Protein Kinase Ca Is Required for Vanilloid Receptor 1 Activation

EVIDENCE FOR MULTIPLE SIGNALING PATHWAYS*

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Zoltan Olah‡, Laszlo Karai, and Michael J. Iadarola

From the Neuronal Gene Expression Unit, Pain and Neurosensory Mechanisms Branch, NIDCR, National Institutes of Health, Bethesda, Maryland 20892

Activation of vanilloid receptor (VR1) by protein kinase C (PKC) was investigated in cells ectopically expressing VR1 and primary cultures of dorsal root ganglion neurons. Submicromolar phorbol 12,13-dibutyrate (PDBu), which stimulates PKC, acutely activated Ca2+ uptake in VR1-expressing cells at pH 5.5, but not at mildly acidic or neutral pH. PDBu was antagonized by bisindolylmaleimide, a PKC inhibitor, and ruthenium red, a VR1 ionophore blocker, but not capsazepine, a vanilloid antagonist indicating that catalytic activity of PKC is required for PDBu activation of VR1 ion conductance, and is independent of the vanilloid site. Chronic PDBu dramatically down-regulated PKCα in dorsal root ganglion neurons or the VR1 cell lines, whereas only partially influencing PKCβ, -δ, -ε, and -ζ. Loss of PKCα correlated with loss of response to acute re-challenge with PDBu. Anandamide, a VR1 agonist in acidic conditions, acts additively with PDBu and remains effective after chronic PKC down-regulation. Thus, two independent VR1 activation pathways can be discriminated: (i) direct ligand binding (anandamide, vanilloids) or (ii) extracellular ligands coupled to PKC by intracellular signaling. Experiments in cell lines co-expressing VR1 with different sets of PKC isozymes showed that acute PDBu-induced activation requires PKCα, but not PKCε. These studies suggest that PKCα in sensory neurons may elicit or enhance pain during inflammation or ischemia.

In dorsal root ganglia, the vanilloid receptor type 1 (VR1) is highly expressed in the small size neurons, which are involved in transmitting noxious heat and chemical stimuli from the periphery. Pungent vanilloids, such as capsaicin (CAP) or resiniferatoxin (1–4), and eicosanoids, combined with low extracellular pH (<6.5) (5, 6) can activate VR1-positive neurons cultured from dorsal root ganglia (DRG), and cells ectopically expressing VR1 (5). In addition, evidence is accumulating that protein kinases, including PKA and PKC, may directly induce or potentiate VR1 activity (7–9). However, the specific PKC isozyme(s) and exact mechanism(s) leading to channel opening are under active investigation. Acute application of phorbol esters sensitizes knee joint sensory nerves (10), and enhances neuronal ionic currents activated by a noxious thermal stimulus (11, 12), but these studies did not connect PKC activation to VR1 activation. PKC also seems to be an important mediator of bradykinin- and epinephrine-induced nociceptor sensitization and hyperalgesia (13–16).

Thus, PKC appears to be an effector in multiple signal transduction cascades in primary afferent neurons. However, PKC is not a “single entity,” but a family of at least 11 serine-threonine kinases with different protein substrate specificities (17–19). Whereas there are functional differences between PKC isozymes, it is not exactly known which isozymes contribute to nociceptive processing. One approach to demonstrate specific involvement of a particular PKC isozyme is targeted gene deletion. Knockout of the PKCα isozyme in mice suggested that this enzyme, which is normally highly expressed in specific second order neurons in spinal inner lamina II, was involved in the proper processing of incoming afferent nociceptive signals (20–23).

Another approach to identify specific isozyme participation is selective down-regulation of PKC isotypes by exposure to active phorbol esters. Acute treatment (i.e. 1 μM PDBu for 10 min) not only activates classical and novel PKC isozymes but also induces enzyme translocation to the plasma membrane. Upon chronic exposure (~24 h) this leads to isozyme-selective proteolysis and differential down-regulation. Either classical PKC isozymes, activated by diacylglycerol and Ca2+, or novel PKC isozymes, activated by diacylglycerol only, can be specifically down-regulated by micromolar doses of PDBu, but to different degrees (24). In primary sensory neurons prepared from 2-day-old rats, Cesare et al. (12) reported expression of only 5 isoforms, PKCβ1, -β11, -δ, -ε, and -ζ. Upon treatment with bradykinin, an endogenous algic peptide, only PKCβ1 was determined to translocate to the plasma membrane in the DRG cultures (12). In contrast to the isozyme pattern detected in sensory neurons from newborns, PKCα, an additional isozyme was positively identified in DRG cultures prepared from 3-month-old rats, which may regulate VR1 in adults (25). Bradykinin was also found to release VR1 from phosphatidylinositol 4,5-bisphosphate-mediated inhibition, a mechanism proposed for pain-specific channel opening (26).

