Cellular/Molecular

Functional Recovery from Desensitization of Vanilloid Receptor TRPV1 Requires Resynthesis of Phosphatidylinositol 4,5-Bisphosphate

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Capsaicin and other naturally occurring pungent molecules have long been used as topical analgesics to treat a variety of chronic pain conditions. The analgesic effects of these compounds involve long-term desensitization of nociceptors after strong stimulation. To elucidate the underlying mechanisms, we studied the recovery from desensitization of the vanilloid receptor TRPV1. We showed that prolonged applications of capsaicin led to nearly complete desensitization of the channel and that its functional recovery from desensitization required a high concentration of intracellular ATP. Nonhydrolyzable ATP analogs did not substitute for ATP to promote recovery. Neither inhibition nor activation of protein kinases prevented recovery of the channel from desensitization. In contrast, blockade of lipid kinases, in particular phosphatidylinositol-4-kinase, abolished recovery, as did activation of membrane receptors that stimulate hydrolysis of phosphatidylinositol 4,5-biphosphate (PIP_2). Additional experiments using the PIP_2-sensitive inward rectifier potassium channel Kir2.1 as a biosensor showed a high degree of temporal correlation between the two channels on both functional suppression after capsaicin stimulation and subsequent recovery. These data suggest that depletion of PIP_2 occurs concomitantly with activation of TRPV1 and its replenishment in the membrane determines recovery of the channel from desensitization. In addition to revealing a new role of phosphoinositide signaling in regulation of nociception, our results provide novel insight into the topical mechanisms of the analgesic effects of capsaicin and the strategies to improve its effectiveness.

Key words: **capsaicin; analgesic; pain; TRP channels; PIP_2; sensory neurons**

Introduction

Responsiveness to capsaicin, the pungent ingredient of hot peppers, is a unique pharmacological trait of a subset of primary afferent neurons (Holzer, 1991). At low suprathreshold doses, locally applied capsaicin stimulates the chemosensitive and thermosensitive nociceptors and elicits pain (Szolcsanyi, 1993). However, at higher doses or with prolonged exposures, it causes desensitization of neurons at both pharmacological and functional levels, resulting in impairment of pain transmission mechanisms (Jancsó, 1968). Systemic capsaicin treatment, in contrast, produces long-term neurotoxicity ranging from ultrastructural damages to degeneration of cells (Jancso et al., 1977; Marsh et al., 1987). The antinociceptive effect of capsaicin on sensory neurons has been exploited for its therapeutic values (Szallasi and Blumberg, 1996).

The molecular target of capsaicin, the vanilloid receptor TRPV1, is a nonselective cation channel of the TRP superfamily (Caterina et al., 1997). The receptor is activated by noxious stimuli such as vanilloids, heat, and acids, and the activity is sensitized by substances that are present in the inflammatory milieu such as nerve growth factor (NGF), prostaglandins, bradykinin, and lipid metabolites (Julius and Basbaum, 2001). Functional studies of mice lacking TRPV1 support a prominent role for the channel in thermal transduction and pain sensation, especially inflammation-induced thermal hyperalgesia (Caterina et al., 2000; Davis et al., 2000).

The cloned vanilloid receptor TRPV1 explains most of the biological actions of capsaicin on sensory neurons, including its antinociceptive effects. Prolonged or repeated applications of capsaicin cause persistent desensitization of TRPV1 (Caterina et al., 1997). Previous studies in cultured neurons show that the desensitization of capsaicin responses is dependent on a variety of factors, including the capsaicin concentration, the period of stimulus, and the presence of extracellular Ca^{2+} (for review, see Szallasi and Blumberg, 1999), and can be reduced by inhibition of protein phosphatase 2B (calcineurin) (Docherty et al., 1996). Recent studies of TRPV1 in heterologous expression systems have implicated other mechanisms such as protein kinase A (PKA) phosphorylation and calmodulin (CaM) binding in regulation of desensitization (Bhave et al., 2002; Mohapatra and Nau, 2003; Numazaki et al., 2003; Rosenbaum et al., 2004). Activation of protein kinase C (PKC), in contrast, is reported to reverse desensitization of capsaicin responses in a Ca^{2+}-imaging assay (Mandadi et al., 2004).

In contrast to the intense studies of desensitization, little is

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known about recovery of TRPV1 from desensitization. The recovery of the channel has been suggested to underlie tachyphylaxis of capsaicin responses, a process that involves successive diminution of responsiveness after brief and repeated capsaicin applications (Liu and Simon, 1996). The molecular mechanisms of the process, however, remain to be elucidated. In this study, we demonstrate that full recovery of TRPV1 from desensitization requires resynthesis of phosphatidylinositol 4,5-biphosphate (PIP_2) by phosphatidylinositol-4-kinase (PI4K). Inhibition of protein kinases or disruption of the putative phosphorylation sites in the channel does not prevent recovery. Neither does truncation of the distal C terminus containing a previously identified PIP_2-binding region (Prescott and Julius, 2003). The effects of PIP_2 on recovery of TRPV1 therefore represent a novel mechanism for phosphoinositide signaling in modulation of TRPV1 activity.

Materials and Methods

Materials. Capsaicin was obtained from Fluka (Buchs, Switzerland). Protein kinase A inhibitor (PKI) 14-22, forskolin, and 1-oleoyl-2-acetyl-sn-glycerol (OAG) were purchased from Calbiochem (La Jolla, CA). PMA was obtained from LC Laboratories (Woburn, MA). Capsazepine was purchased from Precision Biochemicals (Vancouver, British Columbia, Canada). All other chemicals, including wortmannin, phenylarsine oxide (PAO), NGF, bisindolylmaleimide I (BIM), *N*-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride (H89), 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002), ATP, β,γ-methyleneadenosine 5′-triphosphate (AMP-PCP), 5′-adenylylimidodiphosphate (AMP-PNP), and IP_3, were obtained from Sigma (St. Louis, MO).

Water-insoluble reagents were dissolved in either 100% ethanol or DMSO to make a stock solution and diluted into a recording solution at the appropriate concentration before the experiment. The final concentrations of ethanol and DMSO did not exceed 0.17 and 0.3%, respectively.

Mutagenesis. Site-directed mutagenesis was performed using the overlap–extension PCR method. Truncations of the C terminus were done by PCR amplification of primers with the appropriate restriction sites (5′, *Sac*II; 3′, *Xba*I). All recombinant constructs were confirmed by restriction enzyme digestion and by DNA sequence analysis.

