $PI(4,5)P_2$ [41], but incomplete rundown is difficult to interpret; it can either mean very high $PI(4,5)P_2$ affinity or lack of dependence on $PI(4,5)P_2$ or PI(4)P. Since poly-lys inhibits TRPV1 in excised patches even at very high capsaicin concentrations [64], it is feasible that activation by PI and PS are responsible for the partial rundown, since these lipids are unlikely to decrease substantially in excised patches, but they are expected to be chelated by poly-cations.

In conclusion, it is possible that negatively charged plasma membrane phospholipids other than $PI(4,5)P_2$ and PI(4)P may contribute to channel activity at higher stimulation levels, but this hypothesis has not been tested with specific experiments yet.

Phospholipid specificity of other ion channels

Is the promiscuity of TRPV1 for negatively charged lipids unique? Two examples are discussed below: mammalian ATP-sensitive K^+ channels (K_{ATP}) and bacterial K^+ channels. Kir channels are probably the best characterized phosphoinositide dependent ion channels [62]. Most of them show a preference towards $PI(4,5)P_2$ over other phosphoinositides, such as $PI(3,4)P_2$, with the exception of K_{ATP} channels (Kir6.2), which show no selectivity for individual phosphoinositides [91]. K_{ATP} channels, similar to TRPV1, are activated by a variety of negatively charged lipids, including LC-CoA [91], DGS-NTA [51], PI, and PS [26]. This lipid specificity profile is physiologically relevant, because accumulation of LC-CoA under certain pathophysiological conditions was shown to contribute to activation of K_{ATP} [106].

Bacterial membranes do not contain phosphoinositides, but they have high concentrations of other negative phospholipids, such as PG for *Escherichia coli* [88] and PI for *Mycobacterium tuberculosis* [142]. The bacterial ion channel KcsA requires negatively charged lipids for activity, most likely PG [67, 85]. It was also shown that for the bacterial voltage gated K^+ channel K_VAP the negatively charged PG was required for stabilizing the positive charges in the voltage sensor [104]. It is possible that many ancient ion channels depended on the presence of phospholipids with one negative charge such as PG, which is found at high concentrations in bacterial membranes. It is tempting to speculate that in some eukaryotic channels this requirement for a negatively charged lipid evolved into a dependence on phosphoinositides, which have higher charge densities, but found at much lower concentrations. In some cases, such as TRPV1 and K_{ATP}, residual activity of the lipids with low charge densities may have been retained.

How does $PI(4,5)P_2$ interact with ion channels—general considerations

Phosphoinositides bind to proteins mainly via interactions between the negatively charged head group of the phospholipid and positively charged lysines and arginines [97, 99, 112]. Most phosphoinositide binding proteins have well-defined three-dimensional (3D)-binding pockets that are formed by distant parts of the linear protein sequence, such as those found in Pleckstrin homology (PH) domains [56]. Detailed analysis of co-crystal structures of 25 different lipid-binding domains with various phosphoinositides show that positively charged residues are invariably involved in binding, but other, usually aromatic residues (H, Y, F, or W) usually also contribute [99]. The number of residues in direct contact with phosphoinositides varied from 2 to 7 in those co-crystal structures. The only ion channels that have been co-crystallized

with phosphoinositides are two Kir channels: Kir2.2 [34] and Kir3.2 [130]. The $PI(4,5)P_2$-binding pockets in both channels are formed by the proximal N- and C-termini; the residues directly interacting with $PI(4,5)P_2$ overlap between the two structures, but their locations are not completely identical (Fig. 6c).

Phosphoinositide binding sites are not only formed by well-defined 3D structures; almost any short unstructured peptide with 4 or more positive charges will bind to (laterally sequester) the highly negatively charged $PI(4,5)P_2$ [69]. The $PI(4,5)P_2$-binding site in the cytoplasmic MARCKS protein is a prime example for this type of interaction. In the context of membrane proteins, however, such sequence has to be located close to the plasma membrane interface to be able to bind to $PI(4,5)P_2$. Theoretically there is no reason to believe that the two different kinds of binding sites cannot co-exist in one protein, and data on some TRP channels suggest that they do. Due to the minimal structural requirements for charge-mediated binding, proposals for $PI(4,5)P_2$-binding sites based solely on biochemical experiments on isolated protein segments, need to be treated with caution though, since there is no guarantee that a binding site identified that way is in the proper location in the context of a full length protein.

Because $PI(4,5)P_2$ is an obligate co-factor for most ion channels, identifying an interacting residue with mutagenesis is not straightforward. Generally, if a $PI(4,5)P_2$ interacting residue is neutralized, current amplitudes decrease, often dramatically, due to diminished effect of the lipid. Several individual neutralizing point mutations of direct $PI(4,5)P_2$ interacting residues in Kir2.1 for example resulted in essentially non-functional channels [63]. If the mutant channel has measurable activity, its affinity for $PI(4,5)P_2$ is expected to decrease, thus it becomes more sensitive to inhibition by $PI(4,5)P_2$ depletion and the dose–response to $PI(4,5)P_2$ shifts to the right. Having this phenotype of a mutant channel however does not necessarily prove that the given residue directly interacts with $PI(4,5)P_2$, since an allosteric effect changing the conformation of the channel could result in the same phenotype, even if reduced biochemical binding was demonstrated. This problem is discussed in more general terms by Colquhoun in reference [16]. Accordingly, putative $PI(4,5)P_2$ interacting residues identified by carefully controlled mutagenesis experiments in Kir2.1 [63] were only partially confirmed by the subsequent co-crystal structures of $PI(4,5)P_2$ with Kir2.2 [34] and Kir3.2 [130]. Many residues that were concluded by earlier mutagenesis data to interact with $PI(4,5)P_2$ were in direct contact with $PI(4,5)P_2$ on the structures, but some were not, indicating that they may have affected $PI(4,5)P_2$ interactions via allosteric effects. On the other hand, not all the residues coordinating $PI(4,5)P_2$ were in equivalent positions even in the two co-crystal structures, so it is also possible that (some of) the discrepancies were due to differences between the specific channels.