The intracellular signaling pathways upstream of PKC isoforms in nociceptive afferents still represent open questions. Receptors for histamine, peptides, different allergens, and neuronal growth and survival factors and eicosanoids, which are connected via phospholipases (i.e. phospholipases C and D) to PKC signaling, are all good candidates to modulate VR1 activity of sensory neurons. Nerve growth factor (NGF), abundantly synthesized de novo after spinal cord injury and inflammation, was noted to couple to PKC. Administration of

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‡ To whom correspondence should be addressed: Bldg. 49, Rm. 1A19, National Institutes of Health, 49 Convent Dr., MSC-4410, Bethesda, MD 20892-4410. Fax: 301-402-0667; E-mail: zoltan.olah@nih.gov.
* The abbreviations used are: VR1, vanilloid receptor; CAP, capsaicin; CPZ, capsazepine; DRG, dorsal root ganglion; [Ca2+]i, intracellular free calcium; VR1Δ, C-terminal x-tagged vanilloid receptor; NGF, nerve growth factor; MES, 2-(N-morpholino)ethanesulfonic acid; PKC, protein kinase C; PDBu, phorbol 12,13-dibutyrate; ANA, anandamide; MBP, maltose-binding protein; BIS, bisindolylmaleimide.
NGF to neonatal and adult rats in vivo was reported to produce hyperalgesia through activation of PKC (27). Axonal growth and differentiation experiments in cultured PC-12 rat pheochromocytoma cells pointed toward the novel PKCε, a Ca2+-insensitive isotype, as an effector enzyme for NGF-mediated intracellular signaling (28, 29). NGF pretreatment also was noted to potentiate CAP-induced inward currents in voltage-clamped DRG neurons, suggesting either synergy or additively (30). Moreover, other neurotrophins, such as neurotrophins-3 can influence the central terminals of their respective afferent neurons after injury (31). Among several mRNAs, NGF can induce transcription of VR1 in dissociated cultures of adult DRG, as well as VR1 protein levels in undamaged DRG neurons after partial nerve injury (32, 33). Together these observations suggest that NGF may engender hyperalgesia either by de novo transcription and translation or by direct, PKC-mediated activation of VR1. The regulation of VR1 by PKC is also supported by the PKCε-mediated increase in VR1 ion currents in DRG neurons and in cells ectopically expressing recombinant vanilloid receptor (8). In addition, or as an alternative to direct signaling via PKC, NGF was observed to act as a liberator of VR1 from phosphatidylinositol 4,5-bisphosphate-mediated blockade (26).

In the current study, we present evidence suggesting a direct role of PKC in VR1 activation. In particular, full expression of PKCε was found to be essential for acute activation of VR1 with PDBu, a synthetic, stable agonist of classical and novel PKCs. This action was examined in primary DRG cultures as well as NIH 3T3 and HeLa cells stably transfected to express VR1ε, a C-terminal epitope-tagged VR1 recombinant (4). Each system has a different, complementary composition of PKC isozymes, which permitted discrimination of the isoforms that affected VR1 function. NIH 3T3 cells were determined to express classical PKCα, novel PKCθ and ε, and atypical PKCζ isozymes, respectively (34). In contrast, PKCε, a previously proposed upstream regulator of VR1 was not expressed in HeLa and was not required for activation of ectopically expressed VR1. Our findings in DRG cultures, VR1ε-in-NIH 3T3 and VR1ε-HeLa cell lines, strongly suggest that PKCε can sensitize and/or activate VR1 in the absence of endogenous ligands, including anandamide (ANA), an endovanilloid/eicosanoid agonist of VR1 in acidosis (5). In VR1ε-HeLa cells, another permanent line we established, acute PDBu induced ionophore activity of VR1 even in the absence of PKCε. ANA activates VR1 in an additive fashion with PKCε, yet is independent of PKC, because ANA activation is retained after chronic PDBu-mediated down-regulation of PKCε. Similar to ANA (5), the PKCε activation of VR1 requires a drop of extracellular pH < 6.5, indicating that the protonated VR1 conformer serves as a better substrate of PKC. Our observations emphasize the conditional requirements for the actions of endovanilloids and PKC and their capacity to work in a concerted, additive fashion on the nociceptive nerve terminal.