Heterologous expression. Rat TRPV1 and trkA/p75 (wild type and mutants) were generously provided by David Julius (University of California, San Francisco, CA) (Caterina et al., 1997; Prescott and Julius, 2003). Kir2.1 was provided by Lily Jan (University of California, San Francisco, CA) (Kubo et al., 1993). Human embryonic kidney 293 (HEK293) cells were maintained in DMEM plus 10% fetal bovine serum (HyClone, Logan, UT) with 1% penicillin/streptomycin, incubated at 37°C in 5% CO_2, and transfected at a confluence of ~80% using the standard calcium phosphate precipitation method, as described previously (Liu et al., 2004). Either green fluorescent protein or human CD8 lymphocyte antigen (0.5 μg/0.2 ml) was cotransfected as a surface marker. Electrophysiological recordings took place 10–28 h after transfection. For cells cotransfected with CD8, antibody-coated beads were used to visually identify the transfected cells (Dynabeads M450 CD8; Dynal, Lake Success, NY). More than 90% of the bead-decorated cells appeared to express the channel.

Electrophysiology. Conventional whole-cell recording methods were used. Currents were amplified using either an Axopatch 200B (Molecular Devices, Foster City, CA) or a PC-505B (Warner Instruments, Hamden, CT) patch-clamp amplifier, filtered at 1 kHz, and digitized at 5 kHz directly onto the computer hard disk through a BNC-2090/MIO acquisition system (National Instruments, Austin, TX) driven by a custom-designed software (QuB/IcE). Patch electrodes were fabricated from borosilicate glass (Sutter Instruments, Novato, CA). Drug delivery was controlled by a gravity-driven local perfusion system (ALA Scientific Instruments, Westbury, NY) or intracellular dialysis through a patch pipette for 5–10 min after membrane breakthrough. The recording apparatus and perfusion lines were always washed thoroughly with ethanol after the experiments. All experiments were conducted at room temperature (22–24°C).

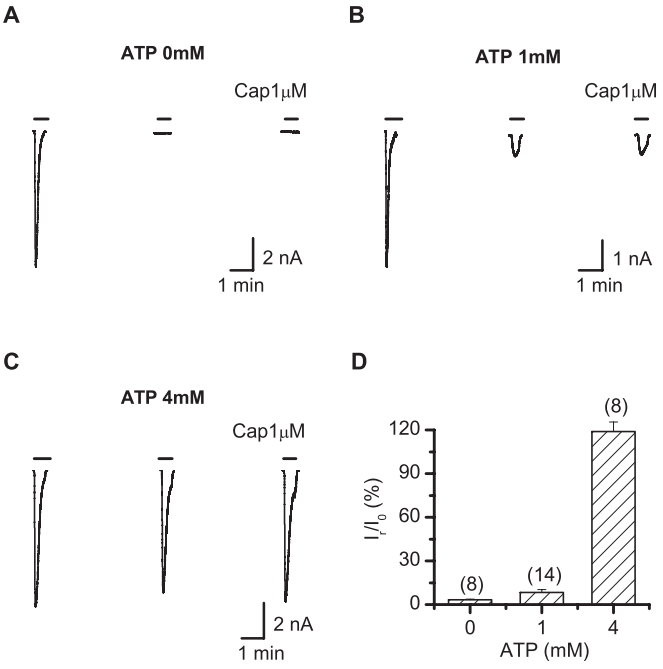

Figure 1. Intracellular ATP promotes recovery of TRPV1 from desensitization. ***A***, Whole-cell recording from a HEK293 cell transiently expressing TRPV1 in response to 1 μM capsaicin in the presence of 1.8 mM Ca^{2+}. The trace includes three repeated applications of capsaicin separated by ~5 min washes with the standard bath solution. Each capsaicin application lasted for a prolonged duration to ensure full desensitization. The holding potential was −60 mV. ***B***, ***C***, The same experiments as in ***A***, except with 1 and 4 mM ATP included in the pipette, respectively. ***D***, Summary graph of recovery from desensitization plotted as a percentage of the maximum current evoked in response to the second and third applications of capsaicin relative to the peak response to the first application (mean ± SEM). The number of cells in each trial is indicated in parentheses above each bar. Cap, Capsaicin.

The control bath solution contained the following (in mM): 140 NaCl, 5 KCl, 1.8 $CaCl_2$, 10 HEPES, and 10–30 glucose, pH 7.4 (adjusted with NaOH). The standard pipette solution consisted of the following (in mM): 140 CsCl, 1 EGTA, and 10 HEPES, pH 7.4 (adjusted with CsOH). For all of the experiments in this study, the pipette solution contained 1 mM EGTA for intracellular Ca^{2+} buffering. In a subset of experiments, the pipette solution was supplemented with 2 mM Mg^{2+}. The reagents were generally applied at their supramaximal concentrations (Davies et al., 2000).

Results

Intracellular ATP confers recovery from desensitization

We studied recovery of TRPV1 from desensitization in transiently transfected HEK293 cells. Desensitization of the channel was induced by a prolonged exposure to 1 μM capsaicin in the presence of 1.8 mM Ca^{2+}. The agonist was applied until the desensitization of the currents reached a relatively steady-state level. The same stimulus was then repeated in a time interval of ~5 min for two to three consecutive times. The extent of recovery was quantified as the peak current attainable during the second or third stimulation relative to the peak current evoked by the first application. Figure 1*A* shows an example of whole-cell recordings obtained with such a protocol. The desensitization of the channel was nearly complete in response to the first application of 1 μM capsaicin. The half-decay time of the desensitization was on the order of seconds ($t_{1/2}$, 6 ± 1 s; $n = 7$). The experiment

included no ATP in the pipette solution. Under this condition, there was virtually no recovery of channel activity within the experimental times (5–30 min). This failure of recovery persisted regardless of the washout duration between successive stimuli, indicating that removal of the agonist did not suffice to reverse desensitization. In addition, the loss of the responsiveness was unlikely caused by channel rundown. Dialysis of cells for a prolonged duration before recording did not incur appreciable suppression of the initial responses, whereas shortening capsaicin exposure durations could extend the responsiveness period. The loss of the responsiveness therefore resulted from desensitization of the channel and reflected a failure of recovery.

