Regulated Exocytosis Contributes to Protein Kinase C Potentiation of Vanilloid Receptor Activity*\(\text{[S]}\)

Received for publication, October 21, 2003, and in revised form, March 22, 2004
Published, JBC Papers in Press, April 5, 2004, DOI 10.1074/jbc.M311515200

Cruz Morenilla-Palao, Rosa Planells-Cases, Nuria García-Sanz, and Antonio Ferrer-Montiel‡

From the Instituto de Biología Molecular y Celular, Universidad Miguel Hernández, Avenida del Ferrocarril s/n, 03202 Elche (Alicante), Spain

The vanilloid receptor-1 (TRPV1) plays a key role in the perception of peripheral thermal and inflammatory pain. TRPV1 expression and channel activity are notably up-regulated by proalgesic agents. The transduction pathways involved in TRPV1 sensitization are still elusive. We have used a yeast two-hybrid screen to identify proteins that associate with the N terminus of TRPV1. We report that two vesicular proteins, Snapin and synaptotagmin IX (Syt IX), strongly interact in vitro and in vivo with the TRPV1 N-terminal domain. In primary dorsal root ganglion neurons, TRPV1 co-distributes in vesicles with Syt IX and the vesicular protein synaptobrevin. Neither Snapin nor Syt IX affected channel function, but they notably inhibited protein kinase C (PKC)-induced potentiation of TRPV1 channel activity with a potency that exceeded the block mediated by botulinum neurotoxin A, a potent blocker of neuronal exocytosis. Noteworthily, we found that PKC activation induced a rapid delivery of functional TRPV1 channels to the plasma membrane. Botulinum neurotoxin A blocked the TRPV1 membrane translocation induced by PKC that was activated with a phorbol ester or the metabotropic glutamate receptor mGluR5. Therefore, our results indicate that PKC signaling promotes at least in part the SNARE-dependent exocytosis of TRPV1 to the cell surface. Taken together, these findings imply that activity-dependent delivery of channels to the neuronal surface may contribute to the buildup and maintenance of thermal inflammatory hyperalgesia in peripheral nociceptor terminals.

TRPV1\(^1\) is a capsaicin-, proton- and heat-sensitive, cation-selective ion channel expressed in nociceptors that participates in the transduction of noxious chemical and thermal stimuli by sensory nerve endings in peripheral tissues (1–3). Heterologous expression of TRPV1 cDNA results in ionic currents that recapitulate most of the functional properties displayed by native capsaicin- and heat-activated currents in sensory neurons (1, 2). For instance, TRPV1 exhibits a time- and Ca\(^{2+}\)-dependent desensitization, a long lasting refractory state during which the receptor does not respond to vanilloids or other stimuli (2). In addition, the channel activity of TRPV1 is remarkably up-regulated by inflammatory mediators through the activation of phospholipase C and protein kinases A and C (PKA and PKC) signaling pathways (4–13). Recent evidence shows that an increase in TRPV1 expression in peripheral nociceptors is critical for the maintenance of inflammatory hyperalgesia (14, 15). The involvement of TRPV1 in heat hypersensitivity is further underscored by the reduced sensitivity of mice lacking TRPV1 (16, 17) and by mice treated with receptor-specific antagonists (18).

TRPV1 belongs to the family of transient receptor potential channels, which structurally resembles the family of voltage-gated potassium or cyclic nucleotide-gated channels (19). Accordingly, these channels are presumed to be tetrameric assemblies of identical subunits (Fig. 1A), although heteromeric assemblies have been reported in heterologous systems (20). Each subunit has six membrane-spanning domains (S1–S6) and cytosolic carboxyl and amino termini. These intracellular domains have consensus sequences for protein kinases. Phosphorylation of the C terminus with PKC remarkably sensitizes TRPV1 channel activity by augmenting the channel open probability (5, 13). Furthermore, PKC-mediated phosphorylation also decreases the heat threshold of channel activation (5, 6). Similarly, phosphorylation of the N terminus with PKA affects TRPV1 channel function by modulating the rate and extent of receptor desensitization (9). In addition, the C- and N termini display the presence of putative lipid-protein and protein-protein interacting domains that may also contribute to the regulation of receptor channel properties. Indeed, the association of the C terminus with phosphatidylinositol 4,5-bisphosphate inhibits the channel activity (7, 8), and its interaction with calmodulin promotes channel desensitization (12). In marked contrast, the identification of proteins that interact with the N-terminal region of TRPV1 has remained elusive despite the fact that this segment contains potential protein-protein interacting domains such as a relatively proline-rich region and three ankyrin repeats (1, 2, 19) (Fig. 1A). Here, we addressed this issue and used the yeast two-hybrid system to identify proteins that associate with the N terminus of TRPV1 (referred to as N-TRPV1; Fig. 1A) and modulate its functional properties.