**EXPERIMENTAL PROCEDURES**

**Preparation of VR1ε Expressing NIH 3T3 Cell Line—**C-terminal-tagged chimeric rat VR1ε was prepared in the pMTH vector plasmid vector (35), as described previously (4). To develop cell lines permanently expressing VR1ε, we used mouse NIH 3T3 fibroblasts and human HeLa adenocarcinoma-derived cells. The parental lines were determined to have low levels of Ca2+ transport that were not altered by treatment with vanilloids, ANA, or PDBu. To avoid toxicity that occurs with VR1 overexpression, VR1ε was expressed in the cell lines using only the basal activity of the metallothionein promoter, as described earlier (5). For the purpose of clarity, in sentences where PKCε is discussed in conjunction with VR1ε (e.g. VR1ε-HeLa) we refer to it as VR1 (e.g. VR1-HeLa).

**DRG Culture—**DRG neuron-enriched cultures were prepared from embryonic rats (E16) (4). Briefly, DRGs were dissected and then processed in fresh dissection medium (Lebowitz medium, Invitrogen) until plated in Dulbecco’s modified Eagle’s medium. The Dulbecco’s modified Eagle’s medium contained 20 mM HEPES (to prevent acidification and stabilize pH at 7.4), 7.5% fetal bovine serum, 7.5% horse serum, 5 mg/ml uridine supplemented with 2 mg/ml 5-fluoro-2-deoxyuridine, and 45 mg/ml ascorbic acid to inhibit cell death and promote survival and differentiation, respectively. Surfaces for cell culture were coated with poly-lysine and laminin. Cells were seeded on 25-mm glass coverslips or on multiwell microtiter plates. Cultures were selected in this medium for 1 week, at which point well differentiated neurons dominated the population. Primary DRG cultures in this stage were used in radioactive microscopic video imaging of Ca2+ uptake assays and on dishes with multidimensional criteria. Cultures were selected in this medium for 1 week, at which point well differentiated neurons dominated the population. Primary DRG cultures in this stage were used in radioactive microscopic video imaging of Ca2+ uptake assays and on dishes with multidimensional criteria. Cultures were selected in this medium for 1 week, at which point well differentiated neurons dominated the population. Primary DRG cultures in this stage were used in radioactive microscopic video imaging of Ca2+ uptake assays and on dishes with multidimensional criteria.

**Down-regulation and Recovery of PKC—**To down-regulate PKC isoforms, DRG neurons and VR1 expressing NIH 3T3 and HeLa cell lines were chronically treated with 2 μM PDBu in complete medium for 24 h. To determine the effect of chronic PDBu on PKC protein levels, individual PKCδs were analyzed with isozyme-specific antibodies. The influence of PKC down-regulation on acute ANA and PDBu-induced Ca2+ uptake was studied in short-term (10 min) experiments, as described above. Recovery of PKCδs from the down-regulated state (2 μM PDBu, 24 h) was monitored in confluent cultures of VR1ε-NIH3T3 cells. Cells were chronically treated in 96-well plates with PDBu (2 μM, 24 h), one set of 12 wells at a time, then changed back into complete growth medium without PDBu and cultured over the 15-day period of the recovery experiment, changing the growth medium every 2-3 days. Western blotting experiments were carried out on nonneuronal cells and chronically treated on the 6th, 7th, 13th, and 15th day before cell harvest. In a parallel set of cultures, the effect of recovery of PKCδs was studied using acute PDBu-induced Ca2+ uptake as the VR1-dependent end point.

**Ca2+ Transport—**45Ca2+ uptake experiments were carried out on 1-week-old primary DRG cultures (3 × 104 cells/well) and on established VR1ε-NIH 3T3 and HeLa cell lines (3 × 104 cells/well), which were seeded 1 day before use. Immediately before the assay, cells were adapted to room temperature (24°C) for 5 min in Hanks’ balanced salt solution (pH 7.4), supplemented with 10 μM Ca2+ and 0.1 mg/ml bovine serum albumin (HCB). 45Ca2+ uptake was performed for 10 min at 24°C in HCB using 0.2 μCi of 45Ca2+ as radioactive tracer in a 200-μl final volume. To determine the pH dependence of the 45Ca2+ uptake, HCB was buffered with 20 mM Tris-HCl, adjusted to the indicated pH with 1 M HCl (HCBTM). To stop 45Ca2+ uptake, cells were rapidly changed back into 1 ml of HCB, washed two additional times with 1 ml of HCB, and then lysed in 200 μl/well of RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 5 mM Ca2+, 0.5% sodium deoxycholate, 1 mM Mg2+, 1 μg/ml aprotinin, 1 mM Na3VO4, 1 mM PMSF, 100 μM sodium fluoride, and 10 μg/ml leupeptin). After 30 min, aliquots of the solubilized cell extracts were counted in a liquid scintillation counter.

