Potentiation of glutamatergic synaptic transmission by protein kinase C-mediated sensitization of TRPV1 at the first sensory synapse

Parul Sikand and Louis S. Premkumar

Department of Pharmacology, Southern Illinois University School of Medicine, Springfield, IL 62702, USA

Sensory input from the periphery to the CNS is critically dependent on the strength of synaptic transmission at the first sensory synapse formed between primary afferent dorsal root ganglion (DRG) and superficial dorsal horn (DH) neurons of the spinal cord. Transient receptor potential vanilloid 1 (TRPV1) expressed on a subset of sensory neurons plays an important role in chronic inflammatory thermal nociception. Activation of protein kinase C (PKC) sensitizes TRPV1, which may contribute to the pathophysiology of chronic pain conditions. In this study, we have examined the modulation of TRPV1-mediated enhancement of excitatory synaptic transmission in response to PKC activation. Miniature excitatory postsynaptic currents (mEPSCs) from embryonic rat DRG–DH neuronal cocultures were recorded by patch clamping DH neurons. Capsaicin potently increased the frequency but not the amplitude of mEPSCs in a calcium-dependent manner, suggesting TRPV1-mediated glutamate release from presynaptic terminals of sensory neurons. Continued or repeated applications of capsaicin reduced the frequency of mEPSCs over time. The PKC activator phorbol 12,13-dibutyrate (PDBu) alone increased mEPSC events to a certain extent in a reversible manner but capsaicin further synergistically enhanced the frequency of mEPSCs. The PKC inhibitor bisindolylmaleimide (BIM) abolished PDBu-mediated potentiation of TRPV1-dependent increases in mEPSC frequency, suggesting modulation of TRPV1 by PKC-induced phosphorylation. In addition, at normal body temperatures (∼37°C) PKC-mediated enhancement of mEPSC frequency is significantly decreased by a specific TRPV1 antagonist, suggesting a physiological role of TRPV1 at the central terminals. Furthermore, bradykinin (BK) significantly potentiated TRPV1-modulated synaptic responses by activating the PLC-PKC pathway. Our results indicate that TRPV1 activation can modulate excitatory synaptic transmission at the first sensory synapse and its effects can further be augmented by activation of PKC. Increased gain of sensory input by TRPV1-induced enhancement of glutamate release and its potentiation by various inflammatory mediators may contribute to persistent pain conditions. Selective targeting of TRPV1 expressed on the central terminals of sensory neurons may serve as a strategy to alleviate chronic intractable pain conditions.

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Corresponding author L. S. Premkumar: Department of Pharmacology, Southern Illinois University School of Medicine, Springfield, IL 62702, USA. Email: lpremkumar@siumed.edu

The first sensory synapse formed between the central terminals of the DRG and spinal cord DH neurons is an important site in the pain pathway. The transmission at the first sensory synapse is highly regulated and can be modulated by both pre- and postsynaptic mechanisms, which can influence the final input reaching the brain (Huettner *et al.* 2002). Thus, this synapse is an important site for activity-dependent synaptic plasticity resulting in increased efficacy of synaptic transmission, a phenomenon referred to as central sensitization, which underlies certain modalities of pain (Li & Zhuo, 1998; Woolf & Salter, 2000; Willis, 2002).

The transient receptor potential vanilloid 1 (TRPV1) is a non-selective, calcium-permeable cation channel that is expressed on both the central and peripheral terminals of small to medium diameter sensory neurons (Caterina *et al.* 1997; Tominaga *et al.* 1998; Nakatsuka *et al.* 2002; Baccei *et al.* 2003). TRPV1 is gated by protons, heat, lipid mediators such as anandamide, *N*-arachidonoyl-dopamine (NADA),

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metabolites of arachidonic acid (Zygmunt *et al.* 1999; Hwang *et al.* 2000; Huang *et al.* 2002; De Petrocellis *et al.* 2004), vanilloid compounds such as capsaicin, the pungent ingredient in hot chilli peppers (Caterina *et al.* 1997), and resiniferatoxin (RTX), an ultra-potent agonist obtained from a succulent plant, *Euphorbia resinifera* (Szallasi & Blumberg, 1999; Raisinghani *et al.* 2005).

TRPV1 is widely implicated in various inflammatory and neuropathic pain conditions (Holzer, 2004). Studies using TRPV1 knock-out mice suggest that it is critical in mediating inflammatory thermal hyperalgesia (Caterina *et al.* 2000; Davis *et al.* 2000). Recently, selective ablation of TRPV1-expressing DRG neuronal cell bodies has been reported to be beneficial in treating pain in canine models of bone cancer and arthritis (Karai *et al.* 2004; Brown *et al.* 2005). These studies underscore the importance of TRPV1 in mediating various thermal inflammatory and chronic pain conditions.

Phosphorylation by protein kinase A (PKA) and protein kinase C (PKC) is known to modulate the properties of TRPV1 (Caterina & Julius, 2001; Holzer, 2004). Continuous agonist application results in dramatic calcium-dependent run-down of TRPV1-mediated currents, designated as acute desensitization. On the other hand, a calcium-dependent decrease in TRPV1 responses with repeated agonist applications is referred to as tachyphylaxis. Interestingly, PKA-mediated phosphorylation of TRPV1 prevents tachyphylaxis (Bhave *et al.* 2002; Mohapatra & Nau, 2003), whereas PKC-mediated phosphorylation is known to potentiate heat-, protons- and agonist-activated TRPV1 currents (Cesare & McNaughton, 1996; Premkumar & Ahern, 2000; Vellani *et al.* 2001; Tominaga *et al.* 2001; Crandall *et al.* 2002; Premkumar *et al.* 2004). Importantly, PKC-activating phorbol esters and the inflammatory mediator bradykinin (BK) have been shown to cause dose-dependent lowering of the thermal activation threshold of TRPV1 to below body temperature (Numazaki *et al.* 2002; Sugiura *et al.* 2002). Furthermore, studies in mice lacking different isoforms of PKC have established the pro-nociceptive role of PKC in the spinal cord. PKC-γ knock-out mice failed to develop neuropathic pain syndromes following partial sciatic nerve ligation but demonstrated normal responses to acute nociceptive stimuli (Malmberg *et al.* 1997). In addition, PKC-ϵ knock-out mice did not develop thermal or mechanical hyperalgesia mediated by administration of epinephrine or acetic acid (Khasar *et al.* 1999).

BK is released at the site of tissue injury or in response to inflammation when protease kallikreins cleave the precursor kininogens (Couture *et al.* 2001). The BK receptor, B_2, is constitutively expressed in both primary afferent sensory neurons and spinal cord neurons (Seabrook *et al.* 1997; Wang *et al.* 2005). The role of BK in sensitizing nociceptors in the periphery is well established (Dray *et al.* 1988; Couture *et al.* 2001). However, a recent study suggests that BK may enhance glutamatergic synaptic transmission between spinal cord neurons (Wang *et al.* 2005). BK has been reported to sensitize TRPV1 in dissociated DRG neuronal cultures via second messengers produced downstream to B_2 receptor activation (Shin *et al.* 2002), mainly by relieving the inhibitory effect of phosphatidylinositol-4,5-bisphosphate (PIP_2) (Chuang *et al.* 2001) and by activating protein kinase C (Cesare & McNaughton, 1996; Premkumar & Ahern, 2000; Sugiura *et al.* 2002). However, whether BK can influence TRPV1-modulated synaptic transmission is still unclear. In the present study, we have examined the role of TRPV1 in modulating glutamatergic synaptic transmission at the first sensory synapse. Our results demonstrate for the first time that phorbol esters and BK by activating PKC potentiate excitatory synaptic transmission at the first sensory synapse by sensitizing TRPV1. Significantly, at normal body temperatures, phosphorylated TRPV1 is active and influences synaptic transmission. Some of these data have appeared in an abstract form elsewhere (Sikand & Premkumar, 2005).

Methods

DRG–DH cocultures

All animal experiment protocols used in this study were approved by the Southern Illinois University School of Medicine Animal Care Committee. The animals were cared for according to the standards recommended by the National Institutes of Health (NIH). Pregnant Sprague–Dawley rats were heavily anaesthetized with a lethal dose of Nembutal (80 mg kg^{-1}, I.P.), and E18 embryos were removed. The embryos were decapitated under hypothermic anaesthesia. DRG and spinal cord DH were dissected and collected in L-15 medium (Invitrogen, Carlsbad, CA, USA) under sterile conditions. The DRG and DH tissue were then triturated to dissociate the neurons in Neurobasal medium (Invitrogen) containing pencillin (50 units ml^{-1}) and streptomycin (25 μg ml^{-1}). The neurons were plated on glass coverslips previously coated with poly D-lysine (10 μg ml^{-1}, Sigma, St Louis, MO). In some dishes, only DH or DRG neurons were plated to obtain DH or DRG neuronal monocultures. Neurons were maintained in Neurobasal culture medium (Invitrogen) that also contained nerve growth factor (2.5S NGF; 100 ng ml^{-1}; Roche Molecular Biochemicals, Indianapolis, IN, USA), 0.5 mM L-glutamine (Invitrogen) and B27 (a serum free supplement) (Invitrogen). The cultures were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2 and the culture medium was replaced weekly.

