Chronic Alteration in Phosphatidylinositol 4,5–Biphosphate Levels Regulates Capsaicin and Mustard Oil Responses

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There is an agreement that acute (in minutes) hydrolysis and accumulation of phosphatidylinositol 4,5-bisphosphate (PIP_2) modulate TRPV1 and TRPA1 activities. Because inflammation results in PIP_2 depletion, persisting for long periods (hours to days) in pain models and in the clinic, we examined whether chronic depletion and accumulation of PIP_2 affect capsaicin (CAP) and mustard oil (MO) responses. In addition, we wanted to evaluate whether the effects of PIP_2 depend on TRPV1 and TRPA1 coexpression and whether the PIP_2 actions vary in expression cells vs. sensory neurons. Chronic PIP_2 production was stimulated by overexpression of phosphatidylinositol-4-phosphate-5-kinase, and PIP_2-specific phospholipid 5′-phosphatase was selected to reduce plasma membrane levels of PIP_2. Our results demonstrate that CAP (100 nM) responses and receptor tachyphylaxis are not significantly influenced by chronic changes in PIP_2 levels in wild-type (WT) or TRPA1 null-mutant sensory neurons as well as CHO cells expressing TRPV1 alone or with TRPA1. However, low concentrations of CAP (20 nM) produced a higher response after PIP_2 depletion in cells containing TRPV1 alone but not TRPV1 together with TRPA1. MO (25 μM) responses were also not affected by PIP_2 in WT sensory neurons and cells coexpressing TRPA1 and TRPV1. In contrast, PIP_2 reduction leads to pronounced tachyphylaxis to MO in cells with both channels. Chronic effect of PIP_2 on TRPA1 activity depends on presence of the TRPV1 channel and cell type (CHO vs. sensory neurons). In summary, chronic alterations in PIP_2 levels regulate magnitude of CAP and MO responses as well as MO tachyphylaxis. This regulation depends on coexpression profile of TRPA1 and TRPV1 and cell type.   © 2011 Wiley-Liss, Inc.

Key words: PIP_2; TRPA1; TRPV1; nociceptor

A plethora of inflammatory mediators affects phosphatidylinositol 4,5-bisphosphate (PIP_2) levels in peripheral sensory neurons by activating $G_{q/11}$–protein-coupled or tyrosine kinase receptors and by elevating intracellular Ca^{2+} (Huang et al., 2006; Akopian et al., 2007; Gosselin et al., 2008; Miller et al., 2009). It has been well documented that acute PIP_2 accumulation or depletion can regulate functional activities of a variety of voltage- and ligand-gated channels, including the TRPV1 and TRPA1 channels (Gamper et al., 2004; Suh and Hille, 2005; Qin, 2007; Rohacs et al., 2008). Two contrasting effects of PIP_2 on TRPV1 and TRPA1 have been reported. Research presented in several publications demonstrates that PIP_2 persistently inhibits the TRPV1 and TRPA1 channel activity (Chuang et al., 2001; Prescott and Julius, 2003; Dai et al., 2007; Kim et al., 2008b). Thus the depletion of PIP_2 levels induced by inflammatory mediators can lead to a release from this inhibition and subsequent sensitization of the TRPV1 and TRPA1 channels by inflammatory mediators (Chuang et al., 2001; Dai et al., 2007). In another set of reports, an enhancing potential of PIP_2 on TRPV1- and TRPA1-mediated responses was illustrated. Thus, PIP_2 was able to activate TRPV1 in excised patches (Stein et al., 2006). PIP_2 also sensitizes TRPA1-mediated mustard oil (MO) responses (Karashima et al., 2008). Substantial elevation of intracellular Ca^{2+} generated by activation of TRPV1 and TRPA1 induces Ca^{2+}–activated PLC isoforms, and the resulting depletion of PIP_2 plays a role in pharmacological desensitization of the TRPV1 and TRPA1 channels to a variety of stimuli (Liu et al., 2005; Akopian et al., 2007, 2008). Rohacs and colleagues suggested that the contradictory data on involvement of PIP_2 depletion in sensitization and desensitization of TRP channels could be dependent on the experimental conditions (Wu et al., 2002; Lukacs et al., 2007; Rohacs et al., 2008). The experimental conditions could be defined by a difference in TRP channel agonist concentrations (Lukacs et al., 2007), basal amounts of PIP_2 in cells, type of cells, or treatment period (minutes vs. hours or days) with low or high concentrations of PIP_2.

The last condition is especially important, in that, in inflammatory pain models and in the clinic, inflammatory mediators bombard nociceptors for long periods of time (hours to days). By doing so, PIP_2 amounts could also be altered within nociceptors for prolonged periods of time.

Regulation of TRPV1 and TRPA1 channels by PIP_2 has often been studied by changing the amount of PIP_2 acutely within TRP-expressing cells. This includes dialysis of PIP_2 into cells (Akopian et al., 2007; Dai et al., 2007), PIP_2 sequestering with antibodies (Chuang et al., 2001; Dai et al., 2007), or application of PIP_2 to excised patches (Stein et al., 2006; Kim et al., 2008b). In this study, we investigated whether the PIP_2 depletion or accumulation within the cells for 1–2 days affects magnitude and pharmacological desensitization of capsaicin (CAP) and MO response. We also examined whether the effects of PIP_2 vary in heterologous expression system (CHO cells) vs. sensory neurons, because the adapter protein *Pirt*, which links TRPV1 and PIP_2, is expressed only in sensory neurons but not in expression systems (Kim et al., 2008a). Finally, the contribution of TRPA1-TRPV1 coexpression on chronic PIP_2 effects was assessed.

MATERIALS AND METHODS

Animals

Primary sensory neuron cultures were generated from trigeminal ganglia (TG) isolated from B6.129S4 (wild-type; WT), B6.129S4-trpV1$^{tml/jul}$ (TRPV1 null-mutant mice provided by Jackson Laboratory, Bar Harbour, ME), or TRPA1 null-mutant mice generated on the B6129P1/F2J background (kindly provided by Dr. Kevin Kwan).

Constructs and Heterologous Expression in CHO Cells and Sensory Neurons

We used the following expression constructs: enhanced green fluorescent protein (pEGFP-N1 from Clontech, Palo Alto, CA); TRPV1 (accession No. NM031982) in pcDNA3 (Invitrogen, Carlsbad, CA); TRPA1 (NM177781) in pcDNA5/FRT (Invitrogen); PIP_2-specific phospholipid 5′-phosphatase (Lyn-PP) plasmid consisting of the 5′-phosphatase of yeast Inp54p; (Stolz et al., 1998), which is expressed as a fusion protein to GFP and to a myristoylation-palmitoylation sequence taken from the Src-family tyrosine kinase Lyn to achieve plasma membrane localization (Raucher et al., 2000); and mouse phosphatidylinositol-4-phosphate-5-kinase-Iβ (PI5-K) in pcDNA 3.1 containing the GFP sequence (Winks et al., 2005).

