Specific Involvement of PKC-ϵ in Sensitization of the Neuronal Response to Painful Heat

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Summary

Pain is unique among sensations in that the perceived intensity increases, or sensitizes, during exposure to a strong stimulus. One important mediator of sensitization is bradykinin (BK), a peptide released as a consequence of tissue damage. BK enhances the membrane ionic current activated by heat in nociceptive neurons, using a pathway that involves activation of protein kinase C (PKC). We find that five PKC isoforms are present in sensory neurons but that only PKC-ϵ is translocated to the cell membrane by BK. The heat response is sensitized when constitutively active PKC-ϵ is incorporated into nociceptive neurons. Conversely, BK-induced sensitization is suppressed by a specific peptide inhibitor of PKC-ϵ. We conclude that PKC-ϵ is principally responsible for sensitization of the heat response in nociceptors by bradykinin.

Introduction

Adaptation to a maintained stimulus is a general feature of sensory systems. The value of adaptation is that it enables a sensory receptor to operate over a wide range of stimulus intensities, by providing an automatic gain control that reduces the receptor sensitivity as the prevailing stimulus level rises. The sensation of pain is an exception, however, as it is clearly important that an organism should not adapt to a stimulus capable of causing damage. Pain-sensitive neurons, or nociceptors, therefore exhibit sensitization, the opposite phenomenon to adaptation, in which intense stimulation leads to an enhanced responsiveness (Meyer et al., 1994). Sensitization appears not to be initiated within the nociceptive neuron itself, as it is not observed in isolated neurons, but is triggered instead by extracellular mediators that are released in vivo from surrounding damaged or inflamed tissue (reviewed by Cesare and McNaughton, 1997). Mediators known to cause sensitization include (among others) prostaglandins, adenosine, serotonin, and bradykinin (Koltzenburg et al., 1992; England et al., 1996; Gold et al., 1996; Mizumura and Kumazawa, 1996; Ahlgren et al., 1997). The diverse range of sensitizing agents probably reflects the need for nociceptors to enhance their sensitivity in response to damage signals from a wide range of extrinsic sources.

When a heat stimulus is applied to the surface of the body, the sensation changes from one of warmth to one of pain at about 44°C, and the intensity of pain increases sharply as the temperature is elevated (Meyer et al., 1994). Prior damage or inflammation increases the intensity of the sensation of heat pain and shifts the threshold for eliciting a sensation of pain to a lower temperature. Recordings from single heat-sensitive nerve fibers show a similar pattern of response (Meyer et al., 1994). There has recently been considerable progress in understanding the molecular basis of these in vivo observations. Heat has been found to directly activate an inward membrane current in nociceptive neurons (Cesare and McNaughton, 1996; Kirschstein et al., 1997; Reichling and Levine, 1997). The heat-sensitive current is activated rapidly but not instantaneously by heat, and the underlying ion channel is a nonselective cation channel. The recently cloned capsaicin receptor, VR1, exhibits many properties of the heat-activated ion channel (Caterina et al., 1997; Tominaga et al., 1998) and is likely to be the molecular substrate for the response to heat. A more recently described homolog, VRL-1, may mediate responses to more extreme heat (Caterina et al., 1999).

Bradykinin, a nonapeptide released by tissue damage and inflammation, causes sensitization of the response to heat by increasing the amplitude of the heat-sensitive membrane current and shifting the threshold for its activation to lower temperatures (Cesare and McNaughton, 1996). There are striking parallels between these in vitro observations on isolated neurons and the effects of inflammation on the response to heat in vivo. Bradykinin has been shown to sensitize the heat response in isolated neurons by a pathway involving activation of protein kinase C (PKC) (Cesare and McNaughton, 1996). Activation of PKC has also been found to cause sensitization of the heat response in an intact preparation (Mizumura and Kumazawa, 1996; Mizumura et al., 1997), showing that this pathway is important at an organismal level. Activation of PKC is therefore important in explaining the lowering of the threshold for heat pain that is observed in vivo after injury.

There are at least 11 different known isoforms of PKC (Dekker and Parker, 1994). Specificity for particular cellular substrates appears to be generated by the existence of specific receptors for activated PKC (RACKs) associated with a target substrate (Mochly-Rosen, 1995). The present study investigates whether particular

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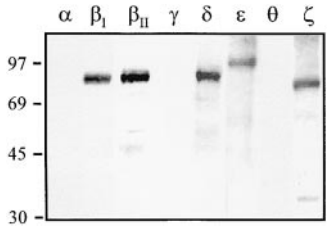

Figure 1. Western Blot of Extract from DRG Neurons, Showing Detection of PKC Isoforms

To the left, the position of molecular weight markers (kD) is indicated. Details of antibodies used in this figure and in Figure 2 are given in the Experimental Procedures.