Generally, the regulation of TRP channels is more diverse and complex than that of most Kir channels; TRPV1 for example is regulated by temperature, chemical agonists, and voltage in a complicated allosteric fashion [53]. Both voltage and capsaicin have been shown to affect the apparent affinity of TRPV1 for $PI(4,5)P_2$ [64]. Similarly, voltage, temperature, and menthol also affected the $PI(4,5)P_2$ sensitivity of the cold-sensitive TRPM8 [92]. This complex regulation poses additional challenges to identifying $PI(4,5)P_2$ interacting residues.

How does $PI(4,5)P_2$ interact with TRPV1-specific data and models

The reliable activation of TRPV1 by phosphoinositides in excised patches and in planar lipid bilayers indicates that these lipids directly interact with the channel. Indirect potentiation via the phosphoinositide interacting protein Pirt was also suggested [46], but the channel had essentially the same phosphoinositide dependence in $Pirt^{-/-}$ and wild-type DRG neurons [123]. Also, the clear effects of phosphoinositides in a wide variety of expression systems and even on purified channels in reconstituted systems argue against significant indirect positive effects of phosphoinositides through Pirt.

Several different parts of TRPV1 have been proposed to interact with phosphoinositides (Fig. 6). Here, I discuss the potential roles of these various regions in the context of the recent side-chain resolution structures of TRPV1 [9, 57].

Prescott et al. proposed that the negative effect of $PI(4,5)P_2$ proceeded through the distal C-terminal 777–820 region [86] (Fig. 6). Deletion of this region had no effect on the apparent affinity of the activating effect of $PI(4,5)P_2$, or on specificity for $PI(4,5)P_2$ over PI(4)P [123], thus it is unlikely to play a role in the positive effect of $PI(4,5)P_2$. Its potential role in the negative effects of $PI(4,5)P_2$ is discussed later. This region is missing from the TRPV1 structure [9], thus it is hard to assess if it is in a location compatible with interacting with $PI(4,5)P_2$.

Ufret-Vincenty et al. identified a proximal C-terminal putative $PI(4,5)P_2$ binding—region that spans from amino acids 711–732 (Fig. 6) and contains five positively charged residues in TRPV1 [123]. This region shows relatively low level of conservation among TRPV channels, but it contains several positively charged residues in all TRP channels from the three major subfamilies [123]. A purified protein fragment (682–725) that includes this region was found to bind to $PI(4,5)P_2$ using a FRET-based method [123]. The authors attempted to determine which positively charged residues play a role in $PI(4,5)P_2$ binding and concluded that: "Although deletion of the proximal C-terminal region and neutralization of >1 basic residue eliminated capsaicin-activated currents in our patches, we could not distinguish whether this was due to prevention of PIP_2 binding to otherwise-normal channels or due to unrelated changes in protein folding, trafficking, or stability." This

A

Region	Sequence	On Structure	Close to PM	Functional data	Binding data	Conserved in other TRPV	Ref.
777-820	LRSGRVSGRNWKNFALVPLLRD ASTRDRHATQQEEVQLKHYTGS	No	?	Involved in inhibition	Yes	No	[8, 31, 86]
711-732 (682-725)	SFLKCMRKAFRSGKLLQVGFTP	Partially (till MRKA)	Yes,	No	Yes	Not very well	[123]
189-221	FVNASYTDSYYKGQTALHIAIE RRNMTLVTLLV	Yes	No	No	Yes	Yes	[31]
688-718	KIAQESKNIWKLQRAITILDTE KSFLKCMRK	Yes includes TRP-box	Yes	No	Yes	Yes	[31]
575-579 694-701	R575, R579 K694, K698, R701, K710,	Yes, PIP_2 docked by modeling	Yes	Yes, (bold) with TRPM8 chimera	No	Yes	[6]
561-579 688	Q561, R575, R579, K688	Yes, PIP_2 docked by modeling	Yes	No	No	Yes	[110],
561-579 688-694	QMGIYAVMIEKMILRDLCR KIAQESK	Yes, PIP_2 docked by modeling	Yes	Yes (bold)	No	Yes	[84]