The expression constructs listed above were delivered into Chinese hamster ovary (CHO) cells using PolyFect (Qiagen, Valencia, CA) according to the manufacturers' protocol. CHO cells were subjected to experimental procedures within 24–48 hr after transfection. Electroporation, by using the Amaxa nucleofector, was carried out according to the manufacturer's protocol to deliver pEGFP-N1, Lyn-PP, and PI5-K into trigeminal ganglion (TG) sensory neurons. In brief, plasimds were mixed with the provided transfection solution and dispersed sensory neurons and then electroporated

at the G013 setting on the nucleofector. Cells were plated onto glass coverslips coated with poly-D-lysine and laminin and cultured in the presence of 100 ng/ml nerve growth factor (NGF-7.02S; Harlan, Indianapolis, IN) as previously described (Diogenes et al., 2007). Sensory neurons were subjected to experimental procedures within 24–48 hr of post-transfection.

Ca^{2+} Imaging

The Ca^{2+} imaging experiments were performed in standard external solution (i.e., SES) as previously described (Diogenes et al., 2006). The net changes in Ca^{2+} influx were calculated by subtracting the basal $[Ca^{2+}]_i$ (mean value collected for 60 sec prior to agonist addition) from the peak $[Ca^{2+}]_i$ value achieved after exposure to the agonists. Ca^{2+} increases above 50 nM were considered positive. This minimal threshold criterion was established by application of 0.1% DMSO as a vehicle. Ratiometric data were converted to $[Ca^{2+}]_i$ (in μM) by using the equation $[Ca^{2+}]_i = K\star (R - R_{min})/(R_{max} - R)$, where R is the 340/380 nm fluorescence ratio. R_{min}, R_{max}, and $K\star$ were measured according to a previously described method (Gamper and Shapiro, 2003).

Electrophysiology

All recordings were made in perforated patch voltage clamp (holding potential (V_h of –60 mV) configuration at 22–24°C from the somata of neurons (15–40 pF) or CHO cells. Data were acquired and analyzed using Axopatch 200B or MultiClamp700 amplifiers and pClamp10 software (Axon Instruments, Union City, CA). Recording data were filtered at 0.5–2.5 kHz and sampled at 2–10 kHz depending on current kinetics. Borosilicate pipettes (Sutter, Novato, CA) were polished to resistances of 7–10 MΩ in the perforated patch pipette solution. Access resistance (R_s) was compensated (40–80%) when appropriate up to the value of 20–25 MΩ. Data were rejected when R_s changed by >20% during recording, leak currents were >70 pA, or input resistance was <200 MΩ. Currents were considered positive when their amplitudes were fivefold larger than displayed noise (in root mean square).

SES contained (in mM): 140 NaCl, 5 KCl, 2 CaCl$_2$, 1 MgCl$_2$, 10 D-glucose, and 10 HEPES, pH 7.4. Bath solution for measurement of voltage-gated Ca^{2+} channels was 160 mM TEA-Cl, 5 mM CaCl$_2$, 10 mM glucose, 10 mM HEPES, pH 7.4 (by TEA-OH). The pipette solution for the perforated patch configurations consisted of (in mM): 140 KCl, 1 MgCl$_2$, 10 HEPES, pH 7.3, and 250 mg/ml amphotericin B (Sigma, St. Louis, MO). In a set of experiments designed to suppress K^+ currents, KCl was equimolarly substituted with CsCl. Drugs were applied using a fast, pressure-driven, computer-controlled, eight-channel system (AutoMate Scientific, San Francisco, CA).

Data Analysis

For statistical analysis, GraphPad Prism 5.0 (GraphPad, San Diego, CA) was used. The data in figures are given as mean $\pm$ SEM, with the value of n referring to the number of analyzed cells. All experiments were performed at least in trip-

licate. The significant difference between groups was assessed by one-way ANOVA with Bonferroni multiple-comparisons post hoc test. A difference was accepted as significant at $P <$ 0.05, $P < 0.01$, or $P < 0.001$ (identified by ⋆, ⋆⋆, and ⋆⋆⋆, respectively).

RESULTS

Positive Control Experiments: Effects of Chronic PIP$_2$ on Run-Down of Voltage-Gated Ca^{2+} Currents in Sensory Neurons

The production of PIP$_2$ in sensory neurons was stimulated by overexpression of PI5-K; whereas PIP$_2$-specific Lyn-PP was selected to reduce plasma membrane PIP$_2$ concentrations in sensory neurons but not other intracellular pools of PIP$_2$ or other plasma membrane phosphatidylinositol phosphates. To evaluate the potential chronic effects of PIP$_2$ on CAP and MO responses, we first employed a positive control to verify functionally relevant overexpression of PI5-K and Lyn-PP in sensory neurons. Previous studies have shown that the depletion of membrane-bound PIP$_2$ contributes to run-down of N-type and P/Q-type Ca^{2+} channels (Wu et al., 2002; Gamper et al., 2004; Suh and Hille, 2005), which are major components of high-voltage-activated (HVA) Ca^{2+} currents (I$_{Ca}$) in sensory neurons (Nowycky et al., 1985; Grigaliunas et al., 2002). Therefore, as a positive control, we evaluated run-down of I$_{Ca}$ in sensory neurons expressing GFP, PI5-K, or Lyn-PP.