We explored a role for ATP in recovery of TRPV1 from desensitization. Intracellular nucleotide analogs have been reported to affect tachyphylaxis of capsaicin responses in native neurons (Koplas et al., 1997). Figure 1*D* summarizes the effects of intracellular ATP on recovery of the channel at different concentrations. When the pipette solution contained 4 mM ATP, the channel recovered completely over a time course of 5–10 min (Fig. 1*C*). When the ATP concentration was decreased to 1 mM, the extent of recovery was dramatically diminished (Fig. 1*B*) and became only slightly higher than in the absence of ATP (Fig. 1*D*). The recovery of TRPV1 from desensitization therefore requires a high concentration of intracellular ATP. In the case of full recovery, the recovered capsaicin responses exhibited rapid acute desensitization with characteristics similar to those of the initial responses. In some patches, the recovery was >100%, suggesting that either the number of functional channels increased on the plasma membrane or the channel activity was potentiated. The intracellular Mg^{2+} appeared to facilitate recovery of the channel (data not shown) but was not required. The same experiments with Na_2ATP produced recovery as strong as with MgATP. The cytosolic ATP is therefore the determinant to promote recovery of the channel from desensitization.

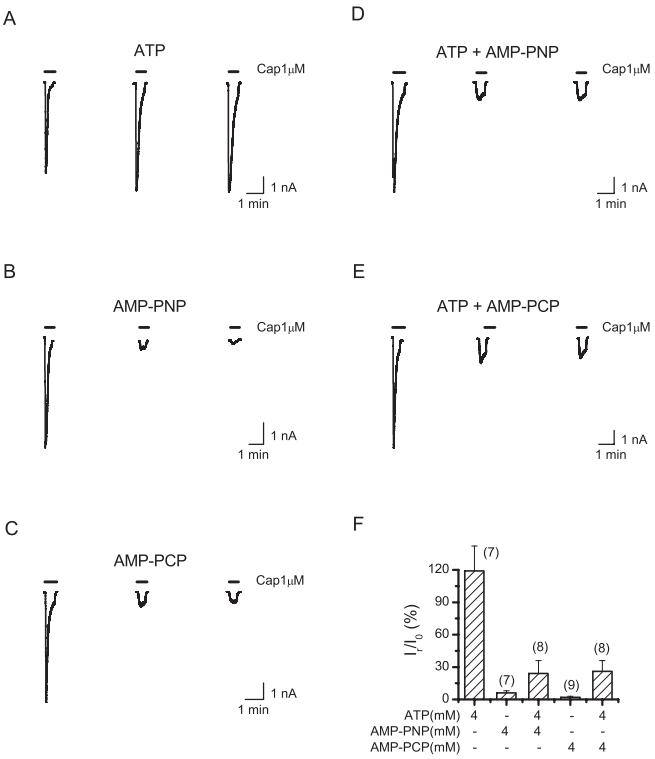

Figure 2. Nonhydrolyzable ATP analogs do not substitute for ATP in recovery. ***A–E***, Whole-cell recordings from HEK293 cells transiently expressing TRPV1, showing the recovery from desensitization obtained under different combinations of ATP and its nonhydrolyzable analogs AMP-PNP and AMP-PCP. Currents were elicited by 1 μM capsaicin in the presence of 1.8 mM Ca^{2+} at $V_h = -60$ mV. The pipette solution contained the following (in mM): 4 ATP (***A***), 4 AMP-PNP (***B***), 4 AMP-PCP (***C***), 4 ATP plus 4 AMP-PNP (***D***), and 4 ATP plus 4 AMP-PCP (***E***). ***F***, Summary of recovery under the various conditions in ***A–E***. The number of cells in each trial is indicated in parentheses above each bar. Cap, Capsaicin.

Nonhydrolyzable ATP analogs do not substitute for ATP

We studied whether hydrolysis of ATP is required for its effects on recovery of TRPV1 from desensitization. Replacement of ATP in the pipette solution with an equimolar amount of the nonhydrolyzable analog AMP-PNP resulted in a nearly complete ablation of recovery (Fig. 2, *B* vs *A*). There remained only small responses to the second and third applications of capsaicin, which had amplitudes similar to the residual currents at the end of the first stimulus, suggesting that they were produced by the remaining nondesensitized channels. Contrary to the pronounced effects on recovery, the nonhydrolyzable analog did not appear to alter desensitization of the channel.

One potential complication with AMP-PNP is that it exists as the lithium salt, and the Li^+ ions have been reported to affect multiple cellular processes in the millimolar range, including phosphoinositide turnover during stimulation of phospholipase C (PLC)-coupled receptors (Jenkinson et al., 1994; Suh and Hille, 2002). To ensure that Li^+ was not underlying the apparent effect of AMP-PNP, we tested another nonhydrolyzable analog, AMP-PCP, which is a sodium salt. As shown in Figure 2*C*, an equimolar substitution of ATP with AMP-PCP caused a similar failure of recovery. The nonhydrolyzablility of the analogs is therefore responsible for their inability to confer recovery.

Additional experiments were performed on the competitiveness of ATP and its nonhydrolyzable analogs. Whereas a full level of recovery occurred with 4 mM ATP (Fig. 2*A*), the addition of an equimolar amount of either AMP-PNP or AMP-PCP in the pipette solution reduced the recovery to only a fractional level (Fig. 2*D*,*E*) (AMP-PNP: 24 ± 12%, $n = 8$; AMP-PCP: 26 ± 10%, $n = 8$). The nonhydrolyzable analogs therefore counteract the stimulatory effects of ATP, which is consistent with their roles as competitive inhibitors on reactions requiring hydrolyzable ATP. Together, these experiments demonstrated that the hydrolysis of phosphate groups from ATP is essential to recovery of TRPV1 from desensitization.