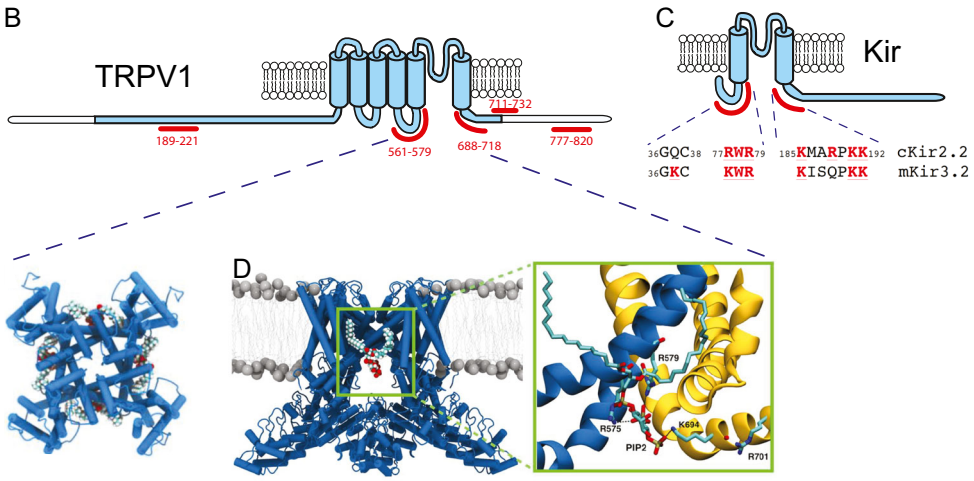

Fig. 6 Proposed $PI(4,5)P_2$ binding sites on TRPV1. **a** Table for regions in TRPV1 proposed to interact with $PI(4,5)P_2$. **b** Cartoon of TRPV1 with putative $PI(4,5)P_2$ interacting regions, *cyan* filling shows parts that are on the high-resolution cryoEM structure, *unfilled areas* are missing from the structure. *Bottom*, model for $PI(4,5)P_2$ interactions in TRPV1 from Ref. [84] with permission. **c** Cartoon of a Kir channel, with regions that were confirmed to play roles in $PI(4,5)P_2$ interactions based of co-crystal structures of $PI(4,5)P_2$ and Kir2.2 and Kir3.2

statement highlights the inherent difficulty to locate binding residues of an obligate co-factor, as discussed earlier.

Grycova et al. measured binding of $PI(4,5)P_2$ to various purified cytoplasmic fragments using fluorescence anisotropy and surface plasmon resonance, but functional measurements were not performed [31]. They identified three potential binding sites (Fig. 6), one in the distal C-terminus, identical to the inhibitory binding site, described earlier (777–820), one in the proximal C-terminus (688–718), overlapping with that discussed in the previous paragraph, and one in the N-terminal ankyrin-repeat domains (ARD) (189–221) that overlaps with a previously identified calmodulin-binding site [58]. The N-terminal binding site does not face the plasma membrane in the structure, but a $PI(4,5)P_2$-binding region in a similar position was proposed recently in TRPV4, based on the co-crystal structure of the ARD of the chicken TRPV4 and IP_3, the head group of $PI(4,5)P_2$ [116].

Several attempts have been made to dock $PI(4,5)P_2$ in silico to various models of TRPV1. The first model by Brauchi et al. was built based on crystal structures of the transmembrane domains of Kv1.2 and the C-terminus of HCN2 [6]. They identified residues R575, R579 in the S4–S5 linker, and K694, K698 and K701 in the proximal C-terminus that may interact with $PI(4,5)P_2$. Functional data were obtained with a chimera based on the cold-sensitive TRPM8 in which a large portion of the C-terminus was replaced with that of TRPV1 (residues 686–753 in TRPV1). Mutation of residues R701 and K710 in the TRP-box region shifted $PI(4,5)P_2$ dose responses of this chimera to the right [6] as expected if they were $PI(4,5)P_2$ interacting residues [95].

A subsequent preliminary molecular dynamics (MD) simulation [110] performed after the publication of the TRPV1 structure confirmed the roles of R575, R579 in the S4–S5 linker and also proposed that Q561 in the same region may also interact with $PI(4,5)P_2$. From the proximal C-terminus they identified K688 as a potential $PI(4,5)P_2$ interacting residue.

The most recent and most thorough attempt to dock $PI(4,5)P_2$ to the TRPV1 structure was performed recently by Poblete et al. (Fig. 6b) [84]. Consistent with the previous two models they found that R575 and R579 from the S4–S5 linker interacted with $PI(4,5)P_2$ 82 % of the 50 lowest energy conformations, whereas K694 from the C-terminus did so in 50 % of the conformations. When these three residues were individually mutated to neutral residues, the $PI(4,5)P_2$ dose responses were shifted to the right [84]. Q561, K571 and K688 were also proposed to interact with $PI(4,5)P_2$, but the functional effects of mutating these residues were not tested. In contrast to earlier suggestions, the K701 reside did not interact

with $PI(4,5)P_2$ in the molecular docking simulations, and accordingly, mutation of this residue did not alter $PI(4,5)P_2$ activation [84].

MD simulations in the same study [84] indicated that $PI(4,5)P_2$ binding induces conformational rearrangements of the structure formed by S6 and the proximal part of the TRP domain, which cause an opening of the lower TRPV1 channel gate. The MD simulations also predicted that $PI(4,5)P_2$ binds stronger to the channel in the presence of capsaicin, which is consistent with earlier results showing a left shift in the $PI(4,5)P_2$ dose–response in the presence of higher capsaicin concentrations [64].

Overall, all molecular simulations point to a model, in which positively charged residues from the very proximal C-terminus, and the S4–S5 linker together form the activating $PI(4,5)P_2$-binding site. The S4–S5 linker in TRP channels is analogous to the proximal N-terminus of the 2 transmembrane domain Kir channels (Fig. 6b, c), thus this model is similar to the activation mechanism of Kir channels, based on co-crystal structure of $PI(4,5)P_2$ with the channel, where residues from the proximal N- and C-termini were in direct contact with $PI(4,5)P_2$ [34, 130].

Note that mutation of the residues in the S4–S5 linker also shifted voltage activation, thus it is possible that they allosterically affected $PI(4,5)P_2$ sensitivity, which was shown earlier to be affected by voltage [64]. Equivalent residues in the cold- and menthol-sensitive TRPM8 were proposed to be involved in voltage dependence, and also affected menthol sensitivity allosterically [128]. Both voltage and menthol were shown to affect $PI(4,5)P_2$ sensitivity of TRPM8 [92].

Overall however, the convergence of experimental work, structural modeling, and the clear parallel with Kir channels, makes this model highly appealing. Additional binding sites are possible, since there are many other positively charged residues located in the vicinity of the plasma membrane in the TRPV1 structure [57]. In Kir channels, a binding site additional to that seen in the co-crystal structure has recently been proposed [55].