PKC isoforms are involved in sensitization of the heat response.

Results

PKC Isoform Expression in DRG Neurons

Experiments were performed on primary sensory neurons isolated from the dorsal root ganglion (DRG) of neonatal rats (see the Experimental Procedures). To determine which PKC isoforms might be involved in sensitization by bradykinin (BK), the first step was to investigate isoform expression in DRG neurons by using specific antibodies. We examined isoform expression using both Western blots of extracts from whole DRG (Figure 1) and immunocytochemistry of cultured DRG neurons (Figure 2A). Five isoforms, PKC-β_I, PKC-β_{II}, PKC-δ, PKC-ϵ, and PKC-ζ, were found to be expressed in these neurons using both Western blots and immunocytochemistry (Figure 1; Figure 2A, left column). Other isoforms of PKC were not present to any significant extent (see details in the Experimental Procedures).

Stimulus-Dependent Translocation of PKC

Most PKC isoforms are activated by binding to diacylglycerol (DAG) generated in cell membranes by physiological stimuli (Tanaka and Nishizuka, 1994). Translocation of PKC from a cytosolic to a membrane-associated location within the cell is therefore a sensitive indicator of activation. In DRG neurons, both PKC-δ and PKC-ϵ were observed to be strongly translocated to the surface membrane by treatment with the PKC activator phorbol-12-myristate-13-acetate (PMA, right column in Figure 2A). While PKC-ϵ was almost exclusively associated with the cell membrane after PMA treatment, PKC-δ was also observed to be strongly translocated by PMA to a central location within the cell, probably the nucleus (see Figure 2A). PKC-β_I and PKC-β_{II} were seen to be partially localized to the plasma membrane in the majority of neurons in the unstimulated state, and treatment with PMA did not appear to change the extent of this membrane localization. The cytoplasmic location of PKC-ζ was unaffected by PMA treatment, in agreement with other work showing that PKC-ζ is not activated by phorbol esters (Ways et al., 1992).

The effect of BK in translocating PKC isoforms was next investigated. BK binds to B_2 receptors in neurons to activate phospholipase C, liberate DAG, and thereby activate PKC (Steranka et al., 1988; Burgess et al., 1989;

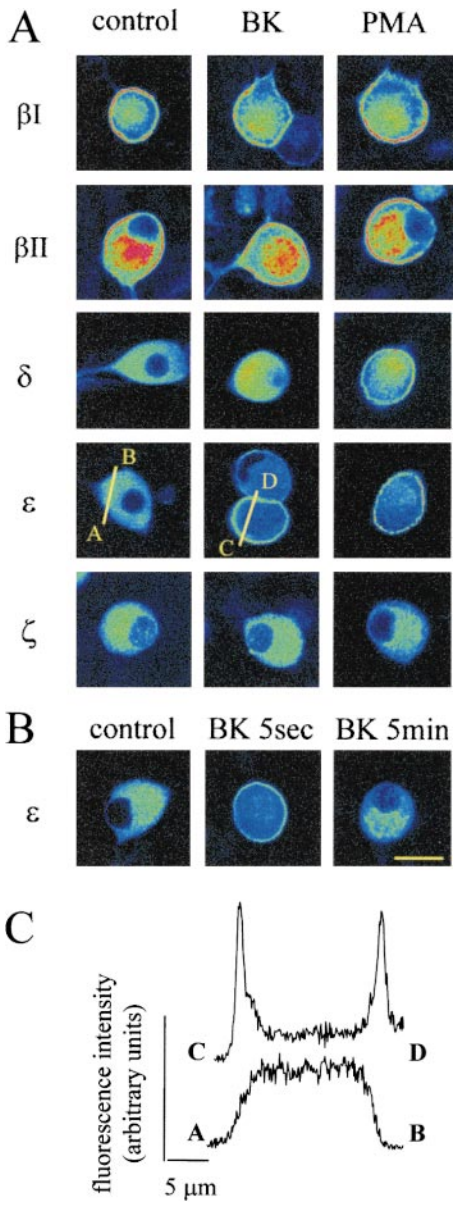

Figure 2. Visualization of PKC Isoforms in Cultured DRG Neurons

(A) Isoform distribution in control neurons (left column), after exposure to BK (10^{-6} M, 30 s, middle column) and after exposure to PMA (2×10^{-7} M, 120 s, right column). False color confocal microscope images in which spectral colors from blue to red represent low to high fluorescence. All images acquired with the same antibody staining and visualization procedures. Yellow bars in PKC-ϵ images show examples of measurement of fluorescence intensity across the neuron (see Figures 2C and 3). Immunoreactivity for other PKC isoforms (PKC-α, PKC-γ, PKC-θ, PKC-λ/ι, and PKC-μ) was also investigated, and little or no specific signal was seen (see the Experimental Procedures).