Patch-clamp recordings from DH neurons in DRG–DH cocultures

Cover slips with the cocultured neurons were placed in a recording chamber and mounted on the stage of an Olympus IMT-2 microscope (Olympus, Lake Success, NY, USA). The DH neurons were distinguished from the DRG neurons based on their morphology and electrophysiological properties. The DH neurons selected were fusiform, pyramidal or multipolar in shape in contrast to the rounded pseudounipolar characteristics of the DRG neurons. Moreover, in our cultures no mEPSCs could be recorded when a DRG neuron was voltage clamped and capsaicin application resulted in an inward whole cell current. However, mEPSCs in control and upon capsaicin application could be recorded when a DH neuron was voltage clamped. The bath solution contained (mM): 150 NaCl; 5 KCl; 2 $MgCl_2$; 0.1 $CaCl_2$; 10 glucose; 10 Hepes; and the pH was adjusted to 7.4 with NaOH. The recording pipettes were made from borosilicate glass (World Precision Instruments, Sarasota, FL, USA), which had the resistance of 3–10 MΩ and filled with a solution that contained (mM): 140 $CsMeSO_3$; 5 CsCl; 10 Hepes; 5 $MgCl_2$; 10 EGTA; 5 Mg-ATP; 1 Li-GTP; and pH was adjusted to 7.4 with CsOH. All experiments were performed at room temperature ($24 \pm 2^{\circ}$C) except when effects of PKC-mediated phosphorylation of TRPV1 were studied at 37°C. In order to record fast excitatory postsynaptic currents, DH neurons were voltage clamped (EPC10, HEKA) at −60 mV (close to E_{Cl}). The capacitance and the series resistance were compensated and the input resistance of the cell was monitored. The data were filtered at 2.5 kHz and digitized at 5 kHz. Adequate voltage clamp was confirmed by recording the mEPSCs at different voltages and determining their reversal potentials. A new coverslip was used for every experiment to prevent run-down of mEPSCs and the activation of second messenger pathways.

Neurons were perfused with standard bathing solution plus lidocaine (10 mM), strychnine (1 μM) and bicuculline (10 μM). A high concentration of lidocaine was used to block both TTX-sensitive and TTX-resistant Na^+ channels. Effects of TRPV1 receptor activation on glutamatergic synaptic transmission were determined by application of capsaicin (10–100 nM). mEPSCs sensitive to block by the AMPA receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) were recorded for 2 min in presence of lidocaine, following which, capsaicin was bath-applied for 30 s and changes in mEPSCs were recorded continuously thereafter. Effects of PDBu (1 μM) and BK (1 μM) were tested following wash of capsaicin for 4–5 min to allow increases in mEPSCs to return to the basal control conditions. Following a 2 min pretreatment with PDBu or BK, capsaicin was applied and changes in mEPSC frequency and amplitude were recorded. In experiments measuring mEPSCs in the absence of extracellular Ca^{2+}, the bath solution was the same as the standard solution except that Ca^{2+} was omitted and 5 mM EGTA was added.

The experiments requiring higher temperature were performed by flowing solutions through a heated coil (TC-324B, Warner Instrument Corp., Hamden, CT, USA). The final temperature of the recording chamber was maintained between 36 and 38°C. The mEPSC events were recorded from voltage clamped DH neurons under these conditions as described above.

Data analysis

Data were analysed using Mini Analysis Program (Synaptosoft, Decatur, GA, USA). The amplitude and frequency of the synaptic events were determined from 20 s data segments to avoid desensitization of responses over time. Only mEPSCs with amplitudes 5 pA or greater were analysed.

All the chemicals used in this study were obtained from Sigma (St Louis, MO, USA). The working concentrations of capsaicin, BK, U73122, U73343, PDBu, BIM and capsazepine were prepared fresh before the experiments from either an ethanol or DMSO stock. *N*-(4-Tertiarybutylphenyl)-4-(3-chloropyridin-2-yl)tetrahydropyrazine-1(2*H*)-carbox-amide (BCTC) was a gift from a pharmaceutical company and was prepared fresh from a DMSO stock. The maximum concentration of ethanol and DMSO were < 0.01 and 0.02%, respectively.

Statistics

Kolmogorov–Smirnov (KS) test was used to compare the cumulative probability curves for interevent intervals and amplitude between various treatment groups. Data are represented as means ± S.E.M. and expressed as a percentage of control, which is scaled to 100%. Student's paired *t* test was used for statistical comparisons and significance was considered at $P < 0.05$.

Results

Enhancement of synaptic transmission by capsaicin at the first sensory synapse

Using DRG–DH neuronal cocultures, mEPSCs were recorded from DH neurons voltage clamped at −60 mV in presence of extracellular solution containing lidocaine (10 mM), bicuculline (10 μM) and strychnine (1 μM) to block tetrodotoxin-sensitive and -resistant sodium channels, $GABA_A$ and glycine receptors, respectively. mEPSC events were recorded in the presence of low extracellular calcium (0.1 mM) to prevent rapid run-down

of synaptic events following application of capsaicin. The AMPA receptor antagonist CNQX (10 μM) completely abolished all synaptic events, suggesting that they are glutamate mediated (data not shown). Application of capsaicin robustly increased the frequency of mEPSCs in a concentration-dependent manner. Overall, the mEPSC frequency expressed as a percentage of control increased significantly to $664.04 \pm 135.2\%$ with 10 nM capsaicin (range 213– 1966%, $n = 17$, $P < 0.05$) and $3714.3 \pm 927\%$ with 100 nM capsaicin (range 309–8700%, $n = 11$, $P < 0.05$) (Fig. 1*A*, *B* and *D*). The mean mEPSC amplitude was not significantly altered (control, 9.5 ± 0.5 pA, $n = 17$; 10 nM capsaicin, 10.9 ± 0.8 pA, $n = 17$ and control, 8.1 ± 0.6 pA, $n = 11$; 100 nM capsaicin, 9.6 ± 1.3 pA, $n = 11$) (Fig. 1*C*). An increase in the mEPSC frequency but not the amplitude suggests that capsaicin acts presynaptically to increase glutamate release from the central terminals of sensory neurons. The facilitatory effect of capsaicin on synaptic transmission was observed in a high percentage (> 80%) of voltage clamped DH neurons in DRG–DH neuronal cocultures. The higher incidence of obtaining synaptic capsaicin responses, even though only 30% of DRG neurons express TRPV1, is probably due to multiple synaptic inputs received by a DH neuron from TRPV1-expressing DRG neurons in our coculture system.

In DH-only neuronal cultures (monoculture), application of capsaicin (100 nM) altered neither the frequency ($92.2 \pm 15.1\%$, $n = 3$) nor the amplitude of mEPSCs (control, 11.3 ± 2.1 pA, $n = 3$; capsaicin, 10.7 ± 2.5 pA, $n = 3$). In DRG-only neuronal cultures, there were no synaptic events, but application of capsaicin resulted in an inward current, when small diameter neurons were voltage clamped. These results suggest that

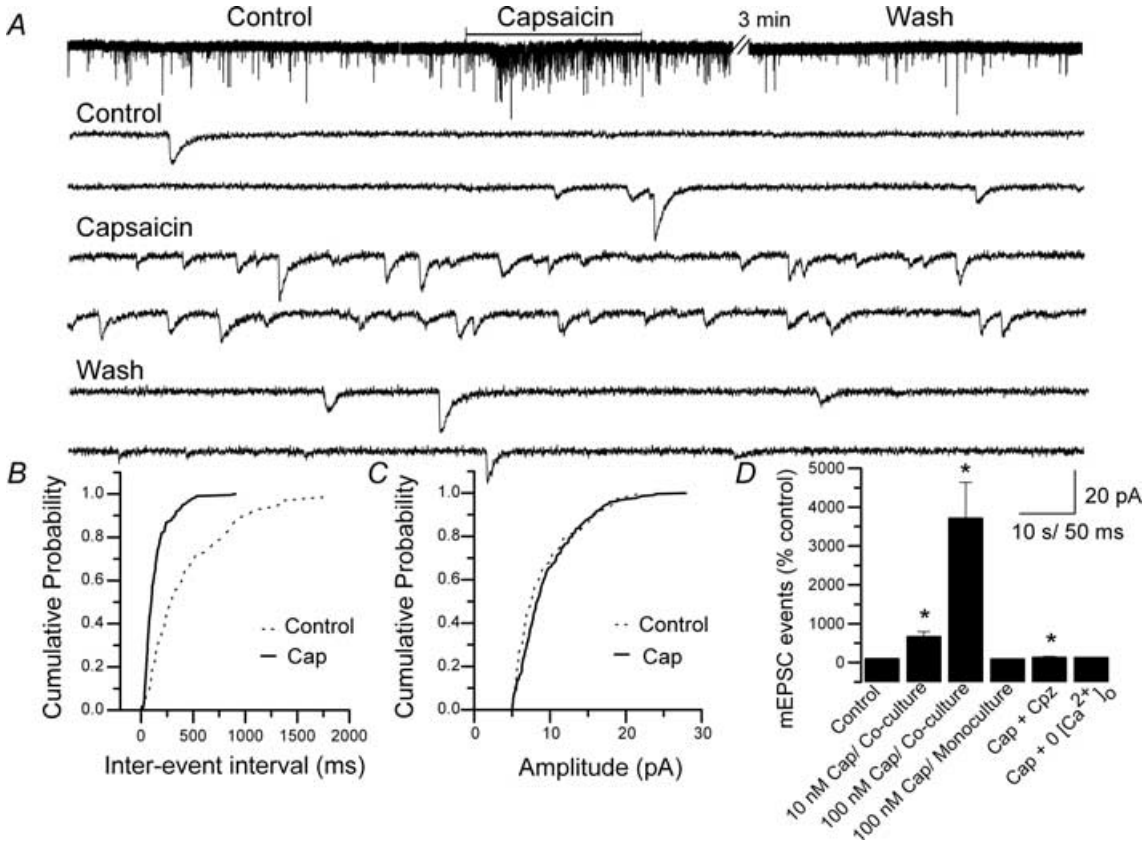

Figure 1. Enhancement of synaptic transmission by activation of TRPV1 by capsaicin at the first sensory synapse

A, application of capsaicin (10 nM) increases the frequency of mEPSCs in a reversible manner. The synaptic events are shown in higher time resolution below. *B*, cumulative probability plot showing decreased interevent intervals representing increased frequency of mEPSCs ($P < 0.0001$, KS test). *C*, the increase in frequency was not accompanied by a change in the amplitude ($P = 0.159$, KS test). *D*, summary graph showing capsaicin-mediated increases in the frequency of mEPSCs in a dose-dependent manner (10 nM, $n = 17$, $P < 0.05$; 100 nM, $n = 11$, $P < 0.05$). The increases in mEPSC events are dependent on extracellular calcium ($n = 3$). Furthermore, the increase in capsaicin-induced synaptic events are blocked by 10 μM capsazepine ($n = 5$, $P < 0.05$). The augmented synaptic transmission upon capsaicin application is only observed in DRG–DH cocultures and not in DH–DH monocultures ($n = 3$).

capsaicin specifically modulates synaptic transmission between the DRG and DH neurons.

