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Prostaglandin E_2 inhibits calcium current in two sub-populations of acutely isolated mouse trigeminal sensory neurons

Stephanie L. Borgland, Mark Connor, Renae M. Ryan, Helen J. Ball * and MacDonald J. Christie †

*Department of Pharmacology, * Department of Pathology and † The Medical Foundation, University of Sydney, NSW, 2006, Australia*

Prostaglandins are important mediators of pain and inflammation. We have examined the effects of prostanoids on voltage-activated calcium currents (I_{Ca}) in acutely isolated mouse trigeminal sensory neurons, using standard whole cell voltage clamp techniques. Trigeminal neurons were divided into two populations based on the presence (Type 2) or absence (Type 1) of low voltage-activated T-type I_{Ca}. The absence of T-type I_{Ca} is highly correlated with sensitivity to μ-opioid agonists and the VR1 agonist capsaicin. In both populations of cells, high voltage-activated I_{Ca} was inhibited by PGE_2 with an EC_{50} of about 35 nM, to a maximum of 30 %. T-type I_{Ca} was not inhibited by PGE_2. Pertussis toxin pre-treatment abolished the effects of PGE_2 in Type 2 cells, but not in Type 1 cells, whereas treatment with cholera toxin prevented the effects of PGE_2 in Type 1 cells, but not in Type 2 cells. Inhibition of I_{Ca} by PGE_2 was associated with slowing of current activation and could be relieved with a large positive pre-pulse, consistent with inhibition of I_{Ca} by G protein $\beta\gamma$ subunits. Reverse transcription–polymerase chain reaction of mRNA from trigeminal ganglia indicated that all four EP prostanoid receptors were present. However, in both Type 1 and Type 2 cells the effects of PGE_2 were only mimicked by the selective EP_3 receptor agonist ONO-AE-248, and not by selective agonists for EP_1 (ONO-DI-004), EP_2 (ONO-AE1-259) and EP_4 (ONO-AE1-329) receptors. These data indicate that two populations of neurons in trigeminal ganglia differing in their calcium channel expression, sensitivity to μ-opioids and capsaicin also have divergent mechanisms of PGE_2-mediated inhibition of calcium channels, with Gi/Go type G proteins involved in one population, and Gs type G proteins in the other.

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Corresponding author M. Connor: Department of Pharmacology, D06, University of Sydney, Sydney, NSW 2006 Australia.
Email: markc@pharmacol.usyd.edu.au

The pain produced in response to many forms of noxious stimulation can be facilitated by prostaglandins (Ferreira, 1972). Prostanoids, including prostaglandin E_2 (PGE_2; Willis, 1969; Greaves *et al.* 1971), are generated in response to injury or inflammation and can increase the excitability of sensory nerves (Handwerker, 1976; Chahl & Iggo, 1977; Mense, 1981) *in vivo* and *in vitro* (Baccaglini & Hogan, 1983; Pitchford & Levine, 1991). The heightened responsiveness to other noxious stimuli produced by PGE_2 is known as sensitization and is reflected at a cellular level by modulation of a number of different ion channels (see below) and the facilitation of neuropeptide release (Nicol *et al.* 1992; Vasko *et al.* 1994; Cui & Nicol, 1995). In sensory neurons from various species, PGE_2 shifts the activation of tetrodotoxin-resistant sodium currents (TTX-R I_{Na}) to more negative potentials (Gold *et al.* 1996; England *et al.* 1996), potentiates capsaicin-induced currents (Pitchford & Levine, 1991; Lopshire & Nicol, 1998) and increases the activation of the hyperpolarization-activated cation current (I_h; Ingram & Williams, 1994, 1996), all of which will increase the excitability of sensory neurons. PGE_2 also attenuates voltage-dependent (Nicol *et al.* 1997) and calcium-activated potassium currents (Fowler *et al.* 1985). The effects of PGE_2 on sensory neuron excitability (Ferreira & Nakamura, 1979) and neuropeptide release (Cui & Nicol, 1995; Hingtgen *et al.* 1995) have largely been attributed to elevation of cAMP. Cyclic AMP acts both directly (Ingram & Williams, 1994, 1996) and *via* protein kinase A to mediate its cellular sensitizing effects (Pitchford & Levine, 1991; England *et al.* 1996; Lopshire & Nicol, 1998; Gold *et al.* 1998; Evans *et al.* 1999). These effects are consistent with the predominant coupling of prostanoid receptors to Gs proteins (Narumiya *et al.* 1999).

High voltage-activated I_{Ca} (HVA I_{Ca}) and low voltage-activated T-type I_{Ca} contribute to the excitability of sensory neurons (e.g. Yoshida *et al.* 1978; White *et al.* 1989; Abdulla & Smith, 1997) and HVA I_{Ca} contributes directly to action potential-dependent neurotransmitter release from primary afferent terminals. The inhibition of sensory neuron voltage-dependent calcium channels at the

primary afferent synapse is thought to be important in mediating the spinal analgesic effects of opioid agonists (Hori *et al.* 1992) and invertebrate neurotoxin analgesics (Xiao & Bennett, 1995). Consistent with its role as a pronociceptive mediator, PGE_2 has been reported to increase HVA I_{Ca} in embryonic avian sensory neurons (Nicol *et al.* 1992), although the mechanism was not determined. In contrast, in rat sympathetic neurons (Ikeda 1992; Ito *et al.* 2000), bovine adrenal chromaffin cells (Currie & Fox, 2000) and rat melanotrophs (Tanaka *et al.* 1998), PGE_2 inhibits calcium channels. In the light of these contrasting findings, we examined the effects of PGE_2 on HVA I_{Ca} in mammalian sensory neurons. We found that PGE_2 inhibited HVA I_{Ca} in almost all acutely isolated mouse trigeminal sensory neurons, and that PGE_2 receptors are differentially coupled to Gi/o- and Gs-type G proteins in separate pharmacologically and electrophysiologically defined populations of sensory neurons.

METHODS

Isolation of trigeminal ganglion neurons

129SvEv/C57BL/6 mice (4–6 weeks old) of either sex were used for this study. All procedures were approved by the Animal Ethics Committee of the University of Sydney, Australia. Mice were anaesthetized with halothane (4%) and decapitated, and the trigeminal ganglia were removed and placed in cold (4°C) physiological saline containing (mM): NaCl 126; KCl 2.5; $CaCl_2$ 2.5; $MgCl_2$ 1.2; NaH_2PO_4 1.2; $NaHCO_3$ 24; and glucose 10, gassed with 95% O_2–5% CO_2. Using a simplified version of the methods outlined in Eckert *et al.* (1997), ganglia were cut up with iridectomy scissors and incubated at 32–34°C for 30 min in physiological saline. The ganglia pieces were then transferred to oxygenated Hepes buffered saline (HBS) containing 20 units ml^{-1} papain and incubated at 32–34°C for 15 min. In a few experiments the ganglia were incubated with 3 mg ml^{-1} collagenase Type IV (Worthington) before incubation with papain. This procedure reduced the amount of trituration needed to release the cells. HBS contained (mM): NaCl 154; KCl 2.5; $CaCl_2$ 2.5; $MgCl_2$ 1.5; Hepes 10; glucose 10; pH 7.2 (NaOH), 330 ± 5 mosmol l^{-1}. The papain digestion was terminated with addition of HBS containing 1 mg ml^{-1} bovine serum albumin (BSA) and 1 mg ml^{-1} trypsin inhibitor. Minced ganglia were washed free of enzyme and enzyme inhibitors with room temperature HBS. Cells were released by gentle trituration through decreasing bore, silanized Pasteur pipettes with fire-polished tips. The cells were plated onto plastic culture dishes and kept at room temperature in HBS. Cells remained viable for up to 10 h after dissociation and could be cultured overnight.

