Regulation of Ca\(^{2+}\)-dependent Desensitization in the Vanilloid Receptor TRPV1 by Calcineurin and cAMP-dependent Protein Kinase*

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The vanilloid receptor TRPV1 is a polymodal nonselective cation channel of nociceptive sensory neurons involved in the perception of inflammatory pain. TRPV1 exhibits desensitization in a Ca\(^{2+}\)-dependent manner upon repeated activation by capsaicin or protons. The cAMP-dependent protein kinase (PKA) decreases desensitization of TRPV1 by directly phosphorylating the channel presumably at sites Ser\(^{116}\) and Thr\(^{370}\). In the present study we investigated the influence of protein phosphatase 2B (calcineurin) on Ca\(^{2+}\)-dependent desensitization of capsaicin- and proton-activated currents. By using site-directed mutagenesis, we generated point mutations at PKA and protein kinase C consensus sites and studied wild type (WT) and mutant channels transiently expressed in HEK293t or HeLa cells under whole cell voltage clamp. We found that intracellular application of the cyclosporin A cyclophilin A complex (CsA-CyP), a specific inhibitor of calcineurin, significantly decreased desensitization of capsaicin- or proton-activated TRPV1-WT currents. This effect was similar to that obtained by extracellular application of forskolin (FSK), an indirect activator of PKA. Simultaneous applications of CsA-CyP and FSK in varying concentrations suggested that these substances acted independently from each other. In mutation T370A, application of CsA-CyP did not reduce desensitization of capsaicin-activated currents as compared with WT and to mutant channels S116A and T144A. In a double mutation at candidate protein kinase C phosphorylation sites, application of CsA-CyP or FSK decreased desensitization of capsaicin-activated currents similar to WT channels. We conclude that Ca\(^{2+}\)-dependent desensitization of TRPV1 might be in part regulated through channel dephosphorylation by calcineurin and channel phosphorylation by PKA possibly involving Thr\(^{370}\) as a key amino acid residue.

The capsaicin receptor TRPV1, a nonselective cation channel expressed predominantly in nociceptive sensory neurons, transduces and integrates various stimuli such as noxious heat (>42 °C), capsaicin, protons, (1, 2), the endogenous cannabinoid anandamide (3), lipoxigenase products, and other lipids related to arachidonic acid (4) and ethanol (5). Studies on TRPV1 gene knock-out mice suggest that TRPV1 is essential for the development of thermal hyperalgesia following inflammation or local injection of bradykinin and nerve growth factor (6–8).

Activation of TRPV1 leads to Ca\(^{2+}\) influx into nociceptive sensory neurons, resulting in membrane depolarization and release of proinflammatory neuropeptides from primary afferent nerve terminals (9). Prolonged or repeated activation of TRPV1 results in desensitization and insensitivity of the receptor to subsequent stimuli (10, 11). The physiological role and importance of TRPV1 desensitization is unknown but speculated to be a process of adaptation and regulation of the peripheral nervous system for the perception of pain. Comparable with other ion channels, desensitization of TRPV1 is at least in part a Ca\(^{2+}\)-dependent process (10, 11). There is growing evidence for the involvement of Ca\(^{2+}\)-dependent phosphorylation and dephosphorylation processes to regulate desensitization and excitability of TRPV1. Previous studies in rat dorsal root ganglion neurons have demonstrated that desensitization is reduced in the presence of inhibitors of the Ca\(^{2+}\)- and calmodulin-dependent protein phosphatase 2B (calcineurin) (12). Conversely, phosphorylation of TRPV1 by Ca\(^{2+}\)-calmodulin-dependent kinase II (CaMKII) seems to be a prerequisite for activation of TRPV1 by capsaicin (13).

Another candidate involved in the mechanisms of Ca\(^{2+}\) negative feedback and Ca\(^{2+}\)-dependent inactivation in many ion channels is the Ca\(^{2+}\) sensor calmodulin (CaM) itself. There is growing evidence that multiple regions of TRPV1 indeed may bind CaM (14, 15).

TRPV1 is also a target for cAMP-dependent protein kinase (PKA)- and protein kinase C (PKC)-dependent phosphorylation. Phosphorylation by PKA sensitizes the channel to heat (16) and capacain (17) and reduces Ca\(^{2+}\)-dependent desensitization of capsaicin- and proton-activated currents (18, 19). Amino acids residues Ser\(^{116}\) and Thr\(^{370}\) are the major substrates for PKA-dependent phosphorylation, although other putative PKA phosphorylation sites might be involved as well. Phosphorylation by PKC sensitizes the channel to capsaicin, protons, and heat (20–22). Here, residues Ser\(^{102}\) and Ser\(^{800}\) are the major substrates for PKC-dependent phosphorylation.