In order to determine whether capsaicin could modulate the inhibitory synaptic transmission mediated by GABAergic and glycinergic interneurons at the first sensory synapse, recordings were made in the presence of the AMPA and NMDA receptor antagonists CNQX (10 μM) and 2-amino-5-phosphonovaleric acid (APV; 20 μM), respectively, at 0 mV, where excitatory currents reverse and inhibitory currents can be recorded in isolation using our experimental conditions. Application of capsaicin (100 nM) failed to enhance the inhibitory transmission in the cocultures (data not shown). These observations suggest that capsaicin selectively enhances excitatory synaptic transmission.

To demonstrate that increase in mEPSCs in response to capsaicin application is due to TRPV1 activation, a TRPV1 selective antagonist, capsazepine (Cpz), was used. Cpz (10 μM) abolished the facilitatory effects of 10 nM capsaicin on mEPSC frequency (Fig. 1*D*). Capsaicin-induced mEPSC frequency decreased from 797 $\pm$ 180% in capsaicin treated cells ($n = 5$, $P < 0.05$) to 132 $\pm$ 29% in presence of Cpz ($n = 5$, $P < 0.05$). There was no change in the amplitude of mEPSCs in the presence of Cpz (9.2 $\pm$ 1.2 pA, $n = 5$), when compared to the control (9.3 $\pm$ 1.2 pA, $n = 5$). These results confirm that capsaicin acts via TRPV1 located on the central terminals of primary afferents to increase glutamate release. In addition, exposure to Cpz alone at room temperature did not affect basal synaptic activity (103.92 $\pm$ 20.1%, $n = 6$), suggesting that there is no tonic TRPV1 activation at this synapse in our coculture system. Further, we determined whether extracellular calcium is necessary for the increase in mEPSC frequency following TRPV1 activation. Recordings were made using a Ca^{2+}-free extracellular solution containing calcium chelator, EGTA (5 mM). Application of capsaicin (100 nM) for 30 s failed to produce a significant increase in the frequency of mEPSCs (105.67 $\pm$ 8.87%, $n = 3$), suggesting that extracellular Ca^{2+} is essential for the observed effects of capsaicin (Fig. 1*D*).

Changes in mEPSCs in response to continuous and repeated application of capsaicin

Capsaicin-induced whole-cell currents have been shown to exhibit calcium-dependent desensitization and tachyphylaxis (Caterina & Julius, 2001; Holzer, 2004). Receptor desensitization is an inherent property of ligand-gated channels, which is usually dependent on the time and concentration of agonist exposure, but is calcium independent. In contrast, desensitization of TRPV1 is a calcium-dependent phenomenon. Continued application of capsaicin decreased the frequency of mEPSCs over time (Figs 1*A* and 2*A* and *B*). Change in mEPSC frequency in successive 5 s intervals immediately following capsaicin application was analysed. Capsaicin-mediated increases in mEPSC events showed a gradual decrease with time. The frequency of mEPSCs in successive 5 s intervals following capsaicin (10 nM) application, expressed as a percentage of control, were 581.65 $\pm$ 112% ($n = 8$); 644.45 $\pm$ 104% ($n = 8$); 447.7 $\pm$ 58% ($n = 8$); and 328.5 $\pm$ 36.7% ($n = 6$) (Fig. 2*B*). The run-down of synaptic events with continuous capsaicin application may be a result of Ca^{2+}-dependent decrease in TRPV1 channel function.

To study the effects of repeated applications of capsaicin on TRPV1-mediated responses, experiments with repeated 30 s capsaicin applications separated by a 4 min wash were analysed. Successive applications of capsaicin (10 nM) showed a steady decrease in the mEPSC frequency (first application, 664.04 $\pm$ 135.2%, $n = 17$; second application, 249.3 $\pm$ 74.8%, $n = 8$; third application, 207.68 $\pm$ 35.04%, $n = 6$; fourth application, 169.4 $\pm$ 64%, $n = 3$; and fifth application, 140 $\pm$ 37% of control, $n = 3$) (Fig. 2*A*, *C* and *D*). The extent of run-down was most pronounced between the first and second capsaicin applications ($P < 0.05$) and then the response stabilized (Fig. 2*D*). This progressive decrease in mEPSC frequency, which is conventionally described as tachyphylaxis, may be a result of calcium-induced receptor desensitization or presynaptic terminal depolarization as a result of TRPV1 activation (Yang *et al.* 1998). As the rundown is not readily reversible within the duration of our experiments and the conditions used, it is most likely due to TRPV1-induced tachyphylaxis. Since the effects of continuous or repeated capsaicin application on mEPSCs represent an indirect measure of TRPV1 channel function, isolating the complex mechanisms underlying the run-down of transmission is beyond the scope of this study.

Enhancement of synaptic transmission by phorbol esters

In order to study the effects of PKC-mediated sensitization of TRPV1 on synaptic transmission, we used the phorbol ester phorbol 12,13-dibutyrate (PDBu). Previous studies have utilized PDBu to activate PKC and examined synaptic transmission in the CNS (Malenka *et al.* 1986; Muller *et al.* 1991; Hori *et al.* 1996; Rhee *et al.* 2002). In this study, application of PDBu (10 nM to 1 μM) alone induced a dose-dependent increase in the frequency but not the amplitude of mEPSCs at this synapse (Fig. 3*A*, *C* and *E*), suggesting presynaptic enhancement of glutamate release. On an average, mEPSC frequency significantly increased in a dose-dependent manner following PDBu application (10 nM, 267.8 $\pm$ 25%,

$n = 6$, $P < 0.05$; 100 nM, 327.6 ± 65.5%, $n = 5$, $P < 0.01$; 1 μM, 400 ± 64%, $n = 10$, $P < 0.001$) (Fig. 3*E*), whereas the mean amplitude remained unaltered (control, 10.3 ± 0.6 pA, $n = 6$; 10 nM PDBu, 11.9 ± 1.8 pA, $n = 6$; control, 11.5 ± 1.1 pA, $n = 5$; 100 nM PDBu, 10 ± 0.9 pA, $n = 5$; and control, 13.5 ± 2.8 pA, $n = 10$; 1 μM PDBu, 13.4 ± 1.3 ($n = 10$). PDBu-induced enhancement of synaptic activity was readily reversible following a 3–4 min wash; the increases in mEPSCs returned to the basal levels in most recordings (10 nM, 106.4 ± 10.3%, $n = 4$; 100 nM, 109 ± 11.2%, $n = 4$; 1 μM, 114 ± 12.4%, $n = 4$). Moreover, unlike the decrease in the frequency of mEPSCs seen with capsaicin, repeated applications of PDBu (100 nM) increased the mEPSC frequency to a similar extent (first application, 258.6 ± 24.5%, $n = 5$; second application, 258.2 ± 28%, $n = 5$; third application, 257.7%, $n = 3$).

Further, to determine whether the synaptic effects of PDBu were due to activation of PKC, we used a specific PKC inhibitor, bisindolylmaleimide (BIM). Cultures were preincubated with BIM (500 nM) for 30 min and synaptic currents were recorded from DH neurons. Interestingly, PDBu (1 μM) still enhanced glutamatergic synaptic transmission in BIM-treated cultures (328.7 ± 65.5%, $n = 7$, $P < 0.05$) (Fig. 3*B* and *E*). BIM application alone did not alter the synaptic transmission (119.2 ± 22.2%, $n = 7$). Thus, the direct effect of PDBu on the excitatory synaptic transmission is independent of PKC-activation.

Potentiation of TRPV1-mediated enhancement of glutamatergic synaptic transmission by PDBu

PKC-mediated phosphorylation robustly potentiates TRPV1 activity and this sensitization plays an important

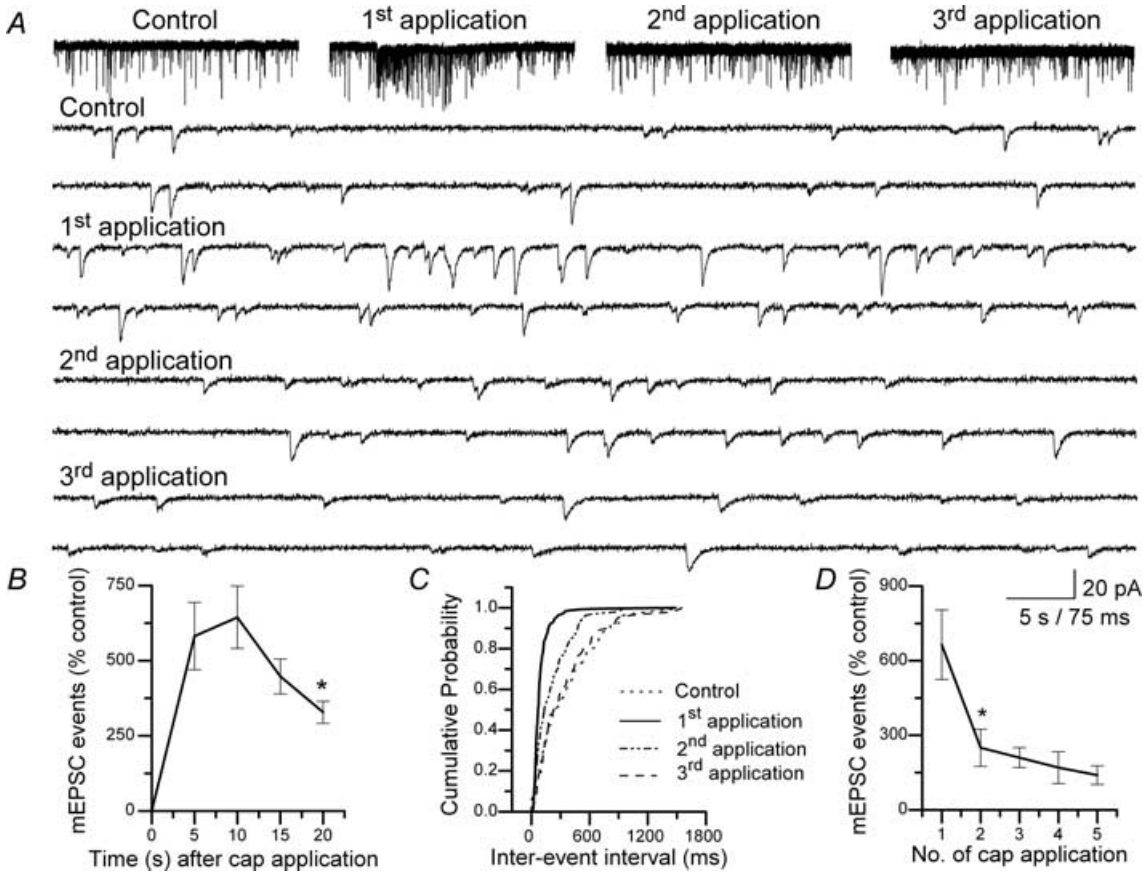

Figure 2. TRPV1-mediated enhancement of synaptic transmission shows run-down upon continuous and tachyphylaxis upon repeated capsaicin application

