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Prostaglandin E_2 Increases Calcium Conductance and Stimulates Release of Substance P in Avian Sensory Neurons

G. D. Nicol, D. K. Klingberg, and M. R. Vasko

Department of Pharmacology and Toxicology, School of Medicine, Indiana University, Indianapolis, Indiana 46202

Prostaglandins are known to lower activation threshold to thermal, mechanical, and chemical stimulation in small-diameter sensory neurons. Although the mechanism of prostaglandin action is unknown, agents known to elevate intracellular calcium produce a sensitization that is similar to that produced by prostaglandins. Consistent with the idea of prostaglandin-induced elevations in calcium, prostaglandins might also stimulate the release of neurotransmitter from sensory neurons. We therefore examined whether prostaglandin E_2 (PGE_2) could enhance the release of the putative sensory transmitter substance P (SP) from isolated neurons of the avian dorsal root ganglion grown in culture. Utilizing the whole-cell patch-clamp recording technique, we also examined whether PGE_2 could alter calcium currents in these cells. Exposure of sensory neurons to PGE_2 produced a dose-dependent increase in the release of SP. One micromolar PGE_2 increased release approximately twofold above basal release, whereas 5 and 10 μM PGE_2 increased release by about fourfold. The release evoked by these higher concentrations of PGE_2 was similar in magnitude to the release induced by 50 mM KCl. Neither arachidonic acid (10 μM), prostaglandin $F_{2\alpha}$ (10 μM), nor the lipoxygenase product leukotriene B_4 (1 μM) significantly altered SP release. The addition of 1 μM PGE_2 increased the peak calcium currents by 1.8-fold and 1.4-fold for neurons held at potentials of −60 and −90 mV, respectively. The action of PGE_2 was rapid with facilitation occurring within 2 min. As with release studies, arachidonic acid, prostaglandin $F_{2\alpha}$, and leukotriene B_4 had no significant effect on the amplitude of the calcium current. These results suggest that PGE_2 can stimulate the release of SP through the activation or facilitation of an inward calcium current. The capacity of PGE_2 to facilitate the calcium current in these sensory neurons may be one mechanism to account for the ability of prostaglandins to sensitize sensory neurons to physical or chemical stimuli.

Prostaglandins (PGs) appear to be important mediators involved in lowering the pain threshold during tissue injury and inflammation, that is, in producing hyperalgesia. Injection of PGE_2 or PGI_2 into the hind paw of the rat or into the knee joints of dogs produces hyperalgesia as indicated by a lowering of the pressure necessary to elicit a withdrawal or by the degree of joint incapacitation, respectively (Willis and Cornelsen, 1973; Ferreira et al., 1978; Taiwo and Levine, 1989). In humans, PGs in micromolar concentrations intensify bradykinin- or histamine-induced pain (Ferreira, 1972). In addition, the accepted mechanism for pain relief produced by aspirin and other nonsteroidal antinflammatory drugs is the inhibition of cyclooxygenase, the enzyme that catalyzes the conversion of arachidonic acid to PGs (Collier, 1971; Smith and Willis, 1971; Vane, 1971).

Presumably, PG-induced hyperalgesia involves a direct action of these eicosanoids on sensory neurons. PGE_1 and PGE_2 sensitize small-diameter sensory nerve fibers to thermal and mechanical stimulation (Handwerker, 1976; Pateromichelakis and Rood, 1982; Martin et al., 1987) and facilitate activation of these neurons by potassium, capsaicin, and bradykinin (Chahl and Iggo, 1972; Baccaglini and Hogan, 1983; Yanagisawa et al., 1986; Schaible and Schmidt, 1988). Although the cellular mechanisms for the action of PGs on sensory neurons remain unknown, early studies suggested that calcium may have an important regulatory role. Agents that are known to alter the intracellular concentration of calcium produce hyperalgesia that is similar to that produced by PGE_1 (Ferreira and Nakamura, 1979). In addition, recent studies utilizing bovine chromaffin cells and NG108-15 neuroblastoma–glioma hybrid cells demonstrate that PGE_2 facilitates the entry of calcium through activation of neuronal membrane calcium channels (Koyama et al., 1988; Miwa et al., 1988; Mochizuki-Oda et al., 1991).

Consistent with this enhanced influx of calcium, it is possible that PGs could initiate the release of neurotransmitter from sensory neurons. The enhanced release of neurotransmitter could in turn contribute to PG-induced hyperalgesia and to PG-induced inflammatory responses. Ueda et al. (1985) and Uda et al. (1990) have proposed that enhanced release of one putative sensory neurotransmitter, substance P (SP), by PGs could be the mechanism to explain the PG-induced hyperalgesia and PG-induced potentiation of the stimulated contraction of iris sphincter muscle. To date, however, no studies have been performed to determine if PGs actually stimulate transmitter release from sensory neurons.

We sought to examine whether PGE_2 or other eicosanoids could alter the release of SP from avian sensory neurons grown in culture. Our studies indicate that PGE_2 can enhance the release of SP from avian sensory neurons grown in culture. In contrast, neither $PGF_{2\alpha}$, arachidonic acid, nor the lipoxygenase metabolite leukotriene B_4 (LTB_4) has any significant effect on release. To examine a possible mechanism for the enhanced release of SP, we utilized the whole-cell patch-clamp technique

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Correspondence should be addressed to Dr. G. D. Nicol, Department of Pharmacology and Toxicology, 635 Barnhill Drive, School of Medicine, Indiana University, Indianapolis, IN 46202.

(Hamill et al., 1981) to examine the actions of PGE_2 and other eicosanoids on calcium currents in these sensory neurons. PGE_2 enhanced the voltage-dependent calcium current, suggesting that the facilitated calcium current might be responsible for increasing release of SP. Again, neither $PGF_{2\alpha}$, arachidonic acid, nor LTB_4 had any significant effect on calcium currents.

Preliminary findings from these studies have appeared in abstract form (Vasko et al., 1989; Nicol and Vasko, 1991).

Materials and Methods

Experiments were performed using primary cultures of neurons harvested from the dorsal root ganglia (DRG) of 8–10 d old chicken embryos. Eicosanoids were purchased from Cayman Chemical Co. (Ann Arbor, MI), peptides from Peninsula Laboratory (Belmont, CA), nerve growth factor from Collaborative Biochemical Products Inc. (Bedford, MA), other supplies for culture medium from GIBCO Laboratories (Grand Island, NY), and routine chemicals from Sigma Chemical Company (St. Louis, MO).

Cell culture. Avian dorsal root ganglia neurons were harvested and grown using a modification of the method of Dichter and Fischbach (1977). Briefly, fertilized chicken eggs were incubated for 8–10 d at 39°C. Embryos were removed and DRG dissected out. The ganglia were placed in a calcium-free, magnesium-free, phosphate-buffered saline solution (Puck's saline) at 4°C. Routinely, 12–20 ganglia were removed from each embryo and 12–18 embryos were dissected at one time. After dissection, the ganglia were transferred to a sterile 15 ml centrifuge tube, washed with Puck's saline, and then incubated at 37°C for 30 min in Puck's saline containing 0.01% crude collagenase. The ganglia were then gently pelleted by centrifugation at 500 × *g* for 5 min, the supernatant discarded, and the ganglia resuspended in growth medium. The growth medium was Dulbecco's Modified Eagle's Medium supplemented with 2 mM glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, 10% (v/v) heat-inactivated fetal calf serum, 5% (v/v) chicken embryo extract (Dichter and Fischbach, 1977), and 100 or 200 ng/ml of 7S nerve growth factor (Collaborative Research Inc., Lexington, MA). Individual cells were dissociated from the ganglia by trituration using a fire-polished Pasteur pipette until a cloudy suspension was observed. The cells were counted using a hemocytometer and diluted in growth medium, and approximately 300,000–400,000 cells were plated on 60 mm collagen-coated Falcon brand culture dishes. For electrophysiological experiments, small plastic coverslips were placed on the bottom of the culture dishes. They were collagen coated, and cells were plated directly onto these slips. The cells are grown for 3 d at 37°C in a 5% CO_2 atmosphere, using growth medium containing either 10 μM cystosine β-D-arabinofuranoside, or 50 μM 5-fluoro-2′-deoxyuridine and 150 μM uridine to minimize growth of non-neuronal cells. After 3 d of exposure to mitotic inhibitors and every other day thereafter, neurons were fed with 2 ml of medium without mitotic inhibitors. Using this protocol, neuronal cultures make extensive neurite processes by the third day in culture, with most non-neuronal cells dying and no longer adhering to the culture dish. Typically by day 6 in culture, approximately 90% of the viable cells are neurons.

