Activation and Recovery of the PGE$_2$-Mediated Sensitization of the Capsaicin Response in Rat Sensory Neurons

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Lopshire, J. C. and G. D. Nicol. Activation and recovery of the PGE$_2$-mediated sensitization of the capsaicin response in rat sensory neurons. J. Neurophysiol. 78: 3154–3164, 1997. Pro-inflam-matory prostaglandins are known to enhance the sensitivity of sensory neurons to various modalities of stimulation, including the excitatory chemical agent, capsaicin. In this report, we examined the capacity of prostaglandin E$_2$ (PGE$_2$) to enhance the capsaicin response recorded from sensory neurons isolated from embryonic rats and grown in culture. Previous work demonstrated that the cyclic adenosine 3',5'-monophosphate pathway mediates initiation of the PGE$_2$-induced sensitization, however, little is known about the pathways regulating the recovery from sensitization. Therefore, we examined the neuronal transduction cascades that control the duration of sensitization. Treatment with PGE$_2$ enhanced the capsaicin-evoked current by two- to threefold, however, this sensitization was transient even in the continued presence of prostaglandin. The duration of sensitization produced by PGE$_2$ was related inversely to the extracellular Ca$^{2+}$ concentration with the shortest recovery times observed in cells exposed to 2 mM Ca$^{2+}$-Ringer. Inclusion of the Ca$^{2+}$ chelator, bis-(o-aminophenoxyl)-N,N,N',N'-tetraace-tic acid, in the recording pipette greatly lengthened the period of sensitization. Pretreatment with either the nitric oxide synthase inhibitor, nitro-L-arginine methyl ester (L-NAME), or the inhibitor of the cyclic guanosine 3',5'-monophosphate (GMP)–depen-dent protein kinase, KT-5823, before the application of PGE$_2$ increased the duration of sensitization even in the presence of 2 mM Ca$^{2+}$. In contrast, after attaining maximal sensitization in 2 mM Ca$^{2+}$-Ringer containing L-NAME, the addition of either nitric oxide donors (3-morpholinosydnonimine or s-nitroso-n-acetylpenicil-lamine) or 8-Br-cyclic GMP led to a rapid decrease in the level of sensitization. In the absence of sensitization, nitric oxide-cyclic GMP modulating agents had no effect on the capsaicin-evoked current. Therefore, these results suggest that capsaicin-induced elevations in intracellular Ca$^{2+}$ levels lead to an enhanced production of cyclic GMP, via the nitric oxide pathway, that ultimately acti-vates cyclic GMP-dependent protein kinase. This protein kinase inactivates or terminates the sensitization produced by PGE$_2$ by an as yet unidentified mechanism.

INTRODUCTION

The sensitivity of mammalian sensory neurons to excitatory chemical agents can be enhanced greatly by exposure to pro-inflammatory prostaglandins (Foreman 1987; Higgs et al. 1984; Salmon and Higgs 1987). This sensitization is observed in behavioral studies as a facilitated perception of pain (Handwerker and Kobal 1993; Handwerker and Reeh 1991) as well as in electrophysiological recordings obtained from isolated sensory neurons. For example, in cultured sensory neurons, treatment with prostaglandin E$_2$ (PGE$_2$) enhances the number of action potentials generated by exposure to either elevated levels of potassium (Baccaglini and Hogan 1983) or the inflammatory mediator, bradykinin (Nicol and Cui 1994). The sensitization produced by PGE$_2$ is mediated by the cyclic adenosine 3',5'-monophosphate (cAMP) transduction cascade because PGE$_2$ increases the intracellular levels of cAMP, exogenous cAMP mimics the effects of PGE$_2$, and inhibition of the cAMP-dependent protein kinase (PKA) blocks sensitization by PGE$_2$ (Cui and Nicol 1995; Hingtgen et al. 1995). In contrast, very little is known about the transduction pathways that lead to the termination of PGE$_2$-initiated sensitization.

A subset of mammalian sensory neurons are stimulated selectively by the vanilloid, capsaicin. These capsaicin-sensitive A-delta fibers are believed to be involved in nociceptive signaling (Holzer 1991). Capsaicin generates an inward current that depolarizes the sensory neuron (Bevan and Forbes 1988; Bevan and Szolcsanyi 1990; Heyman and Rang 1985; Marsh et al. 1987). This inward current results from the opening of a nonselective cationic channel that is largely permeable to Na$^+$ and Ca$^{2+}$ (Bevan and Szolcsanyi 1990; Marsh et al. 1987; Oh et al. 1996; Wood et al. 1988). The capsaicin-mediated influx of Ca$^{2+}$ appears to play a critical role in activating cellular pathways that give rise to the rapid and significant desensitization of the capsaicin response (Cholewinski et al. 1993; Liu and Simon 1996; Yeats et al. 1991). Indeed, recent observations suggest that activation of the Ca$^{2+}$-dependent phosphatase, calcineurin, somehow modifies the activity of the capsaicin-gated channel to produce the desensitization (Docherty et al. 1996).

In addition to inducing desensitization, an influx of Ca$^{2+}$ can lead to the activation of other enzymatic cascades. In rat sensory neurons grown in culture, capsaicin causes an increase in the intracellular levels of cyclic guanosine 3',5'-monophosphate (GMP) (Dymshitz and Vasko 1994; Wood et al. 1989) that is dependent on the presence of extracellular Ca$^{2+}$, however, cAMP levels remain unaffected (Wood et al. 1989). This raises an interesting question as to whether cyclic GMP may play a physiological role in modulating the neuronal sensitization produced by pro-inflammatory prostaglandins. In support of this idea, McGehee et al. (1992) demonstrated that elevated levels of cyclic GMP, as well as receptor occupancy, were required for desensitization of the bradykinin response in rat sensory neurons. Therefore, in this study, we examine the capacity of the pro-inflammatory prostaglandin, PGE$_2$, to sensitize the response to capsaicin recorded from rat sensory neurons grown in culture. Furthermore, we investigate the roles of extracellular Ca$^{2+}$, the nitric oxide-cyclic GMP pathway, and the cyclic GMP-dependent protein kinase (PKG) in reg-
ulating inactivation of the PGE$_2$-induced sensitization. Our findings suggest that activation of PKG via the nitric oxide-cyclic GMP pathway controls the duration of PGE$_2$-induced sensitization.