(B) Time course of translocation of PKC-ϵ by BK. Neurons were exposed for the indicated times to BK (10^{-6} M) before rapid fixation. Scale bar in right image is 20 μm in length and applies to all panels in (A) and (B). Note that the translocation of PKC-ϵ after t = 30 s is shown in (A).

(C) PKC-ϵ specific fluorescence intensity as a function of distance along lines A to B (control) and C to D (BK 10^{-6} M, 30 s) for the two cells shown in (A).

Dray and Perkins, 1993). PKC-ϵ was the only isoform observed to be translocated by BK (middle column of Figure 2A). Detectable translocation was observed in 31% of PKC-ϵ-positive neurons (n = 618). Effects on all neurons would not be expected since only a minority of neurons respond to BK. The figure of 31% of neurons exhibiting PKC-ϵ translocation is similar to the figure of 25% of neurons responding to BK with an elevation in intracellular Ca^{2+} after 3–4 days in culture (Gilabert and McNaughton, 1997).

The PKC-ϵ translocation caused by BK was rapid, with substantial translocation observed in many neurons after only 5 s exposure to BK (Figure 2B). Translocation was complete within 30 s (Figure 2A). At longer times, translocation was gradually reversed even in the maintained presence of BK, and after 5 min, PKC-ϵ was no longer associated with the surface membrane (Figure 2B). The more punctate cytoplasmic distribution observed after longer stimulation may be due to association with subcellular organelles during the process of downregulation (Goode et al., 1995). The rapid time course of translocation of PKC-ϵ to the surface membrane correlates well with the time course of sensitization of the heat-activated membrane current by BK, which is maximal in less than 30 s. The reversal of translocation at longer times also fits well with the transient nature of the sensitization caused by BK, which declines to the prestimulus level with a time constant of 2–3 min even in the continuous presence of BK (Cesare and McNaughton, 1996).

Treatment with PMA caused a similarly rapid translocation to the membrane, but even 20 min after treatment with PMA the PKC-ϵ remained membrane localized (data not shown). The different behavior of PKC-ϵ at longer times after treatment with BK and PMA agrees well with the observation that the sensitization of the heat-activated membrane current is transient after exposure to BK but is maintained for tens of minutes after application of PMA (Cesare and McNaughton, 1996).

BK did not cause translocation of other PKC isoforms to the surface membrane. The lack of effect of BK on PKC-β_I, PKC-β_{II}, and PKC-ζ is expected in view of the lack of effect of PMA in translocating these isoforms, as described above. It is interesting, though, to note that PKC-δ, which is observed to be translocated by PMA, is unaffected by BK (Figure 2A). No significant translocation of PKC-δ to the surface membrane was observed in any neuron. This result suggests that agonists such as BK may be able to activate particular isoforms of PKC selectively.

Quantative Analysis of PKC Translocation

The translocation of PKC isoforms is explored quantitatively in Figures 2C and 3. Figure 2C shows an example of measurement of PKC-ϵ fluorescence intensity as a function of distance across two of the neurons shown in Figure 2A, one unstimulated and the other treated with BK. Treatment with BK causes a clear redistribution of PKC-ϵ from the cytoplasm to the membrane. Collected results from quantitation of PKC-β_I, PKC-β_{II}, PKC-δ, and PKC-ϵ distribution in a number of neurons

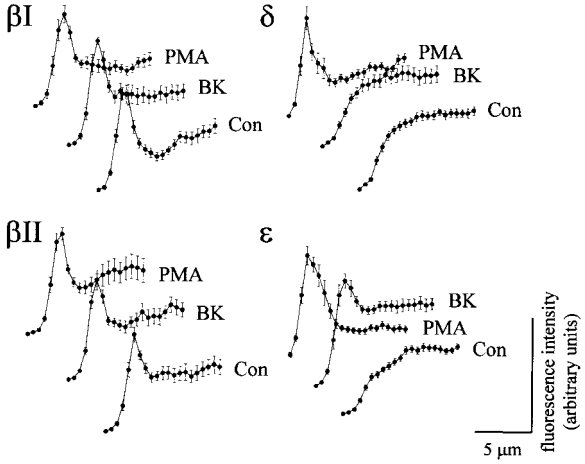

Figure 3. Distribution of PKC Isoforms in DRG Neurons

Distribution of PKC-β_I (top left), PKC-β_{II} (bottom left), PKC-δ (top right), and PKC-ϵ (bottom right) in unstimulated neurons (Con) and in neurons treated with BK (10^{-6} M, 30 s) and PMA (2×10^{-7} M, 120 s). Isoform-specific fluorescence intensity (given on ordinate as arbitrary fluorescence units) was measured along a line across each neuron as shown in Figures 2A and 2C. Average profiles were constructed from between 8 and 16 separate measurements. Bars show ± SEM.

are shown in Figure 3. The averaged half profiles show the distribution of PKC isoforms from the surface membrane to approximately the center of the cell. PKC-ζ was found to be distributed uniformly in the cytoplasm of all neurons, and this distribution was unaffected by either BK or PMA (data not shown).

