Prostaglandin Receptor Subtypes, EP3C and EP4, Mediate the Prostaglandin E₂-induced cAMP Production and Sensitization of Sensory Neurons*

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Although a number of prostaglandin E₂ (PGE₂) receptor subtypes have been cloned, limited studies have been performed to elicit subtypes that subserve specific actions of this eicosanoid, in part because of a paucity of selective receptor antagonists. Using reverse transcription-polymerase chain reaction (PCR) and antisense oligonucleotides, we examined which prostaglandin E₂ receptor (EP receptor) subtypes are expressed in sensory neurons and which mediate the PGE₂-induced increase in cAMP production and augmentation of peptide release. Reverse transcription-PCR of cDNA isolated from rat sensory neurons grown in culture revealed PCR products for the EP1, EP2, EP3C, and EP4 receptor subtypes but not the EP3A or EP3B. Preexposing neuronal cultures for 48 h to antisense oligonucleotides of EP3C and EP4 mRNA diminished expression of the respective receptors by ~80%, abolished the PGE₂-stimulated production of cAMP, and blocked the ability of PGE₂ to augment release of immunoreactive substance P and calcitonin gene-related peptide. Pretreating with individual antisense against the EP2, EP3C, or EP4 receptors or combinations of mismatches had no effect on PGE₂-induced activity. Treatment with antisense to EP3C and EP4 receptor subtypes did not alter the ability of forskolin to increase cAMP or enhance peptide release. These results demonstrate that sensory neurons are capable of expressing multiple EP receptor subtypes but that only the EP3C and EP4 receptors mediate PGE₂-induced sensitization of sensory neurons.

Prostaglandin E₂ receptors (EP receptors)† have been classified into four general subtypes, EP1, EP2, EP3, and EP4, based on cloning and pharmacological manipulations (1, 2). These receptors are G-protein-coupled, and binding of agonists results in activation of various transduction cascades depending on the receptor subtype activated and the cells being studied. Activation of the EP1 receptor in kidney tubule cells increases the concentration of intracellular calcium, phosphoinositide turnover, and PKC activity (3, 4). EP2 and EP4 receptors are coupled through G₄ (1) to increase intracellular cAMP in a number of preparations (5–8). The EP3 receptor undergoes post-transcriptional RNA splicing to produce multiple EP3 isoforms, and activation of these splice variants can increase calcium mobilization or either stimulate or inhibit cAMP production (9–11). Because subtypes of the EP receptor can be linked to different transduction cascades in different types of cells, it is critical to determine which receptors and receptor-associated signal transduction pathways are responsible for specific physiological actions of E-series prostaglandins.

Although PGE₂ has a number of diverse physiological actions (12), its role in pain and inflammation is of primary importance. Indeed, both the analgesic and the anti-inflammatory actions of the nonsteroidal anti-inflammatory drugs are attributed to their ability to inhibit prostaglandin synthesis (13). In addition, PGE₂ is produced at sites of tissue injury (14), and administration of prostaglandins produces vasodilation and edema and augments pain perception in animals and in humans (15). The proinflammatory actions of PGE₂ result, in part, from a direct action of this eicosanoid on sensory neurons. In situ preparations, E-series prostaglandins augment the firing of sensory neurons in response to noxious stimuli (16, 17). Furthermore, exposing sensory neurons in culture to PGE₂ increases cAMP content (18), increases the number of action potentials elicited by a depolarizing stimulus (19), and augments the evoked release of the neuropeptides (18, 20).

Despite the fact that PGE₂-induced sensitization of sensory neurons has been extensively studied, the EP receptors expressed on sensory neurons and which subtypes mediate sensitization remain unknown. Consequently, we used RT-PCR to ascertain which receptor subtype mRNAs are expressed in sensory neurons. We also used antisense oligonucleotides to selectively reduce expression of EP receptor subtypes and examine whether this “knockdown” alters PGE₂-induced increases in cAMP and augmentation of release of immunoreactive substance P (iSP) and immunoreactive calcitonin gene-related peptide (iCGRP). We choose to use antisense, because EP receptor subtypes have highly conserved structures with ligands often activating multiple receptor subtypes (1, 2), and thus there are limited pharmacological tools to distinguish subtypes. Our data provide novel evidence that activation of the EP3C and EP4 receptors mediates the PGE₂-induced sensitization of sensory neurons, suggesting that multiple receptor subtypes can subserve the same function.