**METHODS**

**Isolation and culture of embryonic rat sensory neurons**

The procedures for isolation and culture of rat sensory neurons have been described previously (Vasko et al. 1994). Briefly, pregnant rats (days 15–17 of gestation) were rendered unconscious by placing them in a chamber filled with CO$_2$; animals were then killed by cervical dislocation. The dorsal root ganglia (DRG) from the embryos were dissected free and placed in a dish containing sterile calcium-free, magnesium-free Hank’s balanced saline (HBSS) at 4°C. The DRGs then were incubated in HBSS containing 0.025% trypsin for 25 min at 37°C. The digestion was terminated with the addition of 0.25% trypsin inhibitor; ganglia were washed once with HBSS, centrifuged, and then resuspended in growth medium that consisted of Dulbecco’s modified Eagle’s medium (Life Technologies, Grand Island, NY) supplemented with 2 mM glutamine, 50 µg/ml penicillin and streptomycin, 10% (vol/vol) heat-inactivated fetal bovine serum, 50 µM 5-fluoro-2′-deoxyuridine, 150 µM uridine, and 250 ng/ml 7-sulfuryl nerve growth factor (Harlan Bioproducts for Science, Indianapolis, IN). Individual cells were obtained by mechanical agitation with a fire-polished pipette until a cloudy suspension was observed. Approximately 300,000 viable cells were plated in a collagen-coated culture dish (35 mm) containing small plastic cover slips. Cells were grown at 37°C in a 95% O$_2$–5% CO$_2$ atmosphere and the media changed every 2 days. All procedures were approved by the Animal Care and Use Committee at Indiana University School of Medicine.

**Electrophysiology**

The procedures for whole cell patch-clamp recording from rat sensory neurons have been described in detail previously (Nicol and Cui 1994). Briefly, a cover slip with the sensory neurons (typically after 4 days in culture) was placed in the recording chamber where the neurons were superfused with normal Ringer solution of the following composition (in mM): 140 NaCl, 5 KCl, 2 CaCl$_2$, 1 MgCl$_2$, 10 N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES), and 10 glucose, pH at 7.4 with NaOH. Using the whole cell patch-clamp technique (Hamill et al. 1981), membrane voltages or currents were recorded with a List EPC-7 patch-clamp amplifier (List Electronic, Darmstadt, Germany). Recording pipettes were pulled from borosilicate disposable pipettes and typically had resistances of 2–4 MΩ when filled with the following solution (in mM): 140 KCl, 5 MgCl$_2$, 4 NaATP, 0.3 Na$_3$GTP, 2.5 CaCl$_2$, 5 ethylene glycol-bis(β-aminoethyloxy)ether-N,N,N′,N″-tetraacetic acid (EGTA; calculated free Ca$^{2+}$ concentration of 100 nM), and 10 HEPES, pH at 7.2 with KOH. In those experiments using 10 mM bis-(o-aminophenox)-N,N,N′,N″-tetraacetic acid (BAPTA; tetrapotassium salt), the concentration of KCl was lowered to 100 mM.

The whole cell recording configuration was established in normal Ringer solution. The cell capacitance was compensated by the nulling circuitry of the recording amplifier. The series resistance was compensated (average value 29 ± 0.4%: mean ± SE) and yielded values for the uncompensated series resistance that ranged from 1.6 to 6.6 MΩ (average value 3.4 ± 0.21 MΩ). In voltage-clamp recordings, the membrane was held at −60 mV, which is close to the value of the normal resting potential of about −55 mV (see Cui and Nicol 1994). The currents evoked by capsaicin were recorded on videotape at 3 kHz. Individual responses were played through an 8-pole Bessel filter at 100 Hz and then into the computer using the program Axotape (Axon Instruments, Foster City, CA) where they were sampled at 300 Hz. The traces shown in the figures have been filtered digitally at 40 Hz using the Fetchan program of pClamp 6.0.3 (Axon Instruments).

The procedures for perforated-patch recording are modified from those described by Rae et al. (1991). Briefly, the pipette tip was filled with a solution of the following composition (in mM): 90 K-aspartate, 50 KCl, 1 MgCl$_2$, 10 HEPES, pH adjusted to 7.2 with KOH. The electrode was then backfilled with the same solution containing amphotericin B (240 µg/ml). These electrodes had resistances between 2 and 4 MΩ in normal Ringer solution. After attaining the cell-attached configuration, membrane partitioning of amphotericin B was assessed by applying +10 mV voltage pulses (from −60 mV) at 2-min intervals until a steady state access resistance was obtained (typically 20 min). The mean access resistance was 11.2 ± 0.6 MΩ (n = 12). The series resistance then was compensated (mean value was 55 ± 5%) to yield a final uncompensated series resistance of 4.9 ± 0.6 MΩ. All other conditions were the same as described above.

Capsaicin was applied focally to the neuron by placing a large-bore pipette (5–10 µm diameter) within 20–40 µm of the cell body. The focal pipette was filled with Ringer solution containing the appropriate concentration of capsaicin and 1 mM trypsin blue. During continuous superfusion of the bath with Ringer solution, a pulse of positive pressure (1–2 s) was applied to the pipette to eject the solution where trypan blue served to visualize the ejection. The focal application of trypsin blue alone had no effect on these sensory neurons (see Fig. 2) (Nicol and Cui 1994). In those studies investigating sensitization of the capsaicin response by prostaglandins, two control responses separated by ~2 min were obtained to the focal application of capsaicin. After the application of the sensitizing agent, capsaicin responses were obtained at 2, 6, 10, 14, and 20 min and thereafter at 5-min intervals. Responses from only a single neuron were obtained from each cover slip to avoid any possible desensitization that might occur. All experiments were done at room temperature (−21°C).

Only the results obtained from neurons that satisfied the following criteria are presented in this report. First, neurons had to maintain zero-current potentials more hyperpolarized than −45 mV for ≥4.5 min after establishing the whole cell configuration. Second, in all those experiments examining prostaglandin sensitization, the amplitudes of the two control responses obtained to the focal application of capsaicin must be within ±10% of their mean value or the recording was terminated.