In the case of PKC-β_I and PKC-β_{II} (left two panels in Figure 3), the fluorescence intensity at the membrane even in unstimulated neurons was significantly higher than in the cytoplasm (traces labeled Con), showing that these isoforms are to some extent membrane localized even in the unstimulated state. Stimulation of the cells with BK or PMA did not significantly affect the extent of membrane localization of PKC-β_I and PKC-β_{II} (traces labeled BK and PMA, respectively). PKC-δ was observed to be distributed uniformly throughout the cytoplasm in untreated neurons, a distribution that was unchanged by BK treatment (top right panel in Figure 3). In confirmation of the result shown in Figure 2A, however, activation of PKC-δ by PMA caused a significant migration of PKC-δ to the membrane. PKC-ϵ was also observed to be uniformly distributed in untreated cells (lower right panel in Figure 3), but in contrast to the behavior of PKC-δ, a strong translocation of PKC-ϵ to the membrane was observed in response to BK. A similar but even more marked membrane translocation was observed in response to PMA. These collected results confirm that both PKC-ϵ and PKC-δ are translocated by PMA, but that PKC-ϵ is the only isoform to be translocated by BK.

Activated PKC-ϵ Sensitizes the Heat Response

The above results suggest that PKC-ϵ may be specifically responsible for sensitization of the heat response by BK. As a first step in investigating this possibility, we wanted to test whether active PKC-ϵ alone is capable of

sensitizing the heat-activated current. Activating PKC-ϵ by the use of pharmacological activators such as phorbol esters is not a useful approach as these compounds activate many PKC isoforms nonselectively. We chose instead to activate PKC-ϵ specifically by constructing a constitutively active PKC-ϵ, which was then introduced into the cell via the patch pipette while the membrane current activated by heat was continuously monitored.

PKC isoforms active in the absence of the normal physiological activators can be generated by deleting the internal pseudosubtrate region, which in native PKC binds to and inhibits the catalytic site (Pears et al., 1990; Dekker et al., 1993). Constitutively active PKC-ϵ carrying a pseudosubstrate site deletion, PKC-ϵ-Δ(166–172), was purified from a mammalian expression system (see the Experimental Procedures). Potent kinase activity was associated with this protein and was absent from control preparations obtained from cells transfected with vector alone (see Figure 4A). The phosphotransferase activity of this mutant in the absence of cofactor was 60% of its full activity kinase activity in the presence of the activators phosphatidyl serine and PMA (Figure 4A, right bars), in contrast to native PKC-ϵ whose kinase activity is close to zero in the absence of activators (Schaap et al., 1989). Although the kinase activity of the deletion mutant was lower than that observed for an extensive pseudosubstrate site deletion (Dekker et al., 1993), the limited mutation showed higher recovery and easier extractability and was therefore favored.

The effect of introducing constitutively active PKC-ϵ into heat-responsive neurons via the patch pipette, compared with the effect of control extracts from transfections with vector alone, is shown in Figure 4B. The response to heat was probed repeatedly with brief (400 ms) elevations of temperature to 49°C. When extract from control transfections was included in the patch pipette (Figure 4B, upper panel), the inward current elicited by heat remained constant or declined slightly. Similar results are obtained in experiments using normal patch pipette solution, showing that no modulatory factor had been introduced by the transfection and isolation procedure. Inclusion of active PKC-ϵ, in contrast, caused an increase in the heat-activated current over a period of minutes as the protein diffused into the cell (Figure 4B, lower panel). A slow effect is expected because of the diffusion barrier imposed by the narrow mouth of the patch pipette (Lagnado et al., 1992).

Collected results from a number of experiments are shown in Figure 5B. The difference between the current change caused by active PKC-ϵ (left bar) and extract from control transfections (middle bar) was highly significant ($p = 0.009$, t test). The magnitude of the sensitization induced by active PKC-ϵ is similar to that of sensitization induced by BK itself (compare first and third bars in Figure 5B).