Substance P release. After 5–7 d of growth, release studies were performed using a modification of the method of Mudge et al. (1979). Medium was gently aspirated from the culture dish, and the cells were washed with 1 ml of a modified Krebs' bicarbonate buffer consisting of 135 mM NaCl, 3.5 mM KCl, 1 mM $MgCl_2$, 20 mM $NaHCO_3$, 2.5 mM $CaCl_2$, 3.3 mM dextrose, 100 μM ascorbic acid, 20 μM bacitracin, and 0.5% bovine serum albumin, aerated with 95% O_2, 5% CO_2, pH 7.4–7.5, and maintained at 36–37°C. Cells were then incubated for two consecutive 5 min periods with 1 ml of Krebs' buffer to measure resting release (referred to in the text as basal release), followed by one 5 min exposure to Krebs' buffer containing either 40 mM or 50 mM KCl (substituted for equimolar sodium), PGE_2, $PGF_{2\alpha}$, arachidonic acid, or LTB_4. Cells were then reexposed for two 5 min incubations with Krebs' buffer containing 3.5 mM KCl to reestablish basal release. For all experiments, eicosanoids were initially dissolved in 70% ethanol or in 1-methyl-2-pyrrolidinone (Aldrich Chemical Co., Milwaukee, WI) and diluted to appropriate concentrations with Krebs' buffer. During each 5 min incubation, cells were returned to the incubator. After each 5 min incubation, the Krebs' buffer was carefully aspirated and pipetted into 12 × 75 mm culture tubes containing glacial acetic acid such that the final concentration of acetic acid is 2 *N*. In each experiment, a number of wells were used as controls and consisted of the measurement of the basal release and the release induced by high extracellular potassium. If potassium did not evoke the release of SP, the experiment was discarded. Neither vehicle, ethanol, nor 1-methyl-2-pyrrolidinone, at the concentrations used in the eicosanoid experiments, had any effect on either the basal or potassium-stimulated release of SP. For experiments involving indomethacin, cells were incubated in Krebs' buffer containing 10 μg/ml indomethacin for 30 min prior to initiating the release protocol.

After collection of the release solutions, the samples were lyophilized and reconstituted in an assay buffer of 0.1 M phosphate buffer (pH 7.4–7.5) containing 0.9% NaCl, 0.1% gelatin, and 2 ppm phenol red. Five to nine microliters of 2 *N* NaOH were added to each sample to adjust the pH to approximately 7. Neutralized solutions were centrifuged and supernatant was assayed for SP as described below. In all experiments, release by a test substance was compared to basal release and results were expressed as total picograms of SP released.

Measurement of substance P content in DRG neurons. Following the release protocol, the content of SP in these neurons was determined in each culture dish. One milliliter of 2 *N* acetic acid was added to each dish containing DRG neurons and incubated at room temperature for 15 min. The solution was then removed and the procedure repeated a second time. The extracts were combined and diluted 1:20, 1:50, and 1:100 with 2 *N* acetic acid. Samples were frozen, lyophilized, and assayed for SP as described below.

Radioimmunoassay for substance P. Substance P was assayed using a modification of our previously described radioimmunoassay (Pang and Vasko, 1986). Briefly, the radioimmunoassay was performed by adding 25 μl of the 1:8000 dilution of the anti-substance P serum (57P) and 25 μl of ^{125}I-8-Tyr-SP containing 4000–5000 cpm to 300 μl of 0.1 M phosphate buffer containing known amounts of SP (ranging from 2.5 to 500 pg) or to 300 μl of the reconstituted unknown samples. This mixture was then incubated for 16–18 hr at 4°C. After incubation, 500 μl of a charcoal solution consisting of 1% Norite I charcoal suspended in 0.1 M phosphate buffer (pH 7.5) containing 0.3% NaCl, and 0.1% bovine serum albumin were added to each tube. This solution was centrifuged at 1500 × *g* for 15 min at 4°C to remove unbound peptides. The supernatant was decanted and the radioactivity determined by gamma scintillation spectrometry. The amounts of SP in unknown samples were estimated by comparing unknown samples to the "logit-log" linearized standard curve (Rodbard and Leward, 1970). Using this method, the minimal detectable limit (95% confidence interval) was 5 pg of SP with a 50% displacement of bound peptide at approximately 75 pg.

With each chemical manipulation, standard curves for SP were determined in the presence of the highest concentration of test compound to confirm that the experimental manipulation did not alter the standard curve. Anti-substance P serum was raised in our laboratory from rabbits using thyroglobulin-bound SP (Pang and Vasko, 1986). The antibody used does not cross-react to any significant degree with a number of other peptides including the tachykinins: neurokinin A, neurokinin B, kassinin, eledoisin, or physalaemin; nor does it cross-react with arachidonic acid metabolites.

Chromatography. Neurons were exposed to 1 ml of Krebs' bicarbonate buffer containing 50 mM KCl for 5 min to release SP. This solution was then acidified using acetic acid such that the final concentration was 2 *N*, frozen, and lyophilized. After lyophilization, the sample was resuspended in mobile phase consisting of 0.2 ml of 69% H_2O, 30% acetonitrile, and 1% phosphoric acid (v/v), and the sample was injected onto an 8 mm i.d. 4 μM C18 radial Pak L.C. cartridge (Waters Associates, Milford, MA) that was preequilibrated with mobile phase. Peptide was eluted with the mobile phase at a flow rate of 1 ml/min. Fractions were collected at 30 sec intervals, the organic phase evaporated under a nitrogen stream, and the aqueous phase frozen. Frozen samples were lyophilized, resuspended in assay buffer (see above), and assayed for SP as described above. A parallel run was performed with the SP standard.

Measurement of calcium currents. Avian DRG neurons were plated and cultured on small plastic coverslips as described above. After a period of 5–7 d, a coverslip was removed from the culture dish and transferred to the bottom of a small recording chamber. In order to eliminate sodium and potassium currents, the neurons were bathed in a solution of the following composition (in mM): 105 CsCl, 20 tetraethylammonium chloride, 10 $BaCl_2$, 1 $MgCl_2$, 10 HEPES, 10 glucose, and 1 μM tetrodotoxin (pH adjusted to 7.4 with either free base arginine or