A, capsaicin (10 nM)-induced enhancement of mEPSCs progressively decreases with repeated capsaicin application. Synaptic currents are shown in a higher time resolution below. *B*, cumulative probability plots showing decreased mEPSC frequency as indicated by a progressive increase in the interevent intervals with each subsequent capsaicin application. *C*, summary graph representing a progressive decline in mEPSC frequency with repeated capsaicin application. A significant decrease in frequency is observed only between the first and second capsaicin application (first application, $n = 17$; second application, $n = 8$, $P < 0.05$). *D*, summary graph representing a progressive decrease in the number of mEPSC events analysed in 5 s epochs immediately following capsaicin (10 nM) application (581.65 ± 111.95%, $n = 8$; 644.45 ± 103.95%, $n = 8$; 447.7 ± 57.88%, $n = 8$; 328.5 ± 36.73%, $n = 6$)

role in chronic pain processing (Cesare & McNaughton, 1996; Premkumar & Ahern, 2000; Chuang *et al.* 2001; Vellani *et al.* 2001; Sugiura *et al.* 2002; Bonnington & McNaughton, 2003). Therefore, we studied the influence of PKC on TRPV1-modulated synaptic transmission at the first sensory synapse. Our earlier experiments have established that the first application of capsaicin resulted in a large increase in synaptic transmission with a significant reduction during second application followed by consistent responses thereafter. Therefore, we used the second response as our control and compared the changes in subsequent responses to determine the effect of PKC on synaptic transmission as a result of TRPV1 phosphorylation. The mEPSC frequency (expressed as percent of control) with second application of capsaicin (100 nM) was $311.7 \pm 93.8\%$, $n = 6$ and following PDBu pretreatment, the mEPSC frequency with capsaicin increased to $1280.8 \pm 236.5\%$, $n = 6$, $P < 0.05$ (Fig. 4*A*, *B* and *D*). The change in frequency of mEPSC with PDBu (1 μM) in these experiments was $318.64 \pm 117.5\%$, $n = 6$. The average amplitude remained unchanged (control, 9.6 ± 1 pA, $n = 6$; 100 nM capsaicin, 9.7 ± 1.3 pA, $n = 6$; 1 μM PDBu, 10.1 ± 1.8 pA, $n = 6$; capsaicin after PDBu, 10.3 ± 1.6 pA, $n = 6$). Interestingly, PDBu-mediated direct increase in the synaptic events was seen in all neurons patched even when capsaicin failed to enhance synaptic events. However, further enhancement of capsaicin-mediated synaptic responses following PDBu pretreatment was observed in 66% of capsaicin-sensitive neurons (10 of 15). Of these, 60% of neurons showed profound potentiation and the rest did not demonstrate a statistically significant change with capsaicin after PDBu application. The variability of these responses can be explained by the extent of synaptic connections made to the recording DH neuron. PDBu increases synaptic transmission indiscriminatingly between both DH–DH as well as DRG–DH synapses, whereas capsaicin-mediated increase in mEPSC frequency is specific to TRPV1-expressing DRG and DH neuronal synapses. Thus, the number of synaptic connections between DRG and DH neurons will determine the extent of potentiation observed.

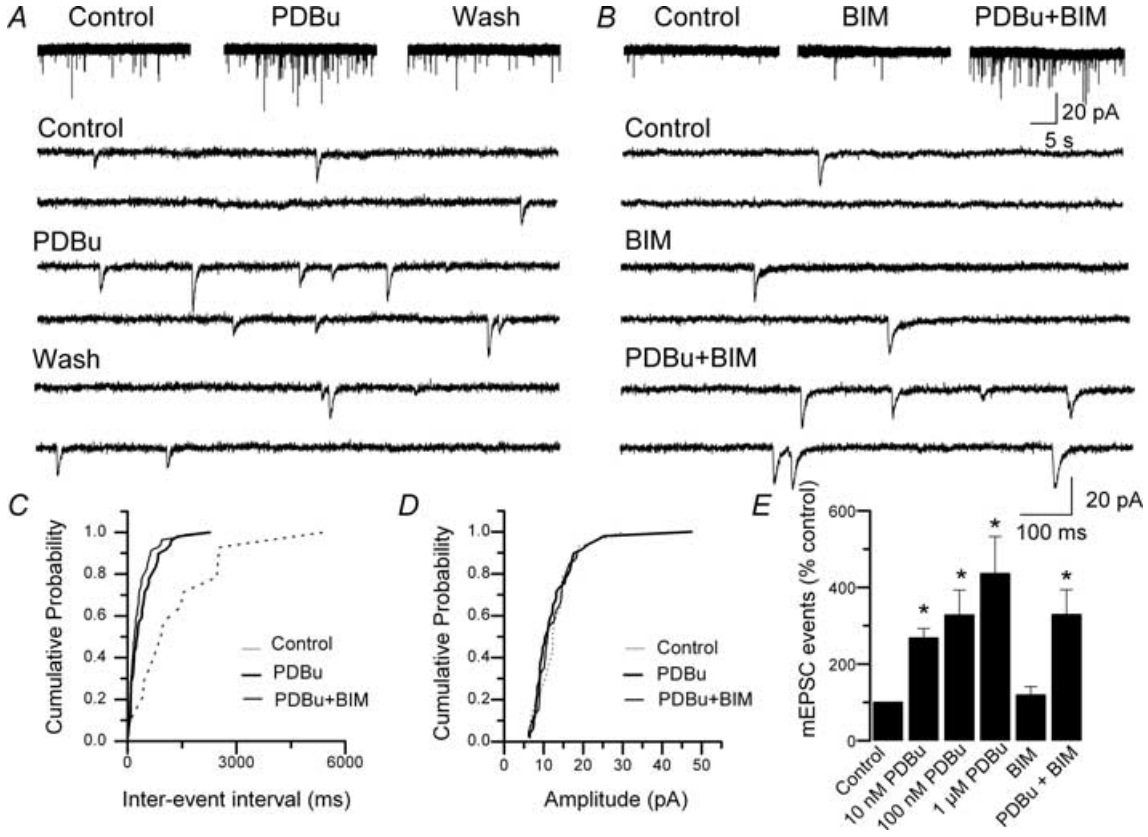

Figure 3. Modulation of synaptic transmission by PDBu
A, PDBu increases the frequency of mEPSCs in a reversible manner. *B*, pretreatment with PKC inhibitor BIM (500 nM) does not inhibit PDBu-mediated increases in the synaptic transmission. Synaptic currents are shown in higher resolution below. *C* and *D*, cumulative probability graphs showing enhanced frequency of mEPSCs ($P < 0.001$, KS test) in response to PDBu without change in their amplitudes ($P = 0.48$, KS test). *E*, summary graphs showing that PDBu-mediated increase in mEPSCs are not inhibited by BIM (10 nM, $n = 6$; 100 nM, $n = 5$; 1 μM, $n = 10$, $P < 0.05$, $n = 7$).

Furthermore, to confirm PKC-mediated phosphorylation of TRPV1 following PDBu application, we recorded synaptic transmission in cultures pretreated with the PKC inhibitor BIM. As reported above, treatment with BIM (500 nM), failed to inhibit direct effects of PDBu on synaptic transmission. However, BIM was able to inhibit PDBu-mediated facilitation of capsaicin responses (second application of capsaicin, $257 \pm 73\%$, $n = 5$; PDBu + BIM, $360 \pm 71\%$, $n = 5$; capsaicin after PDBu + BIM, $315 \pm 78\%$, $n = 5$ (Fig. 5*A*, *B* and *D*). No change in the amplitude of mEPSCs was observed in these experiments (control, 10.1 ± 1.2 pA, $n = 5$; 100 nM capsaicin, 9.9 ± 2.1 pA, $n = 5$; 1 μM PDBu, 10.5 ± 2.2 pA, $n = 5$; capsaicin after PDBu, 10.5 ± 2.4 pA, $n = 5$) (Fig. 5*C*), indicating selective presynaptic modulation of TRPV1-induced synaptic transmission. Inhibition of PDBu-mediated sensitization of TRPV1 by BIM suggests that PKC-mediated phosphorylation is important in this response.