We delivered expression plasmids into sensory neurons by using electroporation with Amaxa nucleofector (Fig. 1A,B). This method is effective, with 5–15% transfection ratio, and leaves undamaged neurons suitable for reliable patch clamp recording. It is noteworthy that Lyn-PP fused to GFP is effectively expressed on the plasma membrane (Fig. 1B). The recording of I$_{Ca}$ was conducted with small to medium-sized neurons (25–35 pF), because the size of cells somewhat affects run-down rate of Ca^{2+} currents (i.e., run-down is lesser in large cells). In GFP-containing neurons, repetitive application of voltage steps produced approximately 25% run-down of I$_{Ca}$ within 30 min (Fig. 1C,D). Accumulation of PIP$_2$ generated by PI5-K overexpression in sensory neurons virtually stopped run-down process for I$_{Ca}$ (Fig. 1C–E). In contrast, putative reduction of PIP$_2$ amounts in sensory neurons transfected with Lyn-PP significantly enhanced run-down rate (70%) of I$_{Ca}$ (Fig. 1C,D,F). Furthermore, I$_{Ca}$ density was substantially reduced by overexpression of Lyn-PP in sensory neurons and was slightly increased by introduction of PI5-K into sensory neurons. Thus, measurements are GFP in TG 69.35 ± 8.54 pA/pF (n = 8), PI5-K in TG 89.5 ± 9.15 pA/pF, and Lyn-PP in TG 37.7 ± 5.62 pA/pF (GFP in TG vs. Lyn-PP in TG, $P < 0.05$; and PI5-K in TG vs Lyn-PP in TG, $P < 0.001$; one-way ANOVA). These data are in accordance with previously published results (Gamper et al., 2004; Suh et al., 2010). In summary, chronic accumulation and depletion of PIP$_2$ in sensory neurons
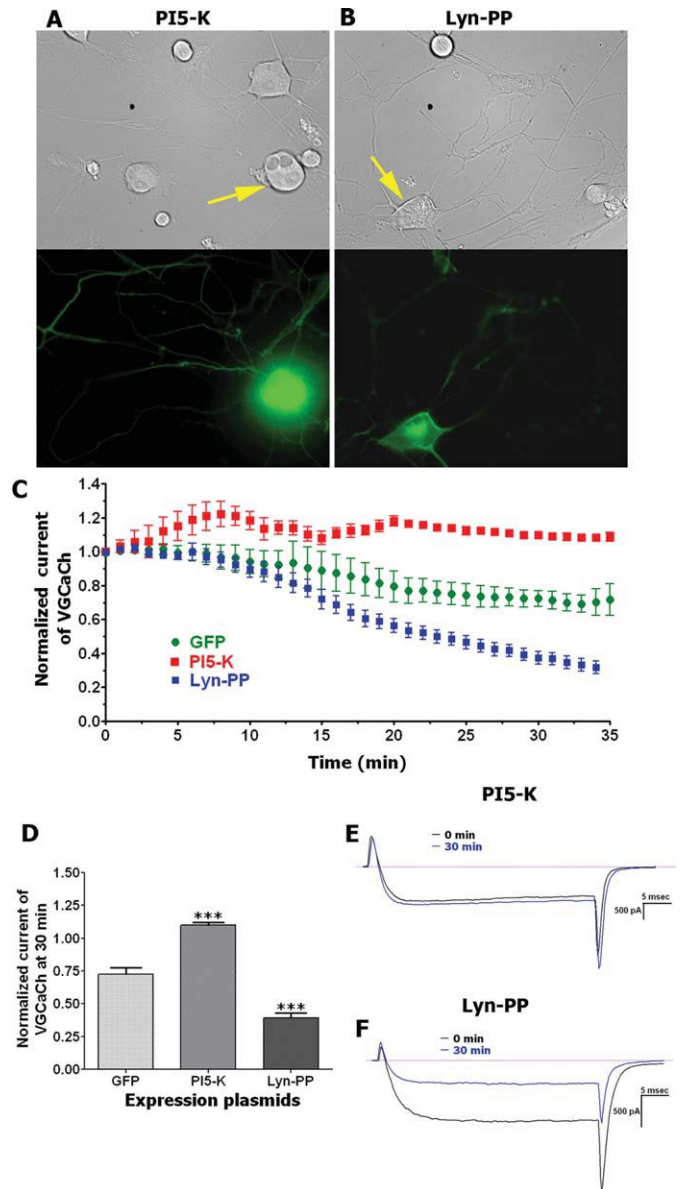

Fig. 1. Positive control experiments: chronic PIP$_2$-dependent run-down of voltage-gated Ca^{2+} channels (VGCaCh) in sensory neurons. Chronic accumulation and depletion of PIP$_2$ affect run-down of VGCaCh in sensory neurons. **A,B:** Expression of GFP-containing PI5-K (A) and Lyn-PP (B) plasmids in sensory neurons transfected by electroporation. Cells containing plasmids are marked with arrows. The pictures were captured under brightfield DIC (upper panels) and in fluorescent modes on a TE2000-U (Nikon) microscope controlled with Metafluor 6.0 software (Molecular Devices, Sunnyvale, CA). **C:** Time-dependent run-down of VGCaCh within 35 min. VGCaCh were activated by voltage step from –60 mV to 0 mV for 40 msec. The voltage step was applied to cells at intervals of 15 sec. Expression plasmids are indicated. Ca^{2+} current was normalized to the peak of Ca^{2+} current recorded at a time point of 0 min; n = 7–9. **D:** Bar graph of normalized voltage-gated Ca^{2+} current recorded at 30 min; n = 7–9. **E,F:** Representative traces of voltage-gated Ca^{2+} current recorded at 0 min and 30 min from sensory neurons expressing PI5-K (E) and Lyn-PP (F) plasmids.

can be induced by overexpression of PI5-K and Lyn-PP, respectively.

Effect of Chronic PIP₂ Depletion and Accumulation on CAP Responses

The modulation of the TRPV1 channel by acute application or depletion of PIP$_2$ has been well documented (Chuang et al., 2001; Liu et al., 2005). However, it appears that acute production and hydrolysis PIP$_2$ have a dual effect on TRPV1 activity (Lukacs et al., 2007). This difference was attributed to experimental conditions, namely, stimulus strength for TRPV1 (Rohacs et al., 2008). Here, we investigated roles of other experimental conditions such as chronic alteration in PIP$_2$ levels, coexpression of TRPV1 with TRPA1, and cell type.

Effects of low concentrations of CAP (20 nM) in cells with chronically low or high amounts of PIP$_2$ were examined by using Ca^{2+} imaging, because a 20-nM CAP-evoked current (I_{CAP}) is too small ($\sim$75 pA), percentage of responsive neurons is low, and the current's noise/flickering is relatively high ($\sim$25 pA) to study its regulation reliably. Figure 2A illustrates that chronic PIP$_2$ accumulation and reduction in CHO cells expressing only TRPV1 result in inhibition and potentiating of CAP-evoked Ca^{2+} influx. This regulation is similar to acute effects of PIP$_2$ on low concentration of CAP responses (Chuang et al., 2001; Lukacs et al., 2007). TRPV1 is extensively coexpressed with TRPA1 in sensory neurons (Kobayashi et al., 2005; Diogenes et al., 2007; Katsura et al., 2007), and both channels undergo an interaction with each other (Salas et al., 2009; Staruschenko et al., 2010). Therefore, we evaluated effects of PIP$_2$ levels on TRPV1 activity in TRPV1-TRPA1-containing cells (responding to both CAP and MO). Coexpression of TRPA1 with TRPV1 led to the eradication of the chronic PIP$_2$ effect on 20-nM CAP responses (Fig. 2A).