In the present study we investigated the influence of cal-

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The abbreviations used are: CaMKII, Ca\(^{2+}\)-calmodulin-dependent kinase II; CaM, calmodulin; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; WT, wild type; CaA, cyclosporin A; CyP, cyclophilin A; CaACyP, cyclosporin A cyclophilin A complex; FSK, forskolin; HEK, human embryonic kidney; PMA, phorbol 12-myristate 13-acetate; OA, okadaic acid; W-7, N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride.
cineurin on Ca\(^{2+}\)-dependent desensitization of capsaicin- and proton-activated currents and examined the interactions of calcineurin and PKA and PKC phosphorylation pathways. We found that Ca\(^{2+}\)-dependent desensitization of TRPV1 might be in part regulated through channel dephosphorylation by calcineurin and channel phosphorylation by PKA possibly involving Thr\(^{370}\) as a key amino acid residue.

**EXPERIMENTAL PROCEDURES**

**Site-directed Mutagenesis and Transient Transfection—**Mutagenesis of rat TRPV1 cDNA was performed with \(5\)'TRPV1-pcDNA3 by means of the transformer site-directed mutagenesis kit (BD Biosciences Clontech, Palo Alto, CA) as described previously (19). Human embryonic kidney (HEK) 293T cells or cells of a human adenocarcinoma-derived cell line (HeLa cells) were transfected with wild type or mutant plasmid (0.75 or 10 \(\mu\)g, respectively) along with reporter plasmid (CD8-pH3m, 1 \(\mu\)g) by the calcium phosphate precipitation method. After incubation for 12–15 h, the cells were replated in 35-mm culture dishes. Transfected cells were used for experiments within 2–3 days. Transfection-positive cells were identified by immunobeads (anti-CD-8 Dynabeads; Dynal Biotech, Oslo, Norway). Transfection efficiency was ~50–70% on average for TRPV1-WT and mutant channels.

**Chemicals and Solutions—**Capsaicin (8-methyl-N-vanillyl-6-nonenamide) and cyclosporin A (CsA; both Sigma-Aldrich) were dissolved in absolute ethanol to give stock solutions of 10 mm. Forskolin (FSK; Calbiochem-Novobiochem GmbH, Bad Soden, Germany), phorbol 12-myristate 13-acetate (PMA; Calbiochem-Novobiochem GmbH), and okadaic acid (OA; Alomone Labs, Ltd, Jerusalem, Israel) were dissolved in dimethyl sulfoxide to give stock solutions of 10, 1, and 1 \(\mu\)m, respectively. Human brain CaM and N-(6-aminoethyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W-7; both Calbiochem-Novobiochem GmbH) were dissolved in double distilled water to give stock solutions of 100 mm. Cyclophilin A (CyP; Sigma-Aldrich) was dissolved in Tris-Cl, pH 7.4, containing HEPES, 1,4-dithio-DL-threitol, phenylmethylsulfonyl fluoride, and sodium azide to give a stock solution of 20 \(\mu\)m. All of the stock solutions were stored at ~20 \(^\circ\)C. Electrodes were pulled from borosilicate glass tubes (TW150F-3; World Precision Instruments, Sarasota, FL) and heat-polished at the tip to give a resistence for experiments as described for A–E. The means were normalized to the current amplitude obtained with first capsaicin application. * indicates a statistically significant difference in the mean amplitude compared with that obtained under control conditions.

**RESULTS**

**Inhibition of Calcineurin Decreases Ca\(^{2+}\)-dependent Desensitization of Capsaicin-activated TRPV1 Currents—**TRPV1 channel exhibits desensitization in a Ca\(^{2+}\)-dependent manner (10, 11). It has been suggested that a rise in cytosolic Ca\(^{2+}\) level caused by TRPV1 activation results in the activation of Ca\(^{2+}\)/calmodulin-dependent protein phosphatases that mediate channel desensitization (12). To test this hypothesis for TRPV1-WT transiently expressed in HEK293T cells, we first studied the effect of various protein phosphatase inhibitors on Ca\(^{2+}\)-dependent desensitization of capsaicin-activated TRPV1-WT currents, specifically on the decreasing current response to successive stimulation (tachyphylaxis). We applied a series of brief (~5 s long) pulses of 1 \(\mu\)m capsaicin at 2-min intervals in Ca\(^{2+}\)-containing solution (2 mm) bath solution without (A) or with 1 \(\mu\)m OA (B), 100 \(\mu\)m CsA (C), or 14 nm CsA + 17 nm CyP in the pipette solution (D), or 14 nm CsA + 17 nm CyP in the pipette solution along with 1 \(\mu\)m OA applied extracellularly (E). After a whole cell voltage clamp was established, the cells were dialyzed for 10 min before the first capsaicin application. The intervals between capsaicin applications were 2 min. All of the data were normalized to the current amplitude obtained with first capsaicin application. * indicates a statistically significant difference in the mean amplitude compared with that obtained under control conditions.

