British Journal of Pharmacology (1997) 122, 1593–1604

Prostanoids synthesized by cyclo-oxygenase isoforms in rat spinal cord and their contribution to the development of neuronal hyperexcitability

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1 The responses of wide dynamic range spinal dorsal horn neurones to noxious mechanical stimulation of the ankle or knee joint were tested before and after spinal administration of the non-selective cyclo-oxygenase (COX) inhibitors, indomethacin and meclofenamic acid. Neither of these drugs altered the responses of these neurones to noxious mechanical stimulation.

2 Wind-up of a spinal nociceptive reflex evoked by electrical stimulation of the sural nerve at C-fibre strength was dose-dependently inhibited by intravenous administration of indomethacin, a non-selective COX inhibitor, and SC58125, a selective COX-2 inhibitor. Intrathecal administration of indomethacin also reduced the wind-up of this nociceptive reflex.

3 Western blot analysis of proteins extracted from normal rat spinal cord revealed the presence of both cyclo-oxygenase (COX)-1 and COX-2 proteins.

4 Immunocytochemistry of sections of normal rat spinal cord with specific COX-1 antiserum revealed little specific COX-1-like immunoreactivity in the grey matter. With the same antiserum, intense COX-1-like immunoreactivity was observed in the cytoplasm, nuclear membrane and axonal processes of small to medium sized ($<1000\ \mu m^2$) dorsal root ganglion (DRG) cell bodies.

5 Immunocytochemistry of sections of normal rat spinal cord incubated with specific COX-2 antiserum showed intense COX-2-like immunoreactivity (COX-2-li) in the superficial dorsal horn of the spinal cord (laminae I and II) and around the central canal (lamina X). COX-2-li was also observed in some neurones in deep dorsal horn and in individual motor neurones in ventral horn. COX-2-li was not observed in the cell bodies of DRG.

6 Superfusion of the lumbar spinal cord of normal rats with artificial CSF and subsequent radioimmunoassay revealed the presence of prostaglandin D_2 (PGD_2) < PGE_2, but not PGI_2 (determined by measurement of the stable metabolite, 6-keto-$PGF_{1\alpha}$) or $PGF_{2\alpha}$.

7 These data suggest that eicosanoids synthesized by an active COX pathway in the spinal cord of normal animals may contribute to nociceptive processing, but only when the spinal cord neurones are rendered hyperexcitable following C-fibre stimulation. Selective inhibition of one or both of the COX isoforms in normal animals may represent a novel target for spinal analgesia.

Keywords: Cyclo-oxygenase, COX-1; COX-2; prostaglandin; nociception; spinal cord; hyperexcitability; NSAID

Introduction

Prostaglandins and thromboxanes, which are synthesized from arachidonic acid by cyclo-oxygenases (COX), have a large number of biological actions including the sensitization of peripheral nociceptors in inflammation (Guilbaud & Iggo, 1985; Heppelmann *et al*., 1986; Schaible & Schmidt, 1988; Grubb *et al*., 1991). Two COX isoforms have been cloned and characterized in different species, e.g. rat (Fletcher *et al*., 1992) and man (Hla & Neilson, 1992; Hla *et al*., 1993), and many tissues express both COX-1, the stably expressed isoform, and COX-2, an inducible isoform. The relative amounts of each COX isoform expressed in individual tissues varies and can be modulated under pathological conditions. COX proteins, for example, are upregulated in synovial tissues in arthritis, probably by increased synthesis of the COX-2 variant (Sano *et al*., 1992; Mitchell *et al*., 1995). Similarly, carrageenan and kaolin injection into the hind-paw of the rat results in an increase in COX-2 mRNA expression in the inflamed tissue (Seibert *et al*., 1994). In contrast to other tissues, normal rat brain expresses more COX-2 mRNA than COX-1 mRNA (Feng *et al*., 1993; Seibert *et al*., 1994), an observation confirmed in immunocytochemical studies which have shown COX-2-like immunoreactivity (COX-2-li) to be present in neurones in several discrete regions of the brain (Breder *et al*., 1992, 1995).

Discrepancies between the doses of some non-steroidal anti-inflammatory drugs (NSAIDs) required to produce anti-inflammatory and analgesic effects suggest that their analgesic actions may arise, at least in part, through a central action (McCormack & Brune, 1991; McCormack & Urquart, 1995). Evidence supporting this hypothesis has come from experiments which have shown that intrathecally applied NSAIDs reduce pain-related behaviour (Malmberg & Yaksh, 1992) and the activity of dorsal horn neurones (Chapman & Dickenson, 1992) in the rat formalin model of inflammation. Furthermore, NSAIDs applied intravenously reduce the responses of rat spinal cord neurones, rendered hyperexcitable by an acute arthritic lesion, to innocuous and noxious stimuli applied to the inflamed knee (Neugebauer *et al*., 1994, 1995). Whilst there is good evidence from studies involving NSAIDs that prostaglandins may be involved in synaptic processing in the spinal cord of rats with peripheral inflammatory lesions, much less is known about the role of eicosanoids in spinal nociceptive processing under normal conditions, i.e. in the absence of peripheral inflammation. This is perhaps surprising given the relative abundance of COX-2 mRNA (compared to COX-1 mRNA) in normal rat brain and the presence of COX-2 mRNA in normal rat spinal cord (Beiche *et al*., 1996; Hay *et al*., 1997), and invites the question of whether COX isoforms are involved in spinal nociceptive processing in normal rat spinal cord.