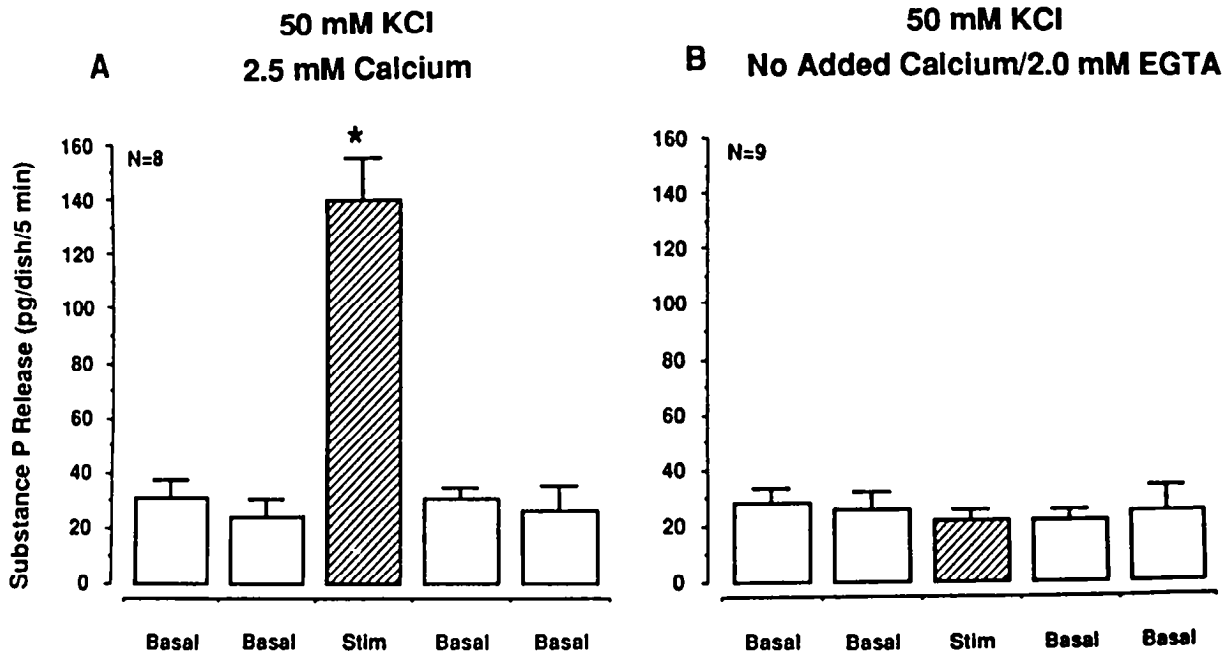

Figure 1. Potassium-stimulated release of SP from avian DRG neurons is dependent on extracellular calcium. The *ordinate* represents the amount of SP released/dish of neurons/5 min incubation. Each column represents the mean ± SEM of the resting or basal release (*Basal, open columns*) or the release in the presence of 50 mM KCl (*Stim, hatched columns*). *A* is the release in buffer containing 2.5 mM calcium, whereas *B* represents the release in buffer containing no added calcium and 2 mM EGTA. The number of dishes examined is indicated in each panel. The *asterisk* indicates statistically significant differences ($p < 0.05$) from the basal release.

CsOH). The recording chamber was then placed on the stage of an inverted microscope for viewing the cells and placement of the recording electrode. The recording chamber had an approximate volume of 500 μl and was superfused by a gravity flow/aspiration setup.

The calcium current (I_{Ca}) was recorded from chick DRG neurons by utilizing the whole-cell patch-clamp technique (Hamill et al., 1981). Membrane currents were recorded with a EPC-7 patch-clamp amplifier (List Electronic, Darmstadt, Germany). The recording pipettes were pulled from borosilicate disposable pipettes and were filled with the following solution (in mM): 50 CsCl, 50 *N*-methyl-D-glucamine chloride, 10 tetraethylammonium chloride, 2 $MgCl_2$, 10 EGTA, 10 HEPES, and 4 Na_2-ATP (pH adjusted to 7.2 with free base arginine or CsOH). The pipette solution had an osmolality of about 275 mOsm. These pipettes typically had resistances of 2–4 MΩ.

The recording pipette was placed against the surface of the DRG neuron, and gentle suction was applied by mouth until a sealing resistance of 2–10 GΩ was obtained. Additional suction was then applied to the pipette until there was a large increase in the pipette capacitance, indicating attainment of the whole-cell configuration. The cell capacitance was compensated nominally by approximately 70% and had typical series resistance values of 3–5 MΩ. The whole-cell current recordings were sampled at 0.5 msec per point. The cells were held at resting potentials of either −60 or −90 mV. Depolarizing voltage steps of 10 mV and 175 msec in duration were computer generated with the software package of pCLAMP (Axon Instruments, Inc., Foster City, CA). Data storage and analysis were accomplished with the pCLAMP package. In all the current traces shown, the capacitive and linear leakage currents have been subtracted by averaging and scaling four hyperpolarizing steps one-fourth the amplitude of the test potential. All experiments were done at room temperature (22–25°C).

Statistics. All release data are presented as the mean ± SEM for four to nine dishes of neurons. To compare basal and stimulated SP release, analysis of variance and Bonferroni's procedure for multiple *t* tests were performed on basal versus stimulated release (Wallenstein et al., 1980). Because the first period of basal release was not significantly different from the second period of basal release in any of the experiments, statistical comparisons were made between the initial basal release and the stimulated release. Student's *t* test was used to compare potassium-evoked release with release evoked by test substance. Significance level for each test was set at 0.05. Evoked release is defined as the amount of SP released during the 5 min of stimulation minus the average of the basal releases prior to stimulation.

Results

Characterization of substance P release

Experiments were first performed to characterize potassium-stimulated release of SP. As can be seen in Figure 1*A*, exposure of DRG neurons to Krebs' buffer containing 3.5 mM KCl results in a resting or basal release of 30 ± 6 and 24 ± 6 pg/dish of cells/5 min for the first two incubation periods, respectively. When the extracellular concentration of KCl is increased to 50 mM, the release of SP is increased approximately four- to fivefold to 138.4 ± 15 pg/dish/5 min (Fig. 1*A*). The potassium-stimulated release does not occur when neurons are incubated with Krebs' buffer containing no added calcium and 2 mM EGTA (Fig. 1*B*), suggesting that the evoked release of SP is calcium dependent. Basal release of SP is not altered by the extracellular calcium concentration. The total content of SP in each dish of cells ranged from 2 to 4 ng, with basal release being approximately 1% of total content, whereas potassium-stimulated release is approximately 4% of total SP content.

To ascertain if the immunoreactive product released from DRG neurons is SP, we determined whether it cochromatographs with authentic SP using reverse-phase HPLC. As shown in Figure 2, the majority of the SP-like immunoreactivity that is released from neurons exposed to Krebs' bicarbonate buffer cochromatographs with authentic SP.

Effect of PGE_2 on substance P release

Because PGE_2 produces hyperalgesia and lowers the threshold of activation in sensory neurons, it seems likely that the eicosa-

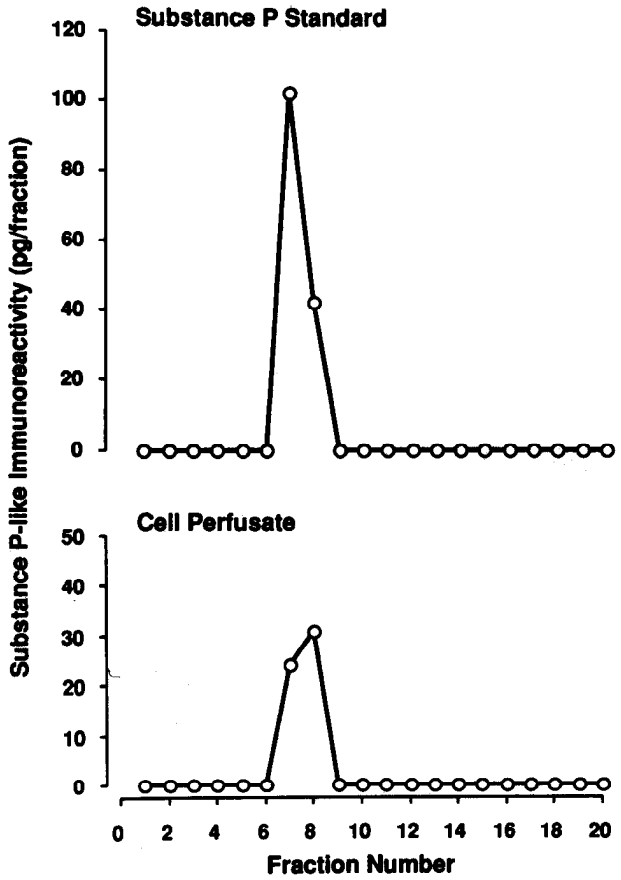

Figure 2. The immunoreactive product released by potassium stimulation of avian DRG neurons coelutes with authentic SP. Samples were chromatographed using HPLC as discussed in the Materials and Methods. The *ordinate* represents the SP-like immunoreactivity in pg/fraction measured by the radioimmunoassay. The *abscissa* represents the fractions collected every 0.5 min.

noid could increase the release of neurotransmitter from these neurons. Consequently, we studied whether PGE_2 could stimulate the release of SP from sensory neurons grown in culture. The release protocol was performed on dishes of neurons where the cells were exposed during the third incubation period to either Krebs' buffer containing 40 mM KCl or buffer containing various concentrations of PGE_2 (3.5 mM KCl). Results of these studies are illustrated in Figure 3. Exposure of cells to PGE_2 produces a dose-dependent increase in the release of SP above basal release. PGE_2 at a concentration of 1 μM stimulates the release of SP approximately twofold over basal release, whereas both 5 and 10 μM PGE_2 increases the release approximately fourfold. The evoked release, that is, stimulated release minus basal release, caused by 1 μM PGE_2 is 23 ± 8 pg/dish/5 min, by 5 μM PGE_2 is 66 ± 7 pg/dish/5 min, and by 10 μM PGE_2 is 63 ± 3 pg/dish/5 min. Evoked release produced by 40 mM KCl is 45 ± 3 pg/dish/5 min (Fig. 3), whereas the release caused by 50 mM KCl is 113 ± 10 pg/dish/5 min (see Fig. 1). For neurons exposed to either 1 μM or 5 μM PGE_2, the release of SP returns to the basal level when the buffer is replaced with Krebs' buffer lacking PGE_2. In contrast, release of SP after exposure to 10 μM PGE_2 remains elevated even after the eicosanoid is removed.