Modulation of synaptic transmission by phosphorylated TRPV1 at normal body temperature

PKC-mediated phosphorylation has been suggested to sensitize TRPV1 resulting in lowering of its activation threshold from 43°C to below body temperature (Sugiura *et al.* 2002). We hypothesize that PKC-mediated phosphorylation of TRPV1 expressed in the central terminals of sensory neurons lowers its activation threshold and enhances synaptic transmission. To test this, mEPSC events were recorded at 37°C in the presence of the control solution and after application of PDBu. A 2 min application of PDBu enhanced the synaptic transmission to $227.8 \pm 36\%$ of the control, $n = 5$ (Fig. 6*A*). Furthermore, to separate the PDBu-mediated direct effects from the TRPV1-modulated component, the selective TRPV1 antagonist *N*-(4-tertiarybutylphenyl)-4-(3-chloropyridin-2-yl)tetrahydropyrazine-1(2*H*)-carbox-amide (BCTC, 100 nM)

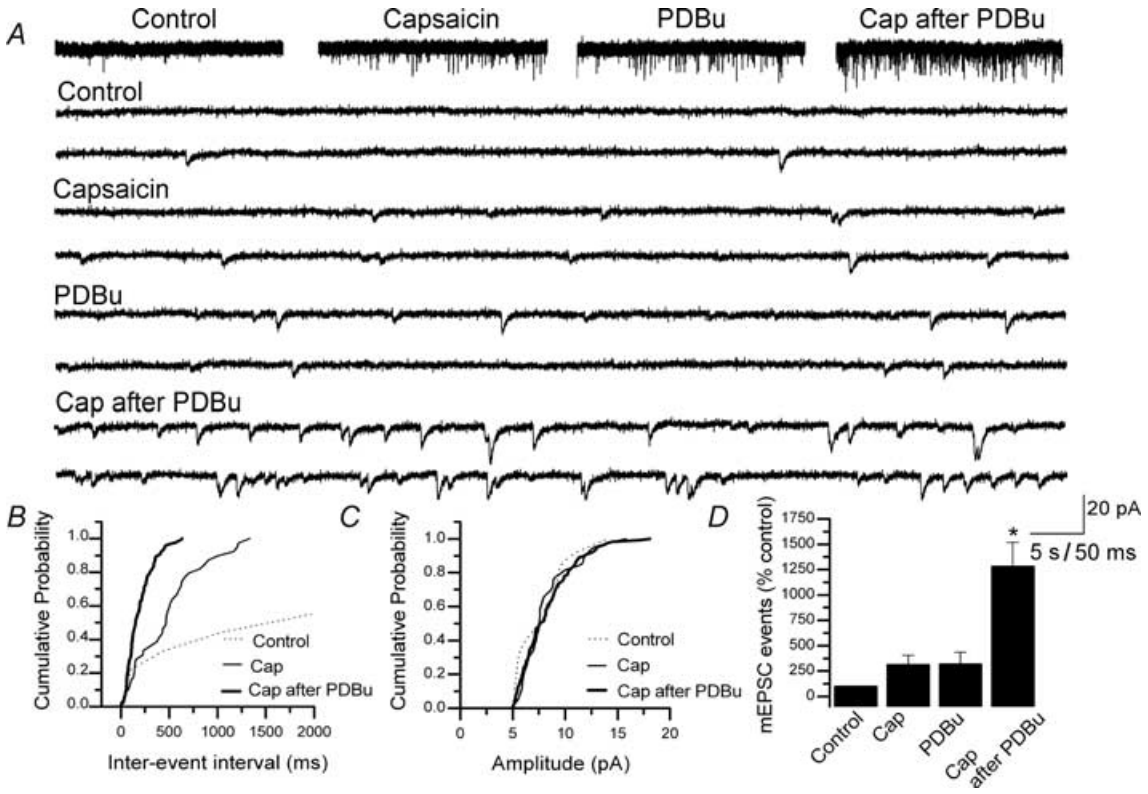

Figure 4. Enhancement of capsaicin-induced changes in synaptic transmission by the PKC-activating phorbol ester PDBu

A, capsaicin-induced increases in mEPSCs were enhanced by pretreatment with PDBu. Synaptic currents are shown in higher time resolution below. *B*, cumulative probability graphs indicating an increase in the frequency of synaptic events with capsaicin (100 nM, $P < 0.02$, KS test), which were further significantly enhanced following PDBu (1 μM) pretreatment ($P < 0.0001$, KS test). *C*, the increase in frequency of events is not accompanied by a change in their amplitude ($P = 0.68$, KS test). *D*, summary graphs showing potentiation of capsaicin-induced increase in mEPSC frequency by PDBu ($n = 6$, $P < 0.05$).

was used. Interestingly, the mEPSC frequency decreased significantly in the presence of BCTC to 125.5 ± 5% of control, $n = 5$, suggesting the involvement of TRPV1 (Fig. 6*A*, *B* and *D*). PDBu and BCTC application did not significantly affect the amplitude of the mEPSC events (Fig. 6*C*). In addition, application of BCTC alone neither affected the basal synaptic transmission at 37°C nor inhibited PDBu-mediated direct responses at room temperature (data not shown). These findings suggest that PKC-mediated phosphorylation of TRPV1 sensitizes the channel and decreases it activation threshold to body temperature. This mechanism may be important in activating TRPV1 expressed in the central terminals of sensory neurons, in the absence of an agonist or in the presence of low potency endogenous agonists.

Enhancement of TRPV1-induced synaptic activity by BK

BK is an important inflammatory mediator known to induce thermal hyperalgesia, at least in part by sensitizing TRPV1 (Cesare & McNaughton, 1996; Premkumar & Ahern, 2000; Chuang *et al.* 2001; Sugiura *et al.* 2002; Shin *et al.* 2002). BK, acting via its B_2 receptor, activates phospholipase C (PLC) to cleave PIP_2 and produce inositol-1,4,5-triphosphate (IP_3) and diacylgycerol (DAG) (Burgess *et al.* 1989; Cesare *et al.* 1999). DAG can activate PKC directly and IP_3 by releasing calcium from internal stores. Furthermore, BK-induced activation of PLC hydrolyses PIP_2 to relieve TRPV1 from its tonic inhibition (Chuang *et al.* 2001). Here, we determined whether BK could induce an increase in TRPV1-modulated excitatory synaptic transmission at the first sensory synapse.

Application of BK (1 μM) produced a direct increase in glutamatergic synaptic transmission. The frequency of mEPSCs increased to 238.4 ± 54% of the control, $n = 8$. The amplitude of the mEPSCs showed variability and was not statistically significant (control, 10.7 ± 0.9 pA, $n = 8$; BK, 12.2 ± 1.2 pA, $n = 8$). The change in the frequency of mEPSCs suggests presynaptic actions of BK, due to activation of BK receptors expressed on the sensory neurons (see also Wang *et al.* 2005).

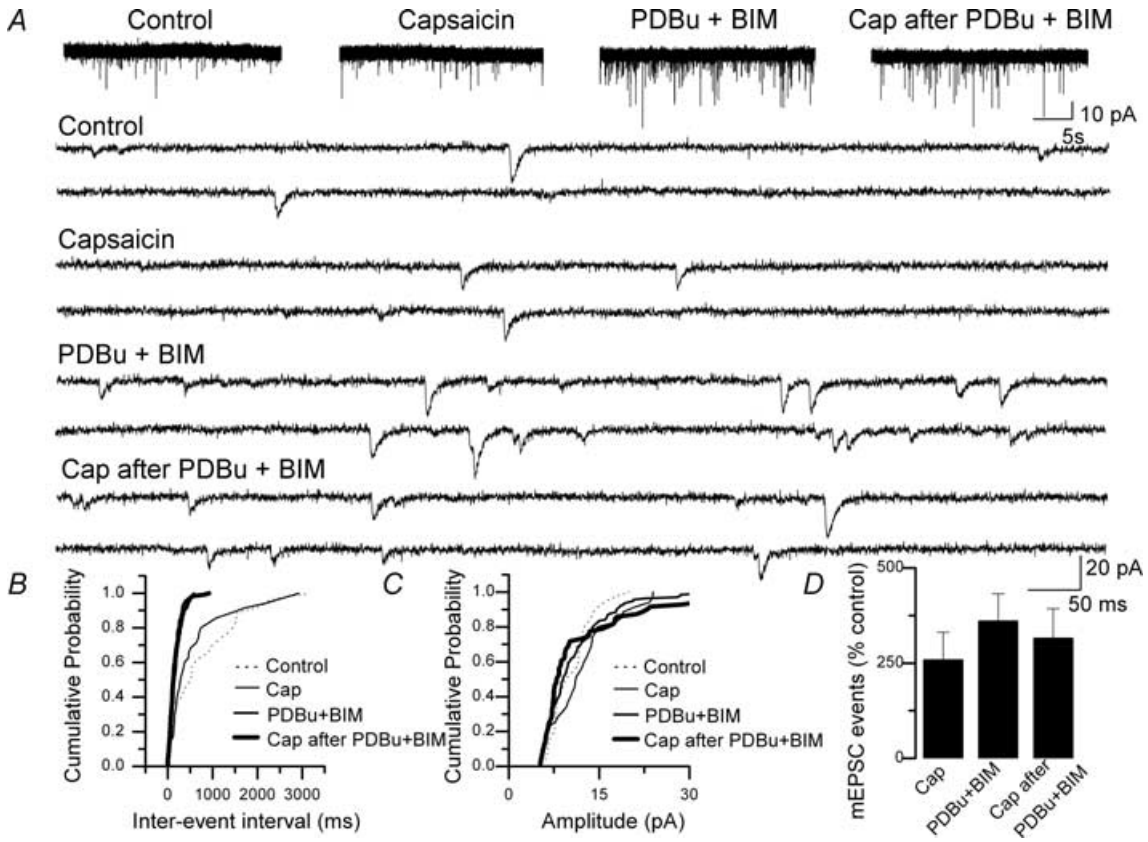

Figure 5. BIM inhibits PDBu-induced potentiation of TRPV1-modulated synaptic responses
A, the specific PKC inhibitor BIM inhibited PDBu-induced potentiation of capsaicin-mediated synaptic activity, without inhibiting the direct effects of PDBu on synaptic transmission. Synaptic currents are shown in higher time resolution. *B* and *C*, cumulative probability curves for the inter-event intervals ($P = 0.23$, KS test) and amplitude ($P = 0.18$, KS test) of mEPSC events are similar with capsaicin before and after PDBu in presence of BIM. *D*, summary graph demonstrating that BIM attenuates PKC-induced potentiation of TRPV1-modulated synaptic transmission ($n = 5$, $P < 0.05$).

To study the influence of BK on TRPV1-modulated synaptic transmission, we determined the effect of BK pretreatment on capsaicin-induced increase in mEPSCs. BK (1 μM) resulted in the potentiation of capsaicin response in 63% of neurons responsive to both capsaicin and BK (5 of 8). The frequency of mEPSC events in response to 10 nM capsaicin increased from $264 \pm 55\%$, $n = 5$ to $890 \pm 83\%$, $n = 5$ after 2 min BK application (Fig. 7*A*, *B* and *D*). In these patches, the direct increases mediated by BK were $202.5 \pm 27\%$, $n = 5$. There was no change in the amplitude of mEPSCs with capsaicin application following BK. The amplitude of mEPSCs with application of capsaicin following BK was 12.1 ± 1.3 pA, $n = 5$, as compared to capsaicin alone, 11.7 ± 1.5 pA, $n = 5$ (Fig. 7*C*). The extent of increase in capsaicin-induced mEPSC frequency mediated by PDBu and BK is variable. As mentioned previously, the variability of these responses can be explained by the extent and unpredictable synaptic connections made to the recording DH neuron. In most experiments, a synergistic response was seen, however, in a few experiments the potentiation was not statistically significant.