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In this study we have used electrophysiological, neuroanatomical and biochemical methods to investigate the role of cyclo-oxygenase isoforms in spinal nociceptive processing. We showed that spinal cord neurones receiving nociceptive input are typically unresponsive to spinally administered NSAIDs, unless the spinal cord has been rendered hyperexcitable by repetitive C-fibre stimulation (a situation with some similarity to afferent input in inflammation). Furthermore COX-1 and COX-2 proteins are present in spinal and primary sensory neurones suggesting that they are involved in spinal nociceptive processing. Some of these data have been published previously in abstract form (Willingale & Grubb, 1996; Gardiner *et al*., 1997).

Methods

Anaesthetic procedures for electrophysiological and superfusion experiments

Male Wistar rats (230–330g) were anaesthetized with sodium thiobutabarbitone (Inactin, Research Biochemical Inc; 100 mg kg^{-1}, i.p.). The depth of anaesthesia was assessed by testing for hindlimb withdrawal and corneal reflexes which had to be absent. Cannulae were placed in the trachea, carotid artery and external jugular vein. Blood pressure was monitored continuously via the carotid arterial catheter and was maintained between 100 and 115 mmHg by administration of additional anaesthetic (i.p.) as required. A gentle jet of oxygen was aimed at the opening of the tracheal cannula to aid oxygenation. Rectal temperature was maintained at 36–38°C by use of a homeothermic blanket system.

Electrophysiological experiments

Rats were supported in a stereotaxic frame by swan-neck clamps placed under the mammillary processes of the exposed lumbar vertebrae. The skin was reflected back and the underlying tissues covered with gauze soaked in 0.9% saline to prevent desiccation. For single-cell recordings, a laminectomy was performed (segments L1-L6). The dura mater was cut longitudinally under paraffin oil and reflected back to reveal the spinal cord. Single-cell recordings were made from slowly adapting, wide dynamic range spinal cord neurones in the superficial and deep dorsal horn by use of glass extracellular microelectrodes (resistance 2–10 MΩ) filled with 0.5 M sodium acetate. These neurones were typically located in spinal segments L4/5 (knee) and L5/6 (ankle).

Wind-up of the sural nerve evoked reflex in the biceps femoris muscle has been studied in several preparations including decerebrate spinalized (Woolf, 1983), spinalized anaesthetized (Hartell & Headley, 1990), and spinally intact anaesthetized rats (Chapman & Dickenson, 1992; Herrero & Cervero, 1996a, b). In our reflex experiments the animals were prepared in a similar manner to that described above, except that a fine polythene catheter (external diameter 15–175 μm) was inserted through a hole made in the dura mater at the rostral end of the laminectomy and advanced until the tip lay over the lumbar enlargement to allow local administration of drugs under the dura mater. An additional incision was made longitudinally on the posterior surface of the right hindlimb. The skin was reflected back to form a pool into which warm paraffin oil was poured. The sural nerve was dissected free from the underlying tissues, cut and the proximal end placed over silver wire stimulating electrodes. A pair of tungsten needle recording electrodes was placed into the biceps femoris muscle to record an extracellular EMG. Square-wave pulses of 500 μs duration were applied to the nerve at frequencies of 0.1–1.0 Hz. To determine the C-fibre threshold of a unit, low frequency stimuli (0.1–0.2 Hz) were applied at increasing voltages until a consistent C-fibre response was obtained. The stimulation intensity was then increased to 1.5 times threshold unless the threshold voltage was unusually high (in the region of 15 V) in which case a stimulus strength of 1.2–1.3 times threshold was used. C-fibre responses were distinguished from A-fibre responses by their latencies: A-fibre responses were considered to be those which appeared within 130 ms of the stimulus artifact, while C-fibre responses were defined as those appearing with a latency greater than this. Single-cell recordings and EMG potentials were discriminated by use of an event counter (Digitimer) and the data were stored with a CED 1401 interface and a microcomputer for off-line analysis.

Stimulation protocols

In experiments where single-cell recordings were made, 15 s noxious (as determined by testing on the experimenters' skin) mechanical stimuli were applied to the ankle joint (meclofenamic acid experiments) or knee joint (indomethacin experiments) with pneumatic callipers (2.5–3.5 bar, calliper contact area = 100 mm^2) every 3–5 min. Stimuli were repeated 6 times during the control period to ensure a reproducible response and then drugs were administered directly onto the spinal cord and the recording and stimulation continued for 30 min. Mechanical stimulation at this intensity did not cause any visible tissue damage over the time course of the experiments (1–2 h recording time).

In reflex experiments, trains of 20 stimuli were applied at a frequency of 0.4–0.8 Hz to elicit wind-up. In preliminary experiments, trains of C-fibre strength stimuli applied at 1 Hz were found to enhance the reflex for up to 3 min. As a consequence, stimuli were applied every 5 min in experiments involving the use of NSAIDs.

Calculations and statistics

The analysis of single-cell recordings was made by comparing the mean response to 6 noxious stimuli before and after drug administration. In reflex experiments, the average size of the reflex elicited during 6 control trains and for 6 trains post-treatment was used to calculate the effects of any drug treatments. It was important to use stimulus frequencies (typically 0.4–0.6 Hz) which would not elicit maximal wind-up to ensure that we could observe positive or negative effects of the drugs on the reflex. These calculations were made for both the A-fibre and C-fibre components of the reflex. The effect of any drug on the reflex was assessed in three ways. Firstly, to obtain a simple measure of the reduction in the magnitude of the A-fibre or C-fibre wind-up, the response to the twentieth stimulus in each train was compared before and after drug treatment. Secondly, a one-way ANOVA (significance = $P<0.05$) was used to compare the total magnitude of the A-fibre or C-fibre responses to all twenty stimuli in each train, i.e. all twenty average responses were summed, before and after drug treatment. Thirdly, the effect of drugs on the magnitude of the baseline reflex, i.e. the response to the first stimulus in each train, was also assessed by one-way ANOVA. Since the response to the first stimulus in a C-fibre train might give only a single action potential, the values in all graphs are calculated as a percentage of the maximum, i.e. twentieth, response obtained during the control trains.