Effects of PGE_2 on calcium currents

The PGE_2-induced increase in SP release observed in DRG neurons may be due to an increase in the influx of calcium since the release of neurotransmitter is usually dependent on calcium influx. Consequently, we determined whether PGE_2 could facilitate the inward calcium current in these neurons. The addition of PGE_2 produced a significant increase in the amplitude of I_{Ca} as recorded in these neurons by utilizing the whole-cell patch-clamp technique. These results are illustrated in Figure 4. Under control conditions, an inward current whose maximum amplitude of −412 pA is obtained at 0 mV (Fig. 4*A,C*). When the perfusate is changed to one containing 1 μM PGE_2, there is a large and rapid increase in I_{Ca} recorded with this voltage protocol (Fig. 4*B,C*). The maximum value of I_{Ca} under these conditions is increased to −854 pA. As in the control recording, the maximal value is obtained at 0 mV, indicating no significant change in the activation profile for this inward I_{Ca}. The PGE_2-induced increase in I_{Ca} is observed after 2 min of exposure to the eicosanoid. However, the increase in I_{Ca} was not sustained and returned to normal levels within 10 min of exposure to the PG (data not shown). The amplitude of I_{Ca} and its dependence on the applied voltage are presented in the current–voltage relation shown in Figure 4*C*. The current–voltage curves for this DRG neuron demonstrate that the addition of PGE_2 enhances I_{Ca} at all voltage steps except where the driving forces are likely to be small (±60 and ±50 mV). The maximum value of I_{Ca} is obtained at 0 mV and undergoes a twofold elevation in the presence of PGE_2.

The capacity of PGE_2 to enhance I_{Ca} was observed in seven of nine DRG neurons studied, and these results are summarized in the current–voltage relations illustrated in Figure 5, *A* and *B*. The data are normalized to the peak values of I_{Ca} under the control conditions. For a holding potential of −60 mV (Fig. 5*A*), the mean peak value of I_{Ca} (n = 4) for each voltage step was normalized to −376 pA at +10 mV. In neurons exposed to PGE_2, the mean I_{Ca} value is enhanced about 1.8-fold (Fig. 5*A*). In a similar manner, when the holding potential was −90 mV (Fig. 5*B*), the peak value of I_{Ca} (n = 3) is increased by about 1.4-fold upon the addition of PGE_2. For both holding potentials, the PGE_2-induced elevation in I_{Ca} was not observed for voltage steps below −20 mV. The lack of any significant increase in I_{Ca} for voltage steps below −20 mV (especially at the holding potential of −90 mV) suggests that PGE_2 is affecting primarily L-type calcium channels rather than the T-type calcium channels (Fox et al., 1987). For a holding potential of −60 mV, exposure to PGE_2 appears to shift the voltage at which the peak value of I_{Ca} is obtained. For the control current–voltage curve, the peak I_{Ca} occurred at +10 mV, whereas the peak value of I_{Ca} in the presence of PGE_2 occurred at 0 mV. This 10 mV shift may not be significant and may reflect the variability of I_{Ca} for the small sample size. This shift was not observed for the −90 mV holding potential. These results demonstrate that PGE_2 can enhance the activation of I_{Ca} in DRG neurons grown in culture.

Effects of arachidonic acid

To determine if the effects of PGE_2 are selective and not the result of formation of other metabolic products of arachidonic acid, we studied whether the metabolic precursor of PGE_2, arachidonic acid, could alter the release of SP or calcium currents in DRG neurons. Release experiments were performed using different groups of cells with or without indomethacin pretreatment. The results from these experiments are shown in Figure 6. When neurons are exposed to 10 μM arachidonic acid, there is no significant increase in the release of SP (Fig. 6*B*). Basal release for the 5 min period prior to exposure to arachidonic

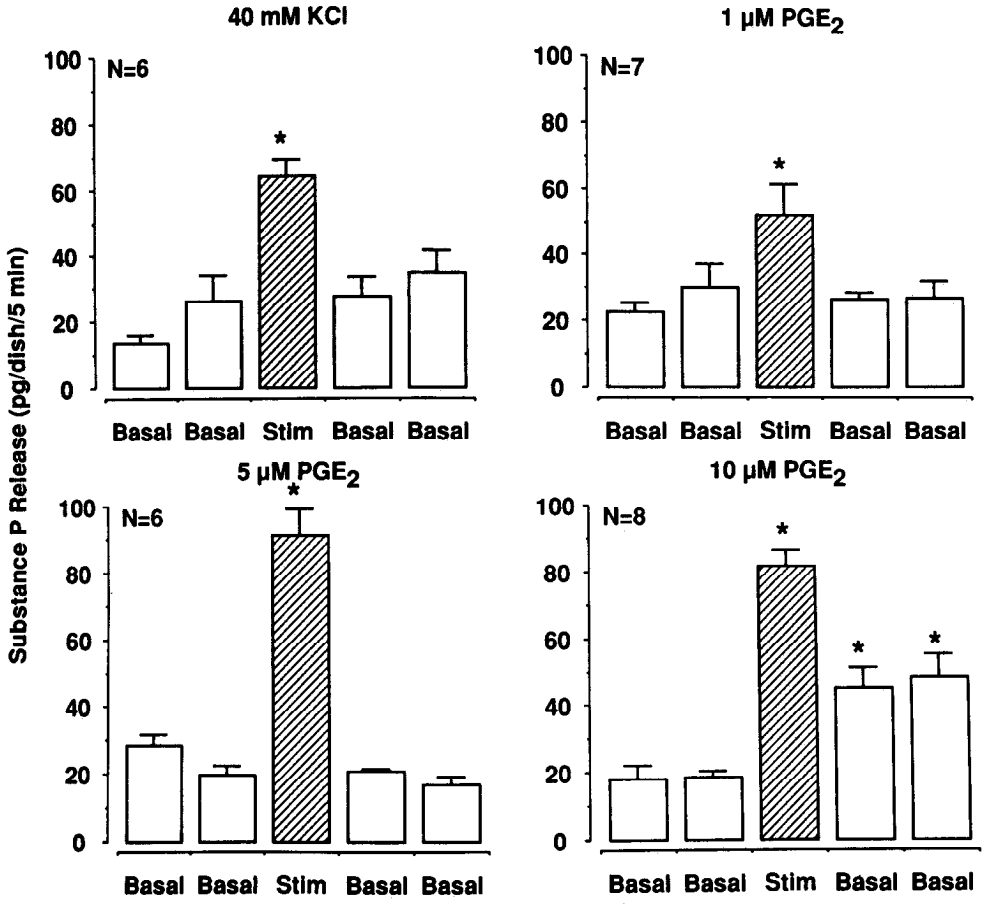

Figure 3. Various concentrations of PGE_2 stimulate the release of SP from avian DRG neurons. The *ordinates* represent the amount of SP released/dish of neurons/5 min incubation. Each column represents the mean ± SEM of the release of SP for each 5 min incubation. In each case, the data are presented showing the basal release during a 5 min exposure to Krebs' buffer containing 3.5 mM KCl (*Basal, open columns*), or release from neurons exposed for 5 min to buffer containing 40 mM KCl, or for 5 min to Krebs' buffer containing 3.5 mM KCl and various concentrations of PGE_2 as indicated (*Stim, hatched columns*). The number of dishes examined is indicated in each panel. The *asterisk* indicates statistically significant differences from basal release.

acid is 26 ± 4 pg/dish/5 min, whereas the release in the presence of arachidonic acid is 31 ± 4 pg/dish/5 min. In neuronal cultures prepared from the same DRG cell harvests, a significant increase in release of SP from 34 ± 3 pg/dish/5 min to 129 ± 14 pg/dish/5 min is observed after exposure to 50 mM KCl (Fig. 6*A*), demonstrating that the absence of any arachidonic acid effect is not due to a lack of neuronal viability. Basal release of SP from neurons exposed to arachidonic acid for 5 min does not return to the levels observed prior to arachidonic acid, but increases to 44 ± 4 and 48 ± 2 pg/dish/5 min, which is significantly higher than the initial basal release. However, this late increase in SP release is not observed when cells are pretreated with 10 μg/ml indomethacin for 30 min (Fig. 6*C*). Basal release for the 5 min period prior to exposure to arachidonic acid is 14 ± 1 pg/dish/5 min. Exposure to 10 μM arachidonic acid does not alter the release and has a value of 15 ± 2 pg/dish/5 min. After arachidonic acid is removed, the release remains similar to that observed for the control condition, having values of 11 ± 1 and 10 ± 2 pg/dish/5 min. Potassium-stimulated release is not altered by indomethacin pretreatment (data not shown). These results suggest that this late rise in SP release observed after exposure to 10 μM arachidonic acid may result from the formation of a product of the metabolism of arachidonic acid by cyclooxygenase.