Pertussis toxin and cholera toxin treatments

Trigeminal ganglion cells were isolated as above. Neurons in HBS were left to attach to plastic culture dishes before changing medium to L15 Lebovitz (Sigma) containing 5% fetal bovine serum (Gibco) and penicillin–streptomycin (50 u, 5 μg ml^{-1}), and incubated at 25°C in a humidified incubator. Pertussis toxin (PTX; 100 ng ml^{-1}) or cholera toxin (CTX; 250 ng ml^{-1}) was added to the culture medium prior to overnight incubation.

Electrophysiological recordings

Ionic currents from mouse trigeminal neurons were recorded in the whole-cell configuration of the patch-clamp method (Hamill *et al.* 1981) at room temperature (22–24°C). Dishes were continually perfused with HBS. For isolating calcium currents, an extracellular solution was used containing (mM): TEACl 140; CsCl 2.5; $CaCl_2$ 2.5; Hepes 10; $MgCl_2$ 1; glucose 10; pH 7.2 (CsOH), 330 ± 5 mosmol l^{-1}. The intracellular solution contained (mM): CsCl 130; Hepes 10; EGTA 10; $CaCl_2$ 2; MgATP 5; NaGTP 0.2; NaCl 5; pH 7.3 (CsOH), 285 ± 3 mosmol l^{-1}.

Recordings were made using an EPC-9 patch-clamp amplifier and corresponding Pulse software from Heka Electroniks (Lambrecht, Germany) or an Axopatch-1D amplifier (Axon Instruments, Union City, CA) using pCLAMP acquisition software (Axon Instruments). Currents were sampled at 20–50 kHz and recorded on hard disk for later analysis. The recordings were filtered at 2.83 kHz (Heka) or 2 kHz (Axopatch). Patch pipettes were pulled from borosilicate glass (AM Systems, Everett, WA, USA). The pipette input resistance ranged between 0.7 and 1.5 MΩ. The capacitance of individual cells ranged between 4 and 40 pF with a series resistance between 1 and 5 MΩ. A series resistance compensation of at least 80% was used in all experiments. Capacitance transients were compensated automatically using a built-in procedure of the Heka amplifier or by using the manual compensation on the Axopatch-1D. Leak current was subtracted on line using a *P*/8 protocol by the Pulse software (Heka Electroniks) unless otherwise noted (see Fig. 1*A*). The average current settling time (from 12 randomly selected cells) was 0.6 ± 0.05 ms. A liquid junction potential of approximately −8 mV has not been corrected for.

Peak HVA I_{Ca} in each cell was determined by stepping the membrane potential from a holding potential of −80 mV to between −60 and +60 mV, for 30 ms, in 10 mV increments. Following this procedure the test current was evoked every 30s and monitored for current stability before drugs were applied. Cells were rejected if the current increased or decreased by more than 2% in the first 90 s after running the *I–V* protocol. Cells were exposed to drugs via a series of flow pipes positioned above the cells. The inhibition by drugs was quantified by measuring the current isochronically from the peak of the control current in the presence and absence of the drug.

RNA isolation and reverse transcription–polymerase chain reaction

Total RNA was isolated from whole trigeminal ganglia of mice using the RNeasy kit (Qiagen, Germany). Total RNA was treated with RQ1 RNase-free DNase (Promega, Madison, WI, USA) at 37°C for 30 min to remove any contaminating genomic DNA. The enzyme was removed by a phenol–chloroform extraction. Optical density readings were performed to estimate the amount of total RNA before it was used in reverse transcription–polymerase chain reaction (RT-PCR).

A total of 1 μg of RNA and 1 μg of oligodT primers (Promega) were incubated at 70°C for 5 min, and then cooled on ice. The RNA was reverse transcribed at 37°C in a 15 μl reaction mixture containing (mM): Tris-HCl 50 (pH 8.5); KCl 75; $MgCl_2$ 3; dithiothreitol 10; and 400 μM each of deoxyadenosinetriphosphate (dATP), deoxythymidinetriphosphate (dTTP), deoxycytidinetriphosphate (dCTP), and deoxyguanosinetriphosphate (dGTP); 20 U of SUPERase•In RNase inhibitor (Ambion Inc., Austin, TX, USA) and 200 U of M-MLV reverse transcriptase (Promega). The RNA from each animal was incubated both with and without M-MLV reverse transcriptase to confirm the absence of contaminating genomic DNA.

PCR was performed in a 20 μl reaction volume containing (mM): Tris-HCl 10 (pH 9); KCl 50; $MgCl_2$ 1.5; 0.1 % Triton X-100; and 200 μM each of dATP, dTTP, dCTP and dGTP; 500 μM of each primer; 1 U of Taq DNA polymerase (Promega) and 5 μl of the appropriate RT product. Amplifications were performed in a Perkin-Elmer 2400 thermal cycler with an initial denaturation step at 94 °C for 1 min followed by 35 cycles. Each cycle consisted of a denaturation step at 94 °C for 30 s, an annealing step at 55 °C for 30 s, and an elongation step at 72 °C for 30 s. This was followed by a final elongation step at 72 °C for 5 min. The PCR products were separated on a 2 % agarose gel stained with ethidium bromide.

Data analysis

All data are expressed as means ± S.E.M. unless otherwise indicated. Concentration response data were pooled for each group and fitted to the Hill equation using the software package GraphPad Prism v. 3. Where noted, significant differences between means were tested, using Student's paired or unpaired, two tailed *t* test.

Drugs and chemicals

PGE_2 was from Cayman Chemical Co. (Ann Arbor, MI, USA). ONO-DI-004, ONO-AE1-329, ONO-AE-248 and ONO-AE1-259 were kindly supplied by Ono Pharmaceuticals Co. Ltd (Osaka, Japan). Baclofen was from Research Biochemicals International (Natick, MA, USA). Buffer salts were from BDH Australia or Sigma Australia. Papain and collagenase were from Worthington Biochemical Corp. (Freehold, NJ, USA). BSA and trypsin inhibitor (chicken egg white ovomucoid, Type II-O), pertussis toxin and cholera toxin were from Sigma Australia. Methionine-enkephalin was from Auspep (Melbourne, Australia).

PGE_2 was dissolved in ethanol and stored at −20 °C. More dilute aqueous solutions were made daily and kept on ice; ethanol alone (up to 0.01 %) did not affect I_{Ca}. Other compounds were dissolved in dimethylsulfoxide (DMSO); DMSO alone (up to 0.01 %) did not affect I_{Ca}. At higher concentrations (up to 0.1 %) of DMSO, the osmotic effects of the solvent were minimized by diluting the recording buffer before addition of the drugs (ONO-AE-248 and ONO-AE1-259) or the DMSO alone. For these higher concentrations of DMSO, the control was always defined as the current in the appropriate concentration of DMSO.

RESULTS

Acute application of prostaglandin E_2 reversibly inhibited HVA I_{Ca} in almost all trigeminal neurons. We have previously characterized two populations of trigeminal sensory neurons based on sensitivity to opioid receptor agonists and capsaicin (Type 1 neurons) or the presence of a prominent T-type calcium current and insensitivity to opioid agonists and capsaicin (Type 2 cells, Borgland *et al.* 2001). In Type 1 neurons PGE_2 inhibited HVA I_{Ca} in 234 of 262 cells examined, with an EC_{50} of 41 nM ($logEC_{50}$ −7.4 ± 0.1) to a maximum of 28 ± 2 % (Fig. 1*C*). In Type 2 neurons PGE_2 inhibited HVA I_{Ca} in 203 of 218 cells examined, with an EC_{50} of 34 nM ($logEC_{50}$ −7.5 ± 0.1) and inhibited HVA I_{Ca} to a maximum of 32 ± 1 % (Fig. 2*C*). In Type 2 neurons that were sensitive to PGE_2, the T-type I_{Ca} itself was not inhibited (Fig. 2*A*; $n = 10$).