**Fluorescent Video Microscopic Determination of [Ca2+]i—**For determination of [Ca2+], an Olympus BX 60 microscope equipped with a low-light fluorescence intensifier system was used, as described earlier (36). DRG cultures were preloaded with 5 μM fluo-4 AM dye for 30 min at 34°C, and then washed three times in HCB to remove excess dye and bring the cells back to dark for at least 15 min before starting experiments. Recordings were carried out in a closed imaging chamber (PH-2, Warner Instrument Corp., Hamden CT), which was perfused with a pump (Minipuls 3, Gilson, France). The emitted fluorescence intensity was calculated from images taken at 10-s intervals by the synapse 3.6e software, an image acquisition and analysis program, as recommended by the manufacturer (Synergy Research, Silver Spring, MD). To monitor vanilloid-induced changes in [Ca2+], after 1 min of baseline recording, DRG neuron cultures were perfused with 1 μM CAP in HCBTM for 30 s, then the medium was changed back to drug-free HCBTM.

**Preparation of VR1 Specific Antibody and Western Blotting—**The hydrophilic C-terminal fragment of rat VR1 (Met684-Lys703), extended with a C-terminal (His6) tag was amplified by the polymerase chain reaction employing GGAAGGATTCACGAATTCATGGGTGAGACCGTCGACAAAGATT as forward primer and TGTCCGACCTGCAGAACTAATGATGTAGTATGATGATGTTTTCCTCCCTGGACCATGGAAATC as reverse primer. The PCR-amplified cDNA fragment was incorporated into the EcoRI and SalI restriction sites from the forward and reverse primers, respectively, and these sites were used to insert the fragment into the pMA-LEc2x (New England BioLabs) prokaryotic expression vector. The peptide was cloned and downstream region for maltose-binding protein (MBP). Escherichia coli cells were transformed with the MBP-VR1(His6) construct and production of the fusion protein was induced by 1 mM isopropyl-1-thio-β-n-galactopyranoside for 3 h. The MBP-VR1(His6) chimeric protein was double affinity purified using the MBP and His tags, consecutively, as recommended by the manufacturers (New England BioLabs and Novagen). The affinity
purified MBP-VR1C(His), protein was used to immunize two rabbits. After 8 weeks of immunizations at biweekly intervals, sera were tested at 1:500 dilutions in Western blotting experiments using SDS extracts prepared from neuron-enriched DRG cultures of E16 embryonic rats.

Materials—ANA (1:4 in a soybean oil:water emulsion), was purchased from Tocris and PDBu from Calbiochem. 45Ca was purchased from ICN. PKC isozyme-specific antibodies were obtained as follows: PKCα, clone M6 mouse monoclonal from Upstate Biotechnology; PKCβ and -ε affinity purified polyclonals from Invitrogen; and PKCγ from Calbiochem. Affinity purified anti-RAKIGQGTKAPEEKTANTISK, a specific PKCα peptide antibody was prepared in rabbits and characterized as described earlier (37).

RESULTS

To study PKC-dependent up-regulation of VR1, permanent cell lines ectopically expressing an e-epitope-tagged recombinant of VR1, were established in both NIH 3T3 (4) and HeLa cells. VR1 specificity and pH dependence of acute (10 min) PDBu-induced 45Ca2+ transport were studied in the parental, immortalized NIH 3T3 mouse fibroblast and VR1e-NIH 3T3 cell lines at pH 5.5 and 7.5, as indicated (Fig. 1). In the parental line, the basal Ca2+ uptake at pH 7.5 was about 4 times higher than at pH 5.5, and addition of PDBu to the assay medium had little or no effect on the basal uptake at either pH. Expression of recombinant VR1 Elevated the baseline compared with parental NIH 3T3 cells at either pH 5.5 or 7.5. At pH 6.5, 7.0, and 7.5 only minor increases in calcium uptake occurred with ascending PDBu concentrations. However, the net change for VR1e-NIH 3T3 cells was not different from the parental cell line at pH 7.5 (open triangles), indicating that these elevations are not dependent on the expression of VR1. The marked transition in ionophore activity occurs with PDBu treatment when the VR1e-NIH 3T3 cells are at pH 5.5. Addition of PDBu at submicromolar concentrations increased Ca2+ transport in a concentration-dependent manner between 3- and 4-fold over the baseline (Fig. 1A and B). BIS (25 μM) added together with progressively increasing concentrations of PDBu completely inhibited the PDBu-inducible portion of 45Ca2+ uptake in VR1e-NIH 3T3 cells, indicating that the catalytic activity of PKC is required for up-regulation of the VR1 channel. Each point represents the mean ± S.E. of triplicate determinations. Similar results were obtained in two independent experiments also performed in triplicate. b, PDBu-activated calcium uptake was inhibited by ruthenium red (RR), a VR1 channel blocker, but not by CPZ, an antagonist at the vanilloid-binding site. Similar results were obtained in two additional experiments carried out in triplicate.