We also examined the effects of arachidonic acid on calcium currents in DRG neurons. The addition of either 1 μM or 10 μM arachidonic acid does not cause any significant increase in I_{Ca}. In experiments utilizing the larger concentration of arachidonic acid, the peak value for the amplitude of I_{Ca} under the control condition is −1313 ± 293 pA (mean ± SEM; n = 7; range, −322 to −2637 pA) whereas 2–5 min after the addition of 10 μM arachidonic acid the peak value of I_{Ca} is −1360 ± 348 pA (mean ± SEM; n = 7; range, −321 to −2761 pA). For both the control and 10 μM arachidonic acid conditions, the peak value of I_{Ca} for each voltage step was normalized to the maximal value of I_{Ca} under its respective control condition. The normalized values were then averaged; these results are illustrated in Figure 7. The current–voltage curves for both the control and arachidonic acid are very similar. The peak values of I_{Ca} for both the control and arachidonic acid conditions occur at 0 mV. Arachidonic acid also does not have any significant effect on I_{Ca} over the long term. In two of the seven neurons where I_{Ca} was examined at 10 and 20 min after the addition of 10 μM arachidonic acid, the peak value of I_{Ca} under the control condition is −1020 pA (mean of two neurons) whereas 10 and 20 min after the addition of arachidonic acid, I_{Ca} is −972 and −936 pA, respectively.

Similar results occur when neurons are exposed to 1 μM arachidonic acid. The peak value for the amplitude of I_{Ca} under the control condition is −674 ± 113 pA (mean ± SEM; n = 9; range, −196 to −1297 pA), whereas 10 min after the addition of 1 μM arachidonic acid the peak value of I_{Ca} is −693 ± 133 pA (mean ± SEM; n = 9; range, −196 to −1308 pA). These results indicate that arachidonic acid has no direct action on the amplitude or voltage dependence of I_{Ca} in these sensory neurons.

Effects of $PGF_{2\alpha}$ and LTB_4

There is evidence that both $PGF_{2\alpha}$ and LTB_4 also produce hyperalgesia, although this action does not appear to be the result

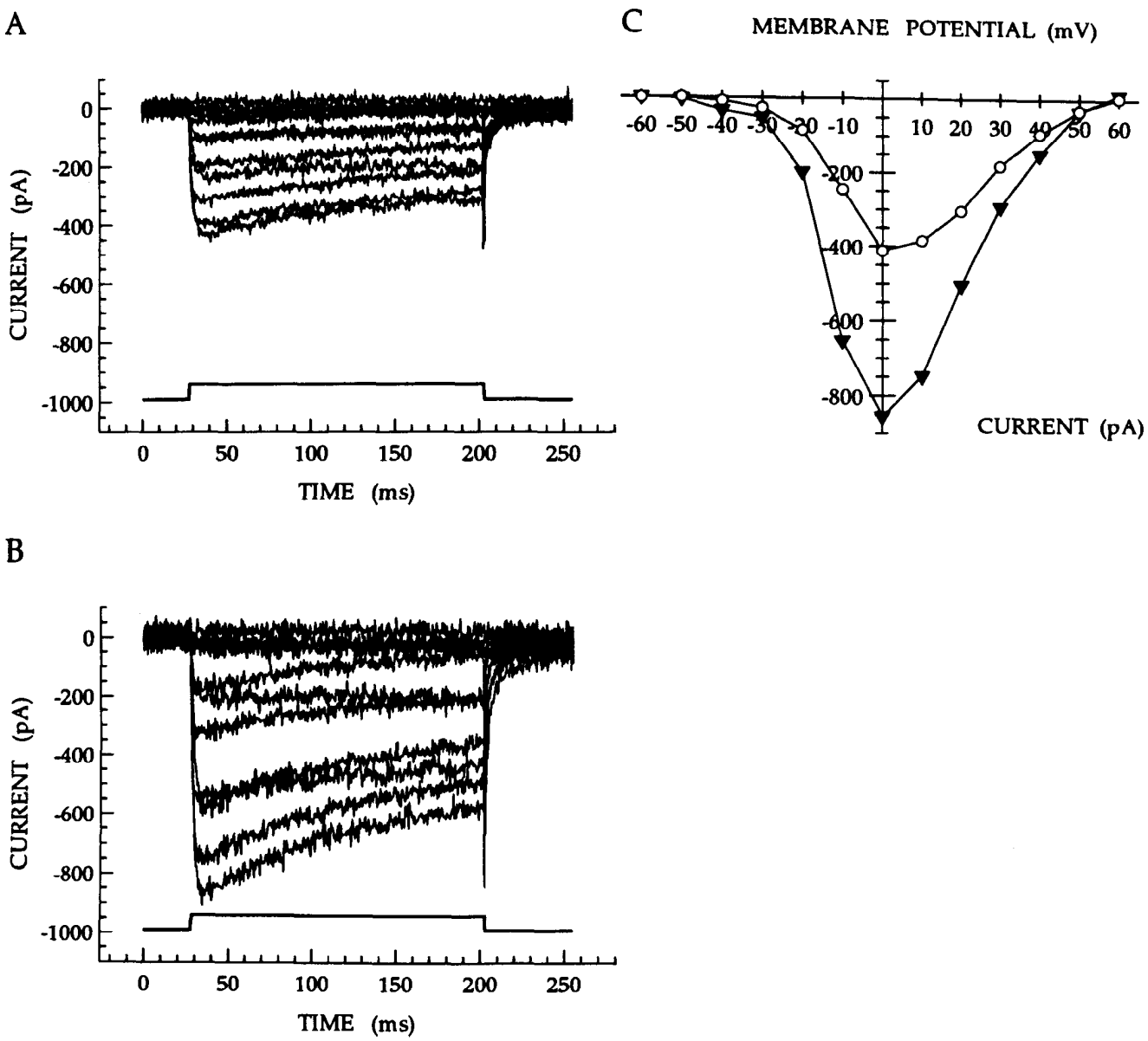

Figure 4. PGE_2 enhances the calcium current in a single avian DRG neuron. For *A* and *B*, the *ordinate* represents the membrane current in pA, whereas the *abscissa* is the time in milliseconds. The cell was held at −60 mV; incremental voltage steps of 10 mV were then applied until the maximal value of +60 mV was reached. The timing and duration of the voltage step are shown in the *bottom trace*. *A* represents recording of I_{Ca} from a DRG neuron in whole-cell mode under control conditions. *B* shows I_{Ca} in the same cell 2 min after the addition of 1 μM PGE_2 to an identical series of voltage steps. This allows sufficient time for the bath volume to be turned over four times. *C* represents the dependence of I_{Ca} on the applied voltage step for both the control condition (*open circles*) and after addition of PGE_2 (*solid triangles*).

of a direct effect on sensory neurons (Levine et al., 1984; Taiwo and Levine, 1986). Based on this finding, and to determine further the specificity of the effects of PGE_2, we also examined the effects of $PGF_{2\alpha}$ and LTB_4 on DRG neurons grown in culture. Release studies were performed as described above. Dishes of neurons were exposed to either Krebs' buffer containing 3.5 mM KCl, buffer containing 50 mM KCl, or buffer containing 3.5 mM KCl and 10 μM $PGF_{2\alpha}$ or 1 μM LTB_4. The results of these experiments are illustrated in Figure 8. As previously observed, exposure of neurons to 50 mM KCl results in an approximate fivefold increase in the release of SP from a basal release of 20 ± 5 pg/dish/5 min to 102 ± 6 pg/dish/5 min (Fig. 8*A*). The addition of 10 μM $PGF_{2\alpha}$ to the Krebs' buffer (KCl concentration of 3.5 mM) has no significant effect on the release of SP. Basal release in these neurons is 23 ± 3 and 19 ± 3 pg/dish/5 min compared to a release of 24 ± 1 pg/dish/5 min in the presence of $PGF_{2\alpha}$ (Fig. 8*B*). In a similar manner, exposure of neurons to 1 μM LTB_4 does not significantly alter release above the basal levels. Basal release in these neurons is 23 ± 4 pg/dish/5 min, whereas the release in the presence of LTB_4 is 28 ± 4 pg/dish/5 min (Fig. 8*C*).