**Chemicals**

Prostaglandins were obtained from Cayman Chemical (Ann Arbor, MI). Nitro-L-arginine methyl ester (L-NAME), s-nitroso-n-acetylpenicillamine (SNAP), 3-morpholinosydnonimine (SIN-1), and KT-5823 were obtained from Calbiochem (La Jolla, CA). All other chemicals were obtained from Sigma Chemical (St. Louis, MO). Prostaglandins, capsaicin, and KT-5823 were dissolved in 1-methyl-2-pyrrolidinone (HPLC grade, Aldrich Chemical, Milwaukee, WI) to obtain concentrated stock solutions. Amphotericin B was dissolved in dimethyl sulfoxide (Aldrich). These stock solutions then were diluted with the appropriate solution to yield the desired concentration. The extracellular concentrations of free Ca$^{2+}$ were calculated from the binding constants described by Caldwell (1970).

**Analysis**

All values are reported as the means ± SE. The concentration-response relation was described by a ligand-binding isotherm of the following form: $\text{CAP}_{\text{p}} = 1.111 + \left( \text{CAP}_{\text{L}} / \text{CAP}_{\text{p}} \right)^{-n}$, where $\text{CAP}_{\text{p}}$ is the fractional response to capsaicin, $\text{CAP}_{\text{L}}$ is the half-maximal effective concentration ($\text{EC}_{50}$) and $n$ is a steepness factor.
FIG. 1. Capsaicin depolarizes and elicits an inward current in embryonic sensory neurons. A, top: current clamp recording from a representative neuron (resting potential of 0 mV) where the focal application of 100 nM capsaicin for 2 s generated a burst of action potentials and depolarized the cell. In a voltage-clamp recording from this same neuron (bottom), capsaicin evoked an inward current (shown as downward) at a holding potential of −60 mV. As shown in B, the reversal potential for the capsaicin-elicited current is between 0 and +10 mV. Capsaicin (100 nM) was applied focally for a period of 1 s. The application of capsaicin is indicated by the bar below/above each trace.

that is analogous to the Hill coefficient in enzyme kinetics (e.g., Davis et al. 1977; McGehee et al. 1992). The time course of sensitization was characterized by two parameters, the time to peak and the time for sensitization to return to half-maximal values ($t_{1/2MAX}$); these values were determined from the time courses of sensitization obtained from individual neurons. We define the time to peak sensitization as the time elapsed from the onset of PGE$_2$ treatment to the maximally sensitized response and $t_{1/2MAX}$ as the time elapsed from the peak of sensitization to the point where sensitization recovered to one-half the maximal response minus the control value. These values then were averaged to obtain the mean ± SE. Statistical differences between the control recordings and those obtained under various treatment conditions were determined by using either a two-tailed Student’s t-test or a one-way analysis of variance (ANOVA). When a significant difference was obtained using ANOVA, a Student-Newman-Keuls (an all-points comparison) or Dunnett’s (control vs. treatment) post hoc test was performed. Values of $P < 0.05$ were judged to be statistically significant.

RESULTS

Characterization of the capsaicin response

In embryonic rat sensory neurons, the voltage response to capsaicin was characterized by a depolarization that was accompanied by the generation of action potentials. As illustrated for the representative neuron in Fig. 1A (top), the focal application of 100 nM capsaicin elicited a barrage of action potentials and a depolarization of 34 mV. In voltage clamp (−60 mV holding potential), the application of capsaicin to this same neuron caused an inward current that had a maximal amplitude of 192 pA (bottom). Note that with the application of capsaicin, there was a large increase in the membrane current noise, suggesting that the inward current resulted from the opening of membrane channels. In a total of eight neurons, capsaicin depolarized sensory neurons by an average value of 20 ± 3 mV. Both the time to peak and the time for recovery are similar in the voltage- and current-clamp recordings, suggesting that the capsaicin-induced inward current gives rise to the depolarization.

To further elucidate the nature of the neuronal response to capsaicin, the reversal potential of the current activated by capsaicin was determined. These results are shown in Fig. 1B. As shown in the bottom trace, at a holding potential of −60 mV, the application of capsaicin produced an inward current. The potential was shifted to −10 mV for ~15 s where the application of capsaicin elicited an inward current of 27 pA. At 0 mV, the current was reduced further (17 pA), but remained noticeably inward. However, at +10 mV, the current clearly became outward in direction (shown as the upward deflection) and had an amplitude of 26 pA. In recordings from three neurons, the average reversal potential for the current elicited by capsaicin was +5 ± 1 mV, which may suggest that the ion channels activated by capsaicin are nonselective for cations.

The amplitude of the inward current evoked by capsaicin was concentration dependent. The concentration-response relationship is shown in Fig. 2 where the points were fit by a ligand-binding isotherm having an EC$_{50}$ value of 163 nM and an $n$ of 3.2. To account for the varying capsaicin sensitivity of these sensory neurons, the current amplitude obtained for a given neuron was normalized to the current obtained for the bath application of 1 µM capsaicin. A concentration of 1 µM was believed to evoke the maximal current because in recordings from the 50 neurons comprising the concentra-
fold enhancement. The sensitization was not sustained and et al. 1988). As the influx of extracellular Ca\(^{2+}\), the current amplitude was increased to 532 pA, a 2.8-fold increase of their respective control responses. After exposure elicited by 100 nM capsaicin was not altered significantly (range 6–14 min) and 8.9 ± 0.6 min (range 6–10 min), respectively. After reaching maximal values, the sensitization produced by PGE\(_2\) began to recover back to control values. As determined from the time courses of individual neurons, the times for sensitization to return to half-maximal values (\(t_{1/2,MAX}\)) for 100 nM (5.5 ± 0.8 min, range 2.4–7.5 min) and 1 \(\mu\)M PGE\(_2\) (3.7 ± 0.5 min, range 1.2–5.5 min) were not significantly different. To determine whether exposure to capsaicin alone contributed to the sensitization or desensitization of these neurons, 100 nM capsaicin was applied focally every 2 min for a period of 30 min. As shown in Fig. 3C, the repeated exposure did not sensitize these sensory neurons as was observed after treatment with PGE\(_2\) (note the smaller scale for the fold-increase). Furthermore, the brief applications of 100 nM capsaicin over time did not appear to desensitize the neurons because the fold-increase relative to the zero time point did not become less than one. Therefore, these results indicate that exposure to PGE\(_2\) transiently sensitizes sensory neurons to the excitation produced by capsaicin.