We report the identification of two vesicular proteins, Syt IX and Snapin, that strongly interact with the TRPV1 channel, suggesting that this channel is located in vesicles that participate in SNARE-dependent exocytosis. Noteworthily, we found that PKC-mediated potentiation of TRPV1 channel activity in

\(1\) This work was supported the Ministry of Science and Technology Grant SAF2003-0509 and Fundación La Caixa Grant 01/085-00 (to A. F.-M.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

\(\text{[S]}\) The on-line version of this article (available at http://www.jbc.org) contains a brief article and supplementary Figs. S1 and S2 in regard to the effects of Syt IX and Snapin.

\(\dagger\) To whom correspondence should be addressed. Tel.: 34-96-665-8727; Fax: 34-96-665-8758; E-mail: afferer@umh.es.

The abbreviations used are: TRPV1, transient receptor potential vanillainoid receptor-1; N-TRPV1, N-terminus domain of TRPV1; Syt, synaptotagmin; DRG, dorsal root ganglion; BoNT A, botulinum neurotoxin A; PKC, protein kinase C; TPA, 12-O-tetradecanoylphorbol-13-acetate; SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptor; VAMP2, vesicle-associated membrane protein-2 (synaptobrevin); ACPD, \(\text{trans}\)-\(\text{S,3R}\)-1-amino-1,3-cyclopentandionesulfonic acid; HEK, human embryonic kidney; \(\alpha\)-Gal, 5-bromo-4-chloro-3-indolyl-\(\alpha\)-\(\beta\)-galactopyranoside.

This paper is available on line at http://www.jbc.org
cells occurs in part by rapid recruitment of vesicular channels to the cell surface by regulated exocytosis. These findings suggest that TRPV1 sensitization by proalgesic agents that activate PKC signaling appears to involve the concerted activation of surface resident channels and the mobilization of receptors from a vesicular pool.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Screen—The yeast two-hybrid screen was performed using the MATCHMAKER GAL4-based two-hybrid system (Clontech) as described by the manufacturer. The full-length cytosolic N-terminal domain of TRPV1 (residues 1–414) was cloned into pGB-9BD and transformed into the PJ69-2A strain of S. cerevisiae. Screening was made by conjugation against strain Y187 pretransformed with the rat brain cDNA library fused to the GAL4 activation domain of plasmid PACT2 (Clontech). Positive interactions were identified by the ability of yeast to grow on selective medium in the absence of histidin and adenine and the presence of 40 μM 3-amino-1,2,4-triazole and Xɑ-Gal for α-galactosidase expression.

Plasmids and Deletions—cDNAs of Syt IX, Syt I, and Snapin were obtained by PCR with specific primers from a human DRG library (21) and cloned into the pcDNAs.1-Myc-His plasmid (Invitrogen). N-TRPV1 was cloned into the pET-22b plasmid (Qiagen) as a His6 fusion protein. Deletion species were generated by inverse PCR (22).

In Vitro His Pull-down Assay—Fusion proteins were affinity-purified on nickel-nitrilotriacetic acid columns (Qiagen). Immobilized fusion proteins (10 μg) were incubated with in vitro translated [35S]Snapin or [35S]Syt IX (Tn7 TT Quick Coupled transcription/translation system, Promega) in binding buffer (10 mM Tris·HCl, 300 mM NaCl, 16 mM 2-mercaptoethanol, 1% Triton X-100, and 20 mM imidazole, pH 8) for 2 h at 22 °C. After three washes with binding buffer, [35S]-bound complexes were eluted from the resin and denaturated with SDS-PAGE sample buffer at 90 °C for 10 min. Protein complexes were resolved by SDS/PAGE on 12% gels, visualized by autoradiography, and quantified as described (23).

Immunoprecipitation and Immunoblotting—HEK293 cells (1.5 × 10⁶) were transfected with 1 μg of full-length TRPV1 and Myc-Snapin, Myc-Syt IX, or Myc-Syt I using the LipofectAMINETM 2000 reagent (Invitrogen). Forty-eight hours after transfection, cells were harvested and solubilized with 900 μl of radioimmune precipitation assay buffer (50 mM Tris·HCl, pH 7.4, 150 mM NaCl, 0.25% sodium deoxycholate, 1% Nonidet P-40, 2 mM phenylmethylsulfonyl fluoride, 2 mM EDTA, and 5 mM iodoacetamide) for 15 min at 22 °C. Cell debris was removed by centrifugation, and the supernatants were incubated overnight at 4 °C with anti-TRPV1 serum (1:200). Thereafter, 30 μl of agarose-protein G beads (Ficoll) and solubilized with 90 °C for 5 min, separated by SDS-PAGE, and analyzed by Western immunoblotting using an anti-c-Myc antibody (1:2,000) (Sigma). Anti-TRPV1 Serum—A synthetic peptide encoding the C terminus of rat TRPV1 (EYFKDFSVMVPGEK, DiverDugs S.L.) was coupled to keyhole limpet hemocyanin and administered to rabbits to raise a polyclonal anti-TRPV1 serum.