The addition of $PGF_{2\alpha}$ to the medium bathing the DRG neurons did not alter the amplitude of I_{Ca}. In the control condition, I_{Ca} has a maximum value of −1179 ± 126 pA (mean ± SEM; n = 12; range, −436 to −2063 pA), whereas after a 2 min exposure to 10 μM $PGF_{2\alpha}$, I_{Ca} has a maximum value of −1259

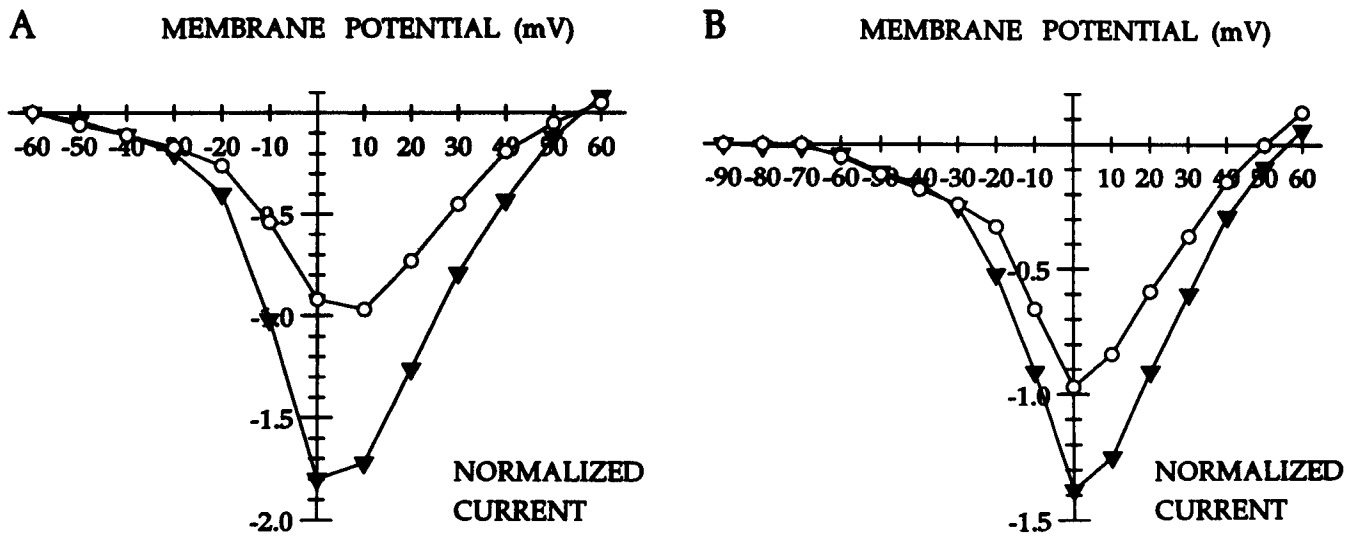

Figure 5. Normalized current–voltage relationships for control and PGE_2-treated DRG neurons. The values of I_{Ca} obtained for each voltage step were normalized to their respective maximal value of I_{Ca} under control conditions. The values of I_{Ca} obtained after the addition of 1 μM PGE_2 (range, 1.5–6 min) were then normalized to their respective peak values for the control I_{Ca}. The *ordinate* represents the normalized current values, and the *abscissa*, the applied voltage step. *A* and *B* represent neurons with holding potentials of −60 mV ($n = 4$) and −90 mV ($n = 3$), respectively. *Open circles* represent I_{Ca} from control cells, and *solid triangles* represent neurons treated with 1 μM PGE_2.

± 143 pA (mean ± SEM; $n = 12$; range, −480 to −2019 pA). For both the control and $PGF_{2\alpha}$ conditions, the peak value of I_{Ca} for each voltage step was normalized to the maximum value of I_{Ca} under its respective control condition. The normalized values were then averaged; these results are shown in Figure 9. The presence of $PGF_{2\alpha}$ has no significant effect on the amplitude of I_{Ca} recorded at all voltage steps.

LTB_4 also does not significantly alter I_{Ca}. The addition of 1 μM LTB_4 has only a slight effect on I_{Ca}. In recordings from two DRG neurons, the peak values of I_{Ca} are −1313 and −1896 pA for a holding potential of −90 mV under control conditions (data not shown). In recordings from nine other neurons held at −90 mV, the peak values of I_{Ca} ranged from −800 to −2500 pA. After 15 min of exposure to 1 μM LTB_4, the peak value of I_{Ca} had increased about 1.1-fold to −1530 and −2067 pA, respectively, for the two neurons. No effect is observed after 1 min. This slight stimulatory action of LTB_4 may reflect a physiological role for the lipoxygenase product of arachidonic acid, although there is little effect compared to the action of PGE_2. More likely, it is a nonspecific effect. The lack of a significant action of LTB_4 within minutes of its administration and the lack of effect of either $PGF_{2\alpha}$ or arachidonic acid are consistent with the notion that the *in situ* metabolic products of either the cyclooxygenase or lipoxygenase pathways are not responsible for the enhanced I_{Ca} observed upon the application of PGE_2.

Discussion

In initial experiments, we characterized the release of SP from avian DRG neurons grown in culture. In a majority of the dishes of cells used, the neurons contained approximately 2–4 ng of

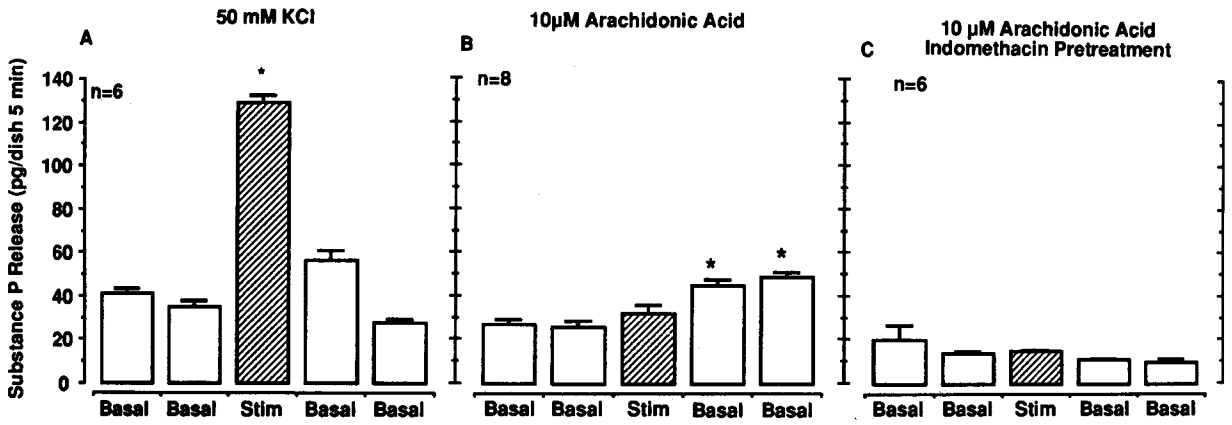

Figure 6. Arachidonic acid does not alter the release of SP from DRG neurons. The *ordinates* represent the amount of SP released/dish of neurons/5 min incubation. Each column is the mean ± SEM for cells exposed to Krebs' buffer containing either the control or the experimental solutions. *A* represents the control experiments with neurons exposed to buffers containing either 3.5 mM KCl (*Basal, open columns*) or 50 mM KCl (*Stim, hatched column*). *B* illustrates the release from neurons exposed to Krebs' buffer containing either 3.5 mM KCl (*Basal, open columns*) or 3.5 mM KCl and 10 μM arachidonic acid (*Stim, hatched column*). *C* represents the release from cells pretreated with indomethacin (10 μg/ml) for 30 min and then exposed to 10 μM arachidonic acid; *open* and *hatched columns* are the same as in *B*. The number of dishes examined is indicated in each panel. The *asterisk* indicates statistically significant differences from the basal release prior to arachidonic acid exposure.