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¶ The abbreviations used are: EP receptor, prostaglandin E₂ receptor; iSP, immunoreactive substance P; iCGRP, immunoreactive calcitonin gene-related peptide; iAMP, immunoreactive cAMP; iGGRP, immunoreactive calcitonin gene-related peptide; PCR, polymerase chain reaction; RT-PCR, reverse transcription-PCR; bp, base pairs, IP receptor prostaglandin I₃ receptor.
**EXPERIMENTAL PROCEDURES**

**Materials—**Dubelco’s modified Eagle’s medium, t-glutamine, penicillin/streptomycin, Hanks’ balanced salt solution, and fetal bovine serum were obtained from Life Technologies, Inc. Nerve growth factor was purchased from Harlan Bioproducts for Science, Inc. (Indianapolis, IN). Prostaglandin E2, butyrophosphatase, 1-OH-PGE2, and sulprostone were obtained from Cayman Chemical Co. (Ann Arbor, MI). Peptides were obtained from Peninsula Laboratory (Belmont, CA). Capsaicin, forskolin, 3-isobutyl-1-methylxanthine (IBMX), and other routine chemicals were purchased from Sigma. Capsaicin and prostaglandins were initially dissolved in 1-methyl-2- pyridylidone (Sigma) to a concentration of 10 μM and then diluted to appropriate concentration in perfusion buffer. In no instances does this vehicle at the dilutions used alter cAMP or neuropeptide release. Substance-P antisera was raised in rabbits with synthetic Substance P coupled to bovine albumin. The antibody was affinity purified on Substance-P coupled to Sephadex G-100. Anti-parvalbumin antisera was raised in rabbits immunized with parvalbumin (Temosol, CA).

**Isolation and Culture of Rat Sensory Neurons—** Cultures of embryonic rat sensory neurons were prepared using a modification of previously described methods (20). Briefly, sensory neurons were dissociated from the dorsal root ganglion of 15–17-day old rat embryos using trypsin and mechanical agitation. Sensory neurons were grown in Dubelco’s modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 250 ng/ml nerve growth factor, 50 μg/ml penicillin and streptomycin, 1% nonmitotic inhibitor, 25 mM HEPES (pH 7.6), and 50 μM uridine (150 μM). Cells were plated in 24-well poly-l-lysine (100 μg/ml)-coated culture plates at ~150,000 cells/well. The cells were maintained at 37 °C in a 5% CO2 atmosphere for 9–11 days, and growth medium was changed every second day. Cultures of adult dorsal root ganglia cells were prepared using a modification of the method of Lindsay (21). Male Harlan Sprague-Dawley rats weighing 150–250 g were sacrificed with CO2. The dorsal root ganglia from each rat were collected in sterile calcium- and magnesium-free modified Hanks’ balanced salt solution. The dorsal root ganglia were digested with 0.125% collagenase in growth medium consisting of F-12 supplemented with 10% horse serum, 2 mM glutamine, 50 μg/ml penicillin and streptomycin, and the mitotic inhibitors 5-fluoro-2-deoxyuridine (50 μM) and uridine (150 μM) at 37 °C for 2 h. Cells were dissociated by mechanical agitation and plated in 24-well culture plates coated with poly-D-lysine and laminin at 48 h with a change of medium and oligonucleotide at 24 h. Cells were dissociated by trypsin and mechanical agitation. Sensory neurons were grown in Dubelco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 250 ng/ml nerve growth factor, 50 μg/ml penicillin and streptomycin, 1% nonmitotic inhibitor, 25 mM HEPES (pH 7.6), and 50 μM uridine (150 μM). Cells were plated in 24-well poly-l-lysine (100 μg/ml)-coated culture plates at ~150,000 cells/well. The cells were maintained at 37 °C in a 5% CO2 atmosphere for 9–11 days, and growth medium was changed every second day. The content of the immunoreactive cAMP (icAMP) was assayed by radioimmunoassay (PerkinElmer Life Sciences) using the nonacetylated protocol. Analysis of variance was used to compare the effects of different treatments on the neuropeptide release and cAMP production, and if a significant difference was observed, the Student-Newman-Keuls post hoc test was performed. The significance level for all tests was set at p < 0.05.

**Immunoblotting—** Cells were washed twice in sterile phosphate-buffered saline and disrupted by freezing in ice to 100–200 μl of lysis buffer containing 125 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 200 mM β-mercaptoethanol, 0.02% bromophenol blue. Equivalent amounts of cell lysates, as determined by using the Bio-Rad protein detection kit, were separated on 12% SDS-polyacrylamide gels and electrophoretically transferred to nitrocellulose. The membrane was blocked with 5% milk in Tris-buffered saline with 0.2% Tween 20 overnight and incubated for 24 h at 4 °C with polyclonal antibody to the EP1, EP2, EP3, EP4, and IP receptor subtypes or to an antibody generated by incubation with the appropriate horseradish peroxidase-conjugated secondary antibody for 1 h. Immunoreactive bands were developed by an ECL kit and visualized by exposure to Eastman Kodak Co. LS X-Omat film. Quantification of the immunoreactive bands was analyzed using NIH-Image software. Student’s t test was used to compare effects of antisense or missense treatment on receptor expression.