Immunocytochemistry—Adult rat DRGs (cervical, thoracic, and lumbar) were isolated, incubated in 0.125% collagenase for 3 h, washed, mechanically dissociated with glass pipettes, and plated on polyornithine- and laminin-coated glass coverslips. Cells were incubated for 72–96 h in Dulbecco’s modified Eagle’s medium plus 10% horse serum before immunocytochemical analysis. Primary cultures were fixed, blocked, permeabilized, and subsequently incubated with the following primary antibodies and dilutions: mouse antibodies to SNAP23 (Chemicon); 1:500 anti-Syt IX (Santa Cruz Biotechnology); and 1:500 anti-VAMP2 (clone C169.1) (Sypaptic Systems). After washing, the cultures were incubated with secondary antibodies, washed, embedded, and analyzed by confocal microscopy (Leica TCS).

Oocyte Electrophysiology—Amphibian oocytes were harvested and microinjected with cRNA encoding the rat TRPV1 channel, Snapin, and Syt IX as described previously (18). Whole-cell currents in oocytes were recorded with a two-microelectrode voltage clamp amplifier. Oocytes were continuously perfused (2 ml min⁻¹) in Mg²⁺-Ringer’s solution (10 mM Hepes, pH 7.4, 115 mM NaCl, 2.8 mM KCl, 0.1 mM BaCl₂, and 2 mM MgCl₂) at 20 °C. TRPV1 currents were activated with acidic solution (McIlwain solution with 10 mM MES, pH 6.5 or capsaicin at the indicated concentrations. The holding potential was kept at −40 mV. Recordings were performed 3–5 days after injection. The recombinant BoNT A catalytic domain was expressed and purified as described (23) with the exception that detergents were not present during the purifi-

Receptor Mobilization by PKC Signaling

FIG. 1. The cytosolic N terminus region of TRPV1 was used as bait for a yeast two-hybrid screen. A, schematic representation of a TRPV1 channel subunit inserted into a lipid bilayer. The depiction of the three ankyrin domains of N-TRPV1 is boxed. An enhancement showing the fragment used as bait is displayed underneath. B, growth of yeast that co-expresses N-TRPV1 fused to the Gal4 activating domain and Snapin or Syt IX linked to the Gal4 binding domain under restrictive conditions. Blue color indicates Xɑ-Gal degradation by the reporter gene α-galactosidase. 

controlation process. Oocytes were microinjected with 1 μM neurotoxin 12 h before current recordings. 12-O-tetradecanoylphorbol-13-acetate (TPA), 4α-TPA (Sigma), and bisindolylmaleimide (Calbiochem) were dissolved in dimethyl sulfoxide.

Biotin Labeling of Surface Proteins—Control and TPA-treated (3 × 30-s pulses of 1 μM TPA) oocytes (30 cells) expressing TRPV1 were incubated with 0.5 μg/ml sulfo-NHS-biotin (Sigma) for 30 min at 4 °C and lysed with radioimmune precipitation assay buffer. Biotin-conjugated cell surface proteins were purified with streptavidin-agarsa (Sigma). Surface TRPV1 was detected by Western blotting with the anti-TRPV1 serum. Immunoblots were digitized and quantified as described (23).

RESULTS

Syt IX and Snapin Interact with the N Terminus of TRPV1—To identify proteins that interact with the N-terminal domain of TRPV1, we used a yeast two-hybrid assay to screen a rat brain complementary DNA library using the rat N-TRPV1 (residues 1–414; Fig. 1A) as bait. A brain-derived library was employed because the receptor is also expressed in this tissue (24, 25). We identified Syt IX and Snapin, two synaptic vesicle proteins involved in regulated exocytosis (26–28), as interacting partners of the N-TRPV1 domain (Fig. 1B). Both proteins were cloned from a cDNA library from human DRGs.

To verify that Snapin and Syt IX bind directly to the N terminus of TRPV1 and to determine the interacting domain on the channel, hexahistidine-tagged fusion proteins of wild type (N-TRPV1-His) and N-TRPV1 deletion mutants were immobilized on nickel-agarose beads and incubated with in vitro translated [35S]Snapin or [35S]Syt IX (Fig. 2). As illustrated (Fig. 2A, lane 2), both proteins were retained specifically by the N-TRPV1-His fusion protein but not by resin alone (Fig. 2A, lane 1). Deletion analysis of the N-TRPV1 domain indicates that Snapin and Syt IX bind preferentially to the ankyrin repeats, as evidenced by the enhanced interaction of both proteins with the construct encompassing residues 200–414 (Δ2–199-His) that contains the ankyrin core structure. Removal of
the three ankyrin repeats notably weakened the interaction, although it did not completely abrogate it, suggesting that neighboring domains may contribute to protein binding. These data demonstrate that Snapin and Syt IX strongly interact in vitro with the N-TRPV1.