To further study the PKC specificity of PDBu-induced, VR1-mediated 45Ca2+ uptake at pH 5.5, bisindolylmaleimide (BIS), an inhibitor directed to the catalytic ATP-binding site of PKC, was co-incubated with increasing concentrations of PDBu (Fig. 2A). BIS (25 μM) added together with progressively increasing concentrations of PDBu completely inhibited the inducible portion of the 45Ca2+ uptake, but had only slight or no effect on baseline uptake in VR1e-NIH 3T3 cells. Studies with VR1 selective inhibitors showed that acute PDBu-induced 45Ca2+ transport was nearly completely inhibited by 10 μM ruthenium red (a blocker of the VR1 ionophore), but not with a high concentration (25 μM) of capsaicin (CPZ, a competitive antagonist at the vanilloid ligand–binding site) (Fig. 2b).
Western blotting with specific antibodies was employed to analyze expression of VR1 and different PKC isoforms in DRG cultures and VR1-NIH 3T3 cells (Fig. 3). The DRG cultures from E16 embryonic rats were enriched for neurons by a 1-week treatment with 5-fluoro-2-deoxyuridine to eliminate dividing cells. Cultures were also treated with NGF to promote neuronal differentiation. Before Western blotting, the DRG cultures were morphologically and functionally characterized with phase-contrast and fluorescence microscopy. After 1 week, the DRG cultures typically contained large (25–30 μm in diameter), medium (~15–25 μm), and small size (7–10 μm) neurons in close to equal ratio. The CAP-induced increase of intracellular [Ca²⁺], in the DRG cultures is one of the markers we used to identify VR1-expressing nociceptors. After loading cultures with fluo-4, a Ca²⁺-sensitive fluorescent dye, cells within the latter two populations (~30% of the total) responded to 1 μM CAP with elevated green fluorescence (Fig. 3, a versus b).

VR1 expression was determined immunochemically by a rabbit antibody, raised against MBP-VR1C(His)₆, a soluble C-terminal fragment of rat VR1. The immune sera recognized a prominent band at 92 ± 4 kDa in DRG extracts (Fig. 3c), which corresponds well with the theoretical molecular mass of rat holo-VR1 (94.9 kDa). In addition to the 94.9-kDa protein, our antibody detected a 114-kDa glycosylated form of VR1 (38), in addition to the 94.9-kDa protein. The CAP-induced increase of intracellular [Ca²⁺], in the DRG cultures is one of the markers we used to identify VR1-expressing nociceptors. After loading cultures with fluo-4, a Ca²⁺-sensitive fluorescent dye, cells within the latter two populations (~30% of the total) responded to 1 μM CAP with elevated green fluorescence (Fig. 3, a versus b).

PKCβ, -δ, -ε, and -ζ isoforms; the latter have been reported previously in cultures from neonatal animals (12). Chronic PDBu treatment had the most dramatic effect on the PKCα isoform, which was almost completely down-regulated in both DRG neurons and VR1-NIH 3T3 cells (Fig. 3, c and d). However, chronic PDBu produced only a partial down-regulation of the PKCβ, -δ, and -ε isoforms in DRG neurons. Similar effects were seen in the VR1-NIH 3T3 cells (Fig. 3, c and d) except that no PKCβ was measured, which is in accordance with previous observations in the parental NIH 3T3 cell line (34) (Fig. 3d). As expected from a nonphorbol-binding isotype, little or no change was noted in the levels of PKCζ in either cell system.