A Specific PKC-ϵ Inhibitor Suppresses Sensitization

A further way of testing for a specific involvement of PKC-ϵ in sensitization is to inhibit its activation without affecting other PKC isoforms. Translocation of PKC-ϵ to its target sites can be specifically inhibited by a peptide identical to the PKC-ϵ V1 region, which acts by competing for PKC-ϵ binding sites on a specific RACK receptor

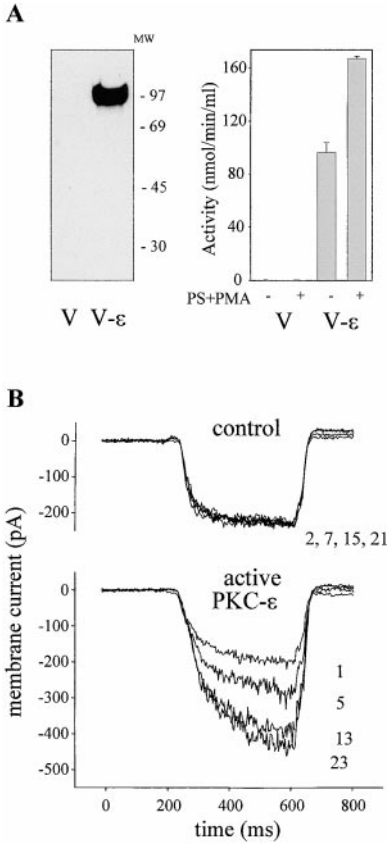

Figure 4. Preparation of Constitutively Active PKC-ϵ-Δ and Its Effect on the Heat-Activated Membrane Current

(A) PKC-ϵ-Δ(166–172) was purified from cells transfected with vector coding for PKC-ϵ-Δ(166–172) (see the Experimental Procedures). The left-hand panel shows immunoreactivity of PKC-ϵ in transfections with the vector coding for PKC-ϵ-Δ(166–172) (V-ϵ), and its absence in parallel preparations from control cells transfected with vector alone (V), assessed by Western blotting using an antibody specific for PKC-ϵ (Schaap et al., 1989). The presence of PKC-ϵ-Δ(166–172) of the correct molecular weight, and the absence of lower molecular weight components, shows that fragments such as the catalytic subunit are not produced during the isolation procedure. The right-hand panel shows that kinase activity is associated with purified PKC-ϵ-Δ(166–172) (V-ϵ) and is absent in control preparations (V). Kinase activity was measured in the absence or presence of the cofactors phosphatidylserine (PS, 1.25 mg/ml) and PMA (1.25 μg/ml), as indicated at bottom. Kinase activity of PKC-ϵ-Δ(166–172) was largely cofactor independent, in contrast to wild-type PKC-ϵ whose activity is dependent on cofactors (Schaap et al., 1989). No free PKC-ϵ catalytic fragment (approximately 45 kDa) is present in the preparation (see left panel), showing that all kinase activity is attributable to PKC-ϵ-Δ(166–172).

(B) Heat-activated membrane current is sensitized by constitutively active PKC-ϵ. A heat stimulus (49°C, applied between 200 and 600 ms) rapidly activates an inward membrane current in heat-sensitive neurons. The amplitude of the heat-activated current changes little during the course of a long recording when control extract is present in the pipette solution (upper traces) but approximately doubles in amplitude when PKC-ϵ-Δ(166–172) was incorporated into the solution used to fill patch-clamp pipettes (lower traces). Neurons were patch clamped at a holding potential of −70 mV, and temperature was rapidly (<20 ms) elevated from 20°C to 49°C for 400 ms as previously described (Cesare and McNaughton, 1996). Time in minutes after attaining the whole-cell configuration, and therefore commencing internal perfusion, is shown adjacent to each trace.

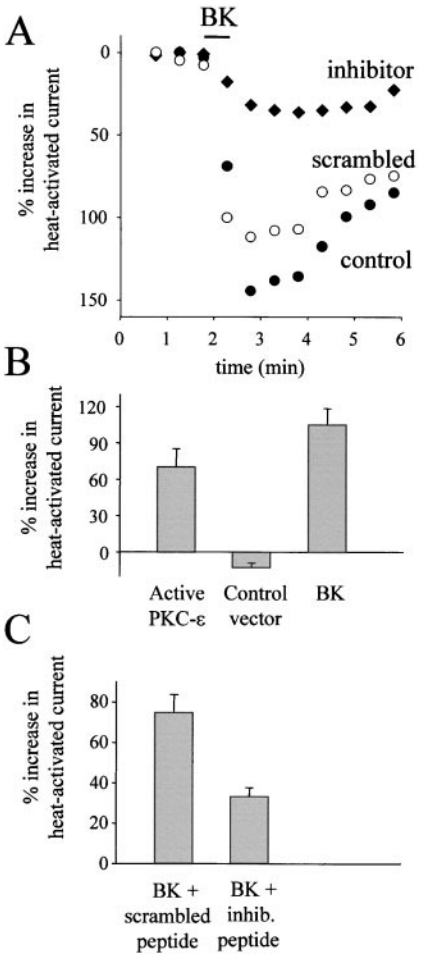

Figure 5. Effect of a Specific PKC-ϵ Inhibitor on Sensitization of Heat-Activated Current by BK