Drug administration

In experiments where single-cell recordings were made, meclofenamic acid (1–100 μM in saline, pH 7.4) and indomethacin (100 μM-1 mM in 1 part 10% sodium bicarbonate and 9 parts PBS, pH 7.4) were applied directly onto the spinal cord in a volume of 40 μl. In reflex experiments, drugs administered intravenously were injected in a volume of 1.0 ml kg^{-1} followed by a 0.2 ml flush with 0.9% saline to clear the catheter. The drugs administered by this route were ketamine (0.5–2.0 mg kg^{-1} in 0.9% saline), SC58125 (1–10 mg kg^{-1} in 5% DMSO, 95% PEG 400) and indomethacin (0.1–5.0 mg kg^{-1} in 1 part 10% sodium bicarbonate and 9 parts 0.9% saline). Indomethacin was also administered through the intrathecal

catheter (10–100 μM in 1 part 10% sodium bicarbonate and 9 parts PBS, pH 7.4) in a volume of 50 μl by slow injection.

Tissue collection

Male Wistar rats weighing 200–650 g were killed by an intraperitoneal injection of sodium thiopentone solution (400 mg kg^{-1}) and transcardially perfused with 0.9% saline prewarmed to 37°C, at a pressure of 100 mmHg, until all blood was removed. Spinal cord segments L1 to S1 were rapidly (3–5 min) exposed and excised. Samples for Western blotting were immediately frozen in liquid nitrogen and stored at −80°C. Samples for immunocytochemistry were embedded in Tissue-Tek (Miles Inc, Elkhart, In), frozen over dry ice/hexane and stored at −20°C.

Western blotting

Whole lumbar spinal cords (segments L1–L6) were chopped on dry ice, homogenized in ice-cold lysis buffer (NaCl 150 mM, Tris pH 7.4 10 mM, EDTA 1 mM, EGTA 1 mM, Triton-X-100 1%, PMSF 0.2 mM, aprotinin 20 μg ml^{-1}) and centrifuged (20,000 r.p.m., 4°C, 10 min). The protein concentration in the supernatant was determined by the Lowry method and samples diluted to make total protein concentrations equal. Denatured samples were separated by SDS-polyacrylamide gel electrophoresis (200 V for 1.5 h). Proteins were transferred to a nitrocellulose membrane by use of a semi-dry transfer unit (Bio-Rad) and incubated for 2 h in buffer containing 10% milk powder in TTBS (Tris Base 50 mM, NaCl 150 mM, Tween-20 0.1%, pH 8). Primary antibody (diluted in TTBS containing 10% milk powder and 0.01% sodium azide) incubation was performed overnight at room temperature followed by a 2 h incubation in peroxidase-conjugated secondary antibody. Protein bands were visualized by means of an ECL-detection kit (Amersham).

Immunocytochemistry

Frozen transverse sections (10–15 μm) were cut from tissue blocks, mounted on pre-subbed slides and air dried. Tissue sections were fixed with a freshly prepared 2% paraformaldehyde solution in Sörensons buffer for 10 min at room temperature and washed in PBS (pH 7.4). Sections were preincubated in blocking solution (PBS containing 10% goat serum and 0.5% Triton-X-100) for 30 min at room temperature, washed in PBS, and then incubated overnight in anti-COX-1 (1 : 200 in PBS containing 10% goat serum) or anti-COX-2 (1 : 200 in PBS containing 10% goat serum). Following a further wash in PBS, fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG (1 : 200 in PBS) or anti-mouse IgG (1 : 200 in PBS) was pipetted onto the sections which were incubated at room temperature for 2 h. Slides were then washed in PBS and excess fluid blotted from around the tissue before it was mounted in Citifluor (UKC Chem Lab, U.K.). All incubations took place in a humidity chamber. In control experiments primary antibody was omitted from the incubation medium to determine the amount of non-specific binding. All sections were examined under epifluorescence or with a confocal laser scanning microscope. When FITC-coupled secondary antibodies are used in conjunction with the confocal microscope, labelled structures appear white and unlabelled structures black, in the digitized images. All labelled structures are described as having COX-1- or COX-2-like immunoreactivity (-li) in case there is non-specific protein binding. Propidium iodide (0.1% w/v in PBS) was used as a nuclear counterstain in some tissue sections.