Lysophosphatidic acid and $PI(4,5)P_2$

Many lipids other than phosphoinositides have also been shown to regulate TRPV1 [72, 110]. While their detailed description is beyond the scope of this review, one lipid, lysophosphatidic acid, (LPA), will be briefly discussed here, because it was proposed to activate TRPV1 via its $PI(4,5)P_2$-binding site [73, 76]. LPA is an extracellular signaling molecule, that exerts most of its effects via GPCRs that couple to Gq and PLC [73]. Activation of these receptors have been shown to sensitize nociceptors [73], similar to many other Gq-coupled receptors. In addition to this indirect effect, Nieto-Posadas et al. [76] convincingly showed that 5 μM LPA also directly activates TRPV1, by demonstrating that it induces large currents via TRPV1 both in the excised inside-out and outside-out configurations. LPA was more active when applied in the inside-out configuration, suggesting an internal site of action [76]. LPA also activated the purified TRPV1 in lipid vesicles, confirming a direct effect [8]. Paw injection of LPA in mice evoked pain, which was reduced, but not eliminated by genetic ablation of TRPV1 [76].

Removal of the 777–820 segment from the channel protein did not affect LPA activation of TRPV1 [76], but the effect of LPA was decreased by 50 % in the K710Q and by 80 % in the K710D mutants of TRPV1 [76]. It was also shown that TRPV1 could be precipitated by LPA-coated agarose beads. Furthermore, $PI(4,5)P_2$ competed with LPA for binding to TRPV1, and LPA binding to the channel was reduced by the K710D mutation. These data were interpreted as LPA activating TRPV1 via its $PI(4,5)P_2$-binding site. The role of the K710 residue however in the effect of $PI(4,5)P_2$ is not very well established. As discussed earlier, the K710Q mutant was shown by Brauchi et al. to reduce $PI(4,5)P_2$ affinity in a chimera between TRPV1 and TRPM8, its effects in the background of the wild-type TRPV1 channel have not been demonstrated yet, and newer models did not support its role in direct $PI(4,5)P_2$ interactions.

LPA induced large TRPV1 currents, which were comparable to those evoked by capsaicin [76]. $PI(4,5)P_2$ on the other hand induced only very small currents in excised patches in the absence of capsaicin [47], mainly at extreme positive voltages [84]. As discussed throughout this review, $PI(4,5)P_2$ can be considered mainly as a co-factor required for channel activity stimulated by its agonists, such as capsaicin. LPA on the other hand is a bona fide agonist that can evoke full activity without any other agent. In contrast to capsaicin the effect of LPA was not prevented by chelating endogenous $PI(4,5)P_2$ with poly-lysine, rather, the dose response was shifted to the left, as if it was relieved from competition by $PI(4,5)P_2$ [76]. Also, diC_8 $PI(4,5)P_2$ shifted the dose response for LPA to the right, again, compatible with the two lipids competing for the same binding site [76]. These data suggest that LPA has complex effects on TRPV1; it acts as a full agonist, but its effect either does not depend on $PI(4,5)P_2$ as a co-factor, or it can also fulfill the co-factor role of $PI(4,5)P_2$.

Evidence for inhibition by phosphoinositides

The inhibitory effect of $PI(4,5)P_2$ is still quite controversial, and I cannot offer a clear conclusion, but will attempt to thoroughly and objectively discuss data in the literature.

Early/original experiments

The original hypothesis is based on four key experiments: First, a bacterial phosphatidylinositol-specific PLC (PI-

PLC) potentiated TRPV1 activity in excised patches [13]. Bacterial PI-PLC enzymes however specifically break down PI, and have negligible activity on PI(4)P or $PI(4,5)P_2$ [36]. PI-PLC had been used earlier in experiments with Na/Ca^{2+} exchangers and Kir channels, where it was shown to inhibit the effect of MgATP in excised inside-out patches, demonstrating that MgATP stimulated activity via replenishing $PI(4,5)P_2$ [37, 113]. We recently tested the effect of PI-PLC on TRPV1 and TRPV6 and found that it eliminated the effect of MgATP in excised patches on both channels [65], as expected if the latter acted via stimulating lipid kinases. PI-PLC however, also induced a small increase in activity of TRPV1 [65], consistently with the original report [13]. PI was reported to inhibit TRPV1 activity in liposomes [8], thus its removal by PI-PLC could potentially stimulate channel activity. When we re-applied PI after PI-PLC treatment, however, it failed to inhibit TRPV1, as would be expected if removal of PI was responsible for potentiation by PI-PLC. Activation of PI-PLC also generates diacyl-glycerol (DAG), which have been shown to potentiate TRPV1 activity either directly [131] or through some of its metabolites [143] which may account for the stimulating effect of the enzyme.

Second, an anti-$PI(4,5)P_2$ antibody potentiated TRPV1 in excised patches [13]. Initial studies on $PI(4,5)P_2$ regulation of ion channels often used this reagent in excised inside-out patches [90], but its popularity is decreasing. Generally, we found that commercially available $PI(4,5)P_2$ antibodies are quite unreliable tools in excised patch experiments [93], and there are published examples from other laboratories where anti-$PI(4,5)P_2$ antibody did not affect ion channels, despite clear effects of $PI(4,5)P_2$ in the same study [23, 61]. Additional experiments also suggest that potentiation by the antibody may have been independent of chelating $PI(4,5)P_2$. It was shown that other $PI(4,5)P_2$ chelating agents such as poly-Lys (Fig. 2a, b) [64, 109] and the purified PH domain of PLCδ1 [49] inhibited, rather than potentiated TRPV1 activity. Furthermore, TRPV1 activity shows clear rundown in excised patches (Fig. 2c) [65], instead of run-up, which would be expected if decreasing $PI(4,5)P_2$ concentration in the patch membrane would potentiate channel activity.