**Fig. 1. Effect of OA, a specific inhibitor of protein phosphatase 1B (calcineurin), on Ca\(^{2+}\)-dependent desensitization of capsaicin-activated TRPV1 currents.** Shown are whole cell current responses of TRPV1 channels to repeated brief (~5 s long) applications of 1 \(\mu\)m capsaicin in Ca\(^{2+}\)-containing (2 mm) bath solution without (A) or with 1 \(\mu\)m OA (B), 100 \(\mu\)m CsA (C), or 14 nm CsA + 17 nm CyP in the pipette solution (D), or 14 nm CsA + 17 nm CyP in the pipette solution along with 1 \(\mu\)m OA applied extracellularly (E). After a whole cell voltage clamp was established, the cells were dialyzed for 10 min before the first capsaicin application. The intervals between capsaicin applications were 2 min. F, mean amplitudes of currents ± S.E. measured in experiments as described for A–E. The means were normalized to the current amplitude obtained with first capsaicin application. * indicates a statistically significant difference in the mean amplitude compared with that obtained under control conditions.

**Fig. 1.**
with the immunosuppressive drug cyclosporin A (CsA; 14 nM) together with the “immunophilin” cyclophilin A (CyP; 17 nM) in the pipette solution (Fig. 1D). CsA and CyP are known to form a drug/immunophilin complex, which associates with and thus inhibits protein phosphatase 2B (calcineurin) (23). In the presence of CsA/CyP, current amplitudes at the second and fourth capsaicin application were 58.4 ± 6.9 and 37.2 ± 6.2% of that at the first application, respectively (Fig. 1F). This is in good agreement with previously reported results obtained in rat dorsal root ganglion neurons using similar concentration of CsA and CyP in the pipette solution (12). Higher concentrations of CsA (up to 1 μM) along with CyP (up to 1 μM) did not lead to any further decrease in tachyphylaxis compared with that observed with 14 nM CsA plus 17 nM CyP (data not shown).

Pretreatment of cells with CsA alone up to concentrations of 100 μM in the pipette solution did not lead to any change in acute desensitization of capsaicin-activated TRPV1-WT currents. The currents had peak amplitudes in the range of 2.4–8.9 nA with a mean of 5.0 ± 1.1 nA, which was not significantly different from control conditions (Table I). The currents reached their peak at 1.9 ± 0.7 s after beginning of activation. Then the currents began to decrease during continuous capsaicin application and reached values of 2.18 ± 0.62, 0.64 ± 0.08, and 0.59 ± 0.05 nA after 10, 20, and 30 s, respectively (Fig. 2B). To describe and compare acute desensitization quantitatively for control conditions and in the presence of CsA/CyP, we measured the areas under the current curves over a time of 30 s and normalized them to an idealized, nondesensitizing current of respective size. The bars represent mean values ± S.E. * indicates a statistically significant difference compared with control.