Sensitization of TRPV1 by BK activating PLC–PKC pathway

To characterize signalling pathways involved in BK-induced potentiation of TRPV1-modulated synaptic transmission, we examined specific pathways known to be activated by BK (Burgess *et al.* 1989). To establish the role of PKC-mediated phosphorylation of TRPV1 following BK application, we used the PKC inhibitor BIM in our experiments. Pretreatment with BIM (500 nM for 30 min) inhibited BK-mediated potentiation of TRPV1-modulated synaptic transmission. The mEPSC frequency (expressed as a percentage of control) with the second application of capsaicin were $193.3 \pm 33.4\%$, $n = 4$, and $133.7 \pm 24\%$, $n = 4$, with capsaicin after BIM + BK treatment, respectively (Fig. 8*A*, *C* and *E*). These effects are significantly lower when compared to

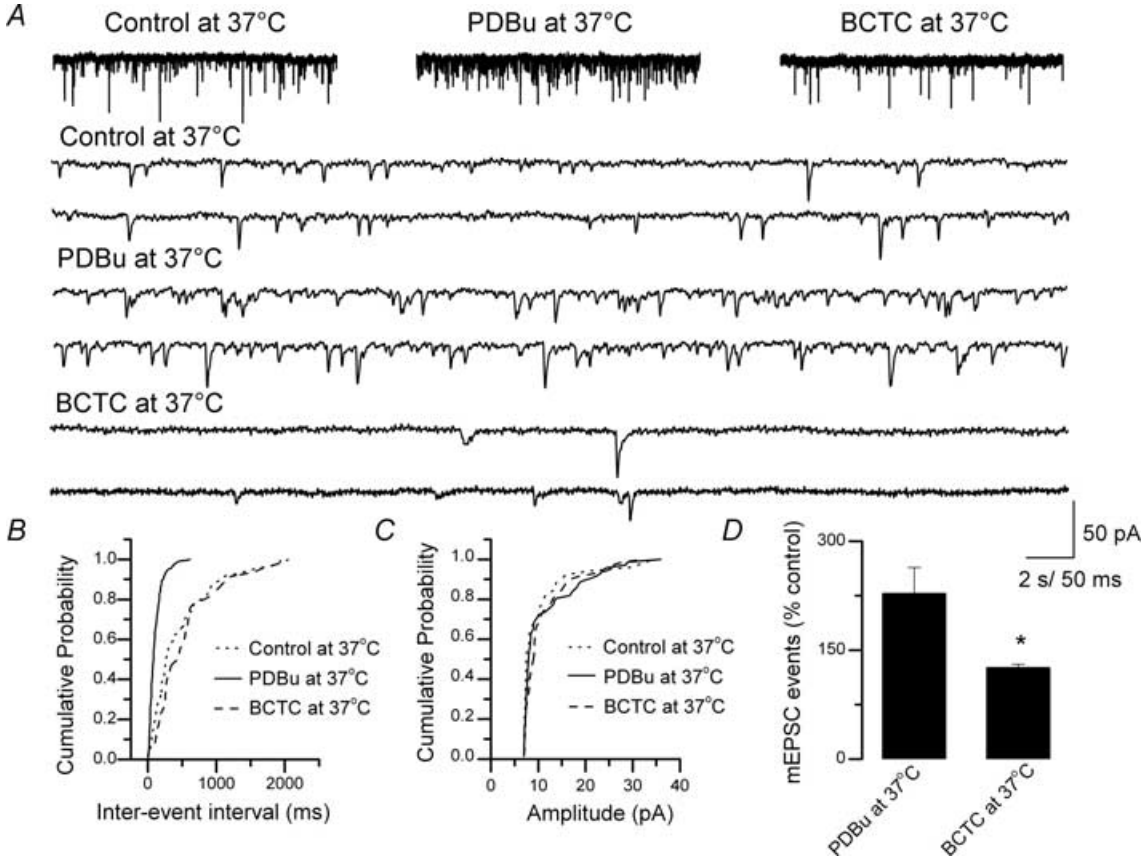

Figure 6. Modulation of synaptic transmission by phosphorylated TRPV1 at normal body temperature
A, PDBu-mediated enhancement in synaptic transmission at 37°C can be partially blocked by the TRPV1 antagonist BCTC (100 nM). *B*, cumulative probability curves demonstrating that PDBu-mediated increases in the mEPSC events ($P < 0.0001$, KS test) are inhibited by BCTC application at 37°C. *C*, cumulative probability graph showing that PDBu and BCTC application does not affect the amplitude of mEPSC events as compared to the control ($P = 0.13$, KS test). *D*, summary graph demonstrating TRPV1-mediated component that is responsible for the increase in the frequency of mEPSC events ($n = 5$, $P < 0.05$).

the frequency of mEPSC events with capsaicin after BK ($890 \pm 83\%$, $n = 5$, $P < 0.05$). The amplitude in these recordings remained unaltered (control, 12.5 ± 2.6 pA, $n = 4$; capsaicin, 11.8 ± 1.6 pA, $n = 4$, BK + BIM, 11.7 ± 1.6 pA, $n = 4$; and capsaicin after BK + BIM, 10.34 ± 0.5 pA, $n = 4$) (Fig. 8*D*). These experiments suggest that BK sensitizes TRPV1-modulated synaptic transmission by activating PKC.

Next, a non-selective PLC inhibitor, U73122 (2 μM), was used to determine the role of PLC-PKC pathway downstream to BK receptor activation in modulating synaptic transmission. Cultures were preincubated with U73122 for 30 min and then used for recording synaptic transmission. Following U73122 treatment, BK (1 μM) failed to enhance the mEPSC frequency ($114.9 \pm 35\%$, $n = 6$). Thus, PLC activation downstream of the BK receptor in the sensory synapses is important for mediating the direct facilitatory effects of BK on the synaptic transmission. Furthermore, in the presence of U73122, BK failed to potentiate capsaicin-modulated synaptic responses. The mEPSC frequency was dramatically reduced ($70.5 \pm 13.8\%$, $n = 6$) with capsaicin after coapplication of BK and U73122 (Fig. 9*A*, *C* and *E*). The average amplitude in the presence of capsaicin after U73122 and BK was 13.4 ± 1.6 pA, $n = 6$, when compared to 12.1 ± 1.5 pA in control, $n = 6$ (Fig. 9*D*). In order to confirm the specificity of action of U73122, an inactive analogue of the phospholipase C inhibitor, U73343 (2 μM), was used. As expected, BK increased the mEPSC frequency to $172.6 \pm 16\%$, $n = 3$, in the presence of U73223 as well as further potentiating capsaicin-modulated synaptic transmission from $195.1 \pm 16\%$, $n = 3$ to $632.9 \pm 55.7\%$, $n = 3$, $P < 0.05$ (Fig. 9*D*). Inhibition of BK-mediated potentiation of capsaicin responses in the presence of U73122 as well as in the presence of BIM suggests that the PLC-PKC pathway is important.

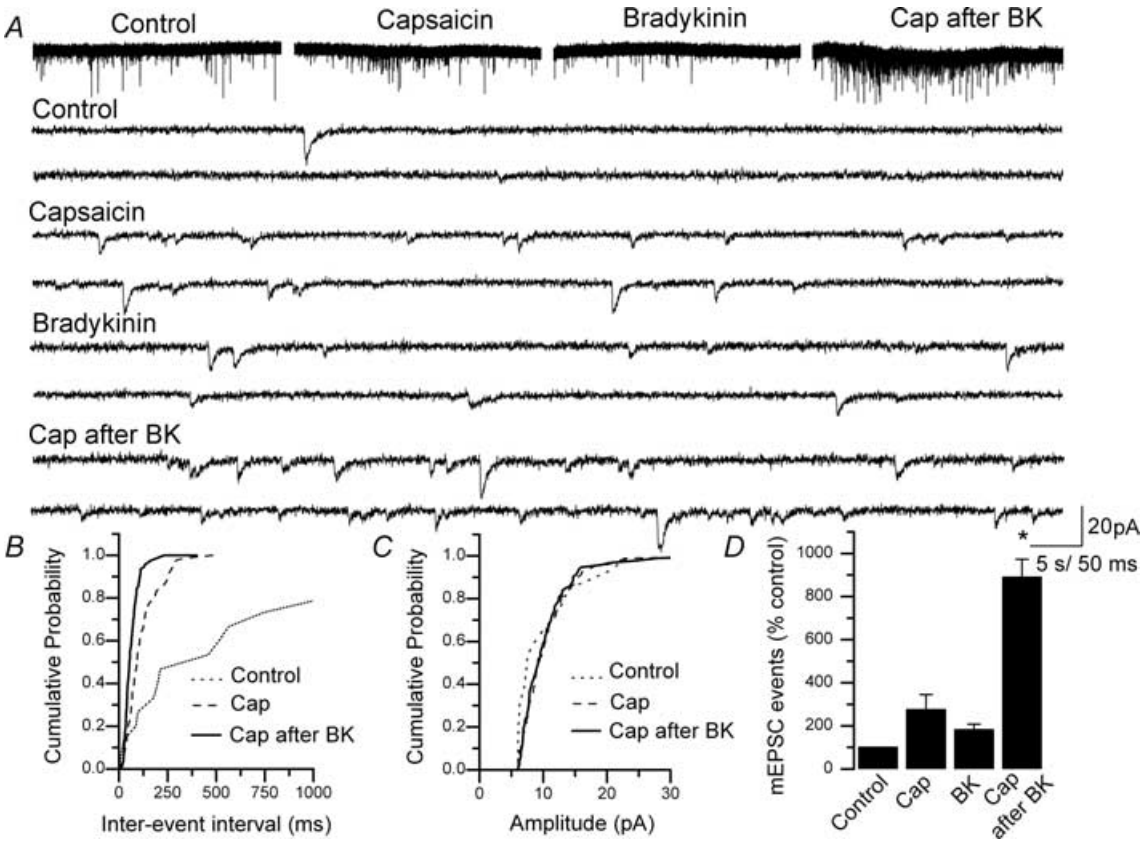

Figure 7. Influence of BK on TRPV1-modulated synaptic transmission
A, capsaicin-induced increases in mEPSCs were enhanced by pretreatment with BK (1 μM). Synaptic currents are shown in higher time resolution below. *B*, cumulative probability curves showing a decrease in inter-event intervals, indicating an increase in the frequency of synaptic events, which were significantly enhanced after treatment with BK ($P < 0.0001$, KS test). *C*, the increase in frequency of mEPSCs is not accompanied by an increase in amplitude ($P = 0.92$, KS test). *D*, summary graph showing capsaicin-induced increase in mEPSC frequency is potentiated ($n = 5$, $P < 0.05$) by BK.