We next examined whether PIP$_2$ affects 100-nM CAP-activated responses (CAP-gated current; I_{CAP}), which are considered as "strong CAP responses" (Rohacs et al., 2008). The results presented in Figure 2B indicate that I_{CAP} generated by 100 nM CAP is not regulated by chronic accumulation and hydrolysis of PIP$_2$ in either TRPV1- or TRPV1-TRPA1-expressing CHO cells. The cell type could influence the regulatory action of chronic PIP$_2$ accumulation and hydrolysis. Accordingly, PIP$_2$ effects on I_{CAP} (100 nM) were investigated in sensory neurons containing TRPV1 with TRPA1 (a subset of WT sensory neurons) and TRPV1 alone (TRPA1 KO sensory neurons). It appears that, as in CHO cells, chronic PIP$_2$ does not regulate I_{CAP} (100 nM) in sensory neurons (Fig. 2B,C).

In a TRPV1 expression system, acute pharmacological blockage of PIP$_2$ hydrolysis can significantly reduce tachyphylaxis generated by 1 μM CAP (Liu et al., 2005). However, this PIP$_2$ action was not observed in sensory neurons, in which CAP tachyphy-
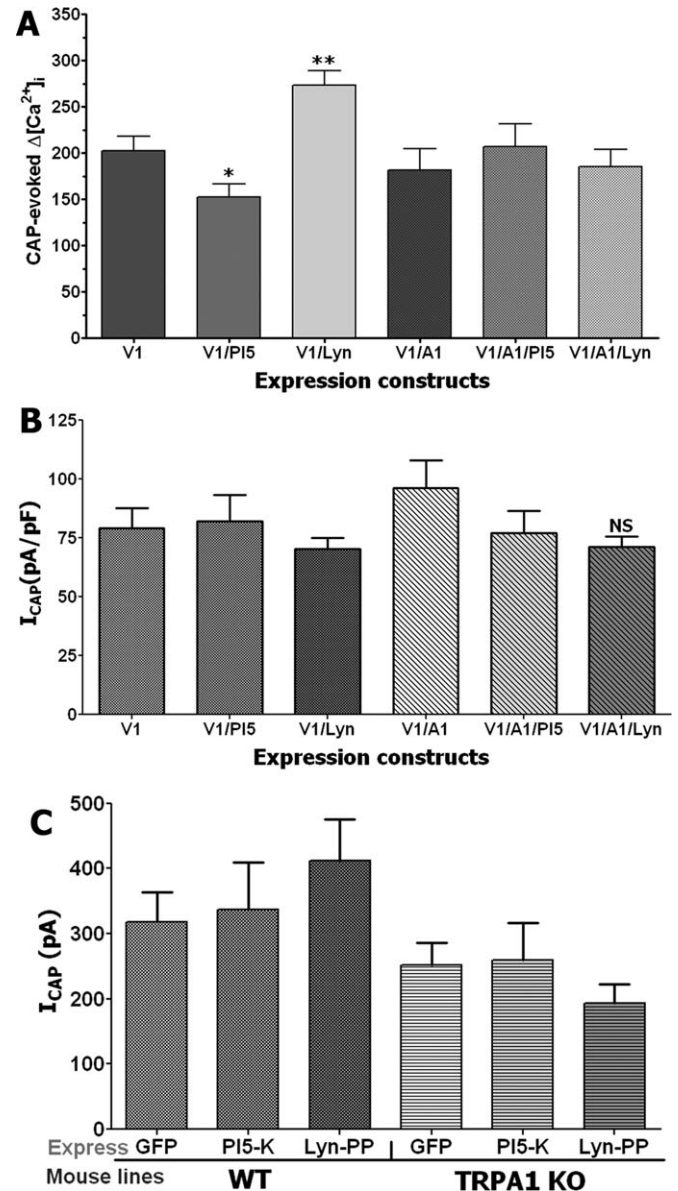

Fig. 2. Chronic effects of PIP$_2$ on CAP responses. Accumulation and depletion of PIP$_2$ for periods of 1–2 days affect CAP responses in certain cell lines. **A:** CAP (20 nM)-evoked Ca^{2+} influx in CHO cells expressing TRPV1 (V1) or TRPV1 with TRPA1 (V1/A1) as well as GFP (visual marker), PI5-K (PI5), or Lyn-PP (Lyn); n = 54–59 for TRPV1 expressing CHO cells; n = 38–56 for TRPV1-TRPA1 coexpressing cells. **B:** CAP (100 nM)-gated current density in CHO cells expressing TRPV1 or TRPV1 with TRPA1 as well as GFP (visual marker), PI5-K, or Lyn-PP; n = 6–10 for TRPV1-expressing CHO cells; n = 7 for TRPV1-TRPA1-coexpressing cells. **C:** CAP (100 nM)-gated current in wild-type (WT) and TRPA1 null-mutant (TRPA1 KO) TG sensory neurons expressing GFP, PI5-K, or Lyn-PP; n = 9–10 for WT sensory neurons; n = 6–8 for TRPA1 KO sensory neurons.

laxis is regulated mainly by dephosphorylation (Koplas et al., 1997; Jung et al., 2004; Akopian et al., 2007). Here, we examined whether chronic accumulation and depletion of PIP$_2$ control I_{CAP} tachyphylaxis generated

by 100 nM CAP in TRPV1- as well as TRPV1-TRPA1-expressing cells. Low concentrations of CAP were not selected for these experiments, because CAP tachyphylaxis was insignificant or absent for <50 nM CAP (Koplas et al., 1997). Neither the accumulation nor the depletion of PIP$_2$ for 1–2 days altered CAP-

A: Normalized I_{CAP} (CAP$_2$/CAP$_1$) — Expression plasmids: V1, V1/PI5, V1/Lyn, V1/A1, V1/A1/PI5, V1/A1/Lyn

B: Normalized I_{CAP} (CAP$_2$/CAP$_1$) — Express: GFP, PI5-K, Lyn-PP | GFP, PI5-K, Lyn-PP; Mouse lines: WT, TRPA1 KO

C: GFP in WT; PI5-K in WT; Lyn-PP in WT

induced tachyphylaxis in TRPV1- and TRPV1-TRPA1-expressing CHO cells (Fig. 3A). Furthermore, overexpression of PI5-K and Lyn-PP in TRPA1 KO and WT sensory neurons expressing both TRPV1 and TRPA1 channels did not change CAP (100 nM) tachyphylaxis (Fig. 3B,C). Altogether, chronic enhanced production and hydrolysis of PIP$_2$ have limited action on TRPV1 activity. Thus, the PIP$_2$ effects were observed only for low-concentration (20 nM) CAP responses in TRPV1- but not in the TRPV1-TRPA1-expressing CHO cells (Fig. 2A). Otherwise, chronic PIP$_2$ does not affect CAP (100 nM) responses and tachyphylaxis in TRPV1- or TRPV1-TRPA1-expressing cells, including sensory neurons (Figs. 2B,C, 3).