(A) Points show the heat-activated membrane current increase observed on application of a brief step to 49°C, using a similar protocol to that shown in Figure 4B, when BK (10^{-6} M) is applied as shown by the bar. The three experiments show the sensitization in a control cell (filled circles), with PKC-ϵ translocation inhibitor incorporated in the patch solution (200 μM; see Johnson et al., 1996; filled diamonds), and with scrambled translocation inhibitor (200 μM, open circles). In all recordings, 10 min was allowed after attaining the whole-cell configuration and before applying BK to facilitate equilibration of pipette contents with the cell interior.

(B) Collected results of experiments similar to those shown in Figure 4B. Bars show percentage change (mean ± SEM) in heat-activated current. From left: incorporation of constitutively active PKC-ϵ-Δ(166–172) sensitizes heat-activated current (n = 18); in control extract, from transfections with vector only, no enhancement was observed (n = 5); BK alone (10^{-6} M) causes sensitization similar to that seen with constitutively active PKC-ϵ-Δ(166–172) (n = 4). Difference between PKC-ϵ-Δ(166–172) (bar 1) and control (bar 2) was highly significant ($p = 9.3 \times 10^{-3}$, t test).

(C) Collected results of experiments similar to those shown in Figure 5A. Bars show percentage change (mean ± SEM) in heat-activated current. When 200 μM scrambled translocation inhibitor peptide is included in patch pipette, no significant reduction in the BK-evoked increase in heat-activated membrane current is observed (bar 1, n = 4) when compared to the effect of BK without scrambled peptide (bar 3 in [B] above). With translocation inhibitor peptide in pipette (bar 2, n = 7), the sensitization is significantly reduced when compared both to BK alone (bar 3 in [B]; $p = 1.5 \times 10^{-4}$) and BK with scrambled peptide (bar 1, this panel; $p = 0.9 \times 10^{-3}$).

protein associated with the target (Johnson et al., 1996). We therefore tested for a specific involvement of PKC-ϵ by observing the effects of this translocation inhibitor peptide on sensitization of the heat-activated current by BK. In control neurons exposed to BK, the heat-activated membrane current increased by a mean of 105% (see Figure 5A and mean data in Figure 5B). When in parallel experiments the translocation inhibitor was included in the patch pipette solution, however, the increase in heat-activated current caused by BK was reduced to 33% (Figures 5A and 5C). As a control, we tested the effect of introducing a scrambled peptide, constructed using the same amino acids as in the inhibitor peptide but in a random order. The sensitization of heat-activated current caused by BK in the presence of the scrambled peptide was not significantly different from that observed with BK alone (Figures 5A and 5C). The sensitization of heat-activated current in the presence of inhibitor was significantly lower than that observed both with no peptide in the pipette ($p = 1.5 \times 10^{-4}$) and with scrambled peptide ($p = 0.9 \times 10^{-3}$). The sensitization caused by BK is therefore substantially reduced when translocation of PKC-ϵ is inhibited.

Discussion

Short-term sensitization is now known to be mediated by two distinct intracellular pathways in nociceptive neurons. In the first mechanism, sensitizing agonists such as prostaglandins, which increase the cellular level of cAMP, lower the threshold for initiation of an action potential and thereby sensitize the neuron to all pain-producing stimuli (England et al., 1996; Gold et al., 1996; Ingram and Williams, 1996; Nicol et al., 1997). In the second, activation of PKC by the proinflammatory peptide bradykinin has the more specific effect of sensitizing the response to painful levels of heat by directly enhancing the amplitude of the membrane current activated by a heat stimulus (Cesare and McNaughton, 1996). The temperature threshold for activating the heat-sensitive current is also lowered, so that normally nonpainful temperatures, such as normal bodily warmth, are capable of activating heat-sensitive current. These observations suggest that activation of heat-sensitive nociceptors may contribute to inflammatory pain in vivo.