When current–voltage (*I–V*) relationships for I_{Ca} were determined in trigeminal neurons, the peak amplitude I_{Ca} of cells with (Fig. 3*B*) or without (Fig. 3*A*) T-type I_{Ca} occurred at either 0 or +10 mV (except in 6 cells with T-type I_{Ca} where the peak was at −40 mV). Inhibition of I_{Ca} by PGE_2 occurred over a range of potentials in both types of cell. The inhibition of I_{Ca} was not due indirectly to a PGE_2-induced increase in an outward current, because PGE_2 did not modulate the current evoked in the presence of 30 μM Cd^{2+} when cells were stepped from −80 mV to either 0 mV (Fig. 3*C*, $n = 9$; 3*D*, $n = 8$) or +60 mV ($n = 13$, data not shown).

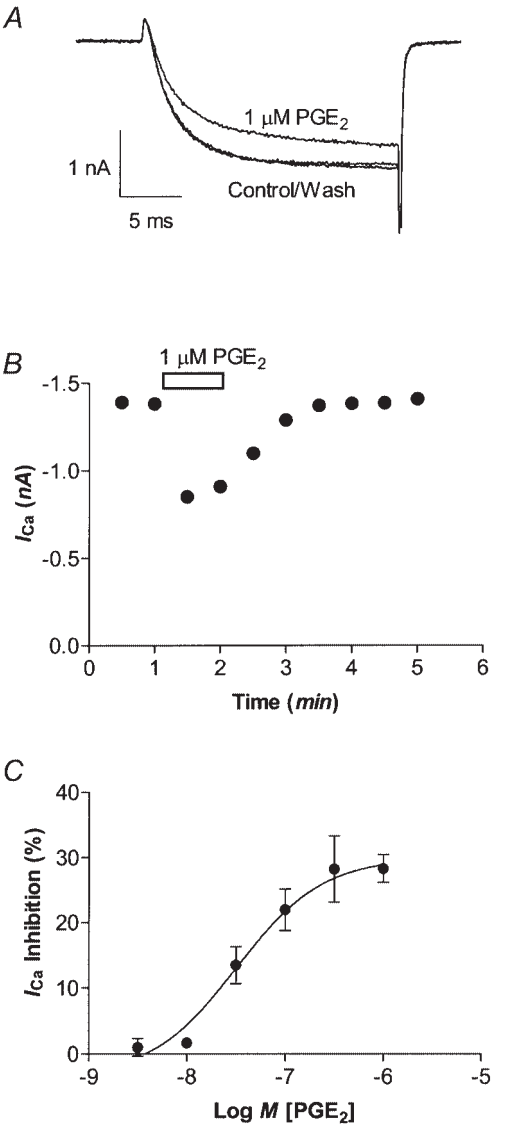

Figure 1. PGE_2 inhibits HVA I_{Ca} in Type 1 mouse trigeminal neurons

A, HVA I_{Ca} elicited by stepping the membrane potential from −80 to 0 mV was inhibited by 1 μM PGE_2 in a Type 1 neuron. *B*, a representative time plot illustrating that inhibition of HVA I_{Ca} by 1 μM PGE_2 completely reversed upon washout of the drug. *C*, Type-1 neurons were superfused with various concentrations of PGE_2 for 1 min and then washed until the current returned to pre-drug treatment amplitude. Each point represents the mean and S.E.M. of 6–12 cells.

PGE_2 inhibits I_{Ca} in two subpopulations of trigeminal sensory neurons by distinct G protein mechanisms

To examine the possible role of G proteins in the PGE_2 inhibition of I_{Ca}, trigeminal neurons were treated overnight with either pertussis toxin (PTX, 100 ng ml^{-1}), an irreversible inhibitor of Gi/o proteins, or cholera toxin (CTX, 250 ng ml^{-1}), an activator of Gs proteins which uncouples Gs α-subunits from their receptors (Fig. 4). PTX treatment significantly attenuated the inhibition of I_{Ca} caused by 1 μM PGE_2 in Type 2 cells (Fig. 4*A*, $P < 0.001$, $n = 8$), but not in Type 1 cells (Fig. 4*A*, $n = 9$). In contrast, CTX did not affect PGE_2-mediated inhibition of I_{Ca} in Type 2 cells ($n = 10$), but it did abolish PGE_2 inhibition of I_{Ca} in Type 1 cells (Fig. 4*A*, $n = 10$). CTX treatment alone did not alter the activation kinetics of the peak I_{Ca} (0–50 % rise times for I_{Ca}: Type 1 cells, 1.6 ± 0.2 ms *vs.* 1.6 ± 0.2 ms after CTX; Type 2 cells, 1.3 ± 0.1 ms *vs.* 1.3 ± 0.1 ms after CTX). In Type 1 cells, PTX treatment abolished inhibition of I_{Ca} by methionine-enkephalin (ME) (Fig. 4*B*, $n = 6–8$ each, and in Type 2 cells PTX treatment abolished the effects of baclofen (30 μM; Fig. 4*C*, $n = 6–8$ each). CTX treatment had no effect on the responses to ME in Type 1 cells or to baclofen in Type 2 cells. (Fig. 4*C*, $n = 6–10$ cells for each).

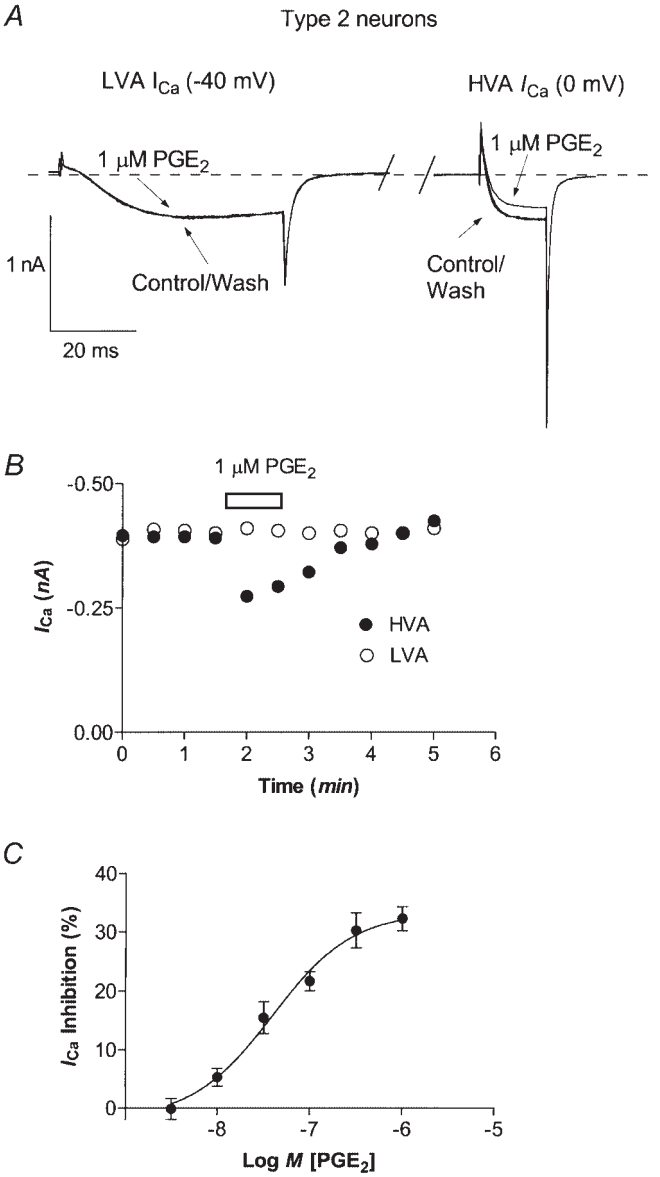

Figure 2. Calcium currents in Type 2 trigeminal neurons are inhibited by PGE_2

A, to elicit the low voltage-activated T-type I_{Ca}, neurons were stepped from −80 mV to −40 mV. Neurons recovered for 80 ms (approximately 65 ms has been removed for clarity) before stepping the membrane potential to 0 mV to elicit HVA I_{Ca}. T-type I_{Ca} was not inhibited by 1 μM PGE_2. This trace was not leak subtracted and zero current is denoted by the dashed line. *B*, time course of inhibition of HVA I_{Ca} by 1 μM PGE_2 in the same cell. *C*, Type 2 neurons were superfused with various concentrations of PGE_2 for 1 min and then washed until the current returned to pre-drug treatment amplitude. Each point represents the mean and S.E.M. of 6–12 cells.