Protein kinases are not required for recovery

The need of hydrolyzable ATP for recovery of TRPV1 indicates possible involvement of kinases. Both PKA and PKC have been implicated in desensitization of TRPV1 (Bhave et al., 2002; Mohapatra and Nau, 2003; Mandadi et al., 2004). We therefore explored whether they might mediate recovery of the channel from desensitization. Figure 3*A–D* summarizes the results on PKA involvement. Several reagents were tested, including two structurally dissimilar inhibitors, H89 and myristoylated PKI, and the adenylate cyclase activator forskolin. As evident from Figure 3, *A* and *B*, neither inhibitor abolished recovery of capsaicin responses in the presence of 4 mM ATP. Conversely, activation of PKA with 10 μM forskolin in the presence of 1 mM ATP did not stimulate recovery (Fig. 3*C*). This failure of recovery was unlikely attributable to an insufficient amount of ATP for PKA phosphorylation, because protein kinase reactions generally require <1 mM ATP (Hilgemann, 1997). In addition, forskolin itself appeared to have no effect on recovery. Increasing the intracellular ATP concentration to 4 mM, while in the presence of 10 μM forskolin, sufficed

to salvage full recovery as observed in control experiments (Fig. 3*D*).

Similar experiments were performed to evaluate the involvement of PKC (Fig. 3*E–H*). BIM, a potent inhibitor of PKC, did not prevent recovery of capsaicin responses from desensitization (Fig. 3*E*). In some cells, the recovery was less complete than in untreated cells, but overall, the recovery was strong and reached 91 ± 6% ($n = 9$) (Fig. 3*F*). In contrast, PMA, a strong activator of PKC, did not induce any significant recovery over a time course of 5–10 min (Fig. 3*G*,*H*). Therefore, either activation or inhibition of PKC produced little effects on recovery of the channel from desensitization. BIM at the micromolar concentration range also inhibits PKA (Bonnington and McNaughton, 2003). Its inability to block the recovery further excludes the involvement of PKA.

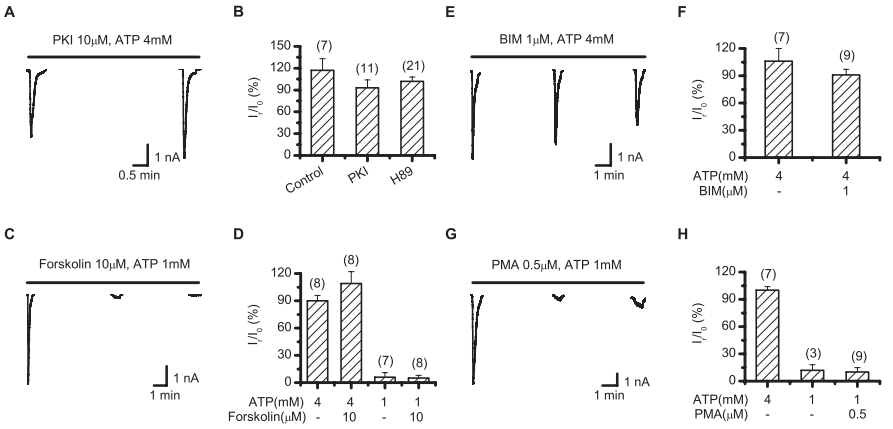

Figure 3. Protein kinases do not affect recovery. ***A***, Whole-cell currents in response to 1 μM capsaicin under inhibition of PKA by the myristoylated peptide inhibitor PKI (10 μM). ***B***, Averaged plot of percentage of recovery from desensitization under control conditions and after applying PKA inhibitors PKI (10 μM) and H89 (10 μM). ***C***, Similar whole-cell recordings on recovery of capsaicin responses, but with stimulation of PKA by the adenylate cyclase activator forskolin (10 μM). The pipette solution contained 1 mM ATP. ***D***, Comparison of recovery with and without stimulation of PKA. ***E–H***, Parallel experiments on PKC involvement. The kinase was activated by 0.5 μM PMA (***G***) and inhibited by 1 μM BIM (***E***). The averaged recovery in each case was compared with same-day controls (***F***, ***H***). In all experiments, the drugs were included in both perfusate and pipette. In a subset of experiments, cells were also exposed to the inhibitors for ~30 min before experimentation. All recordings were made in HEK293 cells transiently expressing TRPV1 held at −60 mV. The number of cells in each trial is indicated in parentheses above each bar.

We also corroborated our pharmacological results with mutagenesis experiments. TRPV1 contains a number of putative phosphorylation sites, including S6, S116, T144, and T370 on the N terminus, S502 between S2 and S3, and S774, S800, and S820 on the C terminus. *In vitro* phosphorylation has confirmed some of these residues capable of PKA and PKC phosphorylation (Bhave et al., 2002; Numazaki et al., 2002). For each putative residue, we constructed the alanine substitution mutant and repeated the corresponding experiments. Figure 4*A–C* shows the representative traces of recordings from sampled constructs. All mutants were functional and exhibited strong, rapid desensitization in response to prolonged applications of 1 μM capsaicin. Furthermore, none of the mutations abolished recovery from desensitization in the presence of 4 mM ATP (Fig. 4*D*). Although the exact level of recovery varied among mutants, the variations were secondary. In all cases, the recovery exceeded 80%, indicating that it was not grossly affected. The mutagenesis results therefore provide additional evidence to support that protein kinase-dependent mechanisms are not responsible for recovery of TRPV1 from desensitization.

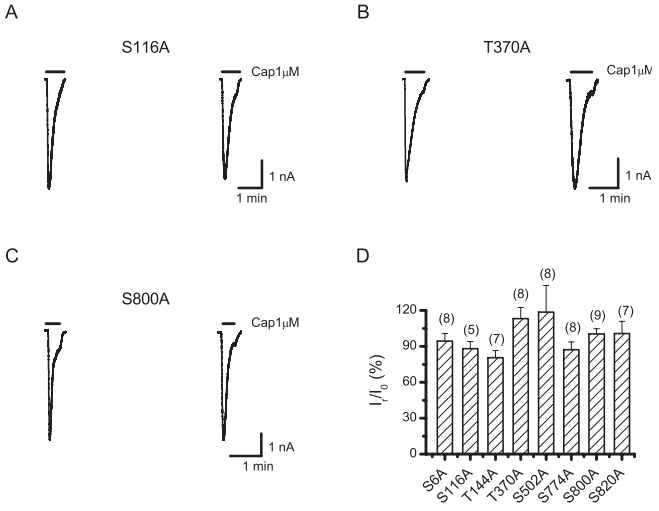

Figure 4. Disruption of putative phosphorylation sites in TRPV1 does not prevent recovery. ***A–C***, Sample recordings of whole-cell responses from HEK293 cells expressing the mutant channels containing alanine substitution at the PKA sites S116 and T370 and the PKC site S800. Currents were evoked by consecutive applications of 1 μM capsaicin separated with a 5–10 min interval of washes. The pipette solution contained 4 mM ATP. The holding potential was −60 mV. ***D***, Summary plot of recovery from desensitization of all mutant channels containing a single alanine substitution at each putative phosphorylation site. The number of cells in each trial is indicated in parentheses above each bar. Cap, Capsaicin.