**RNA Isolation and Reverse Transcription—** Total RNA was isolated from sensory neurons in culture using a QuickPrep Total RNA Extraction Kit (Amersham Pharmacia Biotech). Messenger RNA was reverse-transcribed into cDNA using Superscript II reverse transcriptase (Superscript II RNase H Reverse Transcriptase Kit; Life Technologies). Total RNA (~1–5 μg) and 0.5 μg of oligo(dT)12-18 primer were heated at 70 °C for 10 min. Briefly chilled on ice. After primer annealing, the following were added: 50 μl Tris-HCl, pH 8.8, 75 μl mM MgCl2, 10 μl dithiothreitol, 1 μl dNTP, and 40 units of RNasin ribonuclease inhibitor. The reaction incubated for 50 min at 42 °C and then for 15 min at 70 °C. An aliquot of each reaction was subsequently used as template for a PCR.

**Primer Design and PCR—** The primers were designed to be selective for the PGE2 and PGL2 receptors, and the sequences used were as follows: EP1 (336 base pairs (bp)), 5′-GCAGGATTTCCATCAGCAGA-3′ (nucleotides 865–884) and 5′-CAGCTTGGGAGTCCACCGC-3′ (nucleotides 1182–1201) (23); EP2 (369 bp), 5′-CCGGGCTTGCTACCTTTGCCG-3′ (nucleotides 363–383) and 5′-GCTCGGAAGCTGCATGCCGG-3′ (nucleotides 713–732) (GenBank™ accession number U94708); EP3 (309 bp), 5′-GCTGTCGTGCTGCTGCCT-3′ (nucleotides 471–490) and 5′-GGATATCGGCATGCCTGTA-3′ (nucleotides 958–976); EP4 (453 bp), 5′-CTGTCGCTGGCTCTTCGCG-3′ (nucleotides 567–586) and 5′-GCCAGGGACGCGCCTATT-3′ (nucleotides 1003–1020) (GenBank™ accession number D29969); EP3 (413 bp), 5′-TGTTGGTACCTTTGCTTGCCAACTGGC-3′ (nucleotides 653–680) and 5′-CAAGGAGATGCTGCTGCTTCCGGC-3′ (nucleotides 1038–1065) (nucleotides 423–455); 5′-TTGCTGCTGCTGCTTCCT-3′ (nucleotides 941–963) and 5′-GAGGCTGTTGTCCTGCTGCTGCTG-3′ (nucleotides 1342–1364) (23); IP (431 bp), 5′-GCATCTTGGTGGCCAGCC-3′ (nucleotides 276–295) and 5′-CACGGTGCGCCGTGTTTGTGAC-3′ (nucleotides 688–707) (GenBank™ accession number D28966); and rat histone H3.3 (231 bp), 5′-GCAGAATGCGCGCCTTACTG-3′ (nucleotides 80–100) and 5′-GGCTCCTAGCCTGCTCGCCTAC-3′ (nucleotides 274–290) (PCR primer for rat EP receptors). The EP3 receptor is based on primers for an EP receptor originally classified as EP3B (25); however, the receptor is identical to the mouse EP3 receptor (11). The PCR mixture contained a cDNA template derived from total RNA, 1 unit of recombinant Taq DNA polymerase (Life Technologies, Inc.), 50 pmol each of 5′- and 3′-primers (Life Technologies), 0.2 mM dNTP, in a buffer containing 50 mM Tris-HCl (pH 8.4), 50 mM KCI, 1.5 mM MgSO4, in a volume of 50 μl. The PCR was performed using a PerkinElmer Life Sciences Thermocycler (model 2400) as follows: 94 °C for 120 s and then 37 cycles of 94 °C for 45 s, 60 °C for 45 s, and 72 °C for 120 s followed by 74 °C for 10 min. Samples were applied on 1% agarose gel pretained with 0.5 μg/ml ethidium bromide.

**Phosphorylase Oligonucleotides—** Antisense oligonucleotides were designed to be complementary to the PGE2, IP receptor subtypes (EP2, EP3C, and EP4). Missense control oligonucleotides were synthesized to correspond to antisense sequences except for pairs of bases that have been switched. Oligonucleotide sequences were as follows: EP2, antisense 5′-GCTGTCGTGCTGCTGCCT-3′ (bases 56–70) and missense 5′-CCGGGCTTGCTACCTTTGCCG-3′; EP3C, antisense 5′-GATGGGCTGCCCG-3′ (bases 1101–1125) and missense 5′-CTGTCGCTGGCTCTTCGCG-3′; EP4, antisense 5′-GACTCCGGGAGTGGAG-3′ (bases 4–18) and missense 5′-GACCTTGCGGAGTGA-3′. Oligonucleotides were synthesized (Life Technologies Custom Primers) with phosphorothioate linkages to prevent nuclease degradation.