Third, inhibiting PI4K with phenylarsine oxide (PAO) potentiated TRPV1 activity [86]. PAO is a quite non-specific agent, it covalently modifies SH and OH moieties and it was later shown that it potentiated TRPV1 via direct modification of cysteine residues [14]. Furthermore, inhibition of PI4K by high concentrations of wortmannin reduced, rather than potentiated TRPV1 activity [66], and it also inhibited recovery from desensitization [60].

Fourth, the inhibitory $PI(4,5)P_2$-binding site was proposed to be residues 777–820 in the distal C-terminus (Fig. 6). When this segment was deleted from the channel, sensitization by bradykinin was strongly reduced [86]. It is important to note, however that deletion of this region shifts the capsaicin dose–response to the left [64], and it also contains one of the two important PKC phosphorylation sites, elimination of which by itself also strongly reduced sensitization [79]. Nevertheless, when this region was deleted, it also reduced the inhibition evoked by the overexpression of PIP5K [64], and eliminated inhibition by phosphoinositides on the purified channel in lipid vesicles [8], see later. When the distal C-terminal $PI(4,5)P_2$-binding site was replaced by a putative $PI(4,5)P_2$-binding site from Kir2.1, a channel that binds $PI(4,5)P_2$ with very high affinity, potentiation by EGF was reduced [86]. This was interpreted as a modular binding site for $PI(4,5)P_2$, replacement of which with a higher affinity $PI(4,5)P_2$-binding domain reduces sensitization, because it cannot dissociate from $PI(4,5)P_2$ upon PLC activation [86]. The 207–245 segments in Kir2.1 however was shown to be responsible for the high apparent affinity of the interactions of this channel [138], but was not shown to be a bona fide binding site. Accordingly, this region in the co-crystal structure of Kir2.2 with $PI(4,5)P_2$ is located in a position incompatible with being a direct $PI(4,5)P_2$ interacting region [34].

Overall, even though the data supporting the inhibitory effect of $PI(4,5)P_2$ seemed compelling at the time of publication, many of the experiments can gain alternative interpretations in light of additional data. Subsequent experiments, however, gave further support to a partial inhibition by $PI(4,5)P_2$, as discussed below.

Subsequent experiments supporting an inhibitory role for $PI(4,5)P_2$

Inhibition by $PI(4,5)P_2$ was proposed to play a role in sensitization of TRPV1 by the PLC pathway. Sensitization by GPCR activation induces a left shift in the dose response of most activators, such as heat, capsaicin and low pH [119]. Thus, sensitization enhances currents at low and moderate levels of stimulation, but not at maximal activation, which needs to be kept in mind for the discussion of subsequent data.

As discussed earlier, Lukacs et al. in 2007 [64] showed that $PI(4,5)P_2$ activates TRPV1 in excised patches; the same paper however also found two lines of evidence supporting a concurrent partial inhibitory effect by $PI(4,5)P_2$ in intact cells. (1) Over-expressing the murine PIP5K1β, which generates excess $PI(4,5)P_2$, inhibited capsaicin-induced TRPV1 currents in *Xenopus* oocytes selectively at low stimulation levels. This inhibition was reduced but not eliminated by removal of the putative distal C-terminal $PI(4,5)P_2$-binding site. (2) Rapamycin-induced translocation of a 5′-phosphatase, which dephosphorylates $PI(4,5)P_2$, potentiated TRPV1 currents evoked by low capsaicin concentrations or moderate heat in HEK cells, or low capsaicin concentrations in *Xenopus* oocyes [64]. As discussed earlier, currents evoked by saturating capsaicin concentrations were neither potentiated nor inhibited by

rapamycin. We have concluded then that TRPV1 activity depends on the presence of $PI(4,5)P_2$ or PI(4)P, presumably because these lipids directly stimulate the channel, but there is an additional indirect partial inhibition, resulting in a bell-shaped dependence of channel activity on $PI(4,5)P_2$ [64, 94]. Several subsequent articles, discussed below, were compatible with this view:

Patil et al. [83] examined the effects of chronic manipulations of $PI(4,5)P_2$ levels on TRPV1 activity. They co-expressed the murine PIP5K1β enzyme to increase and a 5′-phosphatase to decrease $PI(4,5)P_2$ levels. Consistent with our earlier results, Ca^{2+} responses to 20 nM capsaicin were potentiated by the 5′-phosphatase and inhibited by PIP5K. Currents induced by higher concentrations of capsaicin were not different, and tachyphylaxis, reduced responsiveness upon repeated capsaicin applications, was not different either [83].

The A-kinase anchoring protein 150 (AKAP150) has been shown to play an important role in TRPV1 sensitization by anchoring protein kinase A (PKA) and PKC to TRPV1 [140]. Jeske et al. [45] found that AKAP150 binds to $PI(4,5)P_2$, and this binding competes AKAP150 away from TRPV1. When $PI(4,5)P_2$ is depleted by PLC, more AKAP is bound to TRPV1, which enhances potentiation [45]. These experiments gave a potential mechanism for an indirect inhibition by $PI(4,5)P_2$.

Neely et al. [75] found that PIP5K1$\alpha^{-/-}$ mice had increased sensitivity to radiant heat and to capsaicin in behavioral assays. This finding is compatible with $PI(4,5)P_2$ exerting a negative effect. Mice heterozygous for the deletion of PIP5K1C (PIP5K1γ) however had the opposite phenotype [132] they were less sensitive to pain. The nomenclature of PIP5K enzymes is somewhat confusing, see [2] for further details. In general, most, but not all experiments with PIP5K and $PI(4,5)P_2$ phosphatase enzymes are compatible with the existence of a partial indirect inhibition by $PI(4,5)P_2$.