Discussion

In the present study, we demonstrate for the first time, an enhancement of glutamatergic synaptic transmission as a result of TRPV1 sensitization by PKC using phorbol esters and BK at the first sensory synapse. We also demonstrate that in the phosphorylated state, TRPV1 can be active at normal body temperature and modulates synaptic transmission. Understanding the physiology of transmission at this synapse is crucial, because increased gain of sensory input to the brain via TRPV1 sensitization may contribute to persistent pain conditions.

Enhancement of glutamatergic synaptic transmission by activation of TRPV1 at the first sensory synapse

In this study, we have shown that application of capsaicin robustly enhances excitatory synaptic transmission at the first sensory synapse using DRG–DH neuronal cocultures. In the coculture system it may not be possible to differentiate synapses formed between DRG neurons and DH neurons of specific laminae. However, similar augmentation of synaptic transmission has been observed with capsaicin in the substania gelatinosa (Yang *et al.* 1998; Nakatsuka *et al.* 2002; Baccei *et al.* 2003), hypothalamus (Sasamura *et al.* 1998), locus coeruleus (Marinelli *et al.* 2002), nucleus tractus solitarii (Doyle *et al.* 2002) and substantia nigra (Marinelli *et al.* 2003) with no effect on GABA- or glycine-mediated synaptic transmission (Yang *et al.* 1998; Marinelli *et al.* 2003). However, a recent study has demonstrated that capsaicin enhanced both glutamatergic and GABAergic transmission in the medial preoptic nucleus of the hypothalamus (Karlsson *et al.* 2005).

The mEPSC frequency increased without affecting the amplitude, suggesting enhanced glutamate release from the terminals of primary afferents upon TRPV1 activation. The TRPV1 antagonist capsazepine by itself did not affect the basal synaptic activity at room temperature, suggesting a lack of tonic TRPV1 activation by endogenous

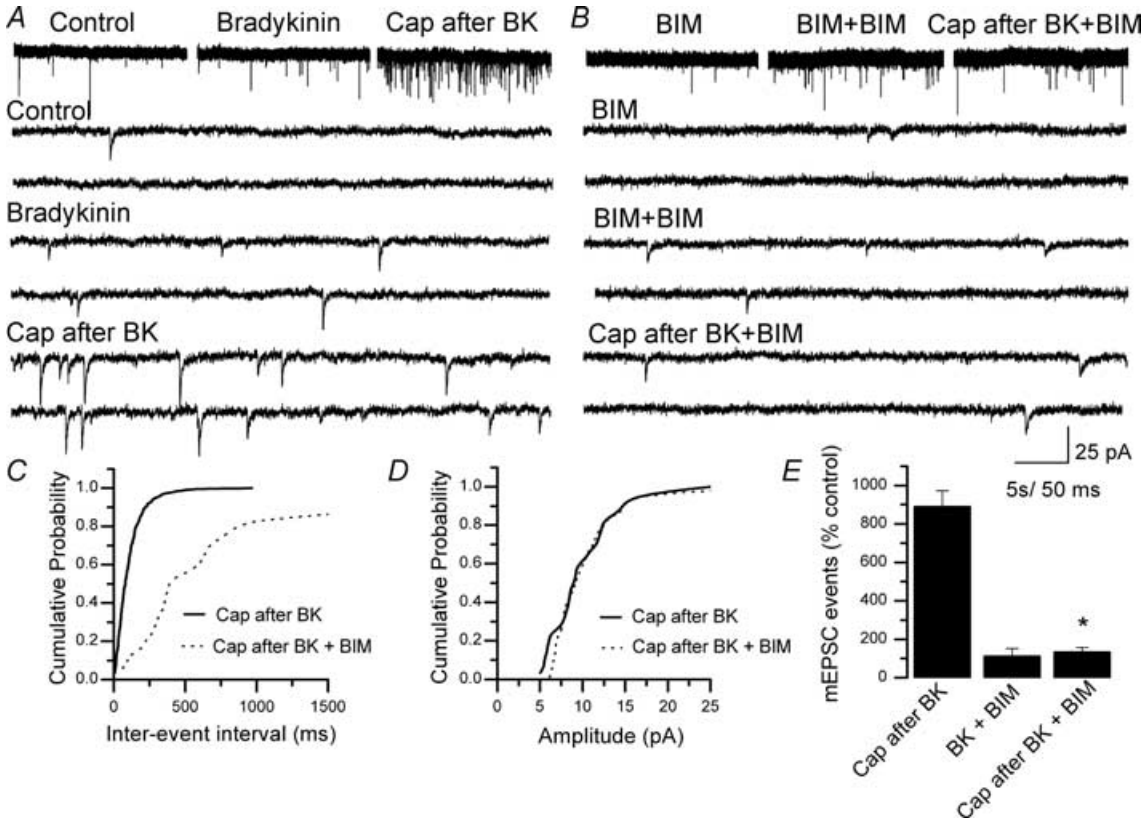

Figure 8. BK potentiates TRPV1-modulated responses by activating PKC
A, traces representing potentiation of capsaicin responses by BK (1 μM). *B*, pretreatment with BIM (500 nM) inhibits BK-mediated potentiation of capsaicin-induced responses. Synaptic currents are shown in a higher resolution below. *C*, decrease in inter-event intervals indicates increase in the frequency of synaptic events with capsaicin after BK. This effect is significantly reduced after application of BIM ($P < 0.0001$, KS test). *D*, the changes in the frequency of events are not accompanied by a change in amplitude ($P = 0.21$, KS test). *E*, summary graph showing that BK-mediated potentiation of capsaicin-induced increases in glutamatergic transmission is abolished by treatment with BIM ($n = 4$, $P < 0.05$).

ligands in our DRG–DH neuronal coculture system. However, capsazepine inhibited capsaicin-modulated transmission, suggesting that the effect is specific to TRPV1 activation. Baccei *et al.* (2003) have demonstrated that capsaicin-induced facilitation of excitatory synaptic transmission in spinal cord slices occurs in the absence of extracellular calcium. Interestingly, in our study, capsaicin-induced increase in mEPSC frequency was dependent on extracellular calcium. This discrepancy could be attributed to a high concentration of capsaicin (2 μM) used in their study. Higher concentrations of capsaicin have been shown to mediate non-specific effects independent of TRPV1 activation (Kawasaki *et al.* 2004).

Changes in mEPSC frequency with continued and repeated activation of TRPV1

TRPV1-mediated whole cell currents show profound calcium-dependent run-down upon continuous and repeated applications of capsaicin (Caterina & Julius, 2001; Holzer, 2004). Phosphorylation at Ser-116 in the amino-terminus of TRPV1 by PKA is important in inhibiting capsaicin-mediated receptor desensitization (Bhave *et al.* 2002; Mohapatra & Nau, 2003). Thr-144, Thr-370 and Ser-502 play a crucial role in PKA-mediated sensitization of TRPV1 (Mohapatra & Nau, 2003). In addition, Ca^{2+}–calmodulin-dependent protein kinase II (CaMKII)-mediated phosphorylation of TRPV1 is crucial for its activation by capsaicin. In contrast, activation of calcium-dependent phosphatase calcineurin mediates dephosphorylation of the receptor resulting in its desensitization (Docherty *et al.* 1996; Jung *et al.* 2004). Thus, CaMKII and calcineurin may control the subtle balance between the phosphorylated and dephosphorylated states of TRPV1 resulting in either its activation or desensitization (Jung *et al.* 2004; Rosenbaum *et al.* 2004). The desensitization observed with TRPV1 is calcium dependent, unlike conventional desensitization observed with ligand-gated channels such as AMPA