**Table I**

| Channel       | Activator | Experimental condition                        | Response | n  | p   |
|---------------|-----------|----------------------------------------------|----------|----|-----|
| TRPV1-WT      | Capsaicin | 100 μM OA in pipette                         | 6.5 ± 1.6| 6  |     |
| TRPV1-WT      | Capsaicin | 100 μM CsA in pipette                        | 9.3 ± 2.6| 6  |     |
| TRPV1-WT      | Capsaicin | 14 nM CsA, 17 nM CyP in pipette              | 6.8 ± 1.2| 6  |     |
| TRPV1-WT      | Capsaicin | 14 nM OA plus 14 nM CsA, 17 nM CyP in pipette| 4.8 ± 1.2| 7  |     |
| TRPV1-WT      | Capsaicin | 10 μM OA in extracellular buffer              | 4.3 ± 1.2| 6  |     |
| TRPV1-WT      | Capsaicin | 14 nM OA in pipette                         | 7.8 ± 1.8| 6  |     |
| TRPV1-WT      | Capsaicin | 14 nM CsA, 17 nM CyP in pipette              | 5.0 ± 1.1| 7  |     |
| TRPV1-WT      | Capsaicin | 100 μM W-7 in pipette                        | 7.8 ± 1.7| 8  |     |
| TRPV1-WT      | Capsaicin | 100 μM CaM in pipette                        | 8.6 ± 2.0| 6  |     |
| TRPV1-WT      | Capsaicin | 10 μM FSK in extracellular buffer            | 5.2 ± 0.9| 9  |     |
| TRPV1-S116A   | Capsaicin | 14 nM CsA, 17 nM CyP in pipette              | 8.9 ± 2.3| 7  |     |
| TRPV1-S116A   | Capsaicin | 14 nM CsA, 17 nM CyP in pipette              | 6.1 ± 1.2| 7  |     |
| TRPV1-T370A   | Capsaicin | 100 μM OA in pipette                         | 2.3 ± 0.6| 6  |     |
| TRPV1-T370A   | Capsaicin | 10 μM OA in extracellular buffer             | 3.2 ± 0.8| 6  |     |
| TRPV1-T144A   | Capsaicin | 14 nM CsA, 17 nM CyP in pipette              | 2.8 ± 0.6| 6  |     |
| TRPV1-T144A   | Capsaicin | 0.1 μM PMA in extracellular buffer           | 5.3 ± 2.3| 6  |     |
| TRPV1-S502A/S800A | Capsaicin | 14 nM CsA, 17 nM CyP in pipette              | 6.2 ± 2.3| 6  |     |
| TRPV1-S502A/S800A | Capsaicin | 14 nM CsA, 17 nM CyP in pipette              | 4.6 ± 1.2| 8  |     |
| TRPV1-WT      | Proton    | 14 nM CsA, 17 nM CyP in pipette              | 3.1 ± 0.6| 6  |     |
| TRPV1-WT      | Proton    | 14 nM OA in extracellular buffer             | 4.2 ± 0.7| 6  |     |
| TRPV1-WT      | Proton    | 14 nM CsA, 17 nM CyP in pipette              | 6.4 ± 1.5| 6  |     |
| TRPV1-WT      | Proton    | 14 nM OA, 17 nM CyP in pipette               | 4.0 ± 0.8| 6  |     |

**Calmodulin Is Not Required for the Decrease in Ca²⁺-Dependent Desensitization by Inhibition of Calcineurin—Calcineurin exerts its phosphatase activity in a Ca²⁺- and calmodulin-dependent manner. CaM itself is a dominant Ca²⁺ sensor for Ca²⁺-dependent inactivation in many ion channels (24–26). There is accumulating evidence that multiple regions of TRPV1 may bind CaM. One putative region was identified in the C-terminal (14), and another was identified in the N-terminal segment (15). We investigated the functional role of CaM in Ca²⁺-dependent desensitization of capsaicin-activated
TRPV1-WT channels. The experiments were performed as described for those shown in Fig. 1. Pretreatment of cells with 100 μM W-7 in the pipette solution, which is a potent noncompetitive antagonist of calmodulin, did not lead to any change in Ca²⁺-dependent tachyphylaxis of capsaicin-activated TRPV1-WT currents (Figs. 3A and C). W-7 also did not have any significant effect on capsaicin-activated peak currents of TRPV1-WT (Table I). This is in good agreement with an earlier report about the ineffectiveness of W-7 on TRPV1 channel desensitization (14). Tachyphylaxis was comparable with that observed in the resting state, TRPV1 is highly phosphorylated, at least when heterologously expressed in CHO-K1 cells (18). PKA is able to phosphorylate TRPV1. However, PKA phosphorylation only becomes obvious in the desensitized state (18). Phosphorylation by PKA partly rescues TRPV1 from desensitization (18). In the resting state, TRPV1 is highly phosphorylated, at least when heterologously expressed in CHO-K1 cells (18).

Phosphorylation by PKA and Dephosphorylation by Calcineurin Regulates Ca²⁺-Dependent Desensitization of TRPV1.—In the resting state, TRPV1 is highly phosphorylated, at least when heterologously expressed in CHO-K1 cells (18). PKA is able to phosphorylate TRPV1. However, PKA phosphorylation only becomes obvious in the desensitized state (18). Phosphorylation by PKA partly rescues TRPV1 from desensitization (18, 19). Because inhibition of calcineurin decreases desensitization of capsaicin-activated TRPV1 currents to a similar extent like activation of PKA, we investigated the interplay between PKA activation and calcineurin inhibition and their effect on Ca²⁺-dependent desensitization of capsaicin-activated TRPV1 currents. As demonstrated previously, pretreatment of cells for 10 min with 10 μM FSK, an activator of adenylate cyclase and thus an indirect PKA activator, led to a significant decrease in tachyphylaxis of capsaicin-activated TRPV1-WT currents (Fig. 3E). Here, the results were quantitatively similar to those obtained with CsA-CyP alone or CsA-CyP along with W-7 in the pipette solution (Figs. 1F and 3C and F).