Inhibition of PIP_2 synthesis prevents recovery

We next investigated whether lipid kinases are involved in recovery of TRPV1 from desensitization. Phosphatidylinositol-3-kinase (PI3K) and PI4K are responsible for production of phosphoinositides that are important in cell signaling. We examined whether inhibition of these kinases might affect recovery of the channel. PAO, a trivalent arsenical that reacts with vicinal thiols in proteins, inhibits all isoforms of PI4K and blocks production of PIP_2 from phosphatidylinositol (Wiedemann et al., 1996). Figure 5, *A* and *B*, shows that exposure of cells to 30 μM PAO profoundly suppressed recovery. The inhibition was relatively slow, taking >5 min to reach a maximal effect in some cells. The effect of PAO was mostly reversed by subsequent applications of the sulfhydryl reagent dithiothreitol (DTT), which reacts with PAO to form an inactive complex (Schaefer et al., 1994).

In addition to PI4K, PAO inhibits other enzymes. To assess whether its effect on recovery of TRPV1 results specifically from inhibition of PI4K, we tested another inhibitor, wortmannin. By irreversibly blocking the PI4K activity, wortmannin has been shown to inhibit replenishment of PIP_2 in the plasma membrane after receptor-mediated hydrolysis, and its prolonged application depletes PIP_2 in unstimulated cells (Varnai and Balla, 1998; Suh and Hille, 2002). Figure 5*C–F* summarizes the results of wortmannin on recovery of TRPV1 from desensitization. At concentrations above 1 μM, it exerted an effect similar to PAO, causing a nearly complete inhibition of recovery (Fig. 5*C*,*D*). In contrast, decreasing the concentration to 0.1 μM rendered it ineffective

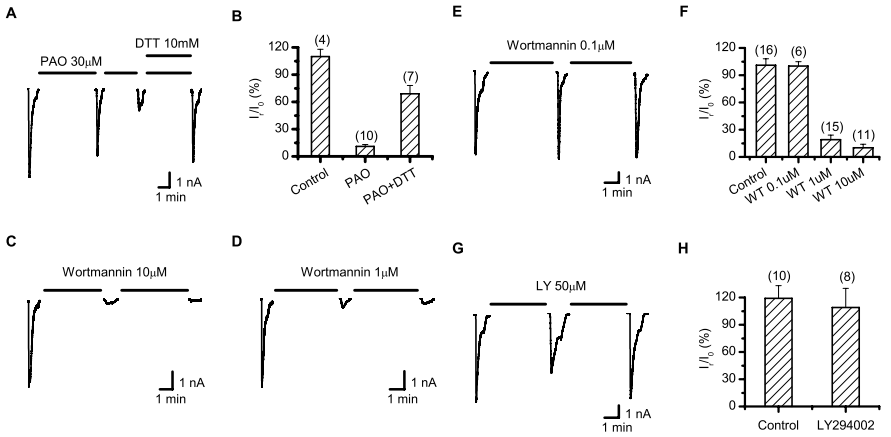

Figure 5. Inhibition of PIP_2 synthesis prevents recovery. ***A***, Representative recording of whole-cell currents in response to 1 μM capsaicin in the presence of PAO. Application of 30 μM PAO for 10 min suppressed recovery. The subsequent addition of 10 mM DTT reversed the effects of PAO. ***B***, Averaged plot of recovery under control conditions and applications of PAO in the absence and presence of DTT. ***C–E***, Whole-cell recordings from cells treated with 10, 1, and 0.1 μM wortmannin, respectively. The recovery of capsaicin responses was inhibited at 1 and 10 μM but not at 0.1 μM. ***F***, Summary of the effects of wortmannin (WT) on recovery of capsaicin responses compared with same-day control experiments. ***G***, Current trace obtained with inhibition of PI3K by LY294002 (LY; 50 μM). ***H***, Plot of percentage of recovery with and without application of the inhibitor LY294002. In all cases, the pipette solution contained 4 mM ATP. Data were recorded from HEK293 cells transiently expressing TRPV1 held at −60 mV. The number of cells in each trial is indicated in parentheses above each bar.

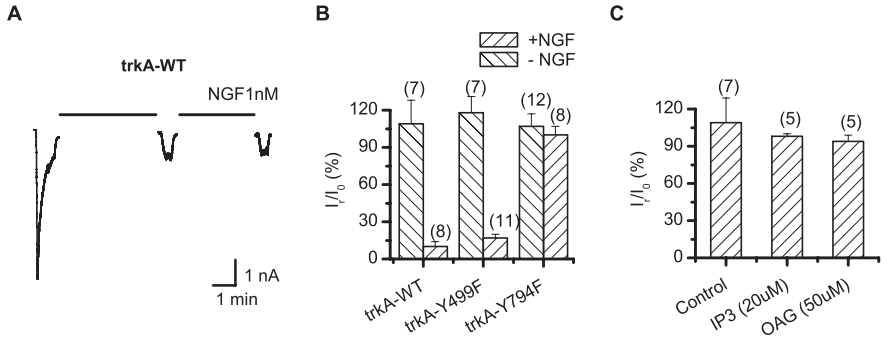

Figure 6. Receptor-mediated hydrolysis of PIP_2 inhibits recovery. ***A***, Representative recording of whole-cell currents from a HEK293 cell coexpressing TRPV1 and wild-type trkA/p75. NGF (1 nM) was applied between successive capsaicin applications. ***B***, Summary plot of percentage of recovery from desensitization for the experiments in ***A*** and those involving the mutant trkA receptors Y499F and Y794 F, with each compared with same-day controls in the absence of NGF. The Y499F and Y794F mutants uncouple the link of trkA to MAPK and PLC-γ, respectively. ***C***, Summary plot of percentage of recovery for the experiments on the signaling pathways downstream of PIP_2 hydrolysis. IP_3 (20 μM) was dialyzed into cells through the patch pipette, and OAG (50 μM), a membrane-permeable analog of DAG, was applied by extracellular perfusion. All data were recorded at −60 mV with 1 μM capsaicin in the presence of 1.8 mM Ca^{2+} as the stimulus. The number of cells in each trial is indicated in parentheses above each bar.WT, Wild type.