Because the transient nature of the PGE\(_2\)-induced sensitization was observed at both 100 nM and 1 \(\mu\)M PGE\(_2\), we examined whether the concentration of focally applied capsaicin influenced the time course of this enhanced sensitivity. When the capsaicin concentration was lowered to 10 nM (see Fig. 4, the 100 nM capsaicin results are included as a point of reference), the magnitude (2.5 ± 0.3, range 1.8–3.6, n = 6), the time to peak (11 ± 2 min, range 6–20 min), and \(t_{1/2,MAX}\) (8.1 ± 2.3 min, range 2.4–19.2 min) for the PGE\(_2\)-induced sensitization were not significantly different from that observed for 100 nM capsaicin. Although the time courses for PGE\(_2\)-induced sensitization observed with these two concentrations of capsaicin were not significantly different, the increased number of points exhibiting a significant difference from the control values for 10 nM implies that the time of sensitization was enhanced with lowered concentrations of capsaicin.

**Calcium dependence of the PGE\(_2\)-induced sensitization**

The results described above imply that activation of fewer capsaicin receptors might lengthen the period of time that these sensory neurons remain sensitized. Previous studies have indicated that the capsaicin receptor-ion channel complex is permeable primarily to Na\(^+\) and Ca\(^{2+}\) (Bevan and Szolcsanyi 1990; Marsh et al. 1987; Oh et al. 1996; Wood et al. 1988). As the influx of extracellular Ca\(^{2+}\) has been implicated in the desensitization of this complex (Cholewinski et al. 1993; Docherty et al. 1996; Liu and Simon 1996; Yeats et al. 1991), we investigated the role of Ca\(^{2+}\) in modulating sensitization of sensory neurons by lowering the concentration of Ca\(^{2+}\) in the Ringer solution (see Fig. 5A). At 30 \(\mu\)M free Ca\(^{2+}\) (2 mM CaCl\(_2\) and 2 mM EGTA) in the absence of PGE\(_2\), the amplitude of the peak inward current elicited by 100 nM capsaicin was not altered significantly compared with that observed for these same neurons in 2 mM Ca\(^{2+}\)-Ringer (268 ± 128 pA compared with 273 ± 153 pA for the 2 mM Ca\(^{2+}\) control, n = 3). In the lowered
Ca\textsuperscript{2+}, 1 \mu M \text{PGE}_2 produced a sensitization (2.9 ± 0.2-fold increase, range 2.7–3.4) that was analogous to that observed in 2 mM Ca\textsuperscript{2+}-Ringer. Although the time to peak in 30 \mu M Ca\textsuperscript{2+} was not significantly different (11.3 ± 1.1 min, range 10–14 min) from that observed in 2 mM Ca\textsuperscript{2+}-Ringer, \( t_{1/2_{\text{MAX}}} \) was increased to 10.3 ± 1.6 min (range 6.8–13.7 min). Lowering the free Ca\textsuperscript{2+} concentration to 100 nM (2 mM CaCl\textsubscript{2} and 3.5 mM EGTA) significantly enhanced the magnitude and duration of sensitization produced by 1 \mu M \text{PGE}_2. The extent of sensitization (4.1 ± 0.9-fold, range 2.0–7.3, \( n = 7 \)), the time to peak (19.6 ± 1.4 min, range 14–24 min), and \( t_{1/2_{\text{MAX}}} \) (19.6 ± 1.7 min, range 14.7–26.4 min) were all increased relative to the values obtained in 2 mM Ca\textsuperscript{2+}-Ringer even though the peak amplitude of the capsaicin-evoked current in the absence of \text{PGE}_2 was not altered significantly in these neurons that were bathed initially in 2 mM Ca\textsuperscript{2+} (93 ± 31 pA compared with 123 ± 20 pA for the 2 mM Ca\textsuperscript{2+}-Ringer control, \( n = 4 \)). In contrast, raising the Ca\textsuperscript{2+} concentration to 10 mM blocked the sensitization in all neurons examined (\( n = 5 \)). In the elevated Ca\textsuperscript{2+}, the amplitude of the capsaicin-elicited current in the absence of \text{PGE}_2 was not altered significantly (260 ± 84 pA compared with 336 ± 172 pA for the 2 mM Ca\textsuperscript{2+} controls). Therefore, these results indicate that the duration of sensitization may depend on elevations in intracellular Ca\textsuperscript{2+} concentration via influx through the capsaicin receptor-ion channel complex.

In another series of experiments, we used the amphotericin perforated-patch recording technique (Horn and Marty 1988; Rae et al. 1991) to determine whether the Ca\textsuperscript{2+} buffering capacity of the pipette contents (2.5 mM CaCl\textsubscript{2}, 5 mM EGTA, calculated 100 nM free Ca\textsuperscript{2+}) used in the whole cell recordings influenced the time course of sensitization. Under control conditions, 100 nM capsaicin evoked a current that had an average amplitude of 213 ± 45 pA (range 76–336 pA, \( n = 5 \)). As shown in Fig. 5B, treatment with 1 \mu M \text{PGE}_2 in 2 mM Ca\textsuperscript{2+}-Ringer produced a transient sensitization (1.8 ± 0.1, range 1.7–2.0, \( n = 5 \)) of the capsaicin-elicited current. The time to peak was 8.4 ± 0.9 min (range 6–10 min) and \( t_{1/2_{\text{MAX}}} \) was 3.3 ± 0.8 min (range 1.9–5.9 min). These values were not significantly different from that obtained in the whole cell recordings. In 100 nM Ca\textsuperscript{2+}-Ringer, the recordings obtained with the perforated-patch were also similar to those obtained with the whole cell configuration. Under low Ca\textsuperscript{2+} conditions, 100 nM capsaicin evoked an average response of 153 ± 30 pA (range 59–247 pA, \( n = 7 \)) and was not significantly different from the response obtained in these same neurons that were bathed initially in 2 mM Ca\textsuperscript{2+} (161 ± 53 pA, range 53–449 pA). Treatment with 1 \mu M \text{PGE}_2 caused a 2.9 ± 0.3-fold increase (range 2.0–4.1, \( n = 7 \)) in the capsaicin response. As determined