The effects of chronic PDBu-induced down-regulation on ⁴⁺Ca²⁺ uptake were studied in a parallel set of DRG cultures as those analyzed above for VR1 and PKC isoform expression. Chronic PDBu almost completely eliminated the acute PDBu-inducible activity as assayed by a short, 10-min re-exposure to PDBu in the presence of ⁴⁺Ca²⁺ (Fig. 4a). Elimination of the acute PDBu effect correlated with the complete down-regulation of PKCα in DRG cultures chronically treated with PDBu (Figs. 3e and 4a). In control DRG cultures, addition of ANA, a vanilloid agonist in acidic conditions, induced ⁴⁺Ca²⁺ uptake at pH 5.5 with similar kinetic parameters as previously characterized (5). After chronic PDBu treatment, the ANA-induced Ca²⁺ transport remained intact, although shifted slightly into a lower affinity region; an EC₅₀ of ~25 μM compared with ~10 μM was obtained in the experiment shown (Fig. 4b). Parallel experiments repeated in VR1-NIH 3T3 cells also resulted in complete inhibition of acute PDBu-induced ⁴⁺Ca²⁺ uptake (Fig. 5a), but left the ANA-elicited ⁴⁺Ca²⁺ transport intact (Fig. 5b).
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property to examine recovery of PKC sensitivity following PDBu-induced down-regulation. Confluent cultures of VR1e-NIH 3T3 cells were treated with 2 μM PDBu for 24 h in complete medium. Recovery of PKCα was monitored for 2 weeks by Western blot analysis using a monoclonal antibody specific for PKCα (Fig. 6, upper panel). No recovery of PKCα from the down-regulated state was detected during the following week (days 6 and 7 after chronic PDBu treatment), however, by 12 days a progressive re-appearance of PKCα was seen and by day 15 nearly complete replenishment had occurred. A set of acute, 0.2 μM PDBu-induced Ca2+ uptake experiments at pH 5.5 were performed in parallel. Coincident with the recovery of PKCα by Western blot, the acute PDBu-induced Ca2+ uptake activity also returned to levels near baseline. These data further support the essential role of PKC in direct activation of VR1.

Activation of VR1 by the endovanilloid ANA and PKC activator PDBu appear to represent two independent and potentially additive mechanisms. The inhibition studies in Fig. 2b show that acute PDBu-induced 45Ca2+ transport is not blocked with 25 μM CPZ, a competitive vanilloid antagonist, but is blocked with 10 μM ruthenium red, which inhibits Ca2+ entry through the VR1 ion channel (99). The VR1 specificity of the 45Ca2+ uptake was analyzed by co-application of either 25 μM ruthenium red or 10 μM CPZ at ascending concentrations of ANA at pH 5.5. Either ruthenium red or CPZ eliminated the ANA inducible portion of 45Ca2+ uptake, indicating that ANA has to specifically interact with VR1 to induce cation transport (Fig. 7). Co-application of 1 μM PDBu with each concentration of ANA shifted the dose-response curve to the left and augmented 45Ca2+ uptake in a manner characteristic of additive and independent routes for VR1 activation (Fig. 7).

A previous study (8) suggested PKCα as a unique upstream regulator of VR1 in DRG nociceptors. The present experiments suggest rather that PKCα is required for intracellular activation of VR1 in DRG neurons. A remarkable correlation between down-regulation of PKCα and loss of acute PDBu-induced, VR1-mediated Ca2+ transport was determined both in embryonic rat DRG cultures and in VR1e-NIH 3T3 cells. To examine

FIG. 5. VR1e-NIH 3T3 cells. Effect of chronic (2 μM, 24 h) PDBu treatment was studied on the same batch of VR1e-NIH 3T3 cells analyzed for VR1 and PKC isozymes with Western blotting. a, chronic exposure completely inhibited the acute effect of PDBu on VR1-mediated 45Ca2+ uptake in NIH 3T3 cells expressing VR1. b, chronic treatment did not affect ANA-induced 45Ca2+ transport. Similar results were obtained in a second independent experiment.

FIG. 6. Effect of PKCα on VR1 after recovery from down-regulation. VR1e-NIH 3T3 cells were seeded and grown to confluency in 96-well plates. Cells were treated with 2 μM PDBu for 24 h in complete medium, and then changed back to medium without PDBu. Acute PDBu-induced Ca2+ uptake experiments were carried after the indicated recovery days and the same batch of cell populations were harvested for Western analysis. PKCα was not detectable 1 week after down-regulation (R6 and R7), however, near complete recovery of PKCα was determined after 2 weeks recovery (R12 and R15). Parallel to the results of Western analysis, the acute PDBu-induced Ca2+ uptake over baseline (B.L.) recovered 70–80% of baseline by the 2nd week (experiments performed in duplicate).