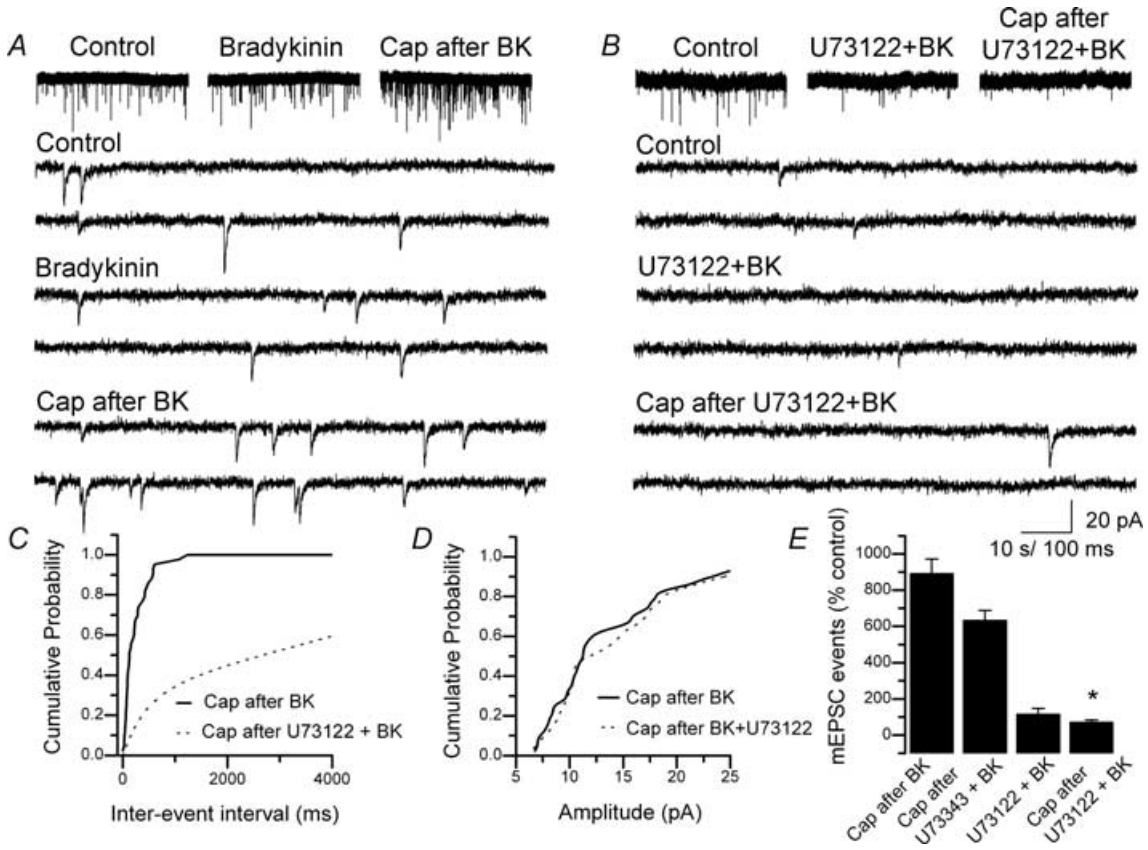

Figure 9. BK-induced sensitization of TRPV1-mediated changes in synaptic transmission involves PLC pathway

A, traces of mEPSCs representing potentiation of capsaicin-mediated synaptic events by BK pretreatment. *B*, the PLC inhibitor U73122 (2 μM) inhibited BK-mediated potentiation of capsaicin responses. Traces are represented in a higher time resolution below. *C* and *D*, graphs showing cumulative probability of inter-event intervals ($P < 0.0001$, KS test) and amplitude ($P = 0.96$, KS test), indicating that U73122 prevents BK induced sensitization of capsaicin responses. *E*, summary graph showing that BK-induced sensitization of TRPV1 is inhibited by the PLC inhibitor U73122 ($n = 6$, $P < 0.05$) but not its inactive analogue, U73343 (2 μM; $n = 3$, $P < 0.05$).

and nicotinic acetylcholine receptors, which is calcium independent, but dependent on the duration of activation and the concentration of agonist.

Change in the mEPSC frequency following capsaicin application is dependent on the influx of calcium through TRPV1. Therefore, an alteration in mEPSC frequency is an indirect measure of TRPV1 function. We have found that both continued and repeated applications of capsaicin decreased the frequency of mEPSCs. The decrease in mEPSC frequency following capsaicin application was long lasting and did not recover upon washout within the time course of our experiments (∼60 min). This decrease in transmission upon capsaicin application could be as a result of either Ca^{2+}-dependent receptor desensitization or primary afferent depolarization (PAD), as a result of TRPV1 activation in the presynaptic terminals of sensory neurons. PAD is considered important in limiting firing of sensory neurons upon repeated capsaicin applications (Yang *et al.* 1998; Doyle *et al.* 2002; Nakatsuka *et al.* 2002; Baccei *et al.* 2003). This involves inactivation of sodium channels due to a relatively depolarized membrane potential secondary to activation of TRPV1. However, whether PAD could affect action potential-independent miniature synaptic events is still unclear. Moreover, this may not be applicable to our study because mEPSCs were recorded in conditions (10 mM lidocaine) that block voltage-gated Na^+ and Ca^{2+} channels. Furthermore, in current-clamp experiments, capsaicin applications resulted in membrane depolarization that was readily reversible. Repeated capsaicin application in these experiments was accompanied by a progressive decrease in response only in the presence of Ca^{2+}, suggesting Ca^{2+}-dependent receptor desensitization (our unpublished data). Therefore, progressive decrease in the synaptic transmission upon either repeated or continuous application of capsaicin is most likely due to Ca^{2+}-dependent desensitization of TRPV1.

Enhancement of synaptic transmission by sensitization of TRPV1 by PKC

Capsaicin-mediated whole-cell currents are robustly potentiated by PKC-activating phorbol esters (PDBu, PMA) in oocytes, in HEK cells heterologously expressing TRPV1 and in native DRG neurons (Premkumar & Ahern, 2000; Vellani *et al.* 2001; Crandall *et al.* 2002; Bonnington & McNaughton, 2003). Two serine residues (Ser-502 and Ser-800) on TRPV1 have been identified as being important in PKC-mediated effects (Numazaki *et al.* 2002). Moreover, inflammatory mediators like BK, prostaglandins (PG), nerve growth factor (NGF) and ATP activate PKC downstream to their G-protein coupled receptors, thereby modulating TRPV1 activity by phosphorylation (Shu & Mendell, 1999; Premkumar & Ahern, 2000; Tominaga *et al.* 2001; Sugiura *et al.* 2002). However, the influence of PKC-mediated phosphorylation of TRPV1 in modulating synaptic transmission at the first sensory synapse has not been studied.

An interesting observation in our study is that PDBu mediates direct (PKC-independent) and PKC-dependent effects on synaptic transmission. Activation of PKC has been shown to facilitate excitatory synaptic transmission in the hippocampus (Malenka *et al.* 1986; Muller *et al.* 1991; Hori *et al.* 1996). However, PDBu has also been reported to increase synaptic transmission by itself to a significant extent in hippocampal slices (Rhee *et al.* 2002). Recent studies have suggested a novel binding site for phorbol ester/DAG on synaptic vesicle priming protein, Munc13–1, which is present only in glutamatergic synapses of the hippocampus (Rhee *et al.* 2002; Brose & Rosenmund, 2002). Activation of Munc13–1 could explain the direct effects of phorbol esters in increasing synaptic transmission. Recently, some studies have suggested that PDBu may enhance synaptic transmission mainly by enhancing the Ca^{2+} sensitivity of synaptic vesicle fusion in the calyx of Held (Lou *et al.* 2005). Thus, PDBu may sensitize the release machinery in the cocultures to enhance synaptic transmission. We have gathered evidence that phosphorylation of TRPV1 plays a role, as the effects we observed with PDBu and capsaicin are synergistic and could be blocked by BIM. However, PDBu-induced direct effect could not be blocked by BIM. These results strongly suggest two different mechanisms for modulation of synaptic transmission by PDBu.

The role of TRPV1 distributed in areas (such as the central terminals of DRG neurons) that are not exposed to temperature ranges required to activate TRPV1 is under debate. Here, we demonstrate that application of PDBu does indeed sensitize TRPV1 and lowers its activation threshold (Numazaki *et al.* 2002; Sugiura *et al.* 2002). At 37°C, PKC activation resulted in an increase in mEPSC events, a significant component of which could be blocked by BCTC, a selective TRPV1 antagonist. However, it should be noted that higher concentrations of BCTC may inhibit other TRP channels, for example recently TRPM8 currents have been shown to be blocked by BCTC (Weil *et al.* 2005; Madrid *et al.* 2006).

This is an important observation as it further bolsters our hypothesis that persistent TRPV1 activation at body temperature following PKC activation in inflammatory or neuropathic pain maybe an important underlying factor contributing to chronic pain conditions. In addition, the inhibitory affects of BCTC on PDBu-mediated responses were not observed when these experiments were performed at room temperature and TRPV1 antagonism at 37°C in the absence of PDBu did not affect the basal synaptic transmission in the cocultures, suggesting that PKC sensitizes the receptor to activate it at body temperature. Thus, it is possible that multiple physical

and chemical stimuli may act synergistically to potently activate TRPV1 and enhance synaptic transmission.

Modulation of synaptic transmission by sensitization of TRPV1 by BK

Inflammatory mediators like BK, PGs, NGF and ATP have been reported to sensitize TRPV1 by activating PKC, PKA and/or CAMKII (Holzer, 2004). We used BK to study its influence on TRPV1-modulated synaptic transmission. Modulation of glutamatergic synaptic transmission by BK has been attributed to activation of its B_2 receptors in spinal cord dorsal horn neurons (Wang *et al.* 2005). We observed that application of BK alone enhanced glutamate release in our cocultures, suggesting a presynaptic locus of action.

BK is known to activate phosholipase C (PLC) resulting in the conversion of PIP_2 to IP_3 and DAG. DAG directly and IP_3 by mobilizing calcium from the internal stores can activate PKC (Burgess *et al.* 1989). BK also activates phospholipase A_2 (PLA2) to produce lipoxygenase metabolites that can activate TRPV1 directly (Hwang *et al.* 2000; Shin *et al.* 2002). We have found that BK synergistically increased capsaicin-induced augmentation of mEPSC frequency, which could be blocked by PKC and PLC inhibitors. These data suggest that BK potentiates TRPV1-modulated synaptic transmission by activating the PLC–PKC pathway.

The source and the stimulus for BK release in the spinal cord during inflammation are not fully understood. However, elevated BK levels have been detected in the spinal cord following peripheral noxious insult (Wang *et al.* 2005). Moreover, administration of BK receptor antagonists has beneficial effects against both acute and chronic pain conditions (Ferreira *et al.* 2002), further supporting the role of BK in pain modulation. Chronic inflammation and/or pathological conditions of the spinal cord/CNS may increase BK levels in the spinal cord, thereby sensitizing TRPV1 by PKC-mediated phosphorylation. Previous studies have demonstrated that BK-induced sensitization of TRPV1 by PKC reduced its activation threshold to well below body temperature (Sugiura *et al.* 2002). Thus, as discussed earlier, under these conditions, TRPV1 could be persistently active in the absence of any other agonist to enhance excitatory synaptic transmission. This facilitation of transmission may result in the hyperexcitability of the postsynaptic dorsal horn neurons. Selective targeting of TRPV1 at the central terminals of sensory neurons could be a beneficial therapeutic strategy to treat chronic, refractory pain arising from inaccessible areas such as viscera, bone, etc.

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