(Fig. 5*E*). This concentration dependence of the effect of wortmannin is consistent with the pharmacological profile of the compound as an inhibitor for PI4K. Wortmannin inhibits both PI3K and PI4K, but at different concentration ranges (PI3K, 10–100 nM; PI4K, 1–10 μM) (Davies et al., 2000). The requirement of a relatively high concentration of wortmannin for its effect therefore supports a role for PI4K in recovery of TRPV1 from desensitization.

The involvement of PI4K rather than PI3K was further differentiated with the specific PI3K inhibitor LY294002, which acts via a different mechanism than wortmannin (Vlahos et al., 1994). As seen in Figure 5*G*, applications of LY294002 at a supramaximal concentration (50 μM) did not inhibit recovery of capsaicin responses in the presence of 4 mM intracellular ATP. Approximately the same size of responses was obtained between the first and the subsequent applications of 1 μM capsaicin (Fig. 5*H*). The acute desensitization of the channel was not blocked either. Together, these results suggest that PI4K is specifically required for recovery of TRPV1 from desensitization.

Receptor-mediated hydrolysis of PIP_2 prevents recovery

The PIP_2 level in the plasma membrane of many cells can be manipulated through surface receptor-mediated hydrolysis. We asked whether depletion of PIP_2 by receptor-mediated hydrolysis might affect recovery of TRPV1. We coexpressed the channel in HEK293 cells with the NGF receptor trkA. Stimulation of trkA by NGF leads to activation of PLC-γ, which cleaves PIP_2 into two second messengers, membrane-bound DAG and soluble IP_3. Figure 6*A* shows a representative recording from a cell transiently coexpressing TRPV1 and trkA/p75. The cell was first exposed to 1 μM capsaicin to desensitize the channel, followed by 1 nM NGF for ~10 min to deplete PIP_2. As evident from the figure, application of NGF significantly suppressed the subsequent capsaicin responses. On average, only ~10% recovery occurred over a time course of 5–10 min, as opposed to a full recovery without application of NGF (Fig. 6*B*).

The trkA receptors are coupled to both mitogen-activated protein kinase (MAPK) and PLC-γ (Stephens et al., 1994). To delineate the two pathways, we examined two trkA mutants, Tyr499Phe and Tyr794Phe, which uncouple, respectively, the MAPK and PLC-γ pathways from the trkA receptor (Obermeier et al., 1993; Stephens et al., 1994; Prescott and Julius, 2003). As summarized in Figure 6*B*, activation of the Tyr499Phe mutant of trkA suppressed recovery of capsaicin responses (17 ± 3%; $n = 11$). In contrast, activation of the Tyr794Phe mutant produced little effects (100 ± 7%; $n = 8$) under a similar condition. These results suggest that the PLC-γ pathway, not the MAPK pathway, was responsible for the apparent effect of NGF.

To further pinpoint whether depletion of PIP_2 itself sufficed to confer inhibition, we tested the downstream products of PIP_2 hydrolysis for their possible effects on recovery. Figure 6*C* summarizes the percentage of recovery from desensitization of capsaicin responses in the presence of 20 μM IP_3 or 50 μM OAG. Neither dialysis of IP_3 nor extracellular perfusion of OAG, a membrane-permeable analog of DAG, altered recovery as application of NGF did. DAG is also known to activate PKC; however, our previous experiments involving inhibition of PKC already excluded a contribution from such a pathway. Together, these results indicate that the inhibitory effects of NGF on TRPV1 recovery result directly from breakdown of PIP_2 rather than downstream signaling molecules and pathways.

Dynamic resynthesis of PIP_2 is involved in recovery

The slow time course of recovery implies that PIP_2 is not only required for but dynamically involved in the process. We hypothesized that PIP_2 is depleted during desensitization, presumably because of the ionotropic Ca^{2+} influx, and that the recovery of the channel from desensitization subsequently requires resynthesis of PIP_2 by lipid kinases. To test this hypothesis, we exploited the inward rectifier potassium channel Kir2.1 (Kubo et al., 1993) as a biosensor to monitor the dynamics of PIP_2 in the plasma membrane. The Kir2.1 channel is exquisitely sensitive to PIP_2 in the membrane (Huang et al., 1998) and has been used successfully to detect PIP_2 changes in *Drosophila* photoreceptors (Hardie et al., 2001).

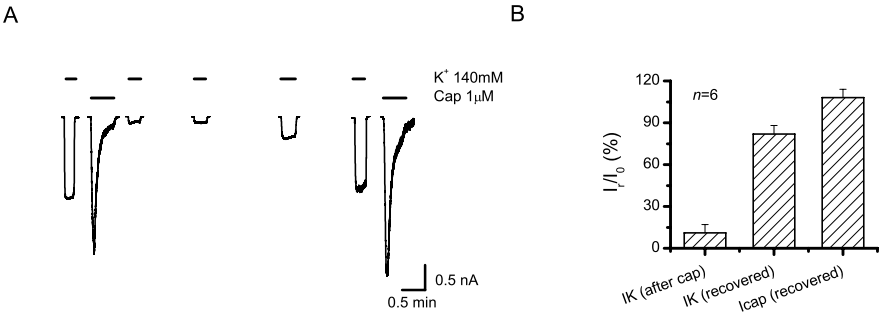

Figure 7. Temporal correlation of TRPV1 and Kir2.1 responses. ***A***, Representative whole-cell recording from a HEK293 cell coexpressing TRPV1 and Kir2.1 held at −60 mV. The Kir channel was activated by exposure to 140 mM K^+, which elicited a sustained inward current. The stimulus was first applied immediately before and after application of capsaicin (Cap) and then repeated intermittently throughout the recovery course of TRPV1. ***B***, Summary bar graph of Kir suppression caused by TRPV1 activation and subsequent recovery of the two channels, all expressed as percentages of their initial responses. IK, 140 mM K^+-induced current; Icap, 1 μM capsaicin-activated current.