![Fig. 3](http://jn.physiology.org/content/113/5/3158/F3.large.jpg)

**Fig. 3.** Prostaglandin E\textsubscript{2} (\text{PGE}_2) enhances the capsaicin response in a time-dependent manner. A: enhancement of the capsaicin-evoked current by 1 \mu M \text{PGE}_2, as obtained from a representative sensory neuron. Bar above each trace indicates the focal application of 100 nM capsaicin. Extent and time courses of the \text{PGE}_2-induced sensitization are summarized in B. Amplitude of the capsaicin response after \text{PGE}_2 was normalized to the average of 2 control responses and represented as the fold-increase relative to the control. Filled circles, those neurons treated with 1 \mu M \text{PGE}_2 (\( n = 6–9 \)); filled squares, those neurons exposed to 100 nM \text{PGE}_2 (\( n = 6 \)). Hatched bar, change to Ringer solution containing \text{PGE}_2. C: results (\( n = 3 \)) obtained for the focal application of 100 nM capsaicin every 2 min for a period of 30 min in the absence of \text{PGE}_2. * Significant difference at \( P < 0.05 \) compared with control.

![Fig. 4](http://jn.physiology.org/content/113/5/3158/F4.large.jpg)

**Fig. 4.** Duration of \text{PGE}_2-induced sensitization is increased with concentrations of focally applied capsaicin <100 nM. Fold-increase in the response to 10 nM capsaicin after sensitization by 1 \mu M \text{PGE}_2 is shown as a function of time. Results obtained with 100 nM capsaicin (Fig. 3B) are presented as a point of reference. Hatched bar, application of \text{PGE}_2. Results for 10 nM capsaicin were obtained from 5–7 neurons. * Significant difference in capsaicin responses after \text{PGE}_2 compared with their respective controls.
from the individual time courses of sensitization, neither the time to peak (13.8 ± 2.4 min, range 10–25 min) nor $t_{1/2\text{MAX}}$ (16.3 ± 3.3 min, range 7.2–28.3 min) were significantly different from values obtained with the whole cell method in 100 nM Ca$^{2+}$. This suggests that with the addition of some amount of Ca$^{2+}$ buffering via the pipette solution (EGTA) there may be less of a change in intracellular Ca$^{2+}$. At present, it is uncertain whether the similar rate of inactivation of sensitization observed in the whole cell recordings at 100 nM Ca$^{2+}$ resulted from the ineffectiveness of EGTA to buffer changes in intracellular Ca$^{2+}$ (Adler et al. 1991; Huang and Neher 1996) or whether the slow relaxation represents a Ca$^{2+}$-independent component of inactivation.

To further examine the notion that changes in the intracellular concentration of Ca$^{2+}$ affected the duration of sensitization, 10 mM BAPTA, a calcium chelator, was included in the pipette solution (see Fig. 5C). The amplitude of the capsaicin response was not altered in the presence of BAPTA (204 ± 36 pA, range 88–383 pA, $n = 9$). Exposure to 1 μM PGE$_2$ produced a 3.0 ± 0.3-fold sensitization (range 2.1–4.9, $n = 9$) of the capsaicin response that had an average time to peak of 17.6 ± 4.1 min (range 6–50 min). Under these conditions, the PGE$_2$-induced sensitization did not recover. In the absence of PGE$_2$, BAPTA had no effect on the amplitude of the capsaicin-elicited current ($n = 3–5$). Therefore, these results suggest that either reduced extracellular Ca$^{2+}$ or increased intracellular Ca$^{2+}$ buffering prevented the activation of a cellular pathway that significantly influenced the time course of sensitization.

To ascertain the specificity of the effects of altered calcium on the capsaicin response in addition to any nonspecific effects induced by exposure to eicosanoids, we examined the capacity of PGF$_{2\alpha}$, which is a prostaglandin that does not sensitize sensory neurons (Hingtgen et al. 1995; Nicol and Cui 1994), to modulate the capsaicin-evoked current at different extracellular Ca$^{2+}$ concentrations. These results are illustrated in Fig. 5D. Under these varied Ca$^{2+}$ concentrations, exposure to 1 μM PGF$_{2\alpha}$ did not alter significantly the response to capsaicin. Thus the current evoked by the focal application of capsaicin was relatively stable during the 30-min time period regardless of the extracellular Ca$^{2+}$ concentration, and the response amplitude was not modified by exposure to a nonsensitizing eicosanoid.

**Activation of the nitric oxide-cyclic GMP pathway mediates the inactivation of sensitization**

Many investigations have established that an influx of Ca$^{2+}$ leads to activation of the nitric oxide pathway with the subsequent generation of cyclic GMP (Bredt and Synder 1989; Garthwaite et al. 1988; Knowles et al. 1989). Because lowering the Ca$^{2+}$ concentration increased the duration of sensitization, the possibility that the persistence of sensitization might depend on the production of nitric oxide was examined by using the inhibitor of nitric oxide synthase, L-NAME. These experiments were performed in 2 mM Ca$^{2+}$-Ringer as this would provide for the greatest possible Ca$^{2+}$-dependent activation of nitric oxide synthase (NOS). Sensory neurons were pretreated with 100 μM L-NAME for 10 min before acquisition of the two control responses to 100 nM capsaicin, and L-NAME was present throughout the exposure to PGE$_2$. A concentration of 100 μM L-NAME was used as this concentration blocked completely the capsaicin-induced increase in cyclic GMP observed in embryonic sensory neurons grown in culture (Dymshitz and Vasko 1994). In the presence of L-NAME, the average amplitude of the
In addition of nitric oxide donors (NOD; 9) was not significantly different from that obtained in 2 sensitization, 8-Br-cyclic GMP was applied to sensory neurons after a 10-min exposure to capsaicin resulted in an increase in the intracellular calcium. Other conditions (i.e., PGE2 and L-NAME) are the exposure to PGE2 (•) throughout the peak time was 12.7 ± 0.6 min (range 2.7–2.9 min, n = 6) or 2.1 ± 0.1 min (range 1.8–2.3, n = 5), respectively. This value is similar to that observed under normal recording conditions (see Fig. 3B). Thus these results demonstrate that an increase in nitric oxide can produce a rapid inactivation of the sensitization produced by PGE2.