Because Syt IX is structurally and functionally related to Syt I, a synaptotagmin isoform abundantly expressed in vesicles that undergo Ca\(^{2+}\)-dependent exocytosis (26), we questioned whether Syt I also interacts with the N-TRPV1. As shown in Fig. 2A, \[^{35}S\]Syt I was retained by an immobilized N-TRPV1-His fusion protein. Similarly, Syt I was preferentially bound to the core ankyrin repeats. We next examined the role of the two Ca\(^{2+}\) binding sites, C2A and C2B, which are present in Syt IX in the interaction with N-TRPV1 (Fig. 2B). Pull-down experiments of Syt IX deletion species in which one of the C2 sites has been removed indicate that the N-TRPV1 domain could interact with both Ca\(^{2+}\) domains, although it seems to bind slightly stronger to the C2A domain. In support of this tenet, a peptide patterned after the C2A domain (C2A peptide) potently inhibited the interaction of N-TRPV1 and Syt IX is abrogated by a 12-mer peptide patterned after the C terminus of neuregulin-1 (22). Peptide concentration was 0.5 mM.

Because Syt IX is structurally and functionally related to Syt I, a synaptotagmin isoform abundantly expressed in vesicles that undergo Ca\(^{2+}\)-dependent exocytosis (26), we questioned whether Syt I also interacts with the N-TRPV1. As shown in Fig. 2A, \[^{35}S\]Syt I was retained by an immobilized N-TRPV1-His fusion protein. Similarly, Syt I was preferentially bound to the core ankyrin repeats. We next examined the role of the two Ca\(^{2+}\) binding sites, C2A and C2B, which are present in Syt IX in the interaction with N-TRPV1 (Fig. 2B). Pull-down experiments of Syt IX deletion species in which one of the C2 sites has been removed indicate that the N-TRPV1 domain could interact with both Ca\(^{2+}\) domains, although it seems to bind slightly stronger to the C2A domain. In support of this tenet, a peptide patterned after the C2A domain (C2A peptide) potently inhibited the interaction of Syt IX with the N-TRPV1-His fusion protein (n = 3, Fig. 2C). Saturation of the fusion protein with an unrelated peptide did not affect the binding capacity of Syt IX (Fig. 2C).

**Syt IX and Snapin Associate with TRPV1**—The association of Snapin and Syt IX with the TRPV1 channel was next evaluated in vivo by co-immunoprecipitation. Full-length, Myc-tagged Snapin and Syt IX were co-expressed with the TRPV1 receptor in HEK293 cells, and cell extracts were immunoprecipitated (IP) with an antibody specific for the TRPV1 channel and probed with an anti-Myc antibody (IB). Each panel depicts the anti-TRPV1 immunoprecipitates (top panels) and the total cell extracts (middle and bottom panels).
Notice, however, that the in vivo association of TRPV1 with Syt I was lower than with Syt IX, which is consistent with the weaker interaction seen in vitro. These results demonstrate that the full-length TRPV1 channel associates with vesicular Syt IX and Snapin proteins in vivo.

**TABLE I**

*Ion channel properties of TRPV1 in the presence of Syt IX and Snapin*

|                      | TRPV1  | TRPV1 + Syt IX | TRPV1 + Snapin |
|----------------------|--------|----------------|----------------|
| $I_{\text{max}}$ (nA) | 485 ± 191 | 548 ± 207      | 385 ± 200      |
| $I_{\text{Cap}}/I_{\text{pH}}$ | 1.4 ± 0.4 | 1.3 ± 0.2      | 1.4 ± 0.2      |
| EC$_{50}$ (capsaicin; μM) | 1.4 ± 0.4 | 1.6 ± 0.3      | 1.6 ± 0.2      |
| IC$_{50}$ (RR; μM) | 0.45 ± 0.05 | 0.32 ± 0.08    | 0.37 ± 0.06    |
| $V_r$ (mV) | −4.5 ± 2.3 | −3.2 ± 3.0      | −6.0 ± 3.7      |
| $\tau_{\text{off}}$ (s) (capsaicin) | 9.7 ± 0.6 | 9.8 ± 2.1      | 9.8 ± 1.4      |
| $\tau_{\text{off}}$ (s) (pH) | 5.7 ± 1.0 | 4.4 ± 1.7      | 3.5 ± 0.6*     |

* Peak current elicited by 10 μM capsaicin at −40 mV.
* Ratio of the inward current elicited by 10 μM capsaicin (Cap) with respect to that evoked by an acidic solution (pH 6).
* EC$_{50}$ is the concentration of capsaicin required to activate the half-maximal response obtained from a dose-response curve (18).
* IC$_{50}$ is the concentration of ruthenium red (RR) needed to block the half-maximal response elicited by 10 μM capsaicin obtained from a dose-response curve (18).
* Reversal potential of the ionic current elicited by an acidic solution (pH 6).
* $\tau_{\text{off}}$ is the time constant of the deactivation process of capsaicin and pH-evoked ionic currents.
* $\tau_{\text{off}}$ is the time constant of the deactivation process of pH-evoked ionic currents.