Phosphorylation by PKA and Dephosphorylation by Calcineurin Regulates Ca²⁺-Dependent Desensitization of TRPV1. Here, the results were quantitatively similar to those obtained with CsA-CyP alone or CsA-CyP along with W-7 in the pipette solution (Figs. 1F and 3C and F).
significant effect on capsaicin-activated peak currents in TRPV1-WT (Table I). Pretreatment of cells for 10 min with 14 nM CsA plus 17 nM CyP in the pipette solution did not lead to any significant decrease in tachyphylaxis compared with that in the presence of 10 μM FSK alone (Fig. 4, B and C). To exclude the possibility that the decrease in desensitization in the presence of CsA-CyP is caused by PKA phosphorylation caused by disinhibition of PKA by CsA-CyP, we investigated the effect of CsA-CyP on channel tachyphylaxis in the presence of the PKA inhibitor KT5720. In these experiments the decrease in desensitization was similar to that obtained with CsA-CyP alone (data not shown).

To address the question of whether the regulation of desensitization by FSK and CsA-CyP are of an additive or synergistic nature, we also measured the effect of submaximal concentrations of FSK and CsA-CyP on channel tachyphylaxis (Table II). Pretreatment of cells for 10 min with 1.4 nM CsA plus 1.7 nM CyP in the pipette solution, current amplitudes at the second and fourth capsaicin application were 21.9 ± 2.6 and 17.5 ± 2.4% of that at the first application, respectively (Fig. 4E). Comparable effects were obtained when cells were pretreated with 0.1 μM FSK alone (21.5 ± 1.6 and 16.2 ± 2.1%; Fig. 4D) or 0.1 μM FSK along with 1.7 nM CsA plus 1.7 nM CyP in the pipette solution (18.3 ± 1.7 and 15.7 ± 2.2%). Pretreatment of cells with 0.1 μM FSK alone with 7 nM CsA plus 8.5 nM CyP in the pipette solution, however, led to an effect similar to that obtained with 10 μM FSK along with 14 nM CsA plus 17 nM CyP in the pipette solution (Fig. 4F).

These results demonstrate that the concentration dependence for the CsA-CyP-mediated decrease in desensitization is rather steep, as expected for effects that require a cascade of reactions rather than simple one-to-one reactions. Thus, we refrained from performing a more detailed quantitative assessment for the interactions of CsA-CyP with FSK. However, the results obtained by simultaneous application of FSK and CsA-CyP exclude a subadditive action on channel desensitization and are in favor of an additive action for two reasons. First, the effect on tachyphylaxis caused by a submaximal concentration of FSK was unaltered by application of CsA-CyP in a concentration that alone was too weak to effect desensitization. Second, simultaneous application of CsA-CyP and FSK both in concentrations that alone caused a significant but submaximal decrease in channel tachyphylaxis led to a maximal decrease in tachyphylaxis, meaning a decrease that could not be further enhanced by higher concentrations of CsA-CyP or FSK.

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**Table II**

| FSK treatment | CsA/CyP treatment | Second current* | Fourth current* | n |
|---------------|-------------------|----------------|----------------|---|
| μM           | nM               | %             | %              |   |
| 0            | 0                | 5.1 ± 2.7     | 3.1 ± 0.4     | 6 |
| 0.1          | 1.4/1.7          | 2.4 ± 0.6     | 1.1 ± 0.3     | 5 |
| 0.1          | 1.4/1.7          | 18.3 ± 1.7    | 15.7 ± 2.2    | 3 |
| 0            | 7/8.5            | 21.9 ± 2.6    | 17.5 ± 2.4    | 8 |
| 0.1          | 7/8.5            | 67.3 ± 5.9    | 56.0 ± 6.8    | 8 |
| 10           | 0                | 62.7 ± 7.8    | 42.8 ± 9.8    | 9 |
| 0            | 14/17            | 58.4 ± 6.9    | 37.2 ± 6.2    | 7 |
| 10           | 14/17            | 64.0 ± 6.8    | 38.9 ± 6.1    | 6 |

*The values given for the second and fourth currents are percentages of the values for the first current.