McGehee et al. (1992) previously reported that the nitric oxide-cyclic GMP pathway played a causal role in the desensitization of the bradykinin response in rat sensory neurons. Based on our finding that in the presence of L-NAME the duration of sensitization was increased and that nitric oxide donors rapidly inactivated sensitization, we examined the capacity of cyclic GMP to modulate the PGE2-induced sensitization. In the presence of L-NAME, the application of 100 μM SIN-1 or SNAP rapidly reduced the sensitization with a 1/2 of 2.1 ± 0.2 min (range 1.7–2.9 min, n = 6) or 2.1 ± 0.1 min (range 1.8–2.3, n = 5), respectively. This value is similar to that observed under normal recording conditions (see Fig. 3B).

Control responses (277 ± 59 pA, range 110–474 pA, n = 9) was not significantly different from that obtained in 2 mM Ca2+-Ringer (449 ± 159 pA, range 100–1,300 pA, n = 9). After exposure to 1 μM PGE2 and L-NAME, the capsaicin response was sensitized and reached a maximal increase of 3.1 ± 1.3-fold (range 1.8–5.9) compared with control values (see Fig. 6A). The time to peak (13.6 ± 2 min, range 10–30 min, n = 9) was similar to that observed in 2 mM Ca2+, however, t1/2 MAX was increased significantly to 14.3 ± 3.8 min (range 5–20 min). In the absence of PGE2, L-NAME had no effect on the relative amplitude of the capsaicin-evoked inward current. These results suggest the activation of NOS and the subsequent generation of nitric oxide are involved in the inactivation of PGE2-induced sensitization.

To explore the possibility that the production of nitric oxide plays a key role in the initiation of inactivation of the PGE2-induced sensitization, we examined the effects of two structurally different nitric oxide donors, SIN-1 and SNAP, on the time course of sensitization. As illustrated in Fig. 6B, exposure of sensitized sensory neurons to either 100 μM SIN-1 or SNAP caused a rapid inactivation of the sensitization. This concentration of donor was used because it produced about a 17-fold increase in the levels of intracellular cyclic GMP (Dymshitz and Vasko 1994). Neurons were pretreated with 100 μM L-NAME to block any residual NOS activity. Exposure to 1 μM PGE2 produced about a twofold enhancement of the capsaicin-evoked current. At the peak of sensitization, the addition of SIN-1 or SNAP rapidly reduced the sensitization with a t1/2 of 2.1 ± 0.2 min (range 1.7–2.9 min, n = 6) or 2.1 ± 0.1 min (range 1.8–2.3, n = 5), respectively. This value is similar to that observed under normal recording conditions (see Fig. 3B).

Thus these results demonstrate that an increase in nitric oxide can produce a rapid inactivation of the sensitization produced by PGE2.

FIG. 6. Nitric oxide-cyclic guanosine 3′, 5′-monophosphate (GMP) pathway modulates the duration of PGE2-induced sensitization. A: inhibition of nitric oxide synthase increases the duration of sensitization. Diamonds, neurons (n = 5–6) that were pretreated with 100 μM nitro-L-arginine methyl ester (L-NAME) in 2 mM Ca2+-Ringer for 10 min before the application of 1 μM PGE2. L-NAME was present (open bar) throughout exposure to PGE2 (■). Filled circles, neurons treated with PGE2 alone (same results as presented in Fig. 3B provided for reference). Filled squares, different group of sensory neurons (n = 5–6) treated with 100 μM L-NAME alone. Error bars are smaller than the symbol. B shows that addition of nitric oxide donors (NOD; ε) inactivates the PGE2-induced sensitization. Open squares and filled circles, different groups of sensory neurons that were exposed to S-nitroso-N-acetylpenicillamine (SNAP; n = 5) and 3-morpholinosydnonimine (SIN-1; n = 6), respectively. Other conditions (i.e., PGE2 and L-NAME) are the same as in A. C: shows that the addition of 100 μM 8-bromo-cyclic GMP (ε) inactivates the PGE2-induced sensitization. Other conditions (i.e., PGE2 and L-NAME) are the same as in A. * Significant difference at P < 0.05 compared with their respective controls.
taken in the presence of KT-5823, which is a selective inhibitor of cyclic GMP-dependent protein kinase (PKG; Ito and Karachot 1990; Kase et al. 1987). These experiments were carried out in 2 mM Ca\(^{2+}\)-Ringer to permit the optimal production of cyclic GMP. Sensory neurons were pretreated with 100 nM KT-5823 for 10 min before acquiring the two control responses to capsaicin, and KT-5823 was present throughout exposure to 1 \(\mu\)M PGE\(_2\). This concentration of KT-5823 was used because concentrations >1 \(\mu\)M inhibit the activity of PKA (Murthy and Makhlof 1995). In the presence of KT-5823, the capsaicin-evoked current had an average amplitude of 248 ± 61 pA (range 30–460 pA, \(n = 6\)). As shown in Fig. 8, KT-5823 did not alter the capacity of PGE\(_2\) to sensitize the sensory neurons (a 2.6 ± 0.2-fold increase, range 2.4–3.4). However, inhibition of PKG led to a sustained sensitization of the capsaicin response. In fact, we were not able to determine a \(t_{1/2\text{MAX}}\) for these neurons because of the prolonged sensitization levels. KT-5823 alone had no effect on the capsaicin response during the 60-min time course of these experiments. Furthermore, in the presence of KT-5823, GMP before the application of PGE\(_2\). In 2 mM Ca\(^{2+}\)-Ringer, pretreatment with 100 \(\mu\)M 8-Br-cyclic GMP for 10 min had no effect on the sensitization produced by 1 \(\mu\)M PGE\(_2\) (see Fig. 7B). These results suggest that an elevation in the level of intracellular cyclic GMP may play a critical role in the inactivation of the sensitization initiated by PGE\(_2\), but only after the capsaicin response has been facilitated by PGE\(_2\).