(Fig. 3C). Notice, however, that the in vivo association of TRPV1 with Syt I was lower than with Syt IX, which is consistent with the weaker interaction seen in vitro. These results demonstrate that the full-length TRPV1 channel associates with vesicular Syt IX and Snapin proteins in vivo.
the interaction of TRPV1 with vesicular SNARE proteins suggests that the channel is sorted into synaptic vesicles in neurons. To address this issue, we investigated the co-distribution of TRPV1 with Syt IX in primary cultures of DRG neurons by immunocytochemistry using specific anti-TRPV1 and anti-Syt IX antibodies. As depicted in Fig. 4A, Syt IX and TRPV1 exhibited a widespread subcellular localization, especially in the soma of the neurons. The overlay of both immunoreactivities revealed a neuronal co-distribution of both proteins, suggesting the presence of TRPV1 in vesicles. This finding was further supported by the codistribution of TRPV1 channels with the synaptic vesicle marker VAMP2, as evidenced by the colocalization of the immunoreactivities of both proteins in DRG neurons (Fig. 4B and C). Vesicular localization was observed in the cell body as well as in neuronal processes (Fig. 4B). Note that the puncta co-stained by TRPV1 and VAMP2 along the neuronal processes were significantly larger (≥200 nm) than the average synaptic vesicles (≈50 nm), suggesting that they may represent the so-called "cytoplasmic transport packets" (29, 30) (Fig. 4C). These results suggest that TRPV1 is sorted into synaptic vesicles and transported to synaptic terminals by vesicular clusters.

Syt IX and Snapin Do Not Modulate TRVP1 Channel Properties—We next evaluated whether Snapin and/or Syt IX modulated the channel activity of TRPV1. The TRPV1 receptor was co-expressed with Snapin or Syt IX in *Xenopus* oocytes, and the receptor channel activity was monitored by a two-microelectrode voltage clamp. As summarized in Table I, the overall channel properties of TRPV1 were not altered by the expression of Syt IX or of Snapin, although Snapin mildly affected the kinetics of pH-induced responses (see also the supplemental data in the online version of this article). These data are consistent with the notion that the interaction of the vesicular SNARE proteins with TRPV1 occurs in vesicles rather than on the cell surface and suggest that these proteins may modulate aspects of receptor trafficking.

Syt IX and Snapin Modulate PKC-induced TRPV1 Potentiation—TRPV1 potentiation by intracellular signaling pathways such as PKC has been primarily attributed to the modulation of channel gating (5, 6, 13). However, the finding that PKC also...
sensitizes regulated exocytosis (31, 32) hints that the potentiation of TRPV1 activity by the kinase could arise, at least in part, by the insertion into the cell surface of active channels located in vesicles. To investigate this hypothesis, we used Xenopus oocytes as a heterologous expression system for TRPV1 because they recapitulate the properties of PKC signaling (5, 33) and SNARE-dependent exocytosis (33, 34). Stimulation of PKC activity was achieved by the incubation of oocytes with 1 μM TPA. Because TPA activates TRPV1 (5), we used a protocol based on three 30-s pulses of TPA interspersed by 2-min washes to minimize receptor activation by the phorbol. As depicted in Fig. 5A, the first TPA pulse evoked a small, desensitizing, inward current that was enhanced >10-fold by a second phorbol instillation (n = 17, Fig. 5, A and F). Treatment of the oocytes with TPA also resulted in a 3-fold potentiation of TRPV1 currents evoked by pH 6 (n = 17; Fig. 5, A and F). TPA-evoked ionic currents were blocked by 1 μM ruthenium red (86 ± 5%, n = 3) and by 5 μM compound DD161515 (18) (79 ± 3%, n = 2). Consistent with other reports (5), TPA-induced potentiation of TRPV1 activity was mediated by PKC activity because no current enhancement was elicited by the inactive analogue 4a-TPA, although this compound was also a potent TRPV1 agonist (Fig. 5, B and F). Similarly, TPA sensitization was prevented by preincubation with the selective PKC inhibitor bisindolylmaleimide I (1 μM, n = 3; Fig. 5F). Thus, these data indicate that TPA-induced potentiation of TRPV1 activity is mediated by PKC.

We next examined whether PKC sensitization of TRPV1 channel activity is modulated by Snapin and Syt IX. Notably, co-expression of either Snapin or Syt IX with TRPV1 attenuated by ≥45% the TPA-induced enhancement of TRPV1 channel activity (p < 0.01, n > 6; Fig. 5, C, D, and F). Because it has been shown that overexpression of SNARE proteins may abrogate SNARE-dependent exocytosis (35), this finding suggests that TRPV1 potentiation by PKC may occur partly by the rapid recruitment of vesicle-associated channel molecules to the cell surface by exocytosis.