Effect of Chronic PIP$_2$ Depletion and Accumulation on MO Responses

There are many parallels between the regulation of TRPV1 and TRPA1 by acute alteration in PIP$_2$ levels. As with TRPV1, both stimulatory and inhibitory effects of acute PIP$_2$ have been reported for the TRPA1 channel (Akopian et al., 2007; Dai et al., 2007; Karashima et al., 2008; Kim et al., 2008b). Here, we examined whether chronic alteration in PIP$_2$ levels, coexpression of TRPA1 with TRPV1, and selection of cell type contribute to regulation of TRPA1 by PIP$_2$. TRPA1 was activated with 25 μM MO, which is a strong stimulus (~EC$_{70}$) for TRPA1-expressing CHO (or HEK) cells (Jordt et al., 2004), but could be considered a moderate stimulus for sensory neurons (Dai et al., 2007; Salas et al., 2009). Data presented in Figure 4A,B suggest that MO responses in TRPA1-expressing CHO cells are significantly increased after chronic PIP$_2$ depletion for 1–2 days. This is very similar to reported acute effects of PIP$_2$ in the expression system (Dai et al., 2007). Coexpression of TRPV1 with TRPA1 abolished the PIP$_2$ effect on MO responses (Fig. 4A,B). Independence of TRPA1 activity on chronic accumulation and depletion of PIP$_2$ was also observed in a subset of wild-type TG sensory neurons that expresses both channels (i.e., responsive for MO and CAP; Fig. 4C,D). However, in TG neurons expressing only TRPA1, PIP$_2$ accumulation
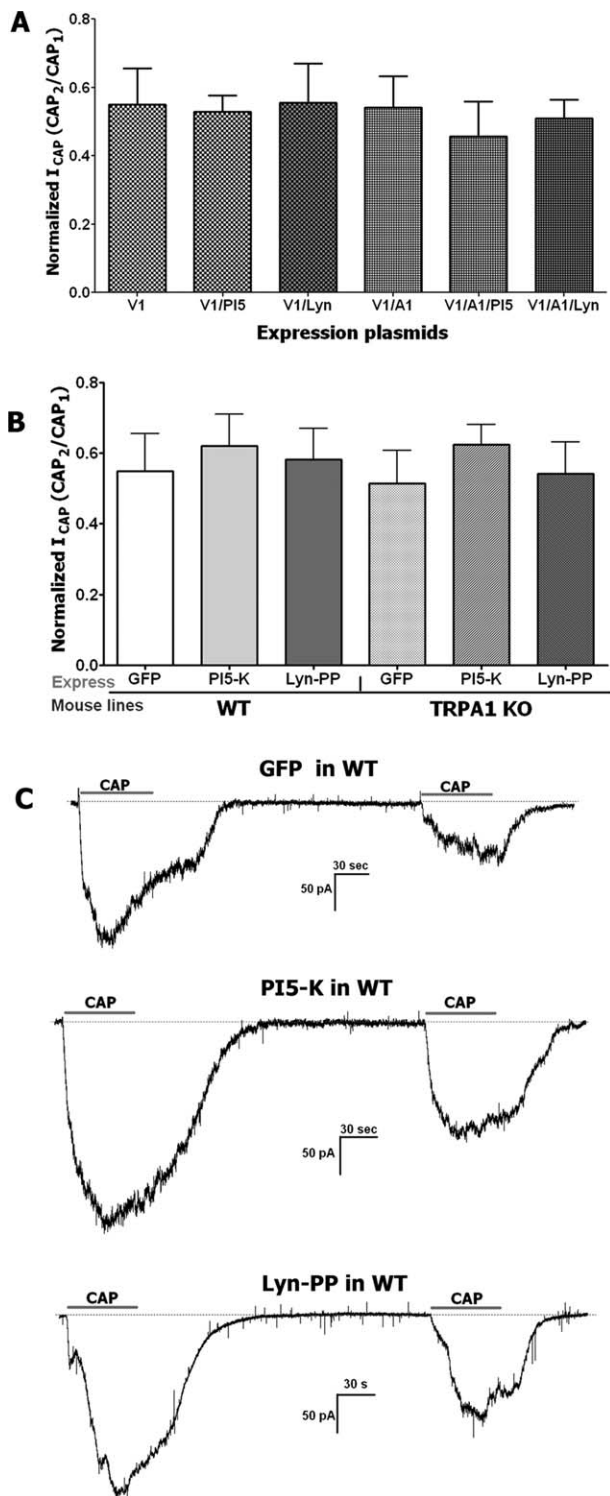
Fig. 3. Chronic effects of PIP$_2$ on CAP pharmacological desensitization (tachyphylaxis). Accumulation and depletion of PIP$_2$ for periods of 1–2 days do not influence CAP tachyphylaxis. **A:** Tachyphylaxis produced by 100 nM CAP in CHO cells expressing TRPV1 (V1) or TRPV1 with TRPA1 (V1/A1) as well as GFP, PI5-K (PI5) or Lyn-PP (Lyn). Four minutes elapsed between CAP applications; n = 7 for TRPV1-expressing CHO cells; n = 7 for TRPV1-TRPA1-coexpressing cells. **B:** Tachyphylaxis produced by 100 nM CAP in WT and TRPA1 KO TG sensory neurons expressing GFP, PI5-K, or Lyn-PP; n = 7 for WT sensory neurons; n = 7–9 for TRPA1 KO sensory neurons. **C:** Representative traces illustrate CAP (100 nM)-induced tachyphylaxis in WT TG sensory neurons expressing GFP, PI5-K, or Lyn-PP. The application period for CAP is marked with a horizontal bar. There was a 4-min interval between CAP applications. Peak responses were used for the ratio.
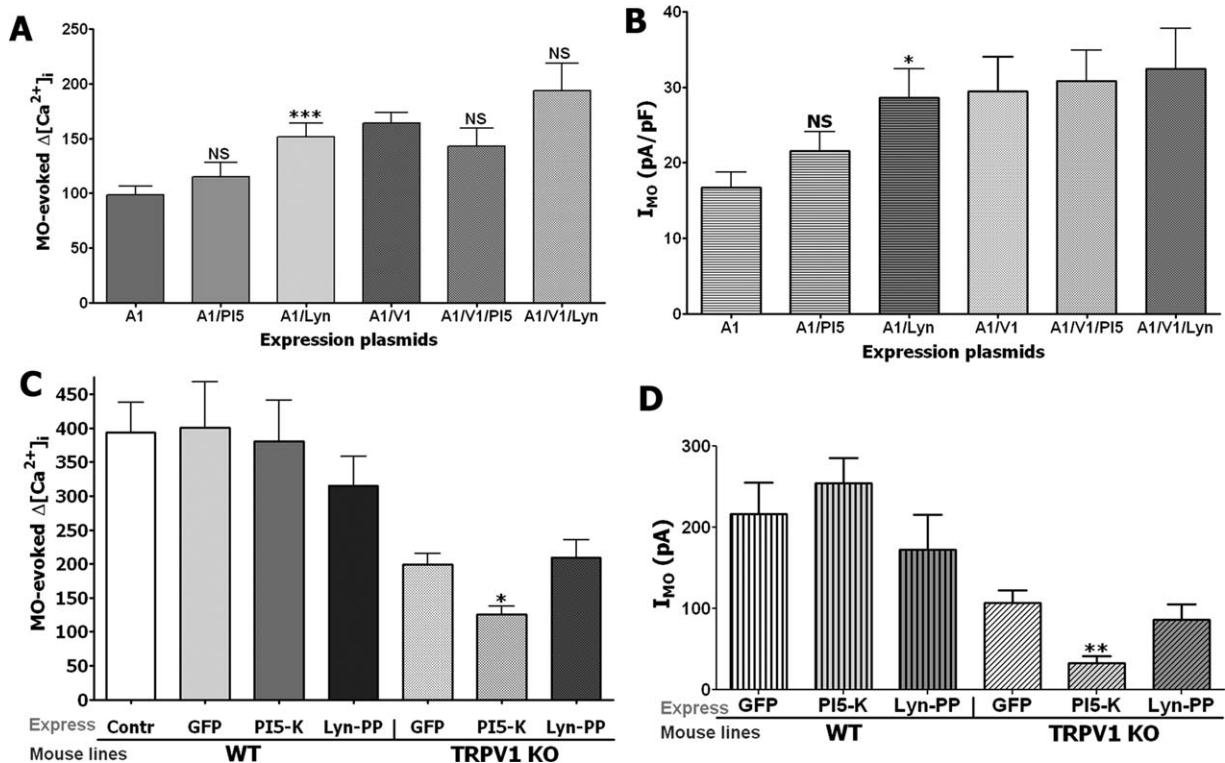
Fig. 4. Chronic effects of PIP$_2$ on MO responses. Chronic accumulation and depletion of PIP$_2$ for 1–2 days affect MO responses. **A:** MO (25 μM)-evoked Ca^{2+} influx in CHO cells expressing TRPA1 (A1) or TRPA1 with TRPV1 (A1/V1), as well as GFP (visual marker), PI5-K (PI5) or Lyn-PP (Lyn); n = 51–84 for TRPA1 expressing CHO cells; n = 61–85 for TRPA1-TRPV1 coexpressing cells. **B:** MO (25 μM)-gated current density in CHO cells expressing TRPA1 or TRPA1 with TRPV1 as well as GFP, PI5-K, or Lyn-PP; n = 7–9 for TRPA1 expressing CHO cells; n = 7–9 for TRPA1- TRPV1 coexpressing cells. **C:** MO (25 μM)-evoked Ca^{2+} influx in WT and TRPV1 null-mutant (TRPV1 KO) TG neurons mock transfected (control), expressing GFP, PI5-K, or Lyn-PP; n = 143 for control in WT TG neurons; n = 20–25 for WT sensory neurons; n = 34–58 for TRPV1 KO sensory neurons. **D:** MO (25 μM)-gated current in WT and TRPV1 KO TG neurons expressing GFP, PI5-K, or Lyn-PP; n = 7–10 for WT sensory neurons; n = 10–11 for TRPV1 KO sensory neurons.