Spinal cord superfusion and sample assay

The rats were positioned in a stereotaxic frame, supported by swan-neck clamps placed under the mammillary processes of vertebrae T9 to L4. A laminectomy exposed the lower thoracic and lumbar spinal segments. In early experiments we found that blind insertion of the catheter into the intrathecal space following a restricted laminectomy significantly increased the chance of damage to blood vessels on the spinal cord surface. Since it was imperative to keep blood products away from the spinal cord surface and to ensure that no damage was done to the spinal cord, a more extensive laminectomy was found to be appropriate. A small hole was made in the dura mater at the rostral end of the laminectomy and two silicon treated polythene catheters, approximately 100 μm in diameter, were introduced into the subdural space. The inflow catheter was advanced caudally until the tip was positioned at the caudal end of the lumbar enlargement. The outflow catheter tip was positioned just inside the hole in the dura mater. Both catheters were attached to a peristaltic pump (Gilson Minipuls 3) and filled with artificial CSF, warmed to 37°C (composition in mM: NaCl 125, KCl 2.5, $NaHCO_3$ 26, NaH_2PO_4 1.25, $CaCl_2$ 2, and $MgCl_2$ 1, gassed with 5% CO_2/95% O_2, pH 7.4). The exposed dura mater was covered with gauze soaked in 0.9% saline. Artificial CSF was perfused over the surface of the spinal cord at a rate of approximately 225 μl min^{-1}. Samples were collected from the outflow catheter at a rate of 200 μl min^{-1} in 1 ml aliquots into siliconised microfuge tubes and placed on ice. Indomethacin (200 μM) was added to each sample to prevent any further prostaglandin synthesis. Samples were stored at −80°C until analysis. The spinal cord was perfused for 1 h prior to sample collection, which continued for a further hour.

A sample of superfusate (100 μl) or prostaglandin standard (100 μl) was mixed with 100 μl prostaglandin antibody (at a dilution which bound 50% of the tracer in the absence of unlabelled prostaglandin) in the presence of 0.15% bovine γ-globulin. The tubes were incubated on ice for 30 min and radiolabelled prostaglandin (100 μl containing 5000 d.p.m.) was added to each tube. The tubes were vortexed and incubated overnight at 4°C. Antibody-bound prostaglandins were separated by the addition of ice-cold polyethylene glycol-8000 (final concentration 12.5% w/v) to each tube. The mixture was vortexed for a total of 30 s, then centrifuged for 1 h at 3,500 r.p.m. at 4°C. The antibody-bound prostaglandins were pelleted by the polyethylene glycol. When tritiated prostaglandins were used, 1 ml of the supernatant was removed, mixed with 1 ml water and 4 ml Ultima-Gold scintillation fluid (Packard, UK) and counted for 3 min. When iodinated prostaglandins were used, the supernatant was discarded and the pellet counted for 1 min. Silicon-treated vials were used throughout the assay procedure. A standard curve was constructed and the minimum assay sensitivity determined by calculating the amount of unlabelled prostaglandin required to shift zero binding by 2 standard deviations. The prostaglandin concentration in each sample was extrapolated from the standard curve, and standardized to pg prostaglandin ml^{-1} min^{-1}.

Compounds and antisera

Unlabelled prostaglandins were kind gifts from Dr P. Birch (Glaxo, Ware, U.K.). Radiolabelled prostaglandins: [^{125}I]PGE_2, [^{3}H]-PGD_2, [^{3}H]-6-keto-$PGF_{1\alpha}$ and [^{3}H]-$PGF_{2\alpha}$ were purchased from NEN, (U.K.). Anti-PGE_2, anti-$PGF_{2\alpha}$, anti-6-keto-$PGF_{1\alpha}$, FITC-conjugated and peroxidase-conjugated anti-rabbit-IgG were purchased from Sigma, (U.K.). Anti-PGD_2 and purified COX-2 were from OBR (Oxford, Mi). Peroxidase-conjugated anti-mouse-IgG was purchased from Amersham, (U.K.). COX-1 and COX-2 antisera were purchased from Cayman Chemical (Ann Arbor, Mi). Manufacturers' instructions indicated that the monoclonal mouse anti-sheep COX-1 antibody bound sheep, mouse, rat and human COX-1 and showed little or no crossreactivity with COX-2 in the three species tested (sheep, mouse, human). The polyclonal rabbit anti-mouse COX-2 antibody bound sheep, mouse, rat, human and guinea-pig COX-2 and showed little or no cross-reactivity with COX-1 in the 3 species tested (sheep, mouse and human). Purified sheep COX-1 protein was purchased from

Cayman Chemical and purified COX-2 protein was purchased from Oxford Biomedical Research Inc (Oxford, Mi). Prestained SDS-PAGE standards were purchased from Bio-Rad (U.K.). All other chemicals were purchased from Sigma (U.K.). SC58125 (1-[(4-methyl-sulphonyl)phenyl]-3-trifluoromethyl-5-(4-fluorophenyl)pyrazole) was a gift supplied by Glaxo Group Research (Ware, U.K.).

Results

Electrophysiological recordings

Single-cell recordings The responses of wide dynamic range neurones in the deep dorsal horn (laminae IV, V, VI) of the spinal cord to noxious mechanical stimuli, applied to the ankle and knee joint, have been described previously (Grubb *et al*., 1993). Briefly, these neurones had large receptive fields which were not restricted to, but had lowest mechanical threshold for activation at, either the ankle or knee joint (depending where the stimulus was to be applied – see Methods). The responses of such neurones to noxious mechanical stimuli were tested before (6 control responses) and after the administration of the non-selective NSAIDs, meclofenamic acid and indomethacin, directly onto the spinal cord. Figure 1 shows a summary of the responses of dorsal horn neurones to spinal administration of meclofenamic acid (vehicle ($n=14$), 10 μM ($n=5$) and 100 μM ($n=16$)) and indomethacin (vehicle ($n=5$), 100 μM ($n=5$)). Neither vehicle nor drug significantly (one-way ANOVA, $P<0.05$) altered the responses of the neurones to noxious mechanical stimulation at these concentrations, nor in the presence of 1 μM indomethacin (data not shown).