PKC Activation Promotes the Exocytosis of TRPV1—To demonstrate that PKC activation facilitates the insertion of new TRPV1 channels into the cell membrane, we determined the effect of loading TRPV1-expressing oocytes with the recombinant BoNT A catalytic domain, a specific blocker of regulated exocytosis (36, 37). As illustrated in Fig. 5E, treatment with BoNT A reduced the extent of TPA-induced stimulation of TRPV1 activity by ~40% (p < 0.05, n = 10; Fig. 5F). Thermally inactivated toxin did not display this inhibitory activity (n = 4; not shown). Furthermore, BoNT A also blocked ~30% of the 2.5-fold potentiation of TRPV1 activity evoked by the activation of metabotropic GluR5, a phospholipase C-coupled receptor that activates PKC (38, 39) (Fig. 6). Note that BoNT A treatment did not affect the receptor channel activity. This lack of effect may be because BoNT A treatment was carried out 48 h after cRNA injection or because the receptor may also be delivered to the cell surface by a SNARE-independent mechanism. Taking together, our observations strongly suggest that PKC signaling promotes TRPV1 trafficking of active channels to the cell surface via SNARE-dependent exocytosis.

PKC Activation Recruits TRPV1 to the Plasma Membrane—We next investigated whether PKC activation by TPA indeed induces a rapid increment in the surface expression of TRPV1 channels. For this purpose, TRPV1-expressing cells were stimulated with 1 μM TPA, surface proteins were biotinylated and purified with streptavidin-agarose, and the presence of TRPV1 was probed with the anti-TRPV1 serum. Fig. 7 shows that PKC activation with TPA promoted an increase in the expression of TRPV1 in the plasma membrane of the cells.

**FIG. 7.** TPA increased the cell surface insertion of TRPV1 through SNARE-dependent exocytosis. A, TPA-evoked translocation of TRPV1 channels to the plasma membrane in control and cells co-expressing TRPV1 and Snapin or Syt IX. B, phorbol ester-induced surface expression of TRPV1 channels in control and BoNT A-treated cells. Surface expression was analyzed by Western immunoblotting of biotinylated surface proteins using an anti-TRPV1 antibody. TRPV1 expression in whole cell extracts (total) prior to incubation with aggregating-NeutrAvidin is also shown. C, TPA treatment increased receptor surface expression by 1.8-fold. BoNT A fully blocked TPA-induced TRPV1 surface expression. Western immunoblots were digitized and quantified as described (23). Values are mean ± S.E., n = 3 (*, p < 0.05; Student’s t test).

Notice that the amounts of receptor in the whole cell extracts before and after the TPA treatment were comparable (Fig. 7, A and B, bottom). Co-expression of TRPV1 with Syt IX modestly affected the TPA-induced surface translocation of the receptor (Fig. 7A). In contrast, Snapin and BoNT A virtually blocked TRPV1 surface expression by the phorbol ester (Fig. 7, A and B). The relative amount of TRPV1 surface expression was augmented by ~1.8-fold upon treatment with TPA (Fig. 7C), as inferred from the quantification of the protein bands shown in the gels (Fig. 7B). Treatment with BoNT A resulted in full abrogation of the TPA-induced increment in TRPV1 surface expression (Fig. 7C). Therefore, these data demonstrate that PKC activation by TPA promotes a rapid recruitment of the TRPV1 channel to the plasma membrane via SNARE-dependent exocytosis.

**DISCUSSION**

The ultimate goal of this study was to identify proteins that interact with the N terminus of TRPV1. We have found that TRPV1 channels associate in vitro and in vivo with the vesicular proteins Syt IX and Snapin, two components of SNARE-dependent exocytosis in excitable cells (26–28). Snapin is a...
vesicular protein that modulates exocytosis by enhancing the interaction of synaptogamin with the SNARE protein SNAP25 (28). On the other hand, Syt IX is a synaptogamin isoform expressed in synaptic vesicles of neuronal cells that regulates Ca\(^{2+}\)-dependent exocytosis (26, 27). The role played by Syt IX and Snapin in SNARE-dependent exocytosis suggests that their interaction with TRPV1 may modulate aspects of TRPV1 trafficking and/or delivery to the plasma membrane. The observation that TRPV1 is located in large puncta present in neuronal processes, presumably the so-called cytoplasmic transport packets (30, 40) similar to those reported for TRPC5 in transit to growth cones (29), substantiates this tenet. Furthermore, our result showing that the interaction of TRPV1 with Syt IX or Snapin does not affect the overall channel properties of the surface-expressed receptor is consistent with a preferential vesicular interaction of these proteins with the channel.

SNARE-dependent exocytosis may be sensitized by protein kinases (31, 32, 41). Moreover, it has been reported the intracellular signaling cascades can promote the recruitment of channels to the cell surface (33). Thus, we reasoned that the potentiation of TRPV1 activity by intracellular signaling cascades may be mediated by activity-dependent mobilization of a vesicular pool of channels. Indeed, we found that the activation of PKC promotes the surface expression of a population of TRPV1 channels. PKC-induced potentiation of TRPV1 channel activity was inhibited by both the overexpression of Syt IX and Snapin and the poisoning of the oocytes with the BoNT A catalytic domain, a highly specific blocker of Ca\(^{2+}\)-dependent exocytosis (36, 37). Furthermore, stimulation of PKC significantly increased the expression of TRPV1 channels in the plasma membrane via SNARE-dependent exocytosis as evidenced by the sensitivity of receptor mobilization to BoNT A treatment as well as co-expression of the receptor with Snapin. However, the inhibition of TPA-induced TRPV1 activity by Syt IX appears to be mediated by a mechanism that does not involve receptor translocation to the plasma membrane. Future experiments will uncover the molecular details of the effect of Syt IX on TRPV1 activity. Taking together, these findings indicate that sensitization of TRPV1 receptors by PKC signaling is due, at least in part, by the regulated exocytosis of channels located in cytosolic vesicles.