The role of phosphoinositides in sensitization

In our recent paper we have also addressed the potential role of an inhibitory effect of $PI(4,5)P_2$ in sensitization [66]. As mentioned earlier, we confirmed our model based on expression system studies for desensitization in native neurons; we found that capsaicin induced a robust depletion of $PI(4,5)P_2$ or PI(4)P and inclusion of either of these lipids in the intracellular solution inhibited desensitization (Fig. 4). On the other hand, inclusion of $PI(4,5)P_2$, but not PI(4)P in the whole cell patch pipette also inhibited sensitization induced by bradykinin (Fig. 7a) [66]. This latter finding is compatible with a partial inhibition specific to $PI(4,5)P_2$ and its depletion playing a role in sensitization. We also found that bradykinin moderately decreased $PI(4,5)P_2$, but not PI(4)P levels in DRG neurons [66]. This explains why bradykinin and other agents that stimulate PLCβ do not inhibit TRPV1: the channel has a high affinity for $PI(4,5)P_2$, and its activity is also supported by PI(4)P thus the decrease of these phosphoinositides does not reach levels that would limit channel activity. On the other hand we also found that the voltage-sensitive 5′-phospatase drVSP did not potentiate TRPV1 activity [66]. This is in contrast to our earlier finding with the rapamycin-inducible 5′ phosphatase, which potentiated TRPV1 at low stimulation levels [64]. We cannot explain this discrepancy, but the drVSP experiment argues that $PI(4,5)P_2$ depletion by itself is not sufficient for potentiating TRPV1. We also found however that depleting $PI(4,5)P_2$ with drVSP potentiated the effect of submaximal activation of PKC by low concentrations (1–10 μM) of the DAG analogue OAG [66].

Based on the findings described here, we proposed a model to integrate the positive and negative effects of phosphoinositides; the two key features of this model are: 1. Bradykinin induces a moderate specific decrease in $PI(4,5)P_2$ levels by PLCβ activation in DRG neurons, which potentiates the effect of PKC during sensitization (Fig. 7b). 2. During desensitization large decreases in both $PI(4,5)P_2$ and PI(4)P, mediated by Ca^{2+} sensitive PLCδ isoforms, limit channel activity [66, 96] (Fig. 4b).

As mentioned earlier, $PI(3,4,5)P_2$, the product of the activation of PI3K enzymes, also activates TRPV1 in excised patches [64], with a similar or slightly lower apparent affinity than $PI(4,5)P_2$ [49]. $PI(3,4,5)P_3$ is undetectable in most cells under resting conditions, and even when PI3K is stimulated, its concentration reaches less than 10 % of that of $PI(4,5)P_2$ [2], thus its direct effect is likely to be overridden by the much more abundant $PI(4,5)P_2$. $PI(3,4,5)P_3$ however may exert another indirect effect on TRPV1 via stimulating its trafficking to the plasma membrane. Nerve growth factor (NGF) acting via its tyrosine kinase receptor stimulates PI3K, thus generating $PI(3,4,5)P_3$. NGF sensitizes TRPV1 channels to capsaicin in a PI3K dependent fashion, by stimulating the insertion of TRPV1 in the plasma membrane thus increasing the maximum current [109, 139]. This is in contrast to activating GPCRs, which shift the capsaicin dose response to the left, without affecting the maximum current [119].

Inhibition of the reconstituted TRPV1 by phosphoinositides

As described before, there is substantial evidence beyond the original experiments by the Julius lab that support an inhibitory role for $PI(4,5)P_2$. Everything discussed so far, was compatible with a general dependence of TRPV1 on phosphoinositides and the existence of an indirect inhibition by $PI(4,5)P_2$ via PKC, AKAP or another $PI(4,5)P_2$ sensitive factor not yet identified. $PI(4,5)P_2$ is a multifunctional signaling lipid, and cells contain a multitude of phosphoinositide binding proteins, many of those could influence TRPV1 activity, offering ample possibility for indirect effects.

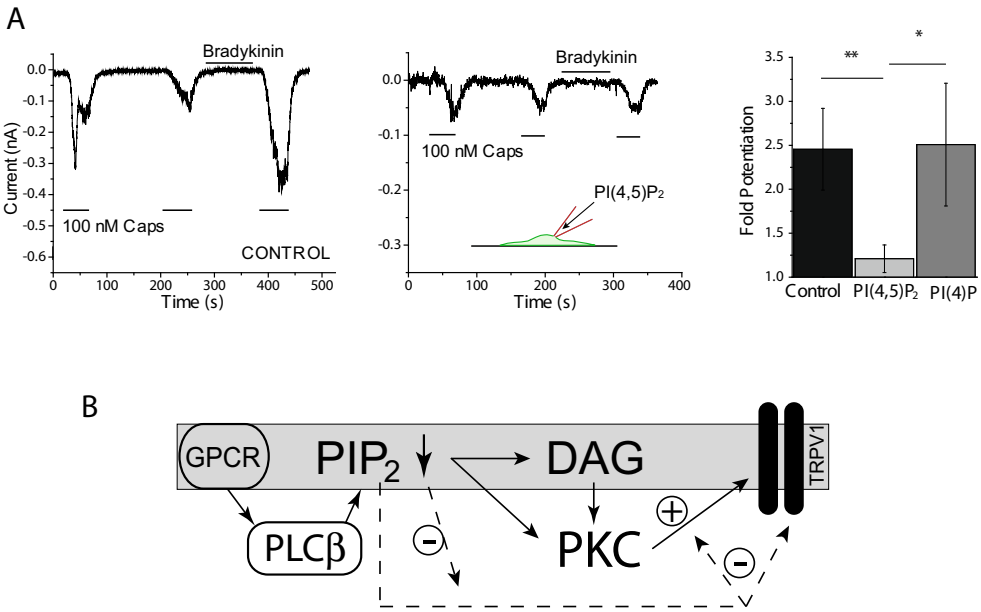

Fig. 7 The role of $PI(4,5)P_2$ in sensitization of TRPV1, from Ref. [66] with permission. **a** Whole cell patch clamp in DRG neurons with and without 100 μM diC_8 $PI(4,5)P_2$ or PI(4)P in the patch pipette, the applications of 100 nM capsaicin and 500 nM bradykinin are shown by the *horizontal lines*. **b** Cartoon showing the model for sensitization, in which GPCR activation leads to stimulation of PLCβ, which leads to a moderate decrease in $PI(4,5)P_2$ but no change in PI(4)P. The decrease in $PI(4,5)P_2$ potentiates the effect of PKC, leading to enhances channel activity. See text for more details