Inhibition of cyclic GMP-dependent protein kinase suppresses the inactivation of sensitization

As discussed above, elevated levels of cyclic GMP appear to inactivate the sensitization produced by PGE\(_2\). To determine whether this effect resulted from cyclic GMP directly or via activation of PKG, the compound KT-5823 was used to block activation of PKG (Ito and Karachot 1990; Kase et al. 1987). These experiments were carried out in 2 mM Ca\(^{2+}\)-Ringer to permit the optimal production of cyclic GMP. Sensory neurons were pretreated with 100 nM KT-5823 for 10 min before acquiring the two control responses to capsaicin, and KT-5823 was present throughout exposure to 1 \(\mu\)M PGE\(_2\). This concentration of KT-5823 was used because concentrations >1 \(\mu\)M inhibit the activity of PKA (Murthy and Makhlof 1995). In the presence of KT-5823, the capsaicin-evoked current had an average amplitude of 248 ± 61 pA (range 30–460 pA, \(n = 6\)). As shown in Fig. 8, KT-5823 did not alter the capacity of PGE\(_2\) to sensitize the sensory neurons (a 2.6 ± 0.2-fold increase, range 2.4–3.4). However, inhibition of PKG led to a sustained sensitization of the capsaicin response. In fact, we were not able to determine a \(t_{1/2\text{MAX}}\) for these neurons because of the prolonged sensitization levels. KT-5823 alone had no effect on the capsaicin response during the 60-min time course of these experiments. Furthermore, in the presence of KT-5823,
the addition of 8-Br-cyclic GMP did not produce the rapid inactivation of PGE₂-induced sensitization (Fig. 8). The peak and plateau values (obtained between 13 and 38 min) for PGE₂-induced sensitization in the presence of KT-5823 or KT-5823/8-Br-cyclic GMP were not significantly different. Therefore, these results indicate that elevated cyclic GMP by itself does not reverse the sensitization, but rather, it is the activation of PKG that subsequently terminates the PGE₂-induced sensitization.

**DISCUSSION**

In embryonic sensory neurons, capsaicin evokes an inward current that has properties similar to those observed in recordings from sensory neurons obtained from adult animals. Examples of this are a reversal potential near 0 mV, desensitization of the response after exposure to high concentrations of capsaicin, and suppression of the response by pretreatment with the capsaicin antagonist, capsazepine (Lopshire, unpublished observations). The concentration dependence for the amplitude of the capsaicin-evoked current has an EC₅₀ value (163 nM) and is similar to values observed for other capsaicin-mediated events. The lower values range from 70 nM for the capsaicin-induced increase in calcium (Cholewinski et al. 1993) or cyclic GMP (Wood et al. 1989) to 155 nM for the capsaicin-evoked release of calcitonin gene-related peptide (Hingtgen and Vasko 1994). Higher values for the EC₅₀ range from 500 nM for the capsaicin-evoked depolarization (Marsh et al. 1987) to 1.1 μM for capsaicin activation of single channel currents (Oh et al. 1996). In addition, activation of the capsaicin receptor/ion channel complex may occur with positive cooperativity because the steepness factor, n, was determined to be 3.2. In support of this observation, previous reports determined Hill coefficients of 1.7 for the binding of resiniferatoxin, a potent analogue of capsaicin, to membranes prepared from the dorsal root ganglion (Szallasi et al. 1993) and 1.8 for activation of the capsaicin-gated ion channel (Oh et al. 1996). Therefore, the values for n and the EC₅₀ imply that activation of the capsaicin receptor/ion channel complex might require the binding of at least two molecules of capsaicin at relatively low concentrations of agonist.

The inward current elicited by capsaicin can be facilitated by two- to threefold after treatment with the pro-inflammatory prostaglandin, PGE₂. This observation is consistent with many previous studies that described the sensitizing actions of PGE₂ on sensory neurons (see INTRODUCTION). Facilitation of the capsaicin response required ~10 min to reach the maximal value, which is similar to the times observed for either PGE₂- or cAMP-induced sensitization of the bradykinin response (Cui and Nicol 1995; Nicol and Cui 1994). Sensitization of the capsaicin response is somewhat different from that observed for sensitization of the bradykinin response. Although the number of action potentials elicited by bradykinin increased threefold after a 10-min exposure to PGE₂, the amplitude of the bradykinin-evoked inward current was unaltered. The bradykinin results suggest that the enhanced excitability likely results from modulation of other conductances regulating the capacity of the neuron to generate an action potential. In contrast, PGE₂ produced a significant enhancement of the capsaicin-evoked current, indicating that the pathway activated by PGE₂ can modulate the capsaicin receptor/ion channel complex to conduct greater currents.

The PGE₂-induced sensitization was transient and returned to baseline within ~10 min after reaching maximal values, independent of the continued presence of prostaglandin. Elevations in intracellular Ca²⁺ concentration modulate the transient nature of the PGE₂-induced sensitization (see Fig. 5). When changes in the intracellular concentration of Ca²⁺ were buffered by internal perfusion with the Ca²⁺ chelator, BAPTA, the sensitization did not inactivate. In contrast, under conditions that provided no buffering of Ca²⁺ (i.e., perforated-patch recordings) or buffering with EGTA, increasing extracellular Ca²⁺ from 100 nM to 2 mM produced a progressive shortening in the duration of sensitization. In 10 mM Ca²⁺-Ringer, sensitization appeared to be blocked completely. At present, it is not clear whether this lack of sensitization in high Ca²⁺ results from inhibition of the effects of PGE₂ or if the capsaicin response had been desensitized by the high Ca²⁺. Although BAPTA and EGTA have about the same affinity for Ca²⁺, BAPTA has a much faster on-rate for Ca²⁺ than EGTA and expected to be much more effective at buffering rapid changes in Ca²⁺ (Adler et al. 1991; Huang and Neher 1996; Stern 1992). The observed differences for the inactivation of sensitization with BAPTA and EGTA suggest that the inactivation might result from transient changes in Ca²⁺ concentration rather than sustained increases.