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Among several putative PKA phosphorylation sites of TRPV1, amino acid residues Ser116 and Thr370 seem to be the most critical ones for PKA-dependent modulation of TRPV1 (18, 19). Substitution of Ser116 or Thr370 with either alanine or aspartate led to mutant channels that could not be modulated by PKA (19). We now investigated the effect of calcineurin inhibition on Ca^{2+}-dependent desensitization in TRPV1 mutant channels S116A and T370A. Experiments were performed as described for those shown in Fig. 1. Under control conditions, TRPV1-S116A clearly showed some tachyphylaxis that was however less pronounced compared with TRPV1-WT (Fig. 5A). Here, the current amplitudes at the second and fourth capsaicin application were 22.6 ± 8.7 and 11.0 ± 4.0% of that of the first application, respectively, and were significantly larger than those for TRPV1-WT under...
control conditions (Figs. 1F and 5C). Pretreatment of TRPV1-S116A for 10 min with 14 nM CsA and 17 nM CyP in the pipette solution, however, led to a significant decrease in tachyphylaxis compared with control conditions (Fig. 5B). Current amplitudes at the second and fourth capsaicin application were 66.1 ± 4.1 and 38.2 ± 7.9% of that of the first application, respectively (Fig. 5C). This indicates that dephosphorylation by calcineurin of a residue other than Ser116 might significantly contribute to desensitization.

As described before (19), TRPV1-T370A is one of the least desensitizing alanine mutations at putative PKA phosphorylation sites under control conditions (Fig. 5D). Here, current amplitudes at the second and fourth capsaicin application were 83.2 ± 5.4 and 59.5 ± 7.9% of that of the first application (Figs. 1F and 5F). Pretreatment for 10 min with 14 nM CsA and 17 nM CyP in the pipette solution, however, had no statistically significant effect on channel tachyphylaxis (Fig. 5E). Here, current amplitudes at the second and fourth capsaicin application were 82.1 ± 3.1 and 56.7 ± 5.5% of that of the first application, respectively (Fig. 5F). Because mutation T370A constitutively shows only weak tachyphylaxis, it could be speculated that inhibition of calcineurin will not produce any measurable effect on tachyphylaxis, even if the channel was a substrate for calcineurin. To exclude this possibility, we investigated the effect of calcineurin inhibition on mutation T144A, which is another channel exhibiting impaired tachyphylaxis (19). In this mutation, pretreatment for 10 min with 14 nM CsA and 17 nM CyP in the pipette solution significantly decreased tachyphylaxis compared with control conditions (Figs. 5, G–I). Pretreatment of cells with CsA-CyP did not have any significant effect on the peak amplitudes of capsaicin-activated currents in any of these mutant channels (Table I). These observations support the idea that amino acid residue Thr370 might be a key site for calcineurin-induced dephosphorylation of TRPV1.

Phosphorylation by PKC Does Not Modulate Ca2+-dependent Desensitization of Capsaicin-activated TRPV1 Currents—In addition to PKA, the ε-isofrom of PKC was demonstrated to directly modify TRPV1 and to sensitize heat- and capsaicin-activated currents (20–22). Here, residues Ser502 and Ser800 were suggested to be the major substrates for PKC-dependent phosphorylation (22). However, there is no evidence for a PKC-dependent modulation of TRPV1 desensitization so far (19). We were interested in whether or not there is any interplay between PKC phosphorylation and calcineurin-modulation of TRPV1. Experiments were performed as described for those shown in Fig. 1.

As demonstrated before, pretreatment of cells with PMA (0.1 μM), an activator of PKC, did not have any effect on channel tachyphylaxis (Fig. 6A). PKC pretreatment also did not have any significant effect on the peak amplitudes of capsaicin-activated currents (Table I). Pretreatment for 10 min with 14 nM CsA and 17 nM CyP in the pipette solution along with 0.1 μM PMA in external buffer significantly decreased channel tachyphylaxis (Fig. 6, B and C).

Calcineurin inhibition by CsA-CyP also significantly decreased tachyphylaxis in double mutation S502A/S800A, in which putative PKC phosphorylation sites were disrupted (Fig. 6, D, E, and G). In the same double mutation S502A/S800A, FSK decreased tachyphylaxis to a similar extent like CsA-CyP (Fig. 6, F and G). Pretreatment of cells with CsA-CyP or FSK did not have any significant effect on peak amplitudes of capsaicin-activated currents in mutation S502A/S800A (Table I). These results confirm that phosphorylation of TRPV1 by PKCε indeed is not involved in the channel desensitization process.