substantially inhibited MO responses (Fig. 4C,D). These data indicate that there is a resemblance in chronic PIP$_2$ regulation of TRPA1 activity in the expression system and sensory neurons: increase in PIP$_2$ production inhibits, whereas PIP$_2$ depletion enhances, MO responses. However, difference between expression system and sensory neurons could be noted. Thus, in sensory neurons, PIP$_2$ accumulation, rather than PIP$_2$ depletion, was required to suppress MO responses (Fig. 3A,B vs. C,D). In summary, chronic PIP$_2$ affects MO responses only in TRPA1-expressing CHO cells and TRPV1 KO TG sensory neurons. Furthermore, PIP$_2$ action varies in CHO cells compared with sensory neurons.

Acute PIP$_2$ regulates MO tachyphylaxis in expression systems (Akopian et al., 2007; Karashima et al., 2008). In sensory neurons, there is a tendency for the same regulation; however, the effect is not statistically significant (Akopian et al., 2007). Furthermore, it appears that substantial Ca^{2+} influx by strong stimuli is required to activate PLC, which brings about PIP$_2$ depletion and pharmacological desensitization of MO

responses (Akopian et al., 2007). Here we stimulated production of and hydrolyzed PIP$_2$ for 1–2 days, which is independent from Ca^{2+} influx. Therefore, chronic PIP$_2$ depletion resulted in significantly stronger MO tachyphylaxis in TRPA1-expressing CHO cells (Fig. 5A) as well as TRPA1-TRPV1-expressing CHO cells and TG neurons (Fig. 5A,B). It should be noted that the effect is less pronounced in WT sensory neurons but is still statistically significant (Fig. 5A,B). In sensory neurons expressing only TRPA1, chronic PIP$_2$ accumulation led to a miniscule I$_{MO}$ (–32.3 ± 9.4 pA; Fig. 4D), which underwent pharmacological desensitization in only four of eight recorded neurons (Fig. 5B,C). Interestingly, all of the "nondesensitizing neurons" had a small I$_{MO}$ (<30 pA). The reasons for small MO responses in TRPV1 KO sensory neurons are not clear. Thus, functional loss of TRPA1 activities could be attributed to either conformational modifications of the TRPA1 channel or reduced density of TRPA1 on the plasma membrane. Overall, cells with depleted amounts of PIP$_2$ have greater MO tachyphylaxis. The exceptions