The inhibition of I_{Ca} by PGE_2 was associated with a significant slowing of the rise time of the evoked current, consistent with direct inhibition of I_{Ca} by G protein $\beta\gamma$ subunits (see Table 1). To address the involvement of G protein $\beta\gamma$ in the PGE_2-mediated inhibition of I_{Ca}, a depolarizing conditioning step designed to reduce affinity of G protein $\beta\gamma$ subunits for the calcium channel was used to test the voltage dependence of inhibition. In these experiments we required different voltage protocols for Type 1 and Type 2 cells to achieve relief of PGE_2-mediated I_{Ca} inhibition. For both cell types I_{Ca} was evoked by two test steps to 0 mV, separated by a conditioning step applied 100 ms after the first test step (S1). A conditioning step to +100 mV for 50 ms was applied to Type 2 neurons and a step to +130 mV for 70 ms was applied to Type 1 neurons (Fig. 5). In Type 2 cells, PGE_2 (1 μM) inhibition of I_{Ca} after the conditioning step was reduced by 50 ± 4 % compared to S1 ($P < 0.01$). After the conditioning step, the slowing of the 0–50 % rise time of I_{Ca} (S2) in the presence of PGE_2 was significantly less than the slowing of the 0–50 % rise time of S1 ($P < 0.01$, Table 1). When Type 1 cells were subjected to the same conditioning step as Type 2 cells, the PGE_2 (1 μM) inhibition of I_{Ca} elicited by the second test step (S2) was only reduced by 7 ± 3 % compared to S1, and there was no change in the rise time of S2 when compared with S1 (0–50 % rise time: S1, 1.04 ± 0.06 ms in control *vs.* 1.17 ± 0.07 ms in PGE_2; S2, 1.05 ± 0.07 ms in control *vs.* 1.15 ± 0.07 ms in PGE_2; $n = 6$). However, when the depolarizing conditioning step applied to Type 1 cells was increased to +130 mV and lengthened to 70 ms, PGE_2 inhibition of I_{Ca} elicited by S2 was reduced by 39 ± 6 % compared to S1 (Table 1). The slowing of the 0–50 % rise time of I_{Ca} by PGE_2, apparent in S1, was no longer apparent following the conditioning step (Table 1). Facilitation of S2 by the depolarizing conditioning step was also observed with baclofen, an agonist that activates Gi/o-coupled

Table 1. Effects of a depolarizing conditioning step on PGE$_2$ modulation of HVA I_{Ca} in mouse trigeminal neurons

	n	Control	1 μM PGE$_2$	*n*	Control	30 μM Baclofen
Type 1						
Ratio S2:S1 peak amplitude	7	1.01 ± 0.07	1.19 ± 0.09*	9	1.00 ± 0.05	1.18 ± 0.07***
0–50 % rise time of S1 (ms)	7	0.95 ± 0.07	1.11 ± 0.05*	9	1.18 ± 0.07	1.29 ± 0.10*
0–50 % rise time of S2 (ms)	7	1.02 ± 0.08	1.05 ± 0.08	9	1.11 ± 0.05	1.14 ± 0.06
Type 2						
Ratio S2:S1 peak amplitude	8	1.07 ± 0.07	1.36 ± 0.09***			
0–50 % rise time of S1 (ms)	8	1.69 ± 0.08	2.35 ± 0.17*			
0–50 % rise time of S2 (ms)	8	1.60 ± 0.15	1.86 ± 0.18†			

Values are means and S.E.M. The S1:S2 ratio and 0–50 % rise times were significantly greater than controls on indicated values (Student's paired, two tailed *t* test) *$P < 0.05$, ***$P < 0.001$. † Significantly different from S1 in PGE$_2$, Student's unpaired *t* test, $P < 0.02$.

GABA$_B$ receptors (Table 1). Baclofen (30 μM) significantly decreased the ratio of S1:S2 (reduced inhibition in the second test pulse) and significantly slowed 0–50 % rise times of S1 in Type 1 cells (Table 1).

The effects of prostaglandin are mediated by EP$_3$ receptors

PGE$_2$ acts predominantly on prostanoid receptors of the EP type. The expression of the four cloned prostaglandin EP receptors in the mouse trigeminal ganglion was

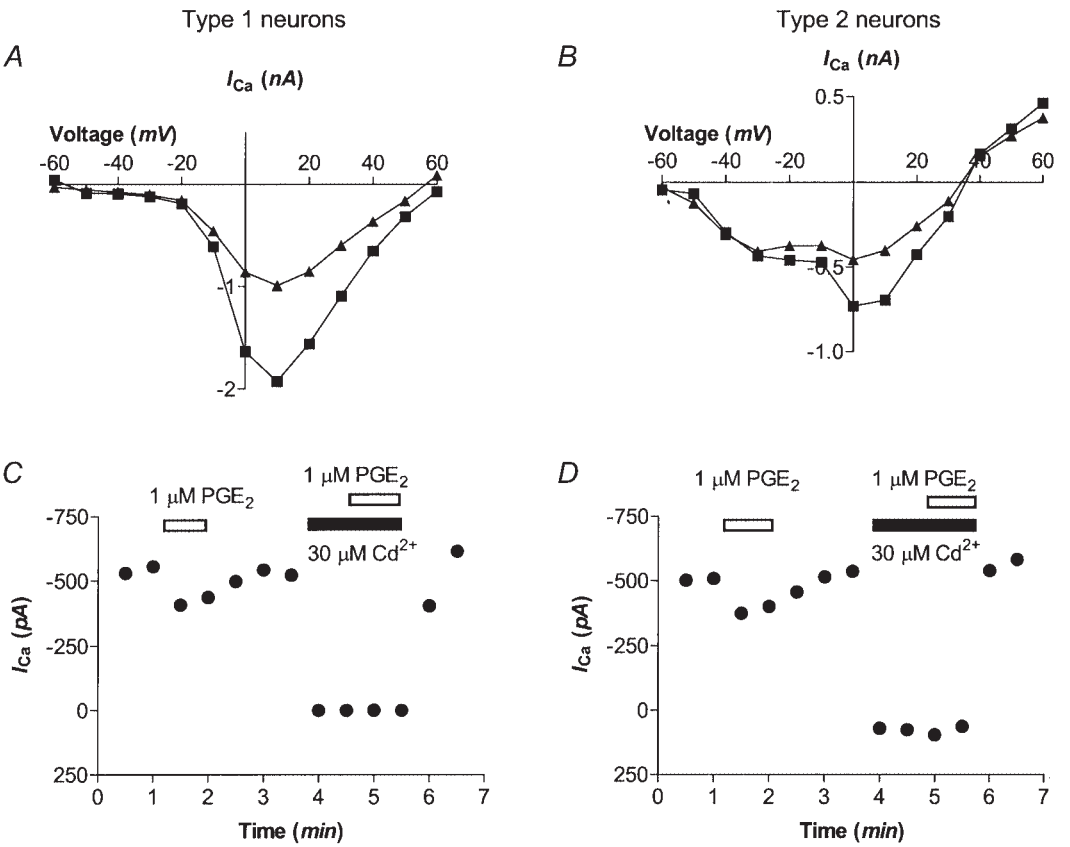

Figure 3. Characteristics of PGE$_2$ modulation of HVA I_{Ca}

A and *B*, HVA I_{Ca} was elicited by stepping the membrane potential from −80 mV to potentials between –60 mV and +60 mV in 10 mV increments (Control, black squares). PGE$_2$ (1 μM; triangles) inhibited I_{Ca} over a range of membrane potentials. *A*, a representative example of a current–voltage relationship of a Type 1 cell. *B*, current–voltage relationship for a Type 2 cell. In Type 1 cells (*C*) and Type 2 cells (*D*) 1 μM PGE$_2$ did not alter Cd^{2+}-insensitive current when HVA I_{Ca} was elicited by stepping the cells from the holding potential to 0 mV.

examined using RT-PCR. Messenger RNA was isolated from the trigeminal ganglion of eight male and five female animals, and PCR was performed using the primers described in Table 2. The primers selectively amplified mRNA fragments of the expected size (Fig. 6, Table 2), and the mRNA for all four prostaglandin EP receptors was found in all animals tested (Fig. 6).