The properties of the flexor withdrawal reflex Repetitive stimulation of the sural nerve produced a dual component single-unit EMG response consisting of a brisk A-fibre response immediately after the stimulus artifact and a long latency C-fibre response. Trains of stimuli applied to the sural nerve at a frequency of 0.2 Hz produced a stable A-fibre and C-fibre reflex response which did not increase in magnitude during the train. When these stimuli were applied at a higher frequency, e.g. 0.6 Hz, there was an increase in the magnitude of the C-fibre response during the stimulus train but little or no change in the A-fibre response. An example of the effects of these different stimulus parameters is shown for a single experiment in Figure 2. Previous studies have shown that NMDA receptors are important in the development of wind-up in spinal cord neurones (Davies & Lodge, 1987; Thompson *et al*., 1990; Woolf & Thompson, 1991; Dickenson & Aydar, 1991) and in order to check that the C-fibre evoked wind-up of the spinal reflex in this preparation was comparable to that seen in other preparations, we used ketamine, a non-competitive N-methyl-D-aspartate (NMDA) receptor antagonist. Ketamine, applied at a dose known to produce selective blockade of NMDA receptors (2 mg kg^{-1}, i.v.), reduced C-fibre evoked wind-up (Figure 2).

The action of NSAIDs on reflex responses Indomethacin (0.5–5.0 mg kg^{-1}, i.v., $n=5$) dose-dependently reduced the magnitude of C-fibre evoked reflex wind-up by reducing both the rate of wind-up and the maximum size of the reflex (Figure 3a). At the end of the stimulus train the C-fibre component of the reflex had been reduced to 46% of control value (control = 36.3 ± 1.1 spikes at 20th stimulus) by 5 mg kg^{-1} indomethacin with vehicle alone having no effect. The total magnitude of the C-fibre response was significantly reduced to 77% of control by 1 mg kg^{-1} indomethacin and to 52% of control by 5 mg kg^{-1} (one-way ANOVA, $P<0.001$). Indomethacin had no significant effect on the baseline reflex at any of the doses tested.

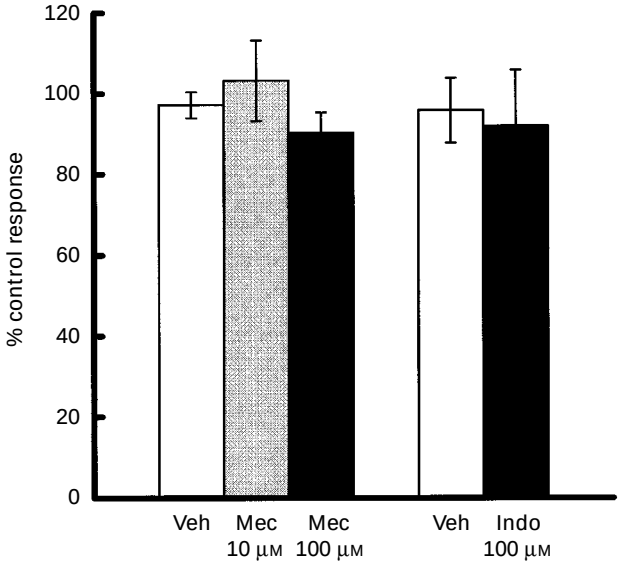

Figure 1 The effect of intrathecally administered NSAIDs on the responses of wide dynamic range dorsal horn neurones to noxious mechanical stimuli applied to the ankle or knee joint of anaesthetized rats. Extracellular recordings were made with sodium acetate-filled extracellular electrodes. Six control noxious mechanical stimuli (applied every 3–5 min (15 s duration) by use of pneumatic callipers) were applied before the administration of meclofenamic acid and vehicle (Mec, 10 μM ($n=5$), 100 μM ($n=16$) and Veh, ($n=14$)) and indomethacin and vehicle (Indo, 100 μM, ($n=5$), Veh, ($n=5$)) directly onto the spinal cord. The responses of the neurones to identical mechanical stimuli were measured for 30 min after drug administration. These drugs had no significant effect on the responses of the cells to noxious mechanical stimulation at the doses tested (one-way ANOVA, $P>0.05$). Values are mean$\pm$s.e.mean.