are TRPA1-alone-expressing TG neurons, in which MO responses were suppressed and MO tachyphylaxis dramatically diminished by overexpression of PI5-K and chronic PIP₂ accumulation.
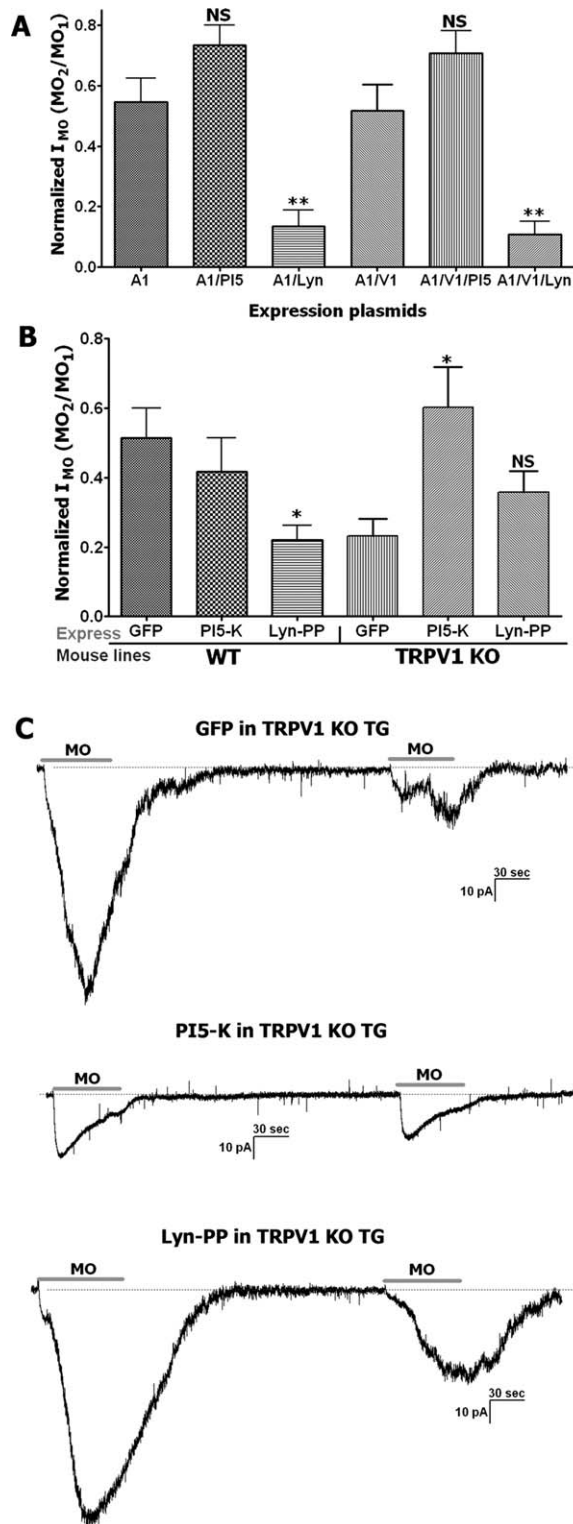

Fig. 5. Chronic effects of PIP₂ on MO desensitization (tachyphylaxis). Accumulation and depletion of PIP₂ for 1–2 days influence MO tachyphylaxis. **A:** Tachyphylaxis produced by 25 μM MO in CHO cells expressing TRPA1 (A1) or TRPA1 with TRPV1 (A1/V1) as well as GFP, PI5-K (PI5), or Lyn-PP (Lyn); n = 6–7 for TRPA1-expressing CHO cells; n = 6–8 for TRPA1-TRPV1-coexpressing cells. **B:** Tachyphylaxis produced by 25 μM MO in WT and TRPV1 KO TG sensory neurons expressing GFP, PI5-K, or Lyn-PP; n = 7–10 for WT sensory neurons; n = 9–10 for TRPV1 KO sensory neurons. **C:** Representative traces illustrate MO (25 μM)-induced tachyphylaxis in TRPV1 KO TG sensory neurons expressing GFP, PI5-K, or Lyn-PP. The application period for MO is marked with a horizontal bar. There was a 5-min interval between MO applications. Peak responses were used for the ratio.

DISCUSSION

A plethora of channels, metabotrophic receptors, adaptor proteins, and enzymes control nociceptive transmission during inflammation. There is agreement that the TRPV1 and TRPA1 channels play critical roles in regulation of certain types of inflammatory hyperalgesia at the peripheral site (Caterina et al., 2000; Davis et al., 2000; Obata et al., 2005; Kwan et al., 2006; Bautista et al., 2006; Dai et al., 2007). In this respect, studies on modulation of TRPA1 and TRPV1 activity by inflammatory mediators are of special interest, because this modulation could underscore molecular mechanisms of inflammatory hyperalgesia. Almost every inflammatory mediator can in principle deplete PIP₂, which is a known regulator of many channels (Suh and Hille, 2005). The TRPV1 and TRPA1 channels are not exceptions to this rule, and the control of TRPA1 and TRPV1 activity by PIP₂ is well documented. However, it appears that these regulations depend strictly on experimental conditions (Lukacs et al., 2007; Rohacs et al., 2008). Keeping this in mind, we have examined regulation of the TRPV1 and TRPA1 activities by PIP₂ under conditions very relevant to peripheral inflammation. In animal models and in the clinic, inflammation usually lasts for many hours or days. However, most of the data on effects of PIP₂ on TRPV1 and TRPA1 activities were obtained after acute (seconds to minutes) alterations of PIP₂ levels (Chuang et al., 2001; Liu et al., 2005; Akopian et al., 2007; Lukacs et al., 2007; Dai et al., 2007; Karashima et al., 2008). Therefore, we have investigated whether changes (i.e., overproduction or hydrolysis) in PIP₂ levels for 1–2 days could affect CAP and MO responses. Inflammation also affects excitability of sensory neurons. However, there are no systematic data addressing difference in PIP₂ regulation of TRPV1 and TRPA1 in expression system vs. sensory neurons. This is especially important, with the recent identification of a novel protein, Pirt, that is expressed specifically in sensory neurons and not in expression systems and is required for the stimulatory effect of PIP₂ on TRPV1 (Kim et al., 2008a). Accordingly, we have examined whether chronic changes in PIP₂ levels regulate TRPV1 and TRPA1 in a similar fashion in an expression system