To determine which EP receptor PGE_2 was acting at to inhibit I_{Ca}, we examined the effects of selective EP receptor agonists on I_{Ca} in Type 1 and Type 2 cells. The effects of

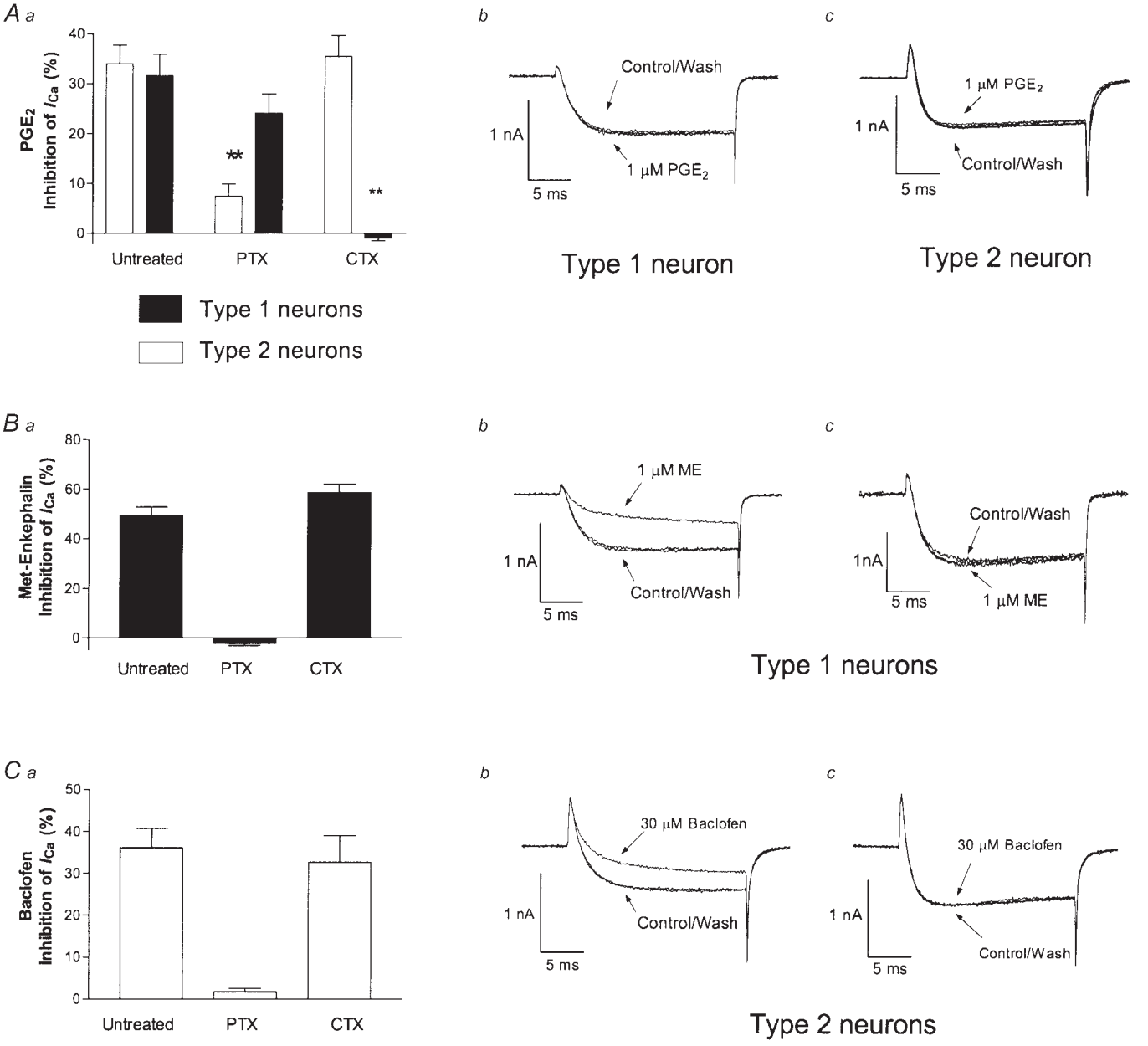

Figure 4. Inhibition of HVA I_{Ca} by PGE_2 is mediated via multiple G proteins

Neurons were treated overnight with either 100 ng ml^{-1} PTX or 250 ng ml^{-1} CTX. *Aa*, CTX selectively abolished PGE_2-mediated inhibition of HVA I_{Ca} in Type 1 neurons, while PTX treatment selectively reduced effects of PGE_2 in Type 2 neurons. Type 2 neurons pre-treated with PTX had significantly less inhibition of HVA I_{Ca} than controls ($P < 0.001$). *Ab*, a representative trace of a CTX treated Type 1 neuron in the presence and absence of 1 μM PGE_2. *Ac*, a representative trace of a PTX treated Type 2 neuron in the presence and absence of 1 μM PGE_2. *B*, in Type 1 neurons, inhibition of I_{Ca} caused by 1 μM ME was abolished by PTX but not CTX. Representative traces of a CTX treated neuron (*Bb*) and a PTX treated neuron (*Bc*) in the presence and absence of 1 μM ME. *C*, I_{Ca} inhibition caused by 30 μM baclofen was abolished by PTX, but not CTX in Type 2 cells. Representative traces of a CTX treated Type 2 neuron (*Cb*) and a PTX treated Type 2 neuron (*Cc*) in the presence and absence of 30 μM baclofen. Records illustrated are superimposed currents recorded before, during and after drug application.

PGE_2 were mimicked by the selective EP_3 receptor agonist ONO-AE-248 (Fig. 7), but not by selective agonists for EP_1 receptors (ONO-DI-004, 100 nM-3 μM), EP_2 receptors (ONO-AE1-259, 300 nM-10 μM) or EP_4 receptors (ONO-AE1-329, 100 nM-3 μM). At the highest concentrations tested the EP_1, EP_2 and EP_4 receptor agonists did not affect I_{Ca} in any cell ($n = 7$–9 for each of Type 1 and Type 2 cells, data not shown).