The results in the present study demonstrate that one isoform of PKC, namely PKC-ϵ, has a vital role to play in this process of sensitization. We find that of the five isoforms of PKC present in nociceptive neurons, only PKC-ϵ is translocated by BK. A role for PKC-ϵ is suggested by the finding that when PKC-ϵ is made constitutively active by removing the pseudosubstrate inhibitor region it is indeed capable of causing sensitization. While this finding does not prove that PKC-ϵ is the only isoform capable of causing sensitization, it does show that PKC-ϵ activation is a sufficient condition for producing sensitization. An important role for PKC-ϵ is confirmed by experiments in which sensitization is found to be largely abolished when PKC-ϵ activation is inhibited by a specific translocation inhibitor. The failure of the inhibitor to abolish completely the sensitization caused by BK may be attributable to a small contribution to sensitization from PKC isoforms other than PKC-ϵ. Alternatively, it may be due to incomplete inhibition of

PKC-ε translocation, perhaps due to failure to achieve complete diffusion of the inhibitor peptide into the cell from the patch pipette. The observation that 70% of the sensitization caused by BK was suppressed by the specific PKC-ε translocation inhibitor does, however, confirm that PKC-ε is the major contributor to BK-induced sensitization. This last observation was made using a cell-impermeable peptide inhibitor of translocation, but the result suggests that if specific cell-permeable inhibitors of PKC-ε can be developed, they may have therapeutic potential in reducing the hyperalgesia caused by the release of BK and other mediators during inflammation.

The ability of BK to cause specific translocation of PKC-ε suggests that mechanisms are in place in the cell for coupling BK receptor activation to a specific isoform of PKC. Of the five isoforms observed in sensory neurons, two, PKC-β_I and PKC-β_{II}, are observed to be located at the membrane in the resting state, so it is perhaps not surprising that no further translocation is observed on application of either BK or PMA. Three isoforms, PKC-δ, PKC-ε, and PKC-ζ, were seen to be uniformly distributed in the cytoplasm in the resting state. Both PKC-δ and PKC-ε are seen to be efficiently translocated by PMA, the first to both the surface membrane and the nucleus, and the second only to the membrane. The response of PKC-δ and PKC-ε to PMA is mediated through binding of PMA to the C1 domain of these isoforms (Hurley et al., 1997). The atypical C1 domain in PKC-ζ does not bind PMA (Hurley et al., 1997), and, accordingly, we did not observe any changes in PKC-ζ location upon PMA treatment.

These results show that both PKC-δ and PKC-ε are capable of undergoing translocation, but upon application of BK, only PKC-ε responds by translocating to the plasma membrane. No translocation of PKC-δ in response to BK was observed in any neuron, while PKC-ε translocation was seen in 31% of neurons, approximately the proportion that responds to BK with an elevation in $[Ca^{2+}]_i$ (Gilabert and McNaughton, 1997). BK receptor activation is therefore specifically coupled to translocation of PKC-ε, but not to PKC-δ. Generation of DAG by activation of phospholipase C, following binding of BK to the B_2 receptor (Burgess et al., 1989), is therefore not a sufficient signal for causing translocation of PKC-δ. These observations suggest that an additional isoform-specific signal is required to induce translocation and that BK receptor activation generates this signal for PKC-ε but not for PKC-δ. The observation that an agonist can target specific isoforms of PKC is interesting in the context of understanding the functions of the specific isoforms in cellular signaling.

Another isoform of PKC, PKC-γ, has recently been shown to have a quite distinct role in enhancing the sensation of pain (Malmberg et al., 1997). Neuropathic pain, which can be induced by peripheral nerve damage, persists in the absence of a stimulus or is elicited only by mild nonnoxious stimuli. This syndrome is found to be greatly reduced in mice lacking the gene for PKC-γ. Changes in the responsiveness of DRG neurons, such as those described here, seem not to be responsible. Rather, the mechanism involves a postsynaptic PKC-γ-dependent upregulation of neurotransmitter and neuropeptide receptors in the dorsal laminae of the spinal cord, and a consequent increase in the responsiveness of second order neurons to both painful and innocuous stimuli. Two different isoforms of PKC therefore have quite distinct roles, and at different stages, in regulating the sensitivity of the pain pathway.

Experimental Procedures

Western Blots

DRGs from 3- to 5-day-old neonatal rats pups were extracted in Laemmli buffer (Laemmli, 1970), and 10 μg of protein was analyzed for the presence of PKC isoforms by SDS-polyacrylamide gel electrophoresis (Laemmli, 1970) and Western blotting (Towbin et al., 1979). The following antibodies were employed for the gel shown: PKC-α, MC5 (Young et al., 1988); PKC-β_I, SC-209 (Santa Cruz); PKC-β_{II}, SC-210 (Santa Cruz); PKC-γ, SC-211 (Santa Cruz); PKC-δ, protein A purified polyclonal antibody (Olivier and Parker, 1991); PKC-ε, protein A purified polyclonal antibody (Schaap et al., 1989); PKC-θ, SC-1875 (Santa Cruz); PKC-ζ, protein A purified polyclonal antibody (Ways et al., 1992). In other experiments, the absence of PKC-α was confirmed with an antibody from Life Technologies (GIBCO). All incubations were performed at 1:1000 dilution. Blots were processed as described (Dekker et al., 1993).