Sensory neurons in culture were exposed to 1 μM antisense or missense oligonucleotide in growth medium or growth medium alone for 48 h with a change of medium and oligonucleotide at 24 h.
Identification of PGE$_2$ Receptor Subtypes in Sensory Neurons—To ascertain which PGE$_2$ receptor subtype mRNAs were found in sensory neurons, total RNA from cultured embryonic or adult sensory neurons was reverse-transcribed and subjected to PCR in the presence of primers for the PGE$_2$ receptor subtypes EP1, EP2, EP3A, EP3B, EP3C, EP4, the PGI$_2$ receptor, IP, or histone protein H3.3. Fig. 1, A and B, depicts the PCR products from embryonic and adult neurons, corresponding to the EP1, EP2, EP3C, and EP4 receptor subtypes. DNA size markers are shown in the lanes marked S. Histone H3.3 (H) was simultaneously amplified with PCR as an external control. C shows detection of PGE$_2$ receptor EP3A (3A) and EP3B (3B) subtype mRNA in kidney (K) but not sensory neurons (SN) by RT-PCR. RNA was extracted from kidney (K) or embryonic sensory neurons (SN). D represents an immunoblot of PGE$_2$ receptor subtypes EP1, EP2, EP3, and EP4 and the IP receptor from protein isolated from embryonic sensory neurons.

RESULTS

Identification of PGE$_2$ Receptor Subtypes Involved in the PGE$_2$-stimulated cAMP Production—We used antisense oligonucleotides that inhibit formation of specific EP receptor subtypes to determine which receptors mediate PGE$_2$-induced increases in the production of cAMP. Sensory neurons in culture were exposed for 48 h to medium alone or to medium containing 1 $\mu$m antisense or missense oligonucleotide to the EP2, EP3C, and/or EP4, and then the PGE$_2$-stimulated production of cAMP was measured. We focused on these receptor subtypes because cloned rat or mouse EP2, EP3C, and EP4 receptors have been shown to increase cAMP production (5, 9–11). In untreated neuronal cultures, exposure to 100 nM PGE$_2$ and 2 mM IBMX for 20 min significantly increased the content of cAMP 2.5-fold over cells treated only with IBMX (Fig. 2, A and B). In cultures treated with individual antisense oligonucleotides directed toward mRNA for EP2, EP3C, or EP4 receptor subtypes or to missense oligonucleotides, 100 nM PGE$_2$ also significantly increased cAMP production (Fig. 2A), suggesting that reducing expression of individual receptor subtypes had no effect on the PGE$_2$-stimulated production of cAMP. Consequently, we further examined whether simultaneously decreasing expression of multiple receptor subtypes would attenuate the actions of PGE$_2$. When neuronal cultures were pretreated for 48 h with 1 $\mu$m of each antisense directed at mRNA for the EP2, EP3C, and EP4 receptors, the effects of 100 nM PGE$_2$ were completely abolished in that cAMP levels were 3.1 $\pm$ 0.5 pmol/well ($n = 17$; Fig. 2B). In a similar manner, when sensory neurons were exposed to antisense oligonucleotides directed against mRNA for only the EP3C and EP4 receptor subtypes, exposure to 100 nM PGE$_2$ did not increase the content of cAMP (3.1 $\pm$ 0.5 pmol/well, $n = 13$). In contrast, antisense oligonucleotides directed toward the EP2 and EP4 receptor subtypes did not significantly attenuate the effect of the prostanoid, since in these cultures cAMP was elevated to 5.8 $\pm$ 0.5 pmol/well ($n = 9$). Exposing sensory neurons to any combination of missense oligonucleotides had no effect on the PGE$_2$-stimulated production of cAMP. These results suggest that simultaneous loss of the EP3C and EP4 receptor subtypes is necessary to prevent PGE$_2$ from increasing the formation of cAMP in sensory neurons.