In Type 1 neurons ONO-AE-248 inhibited I_{Ca} in 25 of 36 cells, with an EC_{50} of 1.4 μM ($\log EC_{50}$ -5.9 ± 0.1) to a maximum of 28 ± 2 % (Fig. 7C). In Type 2 cells ONO-AE-248 inhibited I_{Ca} in all 40 cells tested, with an EC_{50} of 580 nM ($\log EC_{50}$ -6.2 ± 0.1) to a maximum of 30 ± 2 % (Fig. 7C). The inhibition of I_{Ca} by ONO-AE-248 was also associated with significant slowing of the activation kinetics of I_{Ca} in both Type 1 and Type 2 cells. In Type 1 cells, 30 μM ONO-AE-248 resulted in a slowing of the 0–50 % rise time of I_{Ca} from 1.21 ± 0.15 ms to 1.38 ± 0.18 ms ($n = 10$, $P < 0.02$ paired t test). In Type 2 cells 30 μM ONO-248 slowed the 0–50 % rise time from 1.2 ± 0.1 ms to 1.55 ± 0.15 ms ($n = 9$, $P < 0.02$, paired t test). The inhibition of I_{Ca} by ONO-AE-248 was blocked by overnight CTX treatment in Type 1 but not Type 2 cells, whereas PTX pre-treatment blocked the effects of ONO-AE-248 in Type 2 cells but not in Type 1 cells (Fig. 7*D*). These data indicate that ONO-AE-248 inhibits I_{Ca} in a manner similar to that of PGE_2.

PGE_2 inhibits predominantly N- and P/Q-type I_{Ca}

Type 1 and Type 2 trigeminal sensory neurons both express predominantly N- and P/Q-type HVA I_{Ca}, with a lesser amount of R-type current, while Type 1 cells also have some L-type I_{Ca} (Borgland *et al.* 2001). We examined the inhibition of the different types of I_{Ca} in Type 1 and Type 2 sensory neurons by applying PGE_2 in the presence of combinations of calcium channel antagonists. A predominantly N-type I_{Ca} was isolated by using a combination of the P/Q-type I_{Ca} antagonist ω-agatoxin IVA (500 nM) and the L-type I_{Ca} antagonist nimodipine (3 μM). When applied in the presence of these antagonists, PGE_2 (1 μM) inhibited N-type I_{Ca} by 21 ± 3 % ($n = 5$) in Type 1 neurons and 30 ± 4 % ($n = 9$) in Type 2 neurons. A predominantly P/Q-type I_{Ca} was isolated using the N-type I_{Ca} antagonist ω-conotoxin GVIA (1 μM) and nimodipine (3 μM). In the presence of these antagonists PGE_2 (1 μM), inhibited P/Q-type I_{Ca} by 19 ± 3 % ($n = 6$) in Type 1 cells and 15 ± 1 % ($n = 6$) in Type 2 neurons. The residual L-type and R-type I_{Ca} recorded in the presence of ω-agatoxin IVA (500 nM) and ω-conotoxin GVIA (1 μM) were not inhibited by PGE_2 (1 μM) in either Type 1 or Type 2 neurons ($n = 9$).

DISCUSSION

In adult mouse trigeminal sensory neurons, PGE_2 inhibited HVA I_{Ca} in almost all of the cells tested, but did so via two different G protein coupling mechanisms in electrophysiologically and pharmacologically distinct subpopulations of cells. Prostaglandins have been shown to inhibit I_{Ca} in rat sympathetic neurons (Ikeda, 1992; Ito *et al.* 2000), melanotrophs (Tanaka *et al.* 1998) and bovine adrenal chromaffin cells (Currie & Fox, 2000), but the only

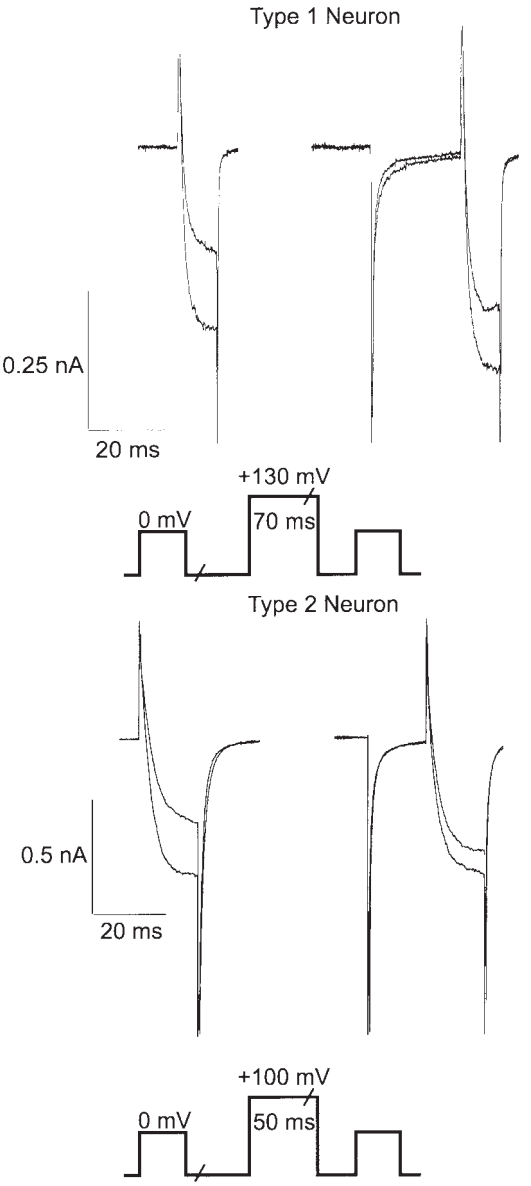

Figure 5. PGE_2 inhibition of HVA I_{Ca} in sensory neurons is relieved by a positive prepulse

Different prepulse protocols were used for Type 1 and Type 2 neurons to achieve relief of I_{Ca} inhibition. Trigeminal neurons were voltage clamped at −80 mV and were stepped initially (S1) to a test potential of 0 mV. In Type 1 neurons, a 70 ms step to +130 mV was applied. Cells recovered for 20 ms before a second test pulse (S2) was applied. In Type 2 neurons, a 50 ms step to +100 ms was applied. Cells recovered for 15 ms before the second test pulse. A section of approximately 80 ms in the current trace has been omitted for clarity. Inhibition of HVA I_{Ca} by 1 μM PGE_2 was relieved after a large positive prepulse in both Type 1 and Type 2 cells, but a greater depolarizing pulse was required to achieve relief in Type 1 neurons.

Table 2. Primer sequences used to amplify mRNA fragments from prostanoid EP receptors EP_{1-4} and the housekeeping enzyme hypoxanthine phosphoribosyltransferase (HPRT)

Receptor			Size (bp)
EP_1	F	GCTTAACCTGAGCCTAGCGGA	294
	R	CGCAGTATACAGGCGAAGCAC	
EP_2	F	CTCAACTACGGGGAGTACGTCC	277
	R	AGGAGAATGAGGTGGTCCGTC	
EP_3	F	CATGATGGTCACTGGCTTCGT	238
	R	ACACTGTCATGGTTAGCCGCA	
EP_4	F	CGTAGTATTGTGCAAGTCGCG	215
	R	CAGATGATGCTGAGACCCGAC	
HPRT	F	GCTACTGTAATGATCAGTCAACGGG	394
	R	CAACATCAACAGGACTCCTCGTA	

F, the forward primer; R, the reverse; Size (bp), the length of the expected PCR product.

previous study to examine the actions of PGE_2 on I_{Ca} in sensory neurons found that PGE_2 increased the calcium current amplitude (Nicol *et al.* 1992). The reason(s) for the very different findings of this study and those of Nicol and colleagues (1992) are not known, but the earlier study was performed in very different cells – embryonic chicken sensory neurons.