Incorporation of phosphoinositides in the lipid mixture however inhibited the purified reconstituted TRPV1 [8], which is a strong evidence for a direct effect. How do we reconcile this with the results discussed before, especially the lack of inhibition by $PI(4,5)P_2$ in excised patches? Cao et al. incorporated relatively high concentrations of PI, PI(4)P and $PI(4,5)P_2$ (4 %) into the lipid mixture, which shifted the capsaicin dose–response to the right, with PI and PI(4)P being slightly more active than $PI(4,5)P_2$ [8]. The effectiveness of PI is quite intriguing and it is also difficult to align with the proposed role of phosphoinositide depletion in sensitization upon PLC activation. The concentration of PI in the plasma membrane exceeds that of $PI(4,5)P_2$ or PI(4)P by at least an order of magnitude [2]. Levels of PI do not change significantly upon PLC activation, thus it is difficult to envision that a decrease in the concentration of the much less abundant $PI(4,5)P_2$ upon PLC activation can significantly stimulate channel activity by relieving an inhibitory effect.

As discussed earlier, the inhibitory effect of $PI(4,5)P_2$ was proposed to be mediated by a distal C-terminal (residues 777–820) $PI(4,5)P_2$-binding site (Fig. 6) [86]. Cao et al. confirmed the role of this domain on the purified reconstituted TRPV1 in lipid vesicles, by showing that removal of this segment eliminated the inhibitory effect of $PI(4,5)P_2$ [8]. They also proposed a mechanistic model in which $PI(4,5)P_2$ anchors the distal C-terminal region to the plasma membrane inducing a conformational change that mediates inhibition. To support this model they tagged the C-terminus of the channel protein with 8 histidine residues (8xHis). Histidines interact in a nickel dependent manner with nitrilotriacetic acid (NTA). When a lipid derivative of NTA (DGS-NTA) was incorporated into the lipid mix, nickel inhibited the channel tagged on the C-terminus with 8xHis [8], showing that bringing the C-terminus of the channel to the membrane inhibits channel activity. These are very elegant experiments that offer a possible mechanism for the role of the distal C-terminus in the inhibitory effect of $PI(4,5)P_2$. It has to be noted however that DGS-NTA, which is highly negatively charged, directly activated TRPV1 channels in excised patches [65].

How do we explain the contradiction that $PI(4,5)P_2$ does not inhibit TRPV1 in excised inside-out patches, but it does so in a reconstituted artificial system? One possible explanation is provided in a recent paper by Senning et al. [105], who found that $PI(4,5)P_2$ inhibited TRPV1 activity when applied to excised outside-out patches, i.e. to the extracellular leaflet of the plasma membrane. This inhibitory effect required higher concentrations of the lipid than the well-characterized positive effect in inside-out patches. Since the lipid vesicles in which TRPV1 was reconstituted were symmetrical, it is possible that phosphoinositides in the extracellular leaflet were responsible for, or contributed to inhibition. Because $PI(4,5)P_2$ is found exclusively in the intracellular leaflet of the plasma membrane, inhibition by extracellular $PI(4,5)P_2$ has limited physiological importance. On the other hand the effect of extracellular $PI(4,5)P_2$ cannot explain the data we discussed earlier that supports inhibition by $PI(4,5)P_2$ in intact cells.

Phosphoinositide effects on other TRP channels

Phosphoinositide regulation is a general feature of TRP channels, which has been reviewed extensively [35, 78, 95, 97]. The vast majority of TRP channels are positively regulated by phosphoinositides, but the dual effects of $PI(4,5)P_2$ are not unique to TRPV1; examples can be found in other members of the TRPV family, and on several TRPC channels, as

discussed below. On the other hand, the complex effects of $PI(4,5)P_2$ are not universal among all TRP channel families, because all TRPM channels tested so far are activated by $PI(4,5)P_2$ and no negative effect has been reported.

TRPV channels

In the TRPV family, TRPV2 [70], TRPV5 [54, 92] and TRPV6 [117, 118] are positively regulated by $PI(4,5)P_2$ [97]. TRPV3 on the other hand was shown to be inhibited by $PI(4,5)P_2$ in excised patches [21], and no positive effect has been published so far. On TRPV4, both positive [30], and negative [116] regulation by $PI(4,5)P_2$ were demonstrated. Garcia Elias et al. [30] showed that activation of TRPV4 by hypotonic stress and heat, but not its chemical agonist 4α-phorbol 12,13-didecanoate (4α-PDD), was inhibited by a rapamycin-inducible 5′-phosphatase. $PI(4,5)P_2$ also was required for heat activation of TRPV4 in excised patches [30]. The binding site responsible for this effect of $PI(4,5)P_2$ was proposed to be the short N-terminal ^{121}KRWRK^{125} segment before the ARD [30]. Takahashi et al. on the other hand, found that $PI(4,5)P_2$ included in the whole cell patch pipette inhibited currents evoked by 4α-PDD [116]. They also co-crystallized the N-terminal ARD of the channels with IP_3, the head group of $PI(4,5)P_2$, and located positively charged residues interacting with $PI(4,5)P_2$. From these two publications it appears that $PI(4,5)P_2$ may have different effects on TRPV4, depending on the activation modality. The dual regulation of TRPV1 thus may not be unique within the TRPV sub-family.