Because it is possible that the inhibitory effect of the combination of antisense oligonucleotides could reflect a lack of specificity or toxicity (31), we examined whether cultures exposed to EP3C and EP4 antisense oligonucleotides were capable of producing cAMP by treating cells with forskolin, a direct activator of adenyl cyclase. In untreated neuronal cultures, a 20-min exposure to 1 $\mu$m forskolin in the presence of 2 mM IBMX increased the content of cAMP from 2.2 $\pm$ 0.3 pmol/well ($n = 20$) to 30.2 $\pm$ 1.9 pmol/well ($n = 20$; Fig. 3). In neuronal cultures pretreated for 48 h with antisense oligonucleotides to the EP3C and EP4 subtype mRNA or missenses, 1 $\mu$m forskolin increased the content of cAMP to 29.3 $\pm$ 3.3 pmol/well ($n = 17$) and to 35.1 $\pm$ 3.8 pmol/well ($n = 12$), respectively. Thus, treating cells with antisense or missense oligonucleotides did not
PGE₂-induced increases in icAMP in sensory neurons. 

hatched columns whereas the Keuls as the stimulated content of icAMP using analysis of variance with Neuman-Keuls test. Treating cells with 100 nM PGE₂ significantly increased cAMP production in sensory neurons. As in the above experiment, preexposure to either antisense or missense to EP3C and EP4 receptor subtypes mRNA as indicated. Asterisks represent a significant difference between basal and forskolin-stimulated levels of icAMP using analysis of variance with Neuman-Keuls as the post hoc test (p < 0.05).

block the ability of the sensory neurons to produce cAMP.

Since our antisense experiments suggested that activation of either the EP3C or EP4 receptor results in the enhanced production of cAMP, we addressed whether the potential activation of these receptors with the putative EP receptor agonists, butaprost, 1-OH-PGE₁, or sulprostone, would similarly enhance cAMP production in sensory neurons. As in the above experiments, treating cells with 100 nM PGE₂ significantly increased icAMP content 1.4-fold from 4.0 ± 0.2 pmol/well (n = 8) to 5.9 ± 0.6 (n = 8). In contrast, exposure to a 100 nM concentration of the putative EP1/EP3 agonist, sulprostone, or butaprost, 1-OH-PGE₁, had no effect on basal icAMP production; icAMP content was 2.7 ± 0.5 pmol/well (n = 8), 3.4 ± 0.2 pmol/well (n = 8), or 4.2 ± 0.4 pmol/well (n = 8), respectively. Exposure to a 100 nM concentration of the EP2 agonist, butaprost, did not increase icAMP production (4.8 ± 0.4 pmol/well, n = 8), whereas exposure to 1 μM butaprost significantly enhanced icAMP levels to 10.6 ± 1.0 pmol/well (n = 8).

Since micromolar concentrations of butaprost can bind to prostaglandin receptor subtypes other than EP2 (57), we addressed whether the increase in cAMP content stimulated by 1 μM butaprost was secondary to actions at the EP2, EP3, or EP4 receptor subtype. Pretreating neuronal cultures for 48 h with 1 μM antisense directed at the EP2 receptor or at the EP3C and EP4 receptors in combination significantly reduced the butaprost-induced increase in icAMP content. Exposure to antisense directed toward the EP2 receptor decreased the 1 μM butaprost-stimulated icAMP content from 9.2 ± 0.8 pmol/well (n = 11) in untreated cells to 5.2 ± 0.3 pmol/well (n = 8), and exposure to antisense toward the EP3C and EP4 receptors also reduced the icAMP content to 6.4 ± 1.1 pmol/well (n = 9). Although the butaprost-induced increase in cAMP content was reduced by either treatment, the agonist still significantly increased the levels of the second messenger above control. Exposing sensory neurons to missense oligonucleotides had no effect on the butaprost-stimulated icAMP.

Antisense Oligonucleotides Diminish the Expression of PGE₂ Receptor Subtypes EP2, EP3, and EP4—To substantiate that exposure to antisense oligonucleotides attenuated expression of EP2, EP3, and EP4 receptors, protein extracts from untreated cells or cells treated with antisense or missense were immunoblotted with antisera raised against specific EP receptor epitopes. Exposing sensory neurons to increasing concentrations of antisense for 48 h resulted in a concentration-dep- ient decrease in EP receptor expression with a maximal inhibition at 1 μM antisense (data not shown). After 48 h of treatment with 1 μM antisense, the optical density of immunoreactive EP2 bands in cells exposed to EP2 antisense was decreased by 71 ± 7% (n = 2), immunoreactive EP3 bands were decreased by 85 ± 9% (n = 2) in EP3C antisense-treated cells, and immunoreactive EP4 expression was decreased 69 ± 8% (n = 2) in cells treated with antisense to EP4 (Fig. 4A). In sensory neurons exposed to 1 μM antisense to the EP3C and EP4 receptor for 48 h, conditions that abolished PGE₂-induced icAMP production, the immunoreactivity of EP3 and EP4 receptor protein was significantly reduced compared with protein from untreated cultures of sensory neurons, whereas immunoreactivity of EP2 and IP receptors was not affected (Fig. 4B).
more, exposure to antisense to the EP2 receptor did not affect EP3C and EP4 receptor expression (data not shown). Immunoreactive EP3 bands were decreased by 83 ± 8% (n = 5), and immunoreactive EP4 expression was decreased 76 ± 6% (n = 5) in cells treated with antisense to EP3C and EP4 receptor simultaneously (Fig. 4B). In contrast, there is no significant reduction in receptor expression in neurons treated with missense oligonucleotides (Fig. 4B). To confirm that equal amounts of protein were loaded onto the gel, the expression of actin also was examined. As illustrated in Fig. 4, A and B, the relative expression of actin did not appear to be affected by exposure to antisense or missense oligonucleotides.