The exposure of DRG neurons in culture to proalgesic agents notably augments heat-evoked currents form these cells (1, 2). This heightened heat sensitivity appears mediated by both a direct and indirect modification of TRPV1 function by intracellular signaling pathways (3). The potentiation of TRPV1 activity by intracellular cascades has been associated primarily with the unmasking of silent channels that are already present on the cell surface (7, 8) and with the modulation of the channel gating properties by direct phosphorylation (5, 9, 10, 13). In addition, an increment in the level of TRPV1 in peripheral terminals has been reported to be important for the maintenance of inflammatory heat hypersensitivity (14). Our discovery that PKC potentiation of TRPV1 activity is partly due to the recruitment of a pool of vesicular receptors to the plasma membrane by regulated exocytosis provides a complementary strategy that may contribute to enhance heat transduction at sensitized nociceptor terminals. It was recently reported that Syt IX is required for export from the perineurial endocytic recycling compartment to the cell surface through the microtubule network (42). Thus, in addition to regulate receptor exocytosis, the interaction of TRPV1 with vesicular protein Syt IX might also be important in the sorting of the receptor into vesicles targeted to the peripheral terminals. Taken together, these data support the tenet that acute and chronic inflammatory heat hyperalgesia results from both post-translational modifications of existing channels and the activity-dependent targeting of receptor molecules to the peripheral terminals (33). It is worth noting that inflammatory sensitization of nociceptor peripheral terminals mechanistically resembles the increase in synaptic strength that occurs during long term potentiation in the central nervous system, where PKC signaling mediates both a modulation of the gating properties and an increase in the density of N-methyl-D-aspartate receptors (33).

In conclusion, the TRPV1 surface expression by inflammatory mediators in nociceptor peripheral terminals might be an important mechanism for both the development and maintenance of inflammatory hyperalgesia. These findings imply that pharmacological modulation of TRPV1 trafficking may be a therapeutic approach for pain management.

Acknowledgments—We are grateful to David Julius for rat TRPV1 cDNA, Joan Blasi for BoNT A catalytic domain, Carmen de Felipe for assistance with immunochemistry, Carolina Garcia for assistance with oocyte electrophysiology, Reme Torres for cRNA preparation and oocyte microinjection, and Cristina Ibanez for recombinant BoNT A preparation. We thank Hugo Cabedo and Marco Caprini for comments and suggestions.