We coexpressed Kir2.1 and TRPV1 in HEK293 cells and measured the Kir activity before and after desensitization of TRPV1. The Kir channel was activated by exposure to a high-K^+ solution at a holding potential of −60 mV. As seen in Figure 7*A*, large currents were evoked initially by either high-K^+ or capsaicin (1 μM). The current profiles of the responses were reminiscent of those of Kir2.1 and TRPV1 when they were expressed alone, suggesting that coexpression of the two channels did not interfere with the function of the other. After desensitization of TRPV1, however, the Kir response was dramatically diminished. Application of capsaicin therefore caused a concomitant suppression of Kir activity, presumably indicating a loss of PIP_2 binding. The same stimulus for Kir was then repeated intermittently throughout the course of recovery of TRPV1. The Kir currents gradually recovered and eventually reached a size comparable with that of the initial response. Immediately after recovery of Kir2.1, application of capsaicin also elicited a large response, indicating that TRPV1 recovered concurrently. Figure 7*B* summarizes the suppression of Kir activity after TRPV1 desensitization and its subsequent recovery compared with that of TRPV1. It was evident that the recovery of the two channels was highly correlated and had a similar time course that presumably reflects the replenishment of PIP_2 in the plasma membrane.

PIP_2 binding in the distal C terminus does not mediate recovery

PIP_2 has been identified to interact with a distal C-terminal region of TRPV1, where the binding causes tonic inhibition of channel activity (Prescott and Julius, 2003). We investigated whether the same structural basis underlies the effect of PIP_2 on recovery of the channel from desensitization. We constructed two truncation mutants, namely TRPV1-Δ50 and TRPV1-Δ62, which lacked the last 50 (788–838) and 62 (776–838) residues, respectively. These truncations either partially remove or completely eliminate the PIP_2-binding domain on the distal C terminus, as well as other functional sites in the region such as the CaM-binding domain (767–801) and the phosphorylation sites Ser800 and Ser820 (Bhave et al., 2002; Numazaki et al., 2003; Mandadi et al., 2004). Figure 8*A* shows representative recordings from cells expressing the truncation mutants. Both constructs were functional and retained pronounced acute desensitization in response to prolonged applications of capsaicin in the presence of 1.8 mM Ca^{2+}. More importantly, their recovery from desensitization remained strong and had a time course similar to that of wild type. Figure 8*B* shows the averaged recovery of the mutant channels versus wild type. There appeared to be a decreasing trend on the level of recovery with the extent of truncation. But the differences were small, and in all cases, the recovery was robust (TRPV1-Δ62: 94 ± 8%, $n = 7$; TRPV1-Δ50: 104 ± 11%, $n = 5$; wild type, 111 ± 22%, $n = 6$). These data indicate that the PIP_2-binding region on the distal C terminus of TRPV1 is not important for functional recovery of the channel; PIP_2 either binds to a different site on the channel or interacts with it indirectly.

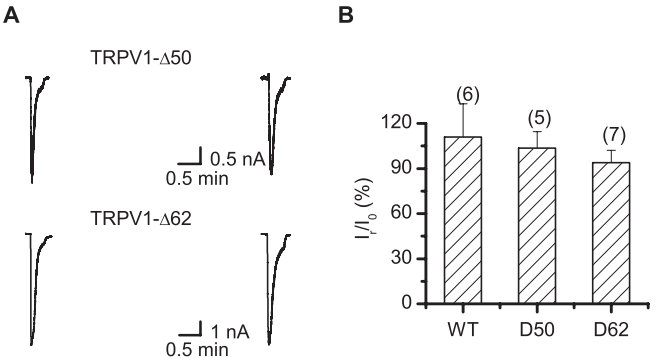

Figure 8. PIP_2 binding in the distal C terminus is not important for recovery. ***A***, Representative recordings of whole-cell responses to 1 μM capsaicin from HEK293 cells expressing the truncation mutants lacking the last 50 (TRPV1-Δ50) and 62 (TRPV1-Δ62) residues, respectively. The holding potential was −60 mV. ***B***, Percentage of recovery of the two truncation mutants versus wild type. The number of cells in each trial is indicated in parentheses above each bar. WT, Wild type.

Discussion

Desensitization to prolonged stimuli after activation is a common phenomenon for surface receptors including ion channels. Not the least, the desensitization protects cells from potential excitotoxicity that arises from excessive activation of a receptor. This is particularly the case for Ca^{2+}-permeable ion channels such as TRPV1. The desensitization of TRPV1, however, is unique on several aspects: (1) it depends on extracellular Ca^{2+}; (2) it requires a whole-cell, although not necessarily neuronal, context; and (3) the channel does not immediately recover from desensitization to the resting states after removal of the stimulus.

Here, we have addressed the problem of recovery of capsaicin responses after desensitization. We showed that the recovery of the channel is conferred by a high concentration of intracellular ATP and is prevented by depletion of PIP_2 through either inhibition of PIP_2 synthesis kinases or receptor-mediated hydrolysis. We also demonstrated that stimulation of TRPV1 causes concomitant suppression of Kir activity in cells coexpressing the two channels and that the recovery of Kir is concurrent with that of TRPV1. From these results, we conclude that the recovery of TRPV1 from desensitization requires resynthesis of PIP_2.

The requirement of PIP_2 synthesis for recovery of TRPV1 is consistent with the dependence of the process on a high concentration of intracellular ATP. Whereas protein kinases have a half-maximal ATP concentration in the range of hundreds of micromolars (Hilgemann, 1997), lipid kinases generally require a significantly higher concentration for maximal activity (~1 mM) (Balla, 1998). The production of phospholipids is also slow and generally takes several minutes, involving a series of steps including phosphorylating phosphatidylinositol lipid on multiple positions (Varnai and Balla, 1998). Both the concentration profile of ATP and the time course of recovery of TRPV1 are in good agreement with those of other reported cellular processes involving resynthesis of PIP_2. The recovery of the KCNQ2/KCNQ3 current from muscarinic suppression, for example, requires 3 mM ATP over a time course of many minutes for a maximal level of recovery (Suh and Hille, 2002).