FIG. 7. ANA and acute (10 min, 1 μM) PDBu activate VR1 calcium flux in an additive manner. Experiments were carried out in VR1e-NIH 3T3 cells in acidic assay medium (pH 5.5). The maximum effect of ANA induced 45Ca2+ uptake ~5 times above the baseline at pH 5.5. An additive effect was seen with co-incubation of 1 μM PDBu and increasing concentrations of ANA (filled triangles). The addition of PDBu elevated the Vmax of Ca2+ transport, and shifted the Emax of ANA from ~10 to ~4 μM in VR1e-NIH3T3 cells. As expected, CPZ, a competitive inhibitor of vanilloid ligand binding, and ruthenium red (RR), a selective channel blocker of VR1, almost completely inhibited ANA action. Each point represents the mean ± S.E. of triplicate determinations. Similar results were obtained in two independent experiments also performed in triplicate.
the requirement for PKCe in VR1 activation, a permanent VR1-expressing HeLa cell line (VR1-HeLa) was established. This cell line exhibits a different PKC isoform composition (notably PKCe is not expressed) (40) than either the NIH 3T3 cells or the DRG neurons. The HeLa line stably expressing VR1 showed approximately a 10-fold elevation of 45Ca2+ uptake above baseline when treated with a maximal dose of CAP (10 μM) or resiniferatoxin (10 nM) at pH 5.5 (Fig. 8a). We corroborated the lack of PKCe in the VR1 expressing HeLa cells (40) with isoform-specific Western blots (Fig. 7b). Similar to parental cells, the VR1-HeLa subclone expresses PKCα, β, γ, and ζ, but not ε (Fig. 8b). As seen in DRG neurons and NIH 3T3 cells, chronic treatment with PDBu almost completely down-regulated PKCα from VR1-expressing HeLa cells. At the same time little or no change in the levels of PKCβ, γ, and ζ or VR1 were detected (Fig. 8). Coincident with the loss of PKCα, the VR1-expressing HeLa cells exhibited inhibition of acute PDBu-induced 45Ca2+ uptake (Fig. 9a), without significant impact on Ca2+ uptake induced by the VR1 agonist ANA (Fig. 9b). As an aside, we note that the VR1-expressing HeLa line showed about 5-fold higher affinity toward ANA and high concentrations of ANA (above 10 μM) caused an inhibition of 45Ca2+ uptake, determined at pH 5.5, which was not seen in VR1-expressing NIH 3T3 cells (Figs. 5b and 6 versus 9b).

**DISCUSSION**

PKC-mediated up-regulation of VR1 was investigated in DRG neurons, NIH 3T3, and HeLa cell lines, with the latter two permanently expressing VR1ε, a C-terminal ε-epitope-tagged recombinant of rat vanilloid receptor type 1. We observed that PDBu, a synthetic agonist capable of binding to the regulatory domain of classical and novel PKC isoforms, activated Ca2+ uptake in the absence of any added vanilloid ligand in all three cell preparations. PDBU-evoked channel activation exhibited a strong pH dependence: VR1ε-NIH 3T3 and VR1ε-HeLa cells responded to less than 1 μM PDBu at acidic pH (pH 5.5), but not at pH 6.5 or higher (up to pH 7.5). We previously observed that ANA activated VR1 45Ca2+ uptake in cells topoically expressing VR1 or in primary DRG cultures only when the assay medium was acidic (pH < 6.5) (5). Protonation of VR1 in an acidic environment does not directly cause channel opening (6) or Ca2+ uptake (Fig. 1), but increases the affinity of the receptor for vanilloid and eicosanoid ligands (5). This observation prompted us and others to suggest that VR1 exists in two distinct conformations with H+ serving as an allosteric switch (5, 41, 42). Between pH 6.5 and 7.5, protons dissociate from VR1, perhaps in the pore loop region (43, 44), yielding a conformer with lower affinity toward vanilloids and vanilloid-like eicosanoids such as ANA (5, 6). However, the protonated form of VR1 (between pH 6.5 and 5.5) displays an increased affinity toward vanilloid ligands, which coincides with a decreased capacity for cation transport (5). The fact that PDBu induces 45Ca2+ transport through VR1 when the medium is acidic is consistent with the hypothesis that the protonated conformer of VR1 is also the preferred substrate of PKC. Sequence analysis of rat and human VR1 indicates 18 intracellular sites that conform to PKC consensus motifs for potential phosphorylation. This highlights the need for determining the exact phosphorylation site(s) involved in VR1 regulation.

Studies with selective inhibitors of VR1 showed that PDBu can induce 45Ca2+ transport in the presence of 25 μM CPZ, a competitive inhibitor of vanilloid ligand binding (Fig. 2b). In contrast to CPZ, ruthenium red, a blocker of the VR1 ion channel (39) can prevent 45Ca2+ uptake activated by both vanilloid agonists or PKC (Figs. 2b and 6). In addition, results with BIS indicate that the catalytic activity of PKC is required for VR1-mediated 45Ca2+ uptake (Fig. 2). BIS eliminates the effect of acute PDBu when they are co-applied to VR1ε-NIH 3T3 and VR1ε-HeLa cells, suggesting that phorbol esters activate VR1 only indirectly, via intact catalytic activity of PKC. In contrast to a previous report (26), but in concert with findings of others, our PKC down-regulation experiments also indicate that PDBu does not directly interact with VR1 (45-47). However, from the literature, it was not clear which isoforms are expressed and mediate VR1 activation in sensory ganglia neu-
Direct Activation of VR1 by PKCα