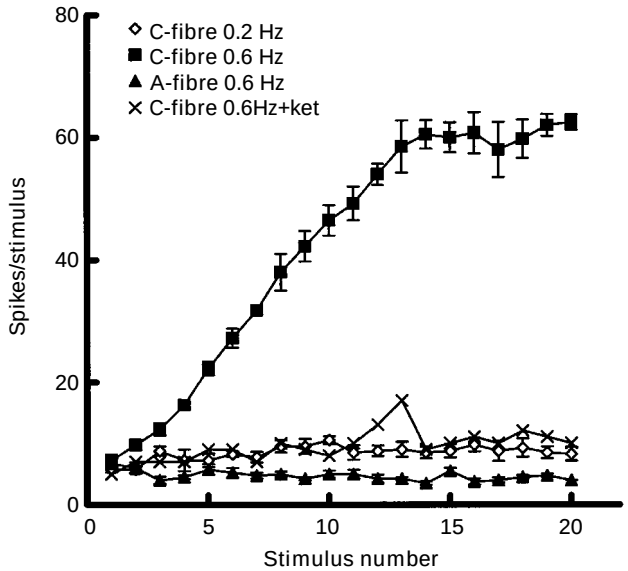

Figure 2 The effect of trains of electrical stimuli (0.6 Hz, 20 stimuli per train, 0.5 ms pulse width, 1.5 × C-fibre threshold) on the magnitude of the A-fibre and C-fibre component of a spinal reflex evoked by stimulation of the sural nerve and recorded in the biceps femoris muscle in a single rat. The magnitude of the C-fibre component increased during the trains ($n=6$) whereas the A-fibre response was virtually unaffected. At lower frequencies (0.2 Hz) trains of identical stimuli ($n=6$) did not induce any change in the magnitude of the C-fibre evoked response. Ketamine (ket), an NMDA receptor channel blocker (2 mg kg^{-1}, i.v.), reduced the magnitude of C-fibre evoked wind-up (0.6 Hz) during the stimulus train immediately following the injection. Values are mean and vertical lines show s.e.mean. The effects of ketamine were short lasting and so a single train exhibiting maximal suppression is shown. Wind-up returned to normal within 5–10 min.

Since intravenous administration cannot rule out the possibility that indomethacin was acting at another site within the CNS (or in the periphery), we administered indomethacin (10–100 μM, pH 7.4) through a subdural catheter in 5 rats. Neither the vehicle alone nor the lower dose of indomethacin altered the magnitude of the C-fibre evoked reflex wind-up. On application of the higher dose of indomethacin (100 μM), both the rate of wind-up and the maximum size of the reflex were reduced. The response to the twentieth stimulus in the train was reduced to 63% of the control value (control = 39.5 ± 11.8 spikes at 20th stimulus) and the total magnitude of the C-fibre responses during all 20 stimuli was reduced to 67% of control (one-way ANOVA, $P<0.05$; Figure 3b). The onset of the indomethacin effect could be observed after 5 min and was maximal after 15–20 min. Recovery was not observed in the short time course of these experiments. Indomethacin had no significant effect on the baseline reflex at any of the doses tested.

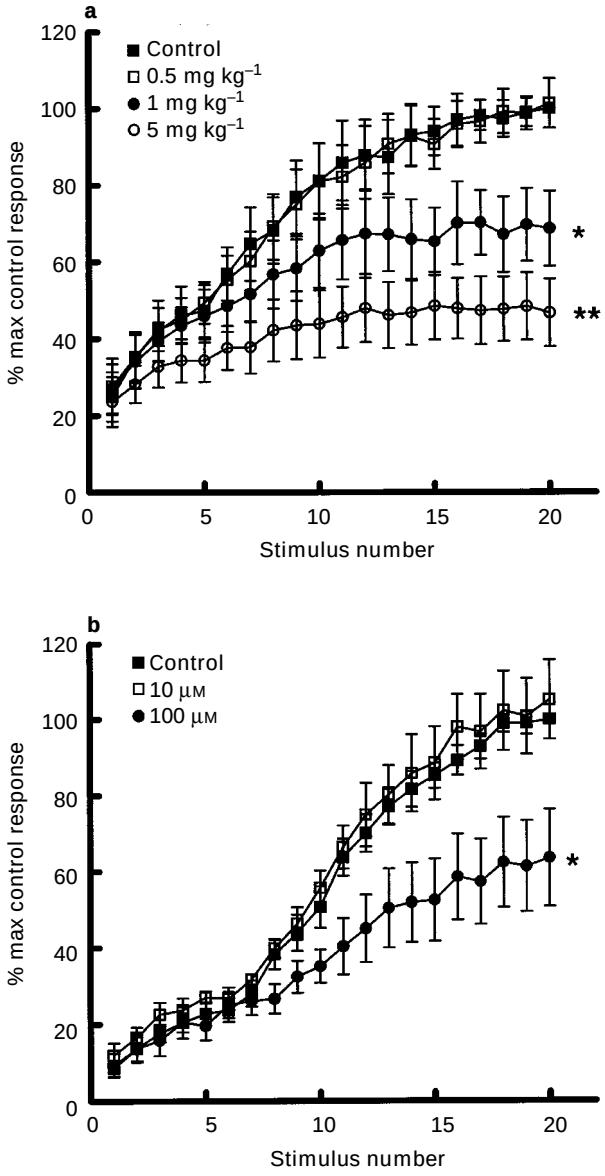

Figure 3 (a) The effect of intravenous indomethacin on C-fibre evoked wind-up of a spinal nociceptive reflex. Control C-fibre responses were calculated from the average biceps femoris EMG responses to 6 successive trains of sural nerve stimulation (20 stimuli per train, 0.5–0.8 Hz, 0.5 ms pulse width, $1.5 \times$ C-fibre threshold; $n=5$). The responses following 0.5 mg kg^{-1}, 1 mg kg^{-1} and 5 mg kg^{-1} indomethacin given at 1 ml kg^{-1} by slow intravenous injection are the averages of the responses to the 6 trains post-injection. Intravenous indomethacin dose-dependently reduced the total magnitude of the C-fibre wind-up (*$P<0.05$ at 1 mg kg^{-1}; **$P<0.005$ at 5 mg kg^{-1}). Intravenous vehicle (data not shown) elicited a small increase in the C-fibre wind-up ($P<0.05$). (b) The effect of intrathecal indomethacin on C-fibre wind-up. Control C-fibre responses were calculated from the average biceps femoris EMG response to 6 successive trains of sural nerve stimulation (parameters as in (a); $n=5$). The responses following an intrathecal injection of 50 μl of 10 μM and 100 μM indomethacin are the average of the responses to the 6 trains post-injection. Intrathecal vehicle (data not shown) and 10 μM indomethacin did not significantly ($P>0.05$) affect the C-fibre wind-up whereas 100 μM indomethacin given intrathecally significantly reduced wind-up (*$P<0.05$). In (a) and (b), the data were calculated by taking the maximal response (i.e. response to the 20th stimulus) during the control trains as the measure (100%) against which other responses were calculated. Values shown are means and vertical lines indicate s.e.mean.