Immunocytochemistry

The dorsal root ganglia (DRG) from 3- to 5-day-old neonatal rats were isolated and neuronal cultures were prepared as previously described (Cesare and McNaughton, 1996; Gilabert and McNaughton, 1997). After culture for 4d, coverslips were exposed to BK or PMA as indicated and then rapidly fixed by immersion in 4% formaldehyde (w/v) plus 4% sucrose (w/v) in 50% PBS (GIBCO) for 20 min at 37°C. Neurons were then permeabilized with 0.1% Triton X-100 in PBS (5 min). Fish skin gelatine (Sigma, 0.2% w/v) was used throughout to reduce nonspecific antibody binding. Fixed neurons were exposed to primary antibodies (detailed below) for 2 hr before washing and labeling with fluorescein-labeled secondary antibodies (goat anti-mouse [Southern Biotechnology Associates] or goat anti-rabbit [Jackson ImmunoResearch Labs], as appropriate). Neurons were visualized using an MRC-600 confocal microscope, and images were stored for later analysis.

Primary antibodies used for each isoform, and results obtained, were as follows (all concentrations, 5 μg/ml unless otherwise stated; all noncommercial polyclonals purified by passing through a protein A sepharose column): PKC-α, MC-5 (Young et al., 1988) and a polyclonal from Life Technologies (GIBCO) were both negative; PKC-β_I, SC-209 (Santa Cruz) was positive (Figure 2A); PKC-β_{II}, SC-210 (Santa Cruz) was positive (Figure 2A); PKC-γ, SC-211 (Santa Cruz) was negative; PKC-δ, a polyclonal (12.5 μg/ml) (Olivier and Parker, 1991) was positive (Figure 2A), as was a monoclonal from Transduction Labs; PKC-ε, a polyclonal (15 μg/ml) (Schaap et al., 1989) was positive (Figures 2A and 2B), as was a monoclonal from Transduction Labs; PKC-ζ, a polyclonal (17 μg/ml) (Ways et al., 1992) was positive (Figure 2A), as was a monoclonal from Transduction Labs; PKC-θ, -λ/ι, and -μ, monoclonals from Transduction Labs were negative. Specificity of labeling by polyclonal antibodies for PKC-δ, -ε, and -ζ was checked by preabsorption with the antigenic peptide used to raise the antibody, and in the case of anti-PKC-β_I, with the whole PKC-β_I protein overexpressed in HEK-293 cells. In each case, the immunoreactivity was completely abolished.

Production of Constitutively Active PKC-ε

The mammalian expression vector pcDNA3 containing a PKC-ε pseudosubstrate site deletion mutant, PKC-ε-Δ(166–172), fused N terminally to a 6-His tag, was introduced into HEK 293 cells by calcium phosphate precipitation (Sambrook et al., 1989). After 60 hr, cells were homogenized and his-PKC-ε-Δ(166–172) was extracted using Ni^{2+}-NTA agarose beads (Qiagen) (Bornancin and Parker, 1996). The final extract containing his-PKC-ε-Δ(166–172) was stored with an equal volume of glycerol at −20°C and before use was dialyzed for 5 hr against the solution used to fill the patch pipette. The control preparation was made by the same procedure using cells transfected with vector pcDNA3 alone. Western blots were

performed as described (Dekker et al., 1993). Kinase activity was measured from incorporation of ^{32}P into a synthetic PKC pseudosubstate peptide in the presence of 0.25% Triton X-100 (Dekker et al., 1993).

Electrical Recording

Neurones were whole-cell patch clamped at −70 mV and step 49°C heat stimuli of rise time 20 ms and duration 400 ms were applied as previously described (Cesare and McNaughton, 1996). Pipettes were back filled with pseudo-intracellular solution containing PKC-ϵ or inhibitor peptide as appropriate, and repetitive test pulses of heat were applied at 30 s intervals. Neurons were classified as heat sensitive if they exhibited a delayed heat-sensitive current increase of 50 pA or greater.

PKC-ϵ Inhibitor Peptide

PKC-ϵ inhibitor peptide ϵV1–2 (EAVSLKPT) was purchased from Calbiochem. The scrambled peptide ϵV1–2 (LSETKPAV) (see Johnson et al., 1996) was synthesized at the ICRF peptide synthesis facility. The peptides were used at a final concentration in the patch pipette of 200 μM.

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