 Previously, HeLa, a human carcinoma cell line, was found not to express PKCε (40) and we confirmed its absence in our clonal VR1-HeLa cell line by Western blot analysis (Fig. 8). The loss of PKCα correlated with loss of the acute activation by PDBu on $^{45}$Ca$^{2+}$ uptake in all three cell cultures. However, direct activation through the vanilloid agonist route remained intact because stimulation by ANA exhibited little alteration (Figs. 4, 5, and 9). These experiments indicate an important role of PKCα isozyme on VR1 function through intracellular signaling pathways. Down-regulation results in DRG and VR1 expressing cell lines suggest that substantially reduced levels of PKCα (Figs. 3, 4, and 8) compromise, whereas the absence of PKCε does not affect acute response to PDBu (Fig. 8a), again emphasizing an essential role for PKCα in VR1 activity. In contrast to the behavioral alteration noted in PKCε mutant mice, results at the cellular levels with PDBu support a more direct role of PKCα in VR1 activation. A PKCε-derived, selective inhibitor peptide decreased the mechanical hyperalgesia only incompletely, produced by epinephrine injection into the hindpaw of normal rats. Together with results of others (8, 16, 26) it seems that PKCε might be involved in multiple nociceptive signaling pathways, including epinephrine-induced hyperalgesia in mechanosensor neurons, which lack VR1 expression (1). The partially attenuated pain phenotype noted in PKCε knock-out mouse nociception models does not rule out a function for other PKC isotypes and PKCε may serve as an alternative upstream regulator of VR1 in nociceptors (15) in addition to PKCα. Taken together, these data suggest that, in addition to heat, at least two independent signaling pathways can regulate the ionophore function of VR1 and hence nociceptive and inflammatory pain signaling. The first pathway utilizes vanilloid ligands or eicosanoid/endovanilloid/endocannabinoid compounds such as ANA, leukotriene B (4), 12- and 15-(S)-hydroperoxyeicosatetraenoic acids, and 5- and 15-(S)-hydroxyeicosatetraenoic acids (5, 6). The second pathway uses PKC, and likely functions as an intracellular second messenger-coupled effector for extracellular inflammatory agents. Both pathways are conditionally dependent on pH and we hypothesize that the protonated form of VR1 is the preferred substrate for PKC. The two pathways produce additive effects on VR1-mediated $^{45}$Ca$^{2+}$ uptake. However, they can be dissociated, as shown by the selective effect of chronic PDBu down-regulation on acute activation of VR1; ANA/vanilloid signaling is preserved, whereas signaling through the PKC pathway is eliminated. Thus, intracellular signaling by PKC isozymes is not necessary for ligand-induced, direct activation of VR1, but PKC isozymes can open the VR1 ionophore in the absence of extracellular vanilloid-like ligands. The importance of the PKCα isozyme in VR1 regulation, in comparison with the $\epsilon$ isoform, was evident in chronic PDBu in VR1 expressing preparations, despite its lack of the PKCε isoform. ANA and PDBu co-incubation experiments highlight the independence of the two pathways as well as the potential for mutual interaction of nociceptive stimuli to reinforce and sustain pain signaling. The number of different signal transducers that are upstream of different PKC isozymes in primary afferent endings is still an open question. Various agonists implicated in pain, including NGF, bradykinin, and substance P have receptors in nociceptors and are coupled to different phospholipases C and D isoforms. These lipases, upon activation, produce diacylglycerol at the plasma membrane (19, 48, 49),

weeks after the down-regulation, further emphasizes the essential role of this isozyme in VR1 expressing cells.
which is able to activate PKC generally, and in particular the classic PKCα in sensory neurons, as suggested here, or novel PKCε isotypes proposed previously by others (12, 15, 16, 29, 50).

In conclusion, PKC-induced activation of VR1 is a novel regulatory pathway, which deserves further investigation. Similar to endovanilloid ligand-induced ionophore activity of VR1, a drop in pH below 6.5 is necessary for PDBu-evoked calcium uptake. PKCε was proposed previously as one of the likely isozymes to activate VR1 by transmembrane signaling. Here we positively identify PKCε in thoroughly characterized DRG neuron cultures from embryonic rats, as a necessary factor for acute PDBu-induced activation of VR1. The PKCε isoform does not appear to be absolutely necessary for VR1 activation as shown by VR1-HeLa, a permanent cell line not expressing PKCε. An interesting aspect of PKC isoform-specific regulation of VR1 is that certain isotypes may be therapeutic targets for novel pain treatments. Specific inhibition or elimination of PKCε in the periphery may result in effective pain management in nerve injuries where upstream regulators of PKC isoforms are overproduced and may contribute to intractable pain states.

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