PGE_2 inhibited I_{Ca} via two different signal transduction pathways in different trigeminal neuron populations. In Type 1 trigeminal neurons PGE_2-mediated inhibition of I_{Ca} was abolished by CTX pre-treatment and unaffected by PTX. In contrast, in Type 2 neurons inhibition of I_{Ca} was blocked by PTX treatment and unaffected by CTX treatment. These results imply that in Type 2 cells the PGE_2 receptors are coupled predominantly through Gi/o proteins, whereas in Type 1 cells PGE_2 receptors couple to calcium channels exclusively through Gs. Rapid, voltage-dependent inhibition of I_{Ca} via native PTX-insensitive

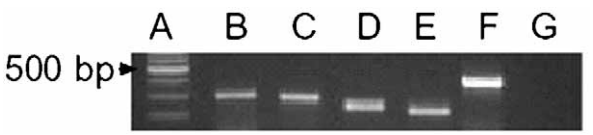

Figure 6. Trigeminal ganglion cells express 4 EP prostanoid receptor subtypes

Total trigeminal ganglion mRNA was extracted and subjected to RT PCR utilizing the primers outlined in Table 1. The resulting PCR products were separated on a 2 % agarose gel stained with ethidium bromide, as illustrated. Lane A is a nucleotide size ladder in 100 bp increments, lanes B, C, D and E contain the amplified DNA fragments corresponding to EP_1, EP_2, EP_3 and EP_4 receptors respectively, lane F is the amplified DNA fragment corresponding to hypoxanthine phosphoribosyltransferase, a housekeeping enzyme, lane G contains all the PCR reaction components without added reverse transcription product. This is representative gel from the ganglia of 1 of 13 mice (8 male, 5 female).

G proteins has been observed in several cell types with agonists such as vasoactive intestinal polypeptide (VIP) (Zhu & Ikeda, 1994; Ehrlich & Elmslie, 1995), histamine and PGE_2 (Currie & Fox, 2000). VIP inhibition of I_{Ca} in sympathetic neurons occurs via a CTX-sensitive, PKA-independent pathway with kinetic characteristics identical to PTX-sensitive pathways in the same cell (Zhu & Ikeda, 1994; Ehrlich & Elmslie, 1995). Similar results were obtained in this study, where the CTX-sensitive PGE_2 inhibition of I_{Ca} appeared to be mediated via G protein $\beta\gamma$ subunits, which also mediated the PTX-sensitive inhibition of I_{Ca} by baclofen (see below). In bovine adrenal chromaffin cells, both histamine and PGE_2 inhibit I_{Ca} via a voltage-dependent, PTX-insensitive pathway, but the involvement of Gs, or other types of G protein, has not been addressed.

In both types of cells the inhibition of I_{Ca} appeared to be consistent with $\beta\gamma$-subunits of the G protein heterotrimers interacting with calcium channels. The rise time of I_{Ca} in the presence of PGE_2 was slowed in both types of cell, and the magnitude of the PGE_2-induced inhibition of I_{Ca} could be strongly reduced by a positive conditioning step; both these features are characteristic of the G protein $\beta\gamma$ subunit-mediated pathway of calcium channel inhibition (Herlitze *et al.* 1996; Ikeda 1996; Zamponi & Snutch, 1998). Similarly, in sympathetic neurons and melanotrophs PGE_2 couples to inhibition of I_{Ca} predominantly via a PTX-sensitive $\beta\gamma$ subunit-mediated pathway (Ikeda 1992; Tanaka *et al.* 1998; Ito *et al.* 2000). The present results do not preclude the possibility that PGE_2 modulates HVA I_{Ca} via other second messenger systems as well.

Different conditioning pulse protocols were required to relieve I_{Ca} inhibition by PGE_2 in Type 1 *versus* Type 2 cells. In Type 1 cells a step to +130 mV was required to observe significant relief of PGE_2-induced inhibition, while a step to +100 mV was sufficient in Type 2 cells. There are several possible explanations for this. Different G protein $\beta\gamma$ subtypes that have a stronger affinity for calcium channels may be coupled to different G proteins in Type 1 *versus* Type 2 cells. Differential voltage-dependent effects on I_{Ca} of different G protein $\beta\gamma$ subunits have been observed in rat superior cervical ganglion neurons, where G protein $\beta\gamma$ subunits derived from G_{oA} or Gi heterotrimers were not equally effective in promoting voltage-dependent inhibition of I_{Ca} (Delmas *et al.* 1999). Recombinant N-type and P/Q-type calcium channels are differentially modulated by different types of G protein β subtypes (Arnot *et al.* 2000), and so association of different $\beta\gamma$ subunits with $Gs\alpha$ *versus* PTX-sensitive G proteins in sensory neurons may explain our observations. However, the identity of the calcium channels and their accessory subunits in Type 1 or Type 2 cells may also be responsible for the need for more robust depolarizations in Type 1 cells

to obtain relief from PGE_2 inhibition. Different splice variants of N-type or P/Q-type calcium channels have been identified and may be differentially affected by G protein $\beta\gamma$ subunits (Lin *et al.* 1997; Bourinet *et al.* 1999; Delmas *et al.* 2000), leading to variable neurotransmitter modulation of otherwise similar channels in native neurons. An example of such variability which may be a result of alternative splicing of the α1B subunit is found in superior cervical ganglion neurons. In these cells, dendrites uniquely expressed an N-type calcium channel which had enhanced interactions with G protein $\beta\gamma$ subunits (Delmas *et al.* 2000), which resulted in the dendrites of these neurons being 'hypersensitive' to neurotransmitter-mediated regulation of I_{Ca} when compared with the cell body. The dendritic N-type channel also showed reduced relief from G protein $\beta\gamma$ inhibition for a given depolarizing step. Similar differences in G protein $\beta\gamma$ subunit-mediated regulation of I_{Ca} can also occur when different calcium channel β-subunits associate with a given pore forming subunit (Canti *et al.* 2000; Feng *et al.* 2001). Although mouse trigeminal neurons appear to have similar amounts of N-type and