Identification of PGE2 Receptor Subtypes Mediating the PGE2-induced Augmentation of Peptide Release—Because EP3C and EP4 receptor subtypes are required for the PGE2-stimulated production of iCAMP, we sought to determine whether these receptor subtypes also mediated PGE2-induced stimulated production of iCAMP, we sought to determine whether these receptor subtypes also mediated PGE2-induced augmented release of neuropeptides. After pretreatment with medium alone (Con) or medium supplemented with 1 μM antisense (AS) or missense (MS) oligonucleotide to EP3C and EP4 receptors simultaneously. Total protein was isolated from sensory neurons grown in culture. 50 μg of the total protein that was extracted from the cultures was separated using a 12% acrylamide gel and transferred to nitrocellulose. After exposure to primary antibodies, immunoreactive bands for the EP2, the EP3, the EP4, and the IP receptor or for actin were detected.

DISCUSSION

Our results using RT-PCR and immunoblotting extend our understanding of which prostaglandin receptor subtypes are expressed in sensory neurons. Previous studies using in situ hybridization revealed mRNA to EP1, EP3, EP4, and IP receptors in dorsal root ganglia neurons (32, 33). Furthermore, the major distribution of PGE2 binding sites and of EP3-like immunoreactivity in the spinal cord is in lamina I and II of the dorsal horn (34, 35), and this coincides with the location of the terminal endings of small diameter sensory neurons that conduct pain signals (36). These studies, however, did not examine the localization of the EP2 receptor; nor were attempts made to define which splice variants of EP3 are found in sensory neurons. Our findings demonstrate that sensory neurons are capable of expressing EP1, EP2, EP3C, EP4, and IP receptors. We could not detect any signal for either EP3A or EP3B receptor mRNA using PCR. We did not examine EP3D receptor expression in sensory neurons, since this receptor subtype has not been cloned from mice or rats. Because we could detect bands for the EP3A and EP3B receptor when we use cDNA from rat kidney and the same PCR protocol, we are confident that the lack of detection of EP3A and EP3B mRNA in sensory neurons is not secondary to methodological problems. While the sizes of the EP1, EP2, EP3, and IP receptors in rat sensory neurons were identical to EP receptors from other species (28–30, 58, 59), the size of the EP4 receptor was similar to the porcine EP4 receptor (30) but not human EP4 receptor (59). This variation in size may reflect glycosylation of the receptor or perhaps peptides by 1.4-fold (Fig. 5). When sensory neurons were pre-exposed to antisense oligonucleotides directed toward the EP3C and EP4 receptor subtype mRNA, the PGE2-augmented release of neuropeptide was abolished. In these neurons, capsaicin-evoked release in the presence of PGE2 was 22.8 ± 1.4 fmol/well/10 min (n = 18) for iSP and 226.0 ± 27.1 fmol/well/10 min (n = 17) for iCGRP. Pretreating cells with missense oligonucleotides did not affect the ability of PGE2 to augment peptide release (Fig. 5). In addition, neither antisense nor missense treatment had any significant effect on capsaicin-stimulated release in the absence of PGE2 (Table I).

In an additional series of experiments, we examined whether the PGE2-augmented release could be blocked by inhibiting expression of either the EP3C or EP4 receptors independently. As can be seen in Table I, exposure to PGE2 in untreated cells did not alter basal release but significantly augmented the capsaicin-stimulated release of iSP and iCGRP by 1.5-fold. Unlike the simultaneous antisense exposure toward the EP3C and EP4 subtypes (Fig. 5), antisense treatment toward either the EP3C or the EP4 receptor subtype individually did not affect the ability of PGE2 to augment the capsaicin-evoked release of iSP and iCGRP (Table I).