TRPC channels

The picture on the TRPC family is complicated, and the parallel with TRPV1 is inescapable. All TRPC channels are activated downstream of PLC, but their activation mechanism is not fully elucidated despite almost two decades of research [87, 96]. TRPC3, TRPC6 and TRPC7 are stimulated by DAG, the hydrolysis product of $PI(4,5)P_2$, which may account for receptor-mediated activation of these channels, but how other members are turned on is less clear. One of the ideas from the very beginning was the relief from inhibition by $PI(4,5)P_2$ upon PLC activation [23], similar to the mechanism proposed for sensitization of TRPV1. There are many reports on $PI(4,5)P_2$ inhibiting various TRPC channels, for reviews see [96, 97]. In some cases, both positive and negative effects on the same TRPC channel have been found in the same article [122]. It is clear by now that relief from inhibition by $PI(4,5)P_2$ cannot fully account for activation of TRPC channels, but it may play some auxiliary role [96]. On the other hand, experiments with inducible phosphoinositide phosphatases show that most members of the TRPC family require $PI(4,5)P_2$ for activity [42, 43, 48, 122], indicating that phosphoinositide dependence is conserved in this family too. Upon maximal receptor-induced PLC activation, decreasing $PI(4,5)P_2$ levels have been proposed to contribute to desensitization of TRPC3/6/7 channels [42, 44]. Overall TRPC channels seem to behave somewhat similar to TRPV1; their activity depend on $PI(4,5)P_2$, but relief from a concurrent inhibition by this lipid may play auxiliary roles in their activation in same cases.

TRPM channels

At variance with TRPV and TRPC channels, the data on TRPM channels is simpler, to my knowledge no negative effects have been published on any TRPM channel, and positive regulation was shown for TRPM2 [120], TRPM4 [77, 141], TRPM5 [61], TRPM6 [133], TRPM7 [32, 100] TRPM8 [59, 92], and in two preliminary reports for TRPM3 [1, 121]. The desensitization mechanism for TRPM8 is similar to that described for TRPV1, i.e. Ca^{2+}-induced activation of PLC and the resulting $PI(4,5)P_2$ depletion limiting channel activity [7, 17, 28, 92, 136]. At this point it seems that all members of the TRPM family are positively regulated by $PI(4,5)P_2$, similarly to Kir channels, thus the complicated regulation by $PI(4,5)P_2$ does not apply to all TRP channel families.

Conclusions

As discussed, the experimental evidence overwhelmingly support the idea that phosphoinositides are positive cofactors required for TRPV1 activity in a cellular environment. Ca^{2+}-induced depletion of $PI(4,5)P_2$ and PI(4)P via PLC activation plays a major role in desensitization upon pharmacological stimulation. This desensitization mechanism is shared by a number of TRP channels, such as TRPM8 [17, 92, 136], TRPV6 [117, 118] or TRPV2 [70]. The dependence on phosphoinositides is also in line with the overwhelming majority of TRPM, TRPV and TRPC channels requiring $PI(4,5)P_2$ for activity [97]. TRPV1, however, is promiscuous and many other negatively charged lipids can also support its activity, usually at much higher concentrations, which may explain the lack of dependence of the activity of the purified reconstituted TRPV1 on phosphoinositides [65, 114].

The picture is less clear on the potential inhibitory role of $PI(4,5)P_2$. The role of PKC in sensitization of TRPV1 is very well established [4, 79, 126], and the lack of evidence for inhibition by $PI(4,5)P_2$ in excised patches argued against the $PI(4,5)P_2$ depletion hypothesis of sensitization. There is substantial additional data however supporting an auxiliary role of a negative $PI(4,5)P_2$ effect in sensitization. There are also other TRPV and TRPC channels on which negative effects of $PI(4,5)P_2$ have been described concurrent with positive effects. It is hard to tell whether the negative effect of $PI(4,5)P_2$ on TRPV1 is direct or indirect. The lack of

inhibition in excised patches strongly suggests an indirect effect; the inhibition of the purified reconstituted TRPV1 by phosphoinositides on the other hand provides a strong argument for direct inhibition [8]. This latter finding however may be explained by the recent report that extracellular $PI(4,5)P_2$ inhibits TRPV1 [105], since the lipid vesicles used by [8] were symmetrical, containing phosphoinositides both in the extracellular and intracellular leaflet. Inhibition by extracellular $PI(4,5)P_2$ however cannot explain the experiments in intact cells pointing to a potential negative regulatory role of $PI(4,5)P_2$. Clearly, more work is needed to understand the role and mechanism of the negative effects of $PI(4,5)P_2$ on TRPV1 described in this review.

The complexity of the regulation of TRPV1 by phosphoinositides with its controversies is puzzling. I attempted to summarize the literature and, wherever possible, find rational explanations for seemingly contradictory results. The story may sound complicated, but biology is inherently complex and seemingly simple questions such as does $PI(4,5)P_2$ inhibit or activate TRPV1 may not always have simple answers. TRPV1 is one of the most promiscuous ion channels; the variety of compounds that modulate it is staggering [72]. $PI(4,5)P_2$ is also one of the most ubiquitous signaling molecules; it affects many cellular processes and binds to a multitude of proteins [2]. When we put them together perhaps we should not be surprised by the resulting complexity. Also I believe this story illustrates how science progresses, rather than one paper permanently settling a question, it is a process that often requires reinterpretation of previous findings when new ones seemingly contradict them. This review reflects my best, yet somewhat simplified, effort to interpret and clarify the results available today, and it is quite possible that future results will change some of the discussions described here.

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Conflict of interest The author declares no conflict of interest.

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