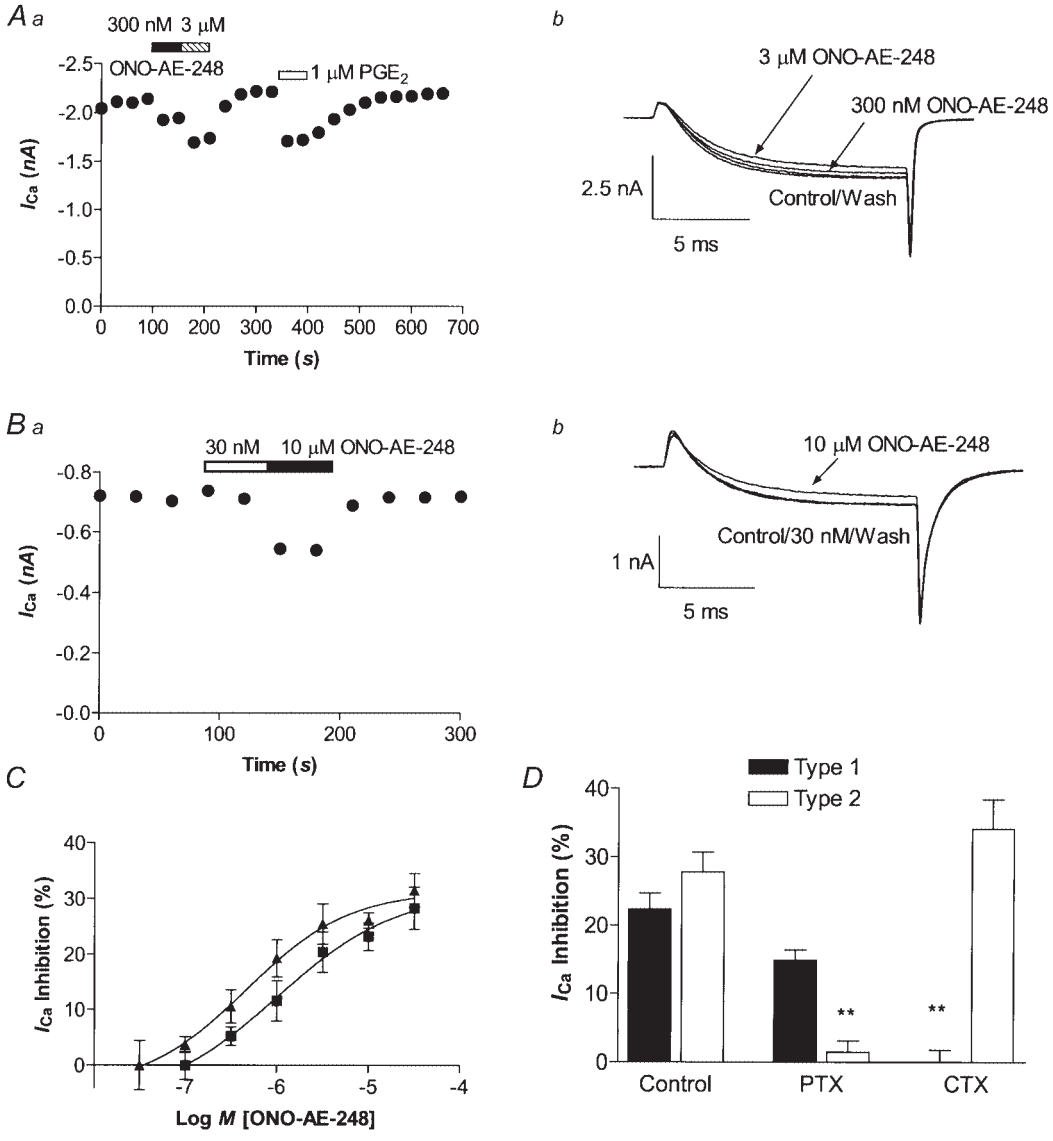

Figure 7. The EP_3 agonist ONO-AE-248 inhibits I_{Ca} in Type 1 and Type 2 trigeminal neurons

Representative time plots of ONO-AE-248 inhibition of peak I_{Ca} in a Type 1 (*Aa*) and Type 2 (*Ba*) neuron. I_{Ca} was elicited by stepping the membrane potential from −80 to 0 mV. Example traces from the Type 1 neuron (*Ab*) and Type 2 (*Bb*) neurons are shown in *Aa* and *Ba*. *C,* the inhibition of I_{Ca} by ONO-AE-248 in Type 1 (triangles) and Type 2 (squares) neurons was concentration dependent, with an EC_{50} of 580 nM and 1.4 μM, respectively. Each point represents the mean and S.E.M. of 6–10 cells. *D,* overnight treatment with PTX (100 ng ml^{-1}) strongly reduced the ONO-AE-248 (10 μM) inhibition of I_{Ca} in Type 2 but not Type 1 cells, whereas treatment with CTX (250 ng ml^{-1}) overnight blocked the ONO-AE-248 inhibition of I_{Ca} in Type 1 but not Type 2 cells (n = 6–15 for each).

P/Q-type calcium channels in Type 1 and Type 2 cells (Borgland *et al.* 2001), our studies cannot detect different splice variants of the α subunit of the channels, or the identity of the calcium channel β subunits they are associated with.

Although we found mRNA for all four types of EP prostanoid receptor in whole trigeminal ganglion, EP_3 receptors appeared to mediate the effects of PGE_2 in both types of trigeminal neuron. Of the selective EP receptor agonists tested, only the EP_3 agonist ONO-AE-248 mimicked the PGE_2 inhibition of I_{Ca}. Agonists selective for EP1 (ONO-DI-004), EP2 (ONO-AE1-259) or EP4 receptors (ONO-AE1-329) did not modulate I_{Ca} in any neurons examined. These agents have been reported to be potent and very selective agonists at native and recombinant mouse EP receptors (Zacherowski *et al.* 1999; Suzawa *et al.* 2000). The inhibition of I_{Ca} by ONO-AE-248 had similar operational characteristics to that caused by PGE_2 in that it was associated with a significant slowing of the activation of I_{Ca} and was largely abolished by CTX treatment in Type 1 cells and PTX treatment in Type 2 cells. ONO-AE-248 was considerably less potent than PGE_2 in inhibiting I_{Ca}. This is consistent with previous findings demonstrating that ONO-AE-248 inhibited forskolin-stimulated adenylyl cyclase activity in cells expressing recombinant murine EP_3 receptors at least 10-fold less potently than PGE_2 (Zacharowski *et al.* 1999). Mouse EP_3 receptor mRNA can undergo multiple alternative splicing events to produce proteins which can couple to either only Gi/Go ($EP_{3\alpha}$ and $EP_{3\beta}$, Sugimoto *et al.* 1993) or to both Gi/Go and Gs ($EP_{3\gamma}$, Irie *et al.* 1993). The relative expression of these isoforms in mouse sensory neurons has not been established, but the results of the present study are consistent with the expression of at least the $EP_{3\gamma}$ isoform in trigeminal neurons.

The presence of multiple EP receptors in mouse trigeminal ganglion is generally consistent with other reports (Oida *et al.* 1995; Narumiya *et al.* 1999), although the presence of EP_2 receptor mRNA has not to our knowledge been reported in mouse. All four EP receptor types are found in rat dorsal root ganglion neurons although one study found only one of the rat EP_3 splice variants (Southall & Vasko, 2001), while another found all three (Donaldson *et al.* 2001). In cultured rat dorsal root ganglion neurons, the Gs-coupled EP_{3c} receptor subtype (together with EP4 receptors), was found to be necessary for full expression of some of the biochemical correlates of PGE_2-mediated sensitization, namely elevation of cAMP and enhanced release of substance P and calcitonin gene-related peptide (Southall & Vasko, 2001).

The present study identified inhibition of HVA I_{Ca} as a novel mechanism by which PGE_2 can modify the activity of virtually all sensory neurons. In many sensory neurons PGE_2 directly enhances excitability (Ingram & Williams, 1996; Gold *et al.* 1996; Evans *et al.* 1999) and sensitizes the cells to a variety of stimuli (Pitchford & Levine, 1991; Cui & Nicol, 1995; Lopshire & Nicol, 1998). These effects occur predominantly via processes that involve activation of adenylyl cyclase via Gs (Ingram & Williams, 1996; England *et al.* 1996; Evans *et al.* 1999). The adenylyl cyclase-dependent sensitization by PGE_2 is likely to occur in the Type 1 trigeminal sensory neurons, which are opioid and capsaicin sensitive, and in which PGE_2 inhibits I_{Ca} via Gs. Although often considered simply an inhibitory effect, the reduction of calcium entry via HVA I_{Ca} may also enhance sensory neuronal excitability by indirectly inhibiting calcium-dependent after-hyperpolarizations, which can allow neurons to attain higher frequencies of action potentials.

We have also identified a significant population of trigeminal sensory neurons in which PGE_2 is unlikely to act as a sensitizing agent, at least when acting through EP_3 receptors. In these Type 2 neurons PGE_2 appears to couple predominantly through inhibitory G proteins. Although we have not established whether other EP receptor subtypes act on other channels to modulate the activity of Type 2 neurons, these neurons also lack one of the major substrates for PGE_2-mediated sensitization, VR1 receptors (Lopshire & Nicol, 1998; Borgland *et al.* 2001). We speculate that the rapid elevations of PGE_2 in the spinal cord that can follow a peripheral noxious stimulus (Yang *et al.* 1996) may act to dampen sensory input from this population of neurons by inhibiting I_{Ca} in the spinal terminals of these cells. In a preliminary study in the substantia gelatinosa of the spinal trigeminal nucleus of the rat, we have reported that PGE_2 inhibited spontaneous miniature glutamatergic synaptic currents (Jennings *et al.* 2000). In contrast, and consistent with the excitatory effects of PGE_2 on sensory neurons, another study found a PGE_2-induced enhancement of glutamatergic synaptic currents in mouse lumbar dorsal horn neurons (Minami *et al.* 1999). These two studies, together with the data reported here, indicate that PGE_2 can act in ways other than simply as a sensitizing agent for sensory neurons, and appears to differentially modulate the sensory information coming from distinct populations of sensory neurons.

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