and sensory neurons. In sensory neurons, inflammation functionally up-regulates both TRPV1 and TRPA1 channels and leads to coexpression of these channels in ~80% neurons (Obata et al., 2005; Diogenes et al., 2007). It is known that TRPA1 and TRPV1 are mutually involved in nociceptive transmission and undergo interaction (Bautista et al., 2006; McMahon and Wood, 2006; Salas et al., 2009; Staruschenko et al., 2010). Furthermore, a PIP_2 interaction domain is closely localized to the tetramerization region on the C-terminal portion of the TRPV1 channel (Prescott and Julius, 2003; Garcia-Sanz et al., 2004). Consequently, here we evaluated regulation of CAP and MO responses in chronic alteration of PIP_2 in TRPA1- or TRPV1-alone-expressing and TRPV1-TRPA1-coexpressing cells, including sensory neurons. Finally, pharmacological desensitization of receptors and channels could dramatically affect responsiveness of neurons to stimuli (i.e., tolerance; Docherty et al., 1996; Bhave et al., 2002; Ruparel et al., 2008; Akopian et al., 2009; Ueda and Ueda, 2009). In this context, we studied not only CAP and MO responses under the different conditions listed above but also pharmacological desensitization of the TRPV1 and TRPA1 to CAP and MO, respectively.

It is well accepted that TRPV1 activity is regulated by phosphorylation/dephosphorylation processes. Thus, many inflammatory mediators, with treatment of sensory neurons, lead to phosphorylation and subsequent sensitization of TRPV1 via different pathways involving protein kinase C (PKC), protein kinase A (PKA), Src-kinase, and PI_3-kinase (Lopshire and Nicol, 1998; Cesare et al., 1999; Numazaki et al., 2002; Sugiura et al., 2002; Zhuang et al., 2004; Zhang et al., 2005). Phosphorylation provides a physiologically relevant mechanism for TRPV1 sensitization, because involvement of these kinases in inflammatory thermal hyperalgesia has been reported multiple times (Aley et al., 2000; Yajima et al., 2003; Zhuang et al., 2004; Dai et al., 2004; Katsura et al., 2007). Pharmacological and functional desensitization of the TRPV1 channel is also regulated by dephosphorylation with PP2B/calcineurin that can be rescued by calcineirin blockers and PKA phosphorylation (Docherty et al., 1996; Bhave et al., 2002; Jung et al., 2004; Jeske et al., 2006; Ruparel et al., 2008). Despite all this compelling evidence, there is still a viewpoint that PIP_2 could also be involved in physiologically relevant sensitization and desensitization of TRPV1 in sensory neurons (Chuang et al., 2001; Liu et al., 2005). Our data generated in sensory neurons expressing both TRPV1 and TRPA1 channels and chronically over- or underproducing PIP_2 suggest that PIP_2's role in regulation of TRPV1 during inflammatory thermal hyperalgesia and pharmacological desensitization of the TRPV1 channel could be minimal if any. Involvement of sensory neuronal PIP_2 in inflammatory hyperalgesia was not directly evaluated by a behavioral test, because it is a technically difficult task. However, indirect evidence suggests that partial involvement of PIP_2 in pain processing from the periphery could still occur. Thus, prostatic acid phospha-

tase (PAP) applied intrathecally modulates several cellular pathways, including reduction PIP_2 levels for 1–2 hr. This PIP_2 depletion correlates with inhibition of thermosensation as well as thermal and mechanical inflammatory hyperalgesia, which have been measured 6–48 hr post-PAP application (Sowa et al., 2010). These data indicate that changes in PIP_2 levels could correlate with initiation of thermal and mechanical hyperalgesia mediated by TRPV1. Similarly, ablation of *Pirt* that serves as a bridge between PIP_2 and TRPV1 only partially reverses bradykinin-induced sensitization of CAP (100 nM) responses (Kim et al., 2008a). The contribution of *Pirt* in inflammatory hyperalgesia has not been examined as yet (Kim et al., 2008a). In summary, the physiological role of PIP_2 in inflammatory thermal hyperalgesia remains an open question.

TRPA1 controls certain aspects of inflammatory hyperalgesia. Unlike the case for TRPV1, there is no agreement on the molecular mechanisms that connect the TRPA1 channel to inflammatory hyperalgesia. However, there are several possible pathways. First, TRPA1 could be sensitized by inflammatory mediator-induced depletion of PIP_2, which provides tonic inhibition of TRPA1 (Dai et al., 2007). Our results indicate that such inhibition can take place in sensory neurons expressing TRPA1 alone but not in WT neurons coexpressing TRPA1 with TRPV1 (Fig. 4D). Second, TRPA1 could be sensitized via the PKA pathway, which leads to increase in membrane levels of the TRPA1 channel (Schmidt et al., 2009). In contrast, it was reported that activation of PKC could not lead to TRPV1 phosphorylation and desensitization (Dai et al., 2007). Third, TRPA1 sensitization could be controlled by associated TRPV1 channels, which are in turn modulated by inflammatory mediators (Akopian, 2010). This pathway in the regulation TRPA1 is a possibility, in so far as TRPA1-mediated responses (such as MO, WIN55,212-2, and especially AM1241) are closely controlled by TRPV1 coexpression (Akopian et al., 2008; Salas et al., 2009).

Pharmacological desensitization of MO engages several mechanisms, including Ca^{2+}-dependent PIP_2 depletion and covalent modification of TRPA1 by agonists (Akopian et al., 2007; Macpherson et al., 2007; Schmidt et al., 2009). Therefore, it is not surprising that MO tachyphylaxis is more pronounced in cells with strong MO responses (Akopian et al., 2007). This could explain the less evident MO tachyphylaxis in TRPA1-alone-expressing sensory neurons (Fig. 5B,C). However, TRPA1 recovers poorly from MO-induced desensitization in cells with depleted PIP_2 (Fig. 5). In native sensory neurons, PIP_2 depletion and MO desensitization occur only after substantial Ca^{2+} influx triggered by strong stimuli such as CAP (Akopian et al., 2007). These data suggest that recovery from desensitization and sensitization of TRPA1 activity during inflammation could require some other pathway independent of PIP_2, because inflammation leads to PIP_2 depletion. Thus, PLC-induced PKA phosphorylation is one possible path-

way (Schmidt et al., 2009). Interestingly, TRPV1 recovery from desensitization is also controlled by PKA (Bhave et al., 2002; Jeske et al., 2006). One confusing point is that the desensitization of TRPV1, but not TRPA1, depends on dephosphorylation (Akopian et al., 2007). In summary, PIP$_2$ could play a role in recovery of TRPA1 pharmacological desensitization in native sensory neurons; however, TRPA1 sensitization during inflammation is probably controlled through some other signaling pathways.

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