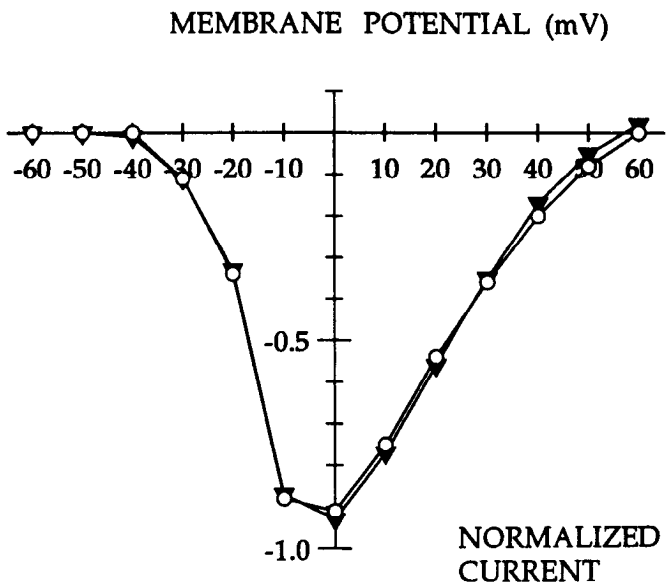

Figure 7. Arachidonic acid has no effect on calcium currents in DRG neurons. The points in the current–voltage relation represent the normalized mean values of I_{Ca} for both the control condition (*open circles*) and after the addition of 10 μM arachidonic acid (*solid triangles*) for seven DRG neurons. The peak values of I_{Ca} for each voltage step under control and arachidonic acid conditions were normalized to the maximum value of I_{Ca} obtained under the control condition for each respective neuron. These normalized values were then averaged and are shown in the current–voltage relation above. The experimental recordings were obtained between 2 and 5 min after the addition of arachidonic acid. The holding potential was −60 mV.

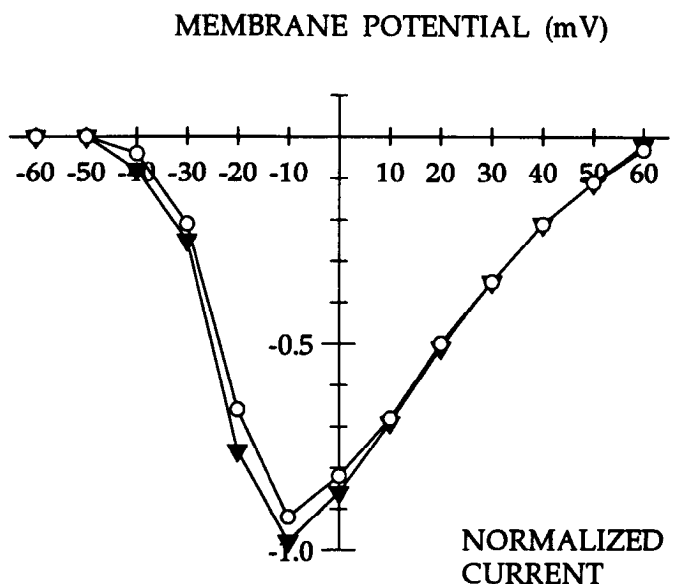

Figure 9. $PGF_{2\alpha}$ does not significantly alter the calcium current in DRG neurons. The points in this current–voltage relation represent the normalized mean values of I_{Ca} for both the control condition (*open circles*) and after the addition of 10 μM $PGF_{2\alpha}$ (*solid triangles*) for 12 DRG neurons. The peak values of I_{Ca} for each voltage step under control and $PGF_{2\alpha}$ conditions were normalized to the maximum value of I_{Ca} obtained under the control condition for each respective neuron. These normalized values were then averaged and are shown in the current–voltage relation above. The experimental recordings were obtained 2 min after the addition of $PGF_{2\alpha}$. The holding potential was −60 mV.

SP per dish of cells. This is similar to the total tissue content reported by Mudge et al. (1979) for avian DRG neurons grown in cell culture. In addition, both the basal (resting) and potassium-stimulated release of SP observed in these experiments are similar in magnitude to values reported by Mudge et al. (1979) and Holz et al. (1988). Basal SP release is approximately 1% of the total tissue content of the peptide, whereas the release evoked by 50 mM KCl represents approximately 4% of the total content. This potassium-stimulated release is dependent on extracellular calcium. These results are comparable to release of SP from other neuronal preparations (Jessell and Iversen, 1977; Gamse et al., 1981; Pang and Vasko, 1986) and suggest that sensory neurons in culture are a valid model for studying transmitter release. The immunoreactive substance released by the cells and measured using our radioimmunoassay appears to be authentic SP because its elution profile on HPLC is similar to that of the SP standard and because the antisera we use in the assay does not cross-react to any significant degree with any known mammalian tachykinin.

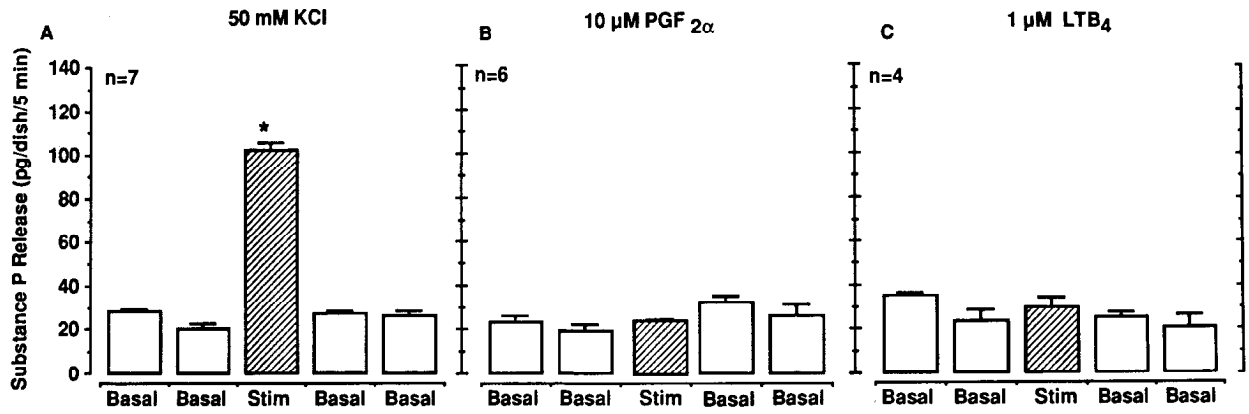

Figure 8. The eicosanoids, $PGF_{2\alpha}$ and LTB_4 do not alter SP release from DRG neurons. The *ordinates* represent the amount of SP released/dish of neurons/5 min incubation. Each column is the mean ± SEM for cells exposed to Krebs' buffer containing either the control or the experimental solutions. *A* represents control experiments for neurons exposed to Krebs' buffer containing either 3.5 mM KCl (*Basal, open columns*) or 50 mM KCl (*Stim, hatched column*) to evoke release. *B* illustrates the release obtained for neurons exposed to either the control conditions of 3.5 mM KCl (*Basal, open columns*) or 3.5 mM KCl and 10 μM $PGF_{2\alpha}$ (*Stim, hatched column*). *C* illustrates the release from neurons exposed to either 3.5 mM KCl (*Basal, open columns*) or 3.5 mM KCl and 1 μM LTB_4 (*Stim, hatched column*). The number of dishes examined is indicated in each panel. The *asterisk* indicates statistically significant differences from the basal release.