Our study here differs from some existing ones in the use of stimulus protocol. Previous studies on desensitization of TRPV1 apply capsaicin only for a brief time, typically shorter than the half-decay time of desensitization. The same stimulus is repeated successively, and the desensitization is studied in the context of tachyphylaxis of capsaicin responsiveness. With such a protocol, the desensitization of TRPV1 has been found to depend on a number of intracellular components, including ATP (Koplas et al., 1997), calcineurin (Docherty et al., 1996), PKA (Bhave et al., 2002; Mohapatra and Nau, 2003), PKC (Mandadi et al., 2004), and CaM (Numazaki et al., 2003). However, the brief stimuli have limitations on the mechanistic interpretation of data, because tachyphylaxis is a phenomenological process convolving multiple factors including channel activation, acute desensitization, slow desensitization (if any), and recovery. Any change of a single component in the complex process could alter the apparent rate of tachyphylaxis. To alleviate such complications, we used prolonged applications of capsaicin at a relatively high concentration to ensure full desensitization of the channel. With this protocol, we found that activation of PKC did not confer recovery of TRPV1 from desensitization. This observation appears to be inconsistent with a previous report that activation of PKC reverses desensitization of Ca^{2+} responses in a Ca^{2+}-imaging assay (Mandadi et al., 2004). One possible explanation is that the brief applications of capsaicin in the previous study may have not caused all channels to be desensitized, so that the rise of Ca^{2+} levels after PKC stimulation could result from the potentiated responses of nondesensitized channels. The differences in the types of experiments may have also contributed to the discrepancy.

PIP_2 has been implicated in a variety of cellular processes and regulations of ion channels and membrane proteins (Hilgemann et al., 2001). Ion channels of the TRP superfamily appear to be particularly amenable to modulation by PIP_2. The mechanisms whereby PIP_2 modulates these channels, however, seem to be disparate. In the case of TRPV1, PIP_2 has been found to exert constitutive inhibition by binding to the distal C terminus of the channel (Prescott and Julius, 2003). Other TRP channels in the V or M subfamily, in contrast, appear to require the presence of PIP_2 to support their functions. Depletion of PIP_2 causes rundown of the spontaneous activity of TRPM7 (Runnels et al., 2002), inhibits activation of TRPM8 (Liu and Qin, 2005), and may contribute to desensitization of TRPM5 (Liu and Liman, 2003). In the TRPC subfamily, the hydrolysis of PIP_2 is a major event leading to TRP-TRPL activation for phototransduction in *Drosophila melanogaster* (Montell, 1999). Regulation of recovery from desensitization as found in this study represents yet another mechanism of PIP_2 on modulation of TRP ion channels.

Our results reinforce an emerging paradigm for PIP_2 regulation of ion channels in which the PIP_2 level in the membrane may be altered by the ionotropic Ca^{2+} influx through the channels. Ion channels are often regulated by PIP_2 through surface receptors. The effect of PIP_2 on recovery of TRPV1, however, requires no surface receptor. Instead, activation of TRPV1 itself suffices to deplete PIP_2, as manifested in the dynamic changes of the Kir activity after TRPV1 stimulation. The channel therefore acts as both a surrogate surface receptor and a downstream PIP_2 target, giving rise to a bidirectional interaction between PIP_2 and the regulation of the channel. The Ca^{2+} entry through the channel is presumably the trigger that initiates the cascade. Ionotropic Ca^{2+} influx has also been reported to regulate PIP_2 levels in *Drosophila* photoreceptors (Hardie et al., 2001), but with an opposite effect as on TRPV1. In *Drosophila* photoreceptors, the Ca^{2+} entry from TRP channels inhibits long-term response inactivation and, as a result, has been suggested to maintain PIP_2 levels by inhibiting PLC activity and facilitating PIP_2 recycling. In the case of TRPV1, the mechanisms that couple Ca^{2+} entry to PIP_2 depletion remains to be elucidated. To this end, it is noteworthy that both PLC and PI3K have been suggested to colocalize with TRPV1 (Chuang et al., 2001; Stein and Gordon, 2004; Zhu and Oxford, 2004). Their close proximity to the channel would make them ideal candidates in sensing local Ca^{2+} signals and mediating depletion of PIP_2.

The slow, PIP_2-dependent recovery of TRPV1 from desensitization provides a mechanistic explanation of tachyphylaxis of capsaicin responses. Previous investigations of sensory neurons suggest that tachyphylaxis stems from a failure of the channel to recover from a desensitization state (Liu and Simon, 1996). Our data provide additional support for this proposition. First, full recovery of capsaicin response is possible under the condition of a high concentration of intracellular ATP. This excludes the existence of a slowly occurring, irreversible desensitization state. Second, prolonged stimulation of TRPV1 causes acute desensitization that is nearly complete and renders the channel irresponsive to subsequent stimuli in the absence of ATP. The acute desensitization itself is therefore irreversible under this condition and may manifest in the form of tachyphylaxis if each application of capsaicin is kept short to give rise to only a partial level of desensitization. Third, recordings in neurons indicate that tachyphylaxis of capsaicin responses can be reduced or prevented by the addition of intracellular nucleotides, suggesting that the two processes of recovery from desensitization and tachyphylaxis share a similar pharmacological profile. In summary, many aspects of tachyphylaxis of capsaicin responses can be explained as a failure of recovery from acute desensitization without invoking an additional, slowly desensitizing state.

The present study also provides new perspectives to understand the physiological modulations of the TRPV1 channel and the roles of phosphoinositides in the pain and inflammation pathways. For example, TRPV1 is essential to inflammatory heat

hyperalgesia induced by proinflammatory substances such as NGF (Shu and Mendell, 1999). Several mechanisms have been suggested, one involving PLC-γ (Chuang et al., 2001) and others implicating PI3K (Bonnington and McNaughton, 2003; Zhuang et al., 2004). Common to these mechanisms is the depletion of PIP_2 by either conversion to other phospholipids or hydrolysis into secondary messengers. It is currently understood that activation of these mechanisms potentiates TRPV1 activity and thereby mediates the proinflammatory effect of NGF. The results presented here, however, add another layer of complexity to the actions of NGF and imply that its stimulatory effect may be accompanied with an inhibitory one on the recovery of desensitized channels. NGF is therefore expected to have distinct, opposing effects on the function of TRPV1. To this end, it is interesting to note that a biphasic effect of NGF on capsaicin responses in native neurons has been reported in which stimulation of cells with NGF causes an initial elevation of the current, followed by a progressive decay (Shu and Mendell, 2001). The decrease of the current after prolonged NGF application may have reflected a failure of recovery of the channel from desensitization. Conceivably, such a bimodal role of NGF may provide a dynamic solution to tune its optimal effects without causing overexcitability of cells. Nevertheless, an unequivocal assessment of the effects of PIP_2 depletion on recovery of the channel from desensitization and their physiological significance awaits additional confirmation in a neuronal context.

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