Activating the production of cAMP with forskolin also augments evoked release of neuropeptides (18). Consequently, as a way of confirming that antisense inhibition of prostaglandin sensitization was not secondary to toxicity, we examined whether this action of forskolin was inhibited by antisense treatment. In untreated cells, exposure to 1 μM forskolin for 10 min prior to and throughout stimulation with capsaicin did not alter basal peptide release but significantly augmented the capsaicin-stimulated release of iSP and iCGRP by 1.6- and 1.7-fold, respectively (Fig. 6). In sensory neurons preexposed to antisense oligonucleotides directed toward the EP3C and EP4 receptor subtypes or to missense oligonucleotides, forskolin still significantly augmented the capsaicin-evoked release (Fig. 6). Thus, antisense treatment inhibits the augmented release of neuropeptides induced by PGE2 but does not affect the ability of forskolin to sensitize sensory neurons.
species variation between rat and human EP4 receptors.

Because multiple EP receptor subtypes are present in sensory neurons, it is important to define which subtypes mediate the biological actions of PGE₂. In the case of sensory neurons, exposure to PGE₂ does not directly activate the neurons but renders them more sensitive to external stimuli. This sensitization results in a reduced threshold for action potential firing (16), an increase in cell firing for a given stimulus (19, 37), and an increase in the amount of neurotransmitter release by a stimulating agent (20, 38). We previously have shown that the PGE₂-induced augmentation of peptide release is mediated by activation of the cAMP transduction cascade (18). In a similar manner, enhanced pain sensitivity (hyperalgesia) and the increase in firing of sensory neurons by PGE₂ also require an increase in cAMP (39–41). Thus, the EP receptor subtypes that potentially mediate sensitivity because they have been linked to an increase in cAMP are the EP2 (5, 6), EP3C (9, 11, 60), and/or EP4 (5). The ability of PGE₂ to increase cAMP levels and to augment peptide release is blocked by reducing expression of the EP3C and EP4 receptors using antisense directed at mRNA for these subtypes. In contrast, antisense to the EP2 receptor or the combination of antisense to the EP2 and EP4 receptor does not alter the effects of PGE₂. These results strongly suggest that the EP3C and EP4 receptors either cooperatively or inde-

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**FIG. 5.** Treatment with antisense oligonucleotide to EP3C and EP4 subtypes abolishes the PGE₂-augmented peptide release. As indicated, cells were exposed to medium alone or medium supplemented with 1 μM antisense or missense oligonucleotides to EP3C and EP4 receptors, simultaneously. The ordinate represents the mean ± S.E. of immunoreactive substance P (iSubstance P: top panel) or of immunoreactive CGRP (bottom panel) released in fmol/well/10 min of incubation. Open columns show release of neuropeptide when cells were exposed to HEPES buffer alone, and hatched columns show release when cells were exposed to HEPES buffer in the presence of 100 nM PGE₂. Shaded columns show release of neuropeptide from cells stimulated with 30 nM capsaicin (CAP) in the absence or presence of PGE₂ as indicated. An asterisk indicates a significant difference from basal release, and a cross indicates a significant difference from capsaicin-stimulated release using an analysis of variance with Neuman-Keuls as post hoc test (p < 0.05).

**TABLE I**

| Treatment                  | n  | iSP (fmol/well/10 min) | iCGRP (fmol/well/10 min) |
|----------------------------|----|-----------------------|--------------------------|
| No antisense               | 12 | 12 ± 1                | 31 ± 1                   |
| 1 μM antisense to EP3C     | 4  | 12 ± 2                | 31 ± 3                   |
| 1 μM antisense to EP3C     | 12 | 11 ± 1                | 43 ± 4                   |
| 1 μM antisense to EP4      | 11 | 13 ± 1                | 43 ± 2                   |
| 1 μM antisense to EP4      | 11 | 13 ± 1                | 43 ± 2                   |

*Statistically significant difference compared with capsaicin alone in the absence or presence of antisense oligonucleotides using analysis of variance with Neuman-Keuls as post hoc test (p < 0.05).
pendently mediate PGE₂-induced sensitization of sensory neurons.

We chose to use antisense to reduce expression of E-type receptors because of the lack of availability of selective antagonists to the receptor subtypes (1, 2). We selected exposure to 1 μM antisense oligonucleotide for a total of 48 h, with a change of medium and oligonucleotide at 24 h, since previous studies have demonstrated a significant loss of protein activity with this protocol (44–46) and our concentration-response results indicated that this protocol achieved significant inhibition of EP receptor expression. Based on our findings, we conclude that antisense oligonucleotides are useful to selectively reduce receptor expression for a number of reasons. First, using antibodies against the EP2, EP3, and EP4 receptors, we showed that antisense but not missense treatment significantly reduced protein expression. In addition, exposure to antisense does not affect the expression of the housekeeping protein, actin, in sensory neurons. Second, treatment with individual antisense or missense oligonucleotides or combinations of missense oligonucleotides did not alter the ability of PGE₂ to increase cAMP or augment peptide release, suggesting that the effects of reducing EP3C and EP4 expression are not mediated by a lack of specificity or toxicity of the oligonucleotides. Finally, although the combination of antisense to EP3C and EP4 receptors abolished the effects of PGE₂, this treatment did not block the forskolin-stimulated production of cAMP or the forskolin-induced augmentation of peptide release from sensory neurons, indicating that the cAMP-dependent sensitization of sensory neurons (18) was not affected.