Extensive experimental evidence suggests that PGE_2 is a direct chemical mediator involved in sensitizing sensory neurons; consequently, we sought to determine if this eicosanoid could enhance neurotransmitter release from sensory neurons. We chose to study the release of SP from DRG cells because abundant evidence suggests that this peptide is an important neuroactive compound in nociceptive sensory neurons (see reviews by Salt and Hill, 1983; Cuello, 1987). In addition, SP appears to be an important mediator of components of the inflammatory response, especially neurogenic inflammation (Payan, 1989). Our results demonstrate that PGE_2 stimulates the release of SP from avian DRG neurons in a dose-dependent manner. Exposure of neurons to 1 μM PGE_2 results in a small but significant increase in SP, whereas at higher concentrations the evoked release is similar to that observed with elevated concentrations of extracellular potassium. The concentrations of PGE_2 utilized in the current studies are similar to those required to sensitize sensory neurons to noxious stimuli as observed in previous reports. For example, Yanagisawa et al. (1986) demonstrated that 0.8–4 μM PGE_2 enhanced the nociceptive reflex in an isolated spinal cord–tail preparation from newborn rat. A lesser concentration of PGE_2 (0.1 μM) potentiated the potassium-induced firing of rat sensory neurons grown in culture (Baccaglini and Hogan, 1983). In addition, micromolar concentrations of PGE_2 are required to increase the influx of calcium in neuroblastoma–glioma hybrid cells (Miwa et al., 1988) and to stimulate catecholamine release from bovine adrenal chromaffin cells (Koyama et al., 1988).

The PGE_2-induced increase in SP release is relatively selective for this prostanoid because neither $PGF_{2\alpha}$, arachidonic acid, nor the lipoxygenase metabolic product LTB_4 significantly enhances SP release upon acute exposure. Neurons exposed to 10 μM arachidonic acid for 5 min show a slight but significant increase in SP release during the two 5 min collections following removal of arachidonic acid, an effect that is attenuated by the indomethacin pretreatment. This latter effect suggests that arachidonic acid is being metabolized to a PG by the cells in culture. In fact, Vesin and Droz (1991) have demonstrated that after a 10 min incubation period with labeled arachidonic acid, avian DRG neurons grown in culture can metabolize arachidonic acid to various PGs, especially PGE_2. Of primary importance, however, is our result demonstrating that arachidonic acid does not have an immediate action (at least within 10 min), supporting the idea that the enhanced release of SP is specific for PGE_2.

The results presented in this study demonstrate that PGE_2 can increase the amplitude of I_{Ca} elicited by voltage steps in these sensory neurons. As with the release studies, the effect of PGE_2 on I_{Ca} appears to be relatively specific since neither $PGF_{2\alpha}$, arachidonic acid, nor the lipoxygenase product LTB_4 enhances I_{Ca} in an analogous manner. The voltage dependence for activation of I_{Ca} does not appear to be affected by PGE_2 since the maximal value of I_{Ca} is obtained at the same voltage step for both the control and experimental conditions. This enhancement of I_{Ca} occurred with a rapid onset, reaching maximal levels within 2 min, but appeared to be a transient phenomenon since the peak value of I_{Ca} had returned to nearly the control value after about 10 min in the PGE_2 solution. A similar time course has been reported for PGE_2-induced I_{Ca} activation in bovine chromaffin cells (Mochizuki-Oda et al., 1991). This result suggests that the neuron has somehow desensitized to the activating effects of PGE_2 and implies either that the transduction cascade has decreased its sensitivity to PGE_2 or that PGE_2-sensitive calcium channels have inactivated. One obvious limitation of the current results is that only one concentration of PGE_2 was examined. We chose to use 1 μM PGE_2 based on the release studies showing that this was the lowest concentration tested that evoked release. Because this low concentration was effective in enhancing I_{Ca}, higher concentrations were not examined.

In both chick and rat DRG neurons, the release of SP is believed to be mediated by L-type calcium channels. The potassium-stimulated release of SP from these neurons can be blocked with the addition of dihydropyridine calcium channel antagonists such as nitrendipine and nifedipine (Perney et al., 1986; Rane et al., 1987). Thus, our findings that PGE_2 can increase both the release of SP and I_{Ca} in chick DRG neurons is consistent with the notion that PGE_2 may be modulating the activity of L-type calcium channels. This is based on the fact that the capacity of PGE_2 to enhance I_{Ca} was similar for holding potentials of either -60 or -90 mV (see Fig. 5*A*,*B*) and this enhancement occurred only for voltage steps that were greater than -20 mV (see Figs. 4*C*, 5*A*). Also, the inactivation of I_{Ca} was relatively slow and lacked the rapid transient relaxation (data not shown) indicative of N-type channel activity (Fox et al., 1987).

It seems likely that the enhancement of I_{Ca} by PGE_2 may contribute to the increase in SP release produced by this eicosanoid. However, this was not specifically determined in our experiments, because not all the neurons grown in cell culture synthesize and release SP. Therefore, it is possible that the observed changes in I_{Ca} are recorded from neurons other than those containing SP. An analogous mechanism for the enhancement of transmitter release by activation of I_{Ca} has been observed in bovine chromaffin cells and may be particularly relevant to these nociceptive sensory neurons. Addition of dopamine to the chromaffin cell stimulates a specific set of calcium channels whose activity appears to be controlled by cAMP and cAMP-dependent protein kinase (Artalejo et al., 1990). This facilitation of I_{Ca} may contribute to the release of more neurotransmitter, that is, a positive feedback mechanism to boost epinephrine levels beyond those normally released. In sensory neurons, PGE_2 may act through a similar type of pathway where PGE_2 acts to enhance I_{Ca} and thus facilitate the release of SP.

The mechanism of action for the increase in transmitter release and the activation of calcium channels remains unknown. It is possible that PGE_2 activates calcium channels directly, although to our knowledge, there is no evidence available to support this possibility. However, PGs are known to modulate the activity of many different types of second messenger systems (see reviews by Halushka et al., 1989; Shimizu and Wolfe, 1990). PGs alter intracellular cAMP content in superior cervical ganglia (Tomasi et al., 1977), neuroblastoma–glioma cells (Sharma et al., 1975), and bovine adrenal medulla (Negishi et al., 1989). In addition, in animal models of pain, the enhanced sensitivity to noxious stimuli produced by PGE_2 may be mediated by cAMP (Ferreira and Nakamura, 1979; Taiwo et al., 1989). In bovine chromaffin cells, the addition of PGE_2 increases the influx of calcium into the cell through potentiation of the calcium current. Presumably, this effect is mediated via PG stimulation of the inositol trisphosphate (IP_3) pathway wherein IP_3 presumably activates the calcium channels directly (Mochizuki-Oda et al., 1991). These results in bovine chromaffin cells are, at this point, speculative because the calcium currents activated by PGE_2 have not been shown to be the same population of currents activated by IP_3. Further studies are warranted to determine whether

PGE_2 activates or recruits additional calcium channels such as is the case for facilitation-type calcium channels (Artalejo et al., 1990) or whether PGE_2 shifts the probability toward the open state for active calcium channels through modulating the activity of a second messenger system.

It is interesting to speculate that the increase in neurotransmitter release and activation of voltage-sensitive calcium channels may be one mechanism to explain the sensitizing action of PGE_2 on sensory neurons. It is largely accepted that PGs are synthesized and released in response to tissue injury, contribute to hyperalgesia, and are involved with the acute inflammatory response (Ferreira, 1983; Higgs and Moncada, 1983; Payan, 1989). The hyperalgesic effect of PGE_2 appears to result from a direct action on sensory neurons because the onset of effect is rapid (Taiwo et al., 1987) and because neither indomethacin pretreatment, polymorphonuclear leukocyte depletion, nor lesion of sympathetic nerves alters PGE_2-induced hyperalgesia (Taiwo and Levine, 1989). In addition, PGE_2 increases the potassium-induced cell firing in rat sensory neurons grown in cell culture (Baccaglini and Hogan, 1983). Consequently, PGE_2 released upon localized tissue trauma may facilitate the release of SP from sensory neurons at both the peripheral and central terminals. The increased release from terminals in the spinal cord would presumably enhance the sequence of neuronal communication for pain sensation, whereas release in the periphery could contribute to neurogenic inflammation (Foreman, 1987). The enhancement of inward I_{Ca} caused by PGE_2 may also partially account for the lower threshold of firing in sensory neurons and for the enhanced sensitivity of these neurons to other chemical mediators of pain and inflammation after exposure to the eicosanoid.

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