REFERENCES
1. Scholz, J., and Woolf, C. J. (2002) Nat. Neurosci. 5, suppl. (1062–1067)
2. Julius, D., and Basbaum, A. I. (2001) Nature 413, 203–210
3. Caterina, M. J., and Julius, D. (2001) Annu. Rev. Neurosci. 24, 487–517
4. Chandall, J. M., Kwaski, J. Y., Wu, and White, G. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 109–117
5. Premkumar, L. S., and Akera, G. P. (2000) Nature 408, 985–990
6. Vellani, V., Mapplebeck, S., Moriondo, A., Davis, J. B., and McNaughton, P. A. (2001) J. Physiol. 534, 813–825
7. Prescott, E. D., and Julius, D. (2003) Science 300, 1284–1288
8. Chuang, H. H., Prescott, E. D., Kong, H., Shields, S., Jordet, S. E., Basbaum, A. I., Chao, M. Y., and Julius, D. (2001) Nature 411, 957–962
9. Bhide, G., Zhu, W., Wang, H., Brasier, D. J., Oxford, G. S., and Gereau, R. W. (2002) Neuron 35, 721–731
10. Numazaki, M., Tominaga, T., Toyooka, H., and Tominaga, M. (2002) J. Biol. Chem. 277, 13275–13277
11. Bonnington, J. K., and McNaughton, P. A. (2003) J. Physiol. 551, 433–446
12. Numazaki, M., Tominaga, T., Takeuchi, K., Murayama, N., Toyooka, H., and Tominaga, M. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 8002–8006
13. Elhage, G., Hu, H.-J., Glauers, R. S., Zhu, W., Wang, H., Brasier, D. J., Oxford, G. S., and Gereau, R. W. (2003) Neuron 35, 721–731
14. Arzubi, M. K., Krause, J. E., Elde, R., Hoobler, R. K., Bamburg, J. R., and Basbaum, A. I., Chao, M. V., and Julius, D. (2001) Nature 411, 957–962
15. Caterina, M. J., Leffler, A., Malmberg, A. B., Martin, W. J., Traffon, J., Petersen- Zeitz, K. R., Kaltzenburg, M., Basbaum, A. I., and Julius, D. (2000) Science 286, 306–313
16. Davis, J. B., Gray, J., Gunthorpe, M. J., Hatcher, J. P., Davey, T. P., Overend, P., Harries, M. J., Latham, J., Clapham, C., Atkinson, K., Hughes, S. A., Rance, K., Grau, E., Harper, J. P., Pugh, L. C., Bogom, S., Randall, A., and Sheard, R. E. (2000) Nature 405, 183–187
17. Garcia-Martinez, C., Humet, M., Planells-Cases, R., Gomis, A., Caprini, M., Viana, F., De La Peña, E., Sanchez-Baeza, F., Carbonell, T., De Felipe, C., Perez-Pay, E., Belmonte, C., Messegueur, A., and Ferrer-Montiel, A. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 2374–2379
18. Clapham, D. E., Runnels, I. W., and Strubbing, C. (2001) Nat. Rev. Neurosci. 2, 387–396
19. Smith, G. D., Gunthorpe, M. J., Kelsell, R. E., Hayes, P. D., Beilby, P., Facer, P., Wright, J. E., Jermann, J. C., Walhin, J. P., Oui, L., Eperton, J., Charles, K. J., Smart, D., Randall, A. D., Anand, P., and Davis, J. B. (2002) Nature 418, 186–190
20. Caprini, M., Gomis, A., Cabedo, H., Planells-Cases, R., Belmonte, C., Viana, F., and Ferrer-Montiel, A. (2003) EMBO J. 22, 3004–3014
21. Cabedo, H., Luna, C., Fernandez, A. M., Gallar, J., and Ferrer-Montiel, A. (2002) J. Biol. Chem. 277, 19065–19112
22. Blanes-Mira, C., Ibanez, C., Fernandez-Ballester, G., Planells-Cases, R., Fernandez-Paya, E., and Ferrer-Montiel, A. (2001) Biochemistry 40, 2234–2242
23. Mazerolles, R., Toth, Z. E., Gerthek, D., Nunn, A. R., Krause, M. R., Krause, J. E., Elde, R., Guo, A., Blumberg, P. M., and Szallasi, A. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 3655–3660
24. Huang, S. M., Bisogno, T., Trevisani, M., Al-Hayani, A., De Petrocellis, L., Fezza, F., Tognett, M., Petros, T. J., Krey, J. F., Chu, C. J., Miller, J. D., Davies, S. N., Geppetti, P., Petersen-Zeitz, K. R., Koltzenburg, M., Basbaum, A. I., Chao, M. V., and Julius, D. (2001) Nature 411, 957–962
25. Greka, A., Navarro, B., Lancea, E., Duggan, A., and Clapham, D. E. (2003) Nat. Neurosci. 6, 837–845
25672

Receptor Mobilization by PKC Signaling

30. Ahmari, S. E., Buchanan, J., and Smith, S. J. (2000) Nat. Neurosci. 3, 445–451
31. Zhu, H., Hille, B., and Xu, T. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 17055–17059
32. Yang, Y., Udayasankar, S., Dunning, J., Chen, P., and Gillis, K. D. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 17060–17065
33. Lan, J. Y., Skeberdis, V. A., Jover, T., Grooms, S. Y., Lin, Y., Araneda, R. C., Zheng, X., Bennett, M. V., and Zukin R. S. (2001) Nat. Neurosci. 4, 382–390
34. Yao, Y., Ferrer-Montiel, A., Montal, M., and Tsien, R. Y. (1999) Cell 98, 475–485
35. Eaton, B. A., Haugwitz, M., Lau, D., and Moore, H. P. (2000) J. Neurosci. 20, 7334–7344
36. Jahn, R., and Südhof, T. C. (1999) Annu. Rev. Biochem. 68, 863–911
37. Schiavo, G., Matteoli, M., and Montecucco, C. (2000) Physiol. Rev. 80, 717–766
38. Hu, H.-J., Bhave, G., and Gereau, R. W., IV (2002) J. Neurosci. 22, 7444–7452
39. Nakara, K., Okada, M., and Nakanishi, S. (1997) J. Neurochem. 69, 1467–1475
40. Nakata, T., Terada, S., and Hirokawa, N. (1998) J. Cell Biol. 140, 659–674
41. Chheda, M. G., Ashery, U., Thakur, P., Rettig, J., and Sheng, Z. H. (2001) Nat. Cell Biol. 3, 331–338
42. Haberman, Y., Grimberg, F., Fukuda, M., and Sagi-Eisenberg, R. (2003) J. Cell Sci. 116, 4307–4318
Regulated Exocytosis Contributes to Protein Kinase C Potentiation of Vanilloid Receptor Activity
Cruz Morenilla-Palao, Rosa Planells-Cases, Nuria García-Sanz and Antonio Ferrer-Montiel

J. Biol. Chem. 2004, 279:25665-25672.
doi: 10.1074/jbc.M311515200 originally published online April 5, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M311515200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2004/04/21/M311515200.DC1

This article cites 42 references, 16 of which can be accessed free at http://www.jbc.org/content/279/24/25665.full.html#ref-list-1