Previous results have demonstrated clearly that increased intracellular Ca²⁺ concentrations play an important regulatory role in desensitization of the capsaicin response (Cholewinski et al. 1993; Liu and Simon 1996; Yeats et al. 1991). We believe that inactivation of sensitization does not result from desensitization of the capsaicin receptor/ion channel complex. This idea is based on several observations. First, we use a short exposure time to 100 nM capsaicin (1–2 s), whereas those studies examining desensitization used long exposure times (20 s to 2 min) and concentrations of capsaicin ranging from 100 nM to 1 μM. Second, under normal recording conditions, the normalized amplitude of the capsaicin response, during a period of 30 min, never falls below a value of one, which would indicate desensitization of the response (see Fig. 3C). Third, inhibition of NOS suppressed the inactivation of sensitization, whereas NOS inhibitors had no effect on desensitization of the capsaicin response (Dockerty et al. 1996).

As described for desensitization, there may be a Ca²⁺-independent component of inactivation. This is suggested by two findings. First, even in the whole-cell recordings with 100 nM Ca²⁺-Ringer, the enhanced capsaicin response recovers to near baseline values, although much more slowly than observed in normal Ca²⁺ (see Fig. 5B). Second, in the presence of L-NNAME (Fig. 6), the sensitization begins to relax back to baseline values after approximately 45 min. Rather than a Ca²⁺-independent process, another possibility might be that buffering extracellular Ca²⁺ at 100 nM is slightly greater than the cytoplasmic concentration and thus permits a capsaicin-mediated influx of Ca²⁺ that over time is sufficient to activate the nitric oxide-cyclic GMP pathway. Consistent with this idea is our finding that sensitization does not inactivate in the presence of 10 mM BAPTA. Alternatively, there may be sufficient release of Ca²⁺ from intrac-
cellular stores (see Fig. 4 of Cholewinski et al. 1993) to activate this pathway.

The duration of the PGE$_2$-induced sensitization was modified by pharmacological manipulations of the nitric oxide/cyclic GMP pathway, which suggested that activation of this cascade plays a critical role in regulating the inactivation of sensitization. This notion is based on our experimental observations where reductions in the influx of extracellular Ca$^{2+}$, as well as inhibition of NOS and PKG, all lengthened the period of sensitization. An antagonistic role for the cyclic GMP transduction cascade is consistent with previous behavioral studies examining the effects of the nitric oxide/cyclic GMP pathway on PGE$_2$-induced hyperalgesia. The heightened sensitivity to pressure applied to the rat paw was reversed by activation of either NOS or guanylyl cyclase (Duarte et al. 1990, 1992). Taken together, these results suggest that elevations in intracellular cyclic GMP levels leading to activation of PKG may play an important role in suppressing the heightened sensitivity to various modalities of noxious stimulation associated with the inflammatory response.

The exact mechanisms for the inhibitory actions of PKG in sensory neurons are unknown presently. It is possible that activation of PKG produces phosphorylation of the capsaicin ion channel either directly or indirectly via some other intermediary. This is the proposed mechanism whereby cyclic GMP and PKG inhibit a nonselective cationic channel from kidney inner-medullary collecting duct (Light et al. 1990) as well as the calcium current in cardiac myocytes (Méry et al. 1991) and rat pinealocytes (Chik et al. 1995). In isolated hippocampal neurons, cyclic GMP appears to inhibit a calcium current through activation of a phosphodiesterase (Doerner and Alger 1988). However, phosphodiesterase activation is not the case in the cardiac myocyte studies because inhibition of phosphodiesterase with isobutylmethylxanthine does not alter the capacity of cyclic GMP to suppress the calcium current (Méry et al. 1991; Wahler and Dollinger 1995). Alternatively, PKG might activate protein phosphatases because inhibition of either PKG with KT-5823 or protein phosphatase 2A with okadaic acid reversed the cyclic GMP-induced enhancement of the activity of the BK potassium channel (White et al. 1993; Zhou et al. 1996). Along these same lines, in the cerebellum a major substrate for PKG (G substrate) appears to be a protein that shares many of the characteristics of protein phosphatase inhibitor 1 (Detre et al. 1985). A recent study determined that PKG is found in sensory neurons at levels that are similar to the high levels found in the cerebellum, however, there was no detectable G substrate in either adult or embryonic sensory neurons (Qian et al. 1996). Thus the cellular mechanisms whereby PKG leads to inactivation of the PGE$_2$-induced sensitization of sensory neurons are clearly an area for future investigation.

We find that modulators of the nitric oxide-cyclic GMP-PKG pathway have no effect on the capsaicin response unless the neurons have been sensitized by prior exposure to PGE$_2$ (see Figs. 6–8). The sensitization produced by PGE$_2$ is mediated by activation of the cyclic AMP transduction cascade and very likely involves PKA-induced phosphorylation of a substrate protein(s) (Cui and Nicol 1995; Hingtgen et al. 1995). Our findings are similar to those reported previously for the actions of PKG on the calcium current recorded from cardiac myocytes. In these cells, the calcium current is suppressed by agents that activate PKG (cyclic GMP or a nitric oxide donor), however, this inhibition occurs only after the calcium current is enhanced by prior exposure to agents that elevate intracellular cAMP levels (Méry et al. 1991; Wahler and Dollinger 1995). This proposed mechanism is consistent with the yin yang hypothesis developed by Goldberg et al. (1975) where cAMP and cyclic GMP have antagonistic influences on the regulation of physiological processes. In addition, our observations may account for the lack of effect that nitric oxide donors and cyclic GMP have on the capsaicin-evoked release of substance P and calcitonin gene-related peptide from nonsensitized sensory neurons (Dymshitz and Vasko 1994). Therefore, taken together, these observations suggest that sensitization of the capsaicin receptor/ion channel complex may result from PKA-dependent phosphorylation. Once modified by PKA, this complex subsequently can be altered by PKG, via an unknown mechanism or pathway, to inactivate the processes contributing to the facilitated conductance. Thus activation of the cyclic GMP pathway may serve an important regulatory role in reversing the heightened sensitivity to noxious stimulation observed in neurogenic inflammation.

We are grateful to Dr. Michael Vasko for discussions about signaling in sensory neurons and a suggestion to try cyclic GMP and to Dr. Angela Evans for providing some of the neuronal preparations. J. C. Lopshire was supported by the Indiana University–Purdue University, Indianapolis Research Investment Fund and a fellowship from the American Heart Association, Indiana Affiliate. This work was supported by National Institute of Neurological Disorders and Stroke Grant NS-30527 to G. D. Nicol.

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Received 9 May 1997; accepted in final form 30 July 1997.

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