Since two isoforms of the COX enzyme are known to exist we used a selective cox-2 inhibitor, SC58125, to examine whether the reductions in C-fibre evoked wind-up of this reflex were due to inhibition of this isoform. SC58125 (1–10 mg kg^{-1}, i.v., $n=5$), but not the vehicle, dose-dependently reduced the rate and maximum size of the wind-up (Figure 4). The response to the twentieth stimulus in the train was reduced to 28% of control value (control = 61.0 ± 9.9 spikes at the 20th stimulus) by 10 mg kg^{-1} SC58125 and the total magnitude of all C-fibre responses during the train was reduced to 29% of control (one-way ANOVA, $P<0.005$). The onset of this effect of SC58125 was very rapid being maximal after 5–10 min. SC58125 had no significant effect on the baseline reflex at any of the doses tested. Unfortunately, problems with drug solubility and vehicle toxicity prevented SC58125 from being administered spinally.

Cyclo-oxygenase isoforms in the spinal cord

Figure 5a shows a protein extract from lumbar spinal cord (segments L1-L6, left lane) run alongside purified sheep COX-1 protein (right lane) in a blot that has been incubated with the

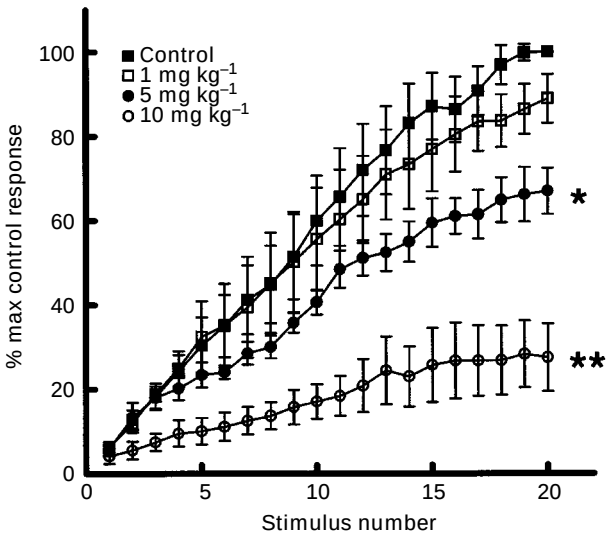

Figure 4 The effect of intravenous SC58125 on C-fibre wind-up. Control C-fibre responses were calculated from the average biceps femoris EMG response to 6 successive trains of sural nerve stimulation (parameters as in Figure 3; $n=5$). The responses following 1 mg kg^{-1}, 5 mg kg^{-1} and 10 mg kg^{-1} SC58125 given at 1 ml kg^{-1} by slow intravenous injection were the averages of the responses to the 6 trains post-injection. Intravenous SC58125 dose-dependently reduced the total magnitude of the C-fibre wind-up (*$P<0.05$ at 5 mg kg^{-1}, **$P<0.005$ at 10 mg kg^{-1}). Intravenous vehicle (data not shown) had no effect on the C-fibre wind-up ($P>0.05$). The data were calculated by taking the maximal response (i.e. response to the 20th stimulus) during the control trains as the measure (100%) against which other responses are calculated. Values are means and vertical lines show s.e.mean.

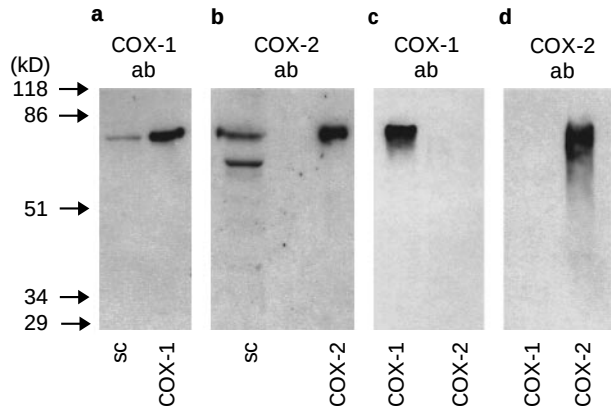

Figure 5 Western blot of proteins extracted from lumbar (segments L1-L6) spinal cord. In (a), spinal cord protein extract (sc; left) and purified COX-1 (right) have been incubated in mouse anti-sheep COX-1 antibody. In (b), the same spinal cord protein sample (left) and purified sheep COX-2 (right) have been incubated in rabbit anti-mouse COX-2 antibody. In (c) and (d) the specificity of the antibody for purified sheep COX-1 and COX-2 proteins (1 μg/lane) are shown, where the blots have been incubated with the COX-1 (c) or COX-2 (d) antibody. COX-1 and COX-2 isoforms ran to approximately 75 kD in a 10% polyacrylamide gel. A second band was present at 68 kD in spinal cord samples with the COX-2 antibody (b).

polyclonal COX-1 antibody. A protein band was present in both lanes at 75 kD consistent with the presence of COX-1 in the sample. Figure 5b is similar and shows the same protein extract (left lane) from rat spinal cord run alongside purified sheep COX-2 protein (right lane) in a blot that has been incubated in COX-2 antibody. A single protein band was present in the left hand lane at 75 kD whilst the right lane showed a double band at 75 kD and 68 kD (see Discussion). The antibody specificity was determined with purified sheep COX-1 and COX-2 by Western blotting. Figure 5c shows that the mouse anti-sheep monoclonal cox-1 antibody detected cox-1 but not COX-2 and that the rabbit anti-mouse COX-2 antibody detected COX-2 but not COX-1 (Figure 5d).