Activation of PKA Increases Capsaicin Sensitivity of TRPV1—To determine the effect of calcineurin inhibition by CsA-CyP and PKA activation by FSK on the sensitivity of TRPV1-WT, TRPV1-S116A, and TRPV1-T370A toward capsaicin, we measured the concentration dependence of capsaicin responses in Ca2+-free bath solutions and determined the half-maximal activating concentrations (EC50) before and after pretreatment with 10 μM FSK in the external solution or 14 nM CsA and 17 nM CyP in the pipette solution. Under control conditions, EC50 for TRPV1-WT was 242 ± 2 nM, the Hill coefficient (h) was 1.9 ± 0.1 (Fig. 7A). These values are in reasonable agreement with values found previously for TRPV1 expressed in HEK293t cells (19). The capsaicin-concentration response curve was significantly shifted leftward by ~4.5-fold after pretreatment with FSK (EC50 = 52 ± 2 nM; h = 1.8 ± 0.1; Fig. 7A) but was not significantly changed after pretreatment with CsA-CyP (EC50 = 190 ± 11 nM; h = 1.8 ± 0.2; Fig. 7A).

These results confirm earlier reports that the PKA pathway not only regulates desensitization of TRPV1 but also sensitizes
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These values are in reasonable agreement with values found previously for TRPV1-S116A [19]. Comparable with WT, in this mutation the capsaicin concentration-response curve was significantly shifted leftward by \( \sim 3\)-fold after pretreatment with FSK (EC\(_{50} = 67 \pm 5 \text{nM}; h = 1.2 \pm 0.1\); Fig. 7B) but was not significantly changed after pretreatment with CsA-CyP (EC\(_{50} = 180 \pm 8 \text{nM}; h = 1.2 \pm 0.1\); Fig. 7B). Earlier, we have demonstrated that in mutation TRPV1-S116, there was a slight reduction in tachyphylaxis after pretreatment with FSK, which, however, was not statistically significant [19]. The data in this study confirm that one or several other residues in addition to Ser\(^{116}\) are very likely involved in the mechanism of PKA-dependent modulation of TRPV1.

For TRPV1-T370A, sensitivity toward capsaicin was significantly lower under control conditions compared with WT and TRPV1-S116A (EC\(_{50} = 41 \pm 3 \text{nM}; h = 1.2 \pm 0.1\); Fig. 7C). In this mutation, pretreatment with FSK or CsA-CyP did not have any significant effect on the EC\(_{50}\) values or Hill coefficients. These observations support the idea that amino acid residue Thr\(^{370}\) might be a key site for PKA-mediated phosphorylation of TRPV1. Concentration-effect experiments were performed in Ca\(^{2+}\)-free solutions to prevent channel desensitization. This way, the ineffectiveness of calcineurin in these experiments can be explained.

**Inhibition of Calcineurin Decreases Ca\(^{2+}\)-dependent Desensitization of Proton-activated TRPV1-WT Currents**—TRPV1 is a multimodal sensor that in addition to vanilloids is also activated by protons and heat [1, 2], anandamide [3], ethanol [5], various lipoygenase products, and other lipids related to arachidonic acid [4]. Although not unequivocal, activation of TRPV1 by protons has been demonstrated to lead to channel desensitization in a Ca\(^{2+}\)-dependent manner [11]. This desensitization can be partly rescued by PKA activation as well [18].

We studied the effect of calcineurin inhibition on desensitization of proton-activated TRPV1 currents. As HEK293 cells were shown to endogenously express an acid sensing ion channel (hASIC1a) [27], channels were transiently expressed in HEK cells for these experiments.

Under control conditions, TRPV1-WT showed pronounced proton-induced tachyphylaxis (Fig. 8, A and C) that was qualitatively similar to capsaicin-induced tachyphylaxis. Proton-induced tachyphylaxis was significantly decreased when cells were pretreated for 10 min with 14 nM CsA plus 17 nM CyP in the pipette solution (Fig. 8, B and C). Similarly, CsA-CyP decreased acute desensitization of TRPV1-WT induced by prolonged application (30 s) of protons (Fig. 8, D–F). Peak amplitude of proton-activated currents were unaffected by CsA-CyP (Table I).