Our findings of the primary role of the EP3C and EP4 receptor in sensitization do not agree with previous work that suggested that the EP2 and EP3A receptor are involved in PGE₂-induced enhanced excitability of sensory neurons (42, 43). One limitation of those studies, however, was that the concentrations of EP agonists used were 10–100-fold greater than the Ki of the drug for the receptor. Thus, the selectivity of the prostaglandin agonist for a specific EP receptor that was used to identify receptor subtypes involved in hypersensitivity is questionable. Furthermore, since the authors’ identification of the EP3A receptor was based on the pharmacological profile of EP receptor agonists (42, 43) rather than on the mRNA or immunological detection of the receptor and since the EP agonist used in that study can activate the EP3C and EP4 receptor subtypes (6, 11, 61), it is possible that these receptors and not the EP3A were being activated.

It is interesting that exposure to a 100 nM concentration of the putative EP1/EP3 agonist, sulprostone, or the EP2/EP4 agonist 1-OH-PGE₁ did not enhance cAMP content in sensory neurons. This lack of effect of sulprostone confirms previous work by Smith et al. (62) using adult sensory neurons. It is possible that higher concentrations of sulprostone or 1-OH-PGE₁ might increase cAMP content; however, the lack of specificity of these agents at higher concentrations could cloud interpretation of results.

FIG. 6. Treatment with antisense oligonucleotides to EP3C and EP4 subtypes does not affect the forskolin-augmented peptide release. As indicated, neurons were exposed to medium alone or medium supplemented with 1 μM antisense or missense oligonucleotides to EP3C and EP4 receptors simultaneously. The ordinate represents the mean ± S.E. of immunoreactive substance P (top panel) and immunoreactive CGRP (bottom panel) released in fmol/well/10 min of incubation. Open columns show release of neuropeptide when cells were exposed to HEPES buffer alone, and hatched columns show release when cells were exposed to HEPES buffer in the presence of 1 μM forskolin. Shaded columns show release of neuropeptide from cells stimulated with 30 nM capsaicin (CAP) in the absence or presence of forskolin, as indicated. An asterisk indicates a significant difference from basal release, and a cross indicates a significant difference from capsaicin-stimulated release using an analysis of variance with Neuman-Keuls as post hoc test (p < 0.05).
In contrast, 1 μM butaprost did increase cAMP, suggesting that the EP2 receptor might mediate the sensitizing actions of PGE₂, and this is inconsistent with the results from antisense studies. The findings with butaprost could be explained by a lack of specificity of the agent. Although antisense to EP2 reduces the effects of butaprost on cAMP, it does not completely block the action. Furthermore, antisense to both the EP3C and the EP4 receptors also significantly reduces the effects of butaprost, although these antisenses do not reduce EP2 expression. Thus, the effect elicited by 1 μM butaprost is not restricted to the EP2 receptor and may be mediated by activation of other prostanoid receptors. In addition, butaprost at micromolar concentrations binds to the mouse IP receptor (57), and IP receptor agonists increase cAMP content in sensory neurons (18, 62).

Since IP receptor expression was not affected by antisense treatment to EP3C and EP4, there are sufficient IP receptors available to potentially mediate the actions of butaprost. It also suggests that the EP2 receptor is not critical to the actions of PGE₂, whereas EP2 receptors appear to have little or no functional role in PGE₂-induced cAMP production (52). An analogous situation might exist in sensory neurons. It would be interesting to determine whether the EP3C and EP4 receptor subtypes are equally expressed in cultures of sensory neurons and whether the expression of EP1 and EP2 in sensory neurons is of a similar magnitude.

Further studies are warranted to ascertain whether EP receptor subtypes are differentially expressed in the same neuron or exist in separate subpopulations of sensory neurons. However, if multiple receptors mediate sensitization of sensory neurons in situ, then attempts to inhibit a single receptor to block the inflammatory actions of PGE₂ might be ineffective. Furthermore, although transgenic mice with knockouts of single EP receptor subtype have been developed (53–56), the redundancy of EP receptor functions, which we have reported in the present study, could limit the value of these single receptor transgenic models of nociceptive transmission. Future studies using double knockout mice might be more appropriate in studying the mechanisms of pain and inflammation.

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