**DISCUSSION**

In this study, we show that specific inhibition of calcineurin (protein phosphatase 2B) significantly decreases Ca\(^{2+}\)-dependent desensitization of capsaicin- and proton-activated TRPV1 currents. This effect is qualitatively and quantitatively similar to but independent from that obtained by extracellular application of FSK and cannot be further enhanced by simultaneous application of FSK and CsA-CyP. In mutation T370A, but not mutations S116A and T144A, desensitization properties are unaffected by calcineurin inhibition. In double mutation S502A/S800A, in which putative PKC phosphorylation sites are disrupted, both calcineurin inhibition and PKA activation decrease desensitization of capsaicin-activated currents similar to WT channels. We conclude that Ca\(^{2+}\)-dependent desensitization of TRPV1 might be in part regulated through channel dephosphorylation by calcineurin and channel phosphorylation by PKA, possibly involving Thr\(^{370}\) as a key amino acid residue.

**Dephosphorylation and Desensitization**—Protein phosphorylation and dephosphorylation is a major mechanism in mamm-
Calcineurin inhibition not only decreased channel tachyphylaxis but also decreased acute desensitization elicited by a 30-s-long capsaicin application, an effect that could not be observed with PKA activation in an earlier study (19). This disparity in effects between calcineurin inhibition and PKA activation suggests that dephosphorylation might be a faster process as compared with rephosphorylation. Moreover, dephosphorylation might not require a closed or ligand-free channel, as hypothesized for the process of rephosphorylation.

Calcineurin inhibition also decreased desensitization of proton-activated TRPV1 currents under our experimental conditions. Unlike suggested by others (13), we conclude that activation by capsaicin and activation by protons most probably initiate comparable mechanisms of Ca\(^{2+}\)-dependent desensitization.

The Role of CaM—Calcineurin acts in a Ca\(^{2+}\)- and calmodulin-dependent manner. CaM is a dominant Ca\(^{2+}\) sensor for Ca\(^{2+}\)-dependent inactivation in many ion channels (24–26). There is accumulating evidence that multiple regions of TRPV1 may bind CaM. One putative region was identified in the C-terminal (14), and another was identified in the N-terminal segment (15).

In our experiments both CaM and the calmodulin antagonist W-7 did not have any effect on TRPV1 desensitization or peak amplitudes of capsaicin-activated currents. The ineffectiveness of the CaM inhibitor is in good agreement with an earlier report (14) and might suggest that CaM is not involved in Ca\(^{2+}\)-dependent desensitization of capsaicin-activated TRPV1 currents. However, CaM inhibitors would only be expected to inhibit desensitization if CaM acted as a free molecule (32, 33). This, however, might not be the case as suggested by recent reports (15, 22).

CaM was demonstrated to mediate Ca\(^{2+}\)-inhibition of TRPV1 in inside-out excised patches of Xenopus oocytes and HEK293 cells expressing TRPV1. In that study, CaM was applied to the intracellular site of the channel together with 50 μM free Ca\(^{2+}\). We cannot exclude that the ineffectiveness of CaM in our study is due to the rather uncontrolled intracellular free Ca\(^{2+}\) concentration under our experimental conditions. Thus, from our data, we cannot derive profound evidence for or against a role of CaM in TRPV1-WT channel desensitization.

Functional Coupling of Calcineurin and PKA—The decrease in desensitization of capsaicin-activated currents by calcineurin inhibition in this study was qualitatively and quantitatively similar to that obtained by PKA activation. Simultaneous application of FSK and CsA-CyP in submaximal and maximal concentrations suggest additive actions of FSK and CsA-CyP rather than subadditive or synergistic actions. In mutation T370A, in which a putative PKA phosphorylation site is disrupted, desensitization properties were unaffected by calcineurin inhibition. Calcineurin inhibition, however, did reduce desensitization in both mutations S116A, in which another critical PKA phosphorylation site is disrupted (18, 19), and mutation T144A, which shares similar desensitization properties with mutation T370A (19). These results suggest that Ca\(^{2+}\)-dependent desensitization of TRPV1 might be in part regulated through channel dephosphorylation by calcineurin and channel phosphorylation by PKA possibly involving Thr\(^{370}\) as a key amino acid residue. A functional coupling of calcineurin and PKA was proposed before in mouse ventricular myocytes to control Ca\(^{2+}\) influx through Ca\(^{2+}\) channels and Ca\(^{2+}\) release through ryanodine receptors (34). In these cells, immunofluorescence also revealed colocalization of calcineurin and PKA.

We hypothesize that a similar mechanism could control excitability of nociceptive sensory neurons by regulating desensitization and thus channel availability. The interplay between...
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CaMKII and PKA remains to be characterized in future studies.

TRPV1 channel phosphorylation by PKA (16) and PKC (22) to control activation thresholds and TRPV1 channel phosphorylation/dephosphorylation by PKA (18, 19), CaMKII (13), and calcineurin to regulate desensitization/availability might allow fine tuning of the nociceptor in response to a noxious environment.

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