Immunocytochemistry

COX-1-li was generally absent from the rat spinal cord except for a few fibres which were observed in the dorsal root entry zone and in the superficial dorsal horn. In the dorsal roots (which were not trimmed away from the spinal cord before sectioning) many axons showed COX-1-li which led us to examine the COX proteins present in dorsal root ganglia (DRG). Figure 6a shows the random distribution of cox-1 immunopositive neuronal cell bodies in an L5 DRG. Figure 6b, c & d are typical examples of this COX-1-li which was present mainly in small to medium sized cell bodies (<1000 μm^2). The distribution of COX-1-li in these cells was typically cytoplasmic although the nuclear membrane was also densely labelled in many cell bodies (Figure 6c) as well as a number of axonal projections (Figure 6d). Omission of the primary antibody from the incubation medium resulted in a reduction of immunofluorescent labelling to background levels (Figure 6e).

COX-2 immunolabelling showed a different distribution from COX-1 immunolabelling in that it was not present in DRG but was present in the spinal cord. Figure 7 shows two montages of rat spinal cord from spinal segments L3 (Figure 7a and L5 (Figure 7b) which were incubated with a COX-2 polyclonal antibody. The antibody labelling, demonstrated in Figure 7a was symmetrical and showed COX-2-li present in the superficial dorsal horn, (laminae I and II) on both the left and right sides, and around the central canal (lamina X). Some diffuse labelling was also seen in the deep dorsal horn around the medial aspect of laminae V and VI (Figure 7a). In Figure 7b the distribution of COX-2-li differs from that in Figure 7a as a result of the change in shape of the grey matter in this segment. Pronounced COX-2-li was again observed in the superficial dorsal horn, although in this section the labelling around the central canal was much less intense. Omission of the primary antibody from the incubation medium resulted in a reduction of immunofluorescent labelling to background levels (Figure 7c).

COX-2-li was restricted mainly to the cytoplasm of individual neurones as shown in Figure 8a, c, d, e. The high density of neurones in the superficial dorsal horn made it difficult to distinguish single neurones but in regions where the neurones were less densely packed, e.g. ventral horn (Figure 8a, e), lamina X (Figure 8c) and deep dorsal horn (Figure 8d), COX-2-li was clearly present in individual neuronal cell bodies. In some motoneurones a halo was observed round individual cell bodies which may represent either plasma membrane labelling or the enhancement of labelling at the edge of cells within the section. In addition to specific neuronal labelling some diffuse interstitial labelling was also present throughout grey matter. This was more intense than the background labelling observed in the white matter and may represent the labelling of glial cells in the grey matter. Propidium iodide was used in some sections (Figure 8c, d, e) to indicate the position of individual nuclei which appear as the diffuse, high intensity punctate staining present in these sections.

Basal prostaglandin release from the spinal cord

The presence of both COX-1 and COX-2 isoforms in the spinal cord suggested that prostaglandins are synthesized and released from the spinal cord since no mechanism is known to exist for the storage of such lipophilic molecules. Superfusion of the spinal cord in the absence of any intentional stimulus revealed the presence of prostaglandins within the spinal superfusate. Table 1 shows the results of assays of spinal cord superfusates obtained from normal rats. The first column shows the minimum assay sensitivity and the second column, the absolute mean concentration of each prostaglandin species in each tube determined by radioimmunoassay. Since the flow rate of the superfusion varied between experiments, the final column gives the prostaglandin concentration expressed per unit time. These experiments showed that PGE_2 and PGD_2 are present in measurable concentrations in the spinal cord superfusates, but that $PGF_{2\alpha}$ and PGI_2 (measured as the metabolite, 6-keto-$PGF_{1\alpha}$) are present at lower concentrations which were close to the minimum sensitivity of the assay.

Discussion

The results presented in this paper illustrate several important points. Firstly, the wind-up of a spinal nociceptive reflex (but not the responses of individual spinal cord neurones to noxious mechanical stimuli) is reduced by intravenous and intrathecal administration of the non-selective COX inhibitor, indomethacin, and by intravenous administration of a selective COX-2 inhibitor, SC58125. Secondly, COX proteins were found to be present in the spinal cord, and in the case of COX-2, in locations consistent with an involvement in nociceptive processing, i.e. superficial dorsal horn. Thirdly, COX-1-li was shown to be present in the small to medium sized cell bodies in the DRG. Fourthly, measurable concentrations of PGE_2 and PGD_2 are released from the spinal cord indicating an active synthetic pathway.

Electrophysiological experiments

Pain-related behaviours (Malmberg & Yaksh, 1992) and dorsal horn neurone activity (Chapman & Dickenson, 1992) in the formalin paw model of inflammation, and the responses of hyperexcitable dorsal horn neurones in kaolin and carrageenan-induced arthritis in the knee (Neugebauer *et al*., 1994), are all reduced by a central action of non-selective NSAIDs, i.e.

those which do not show marked selectivity in their ability to block either COX isoform. However, in a recent study the latency of the hot plate test was unaffected by intrathecally administered indomethacin and NS398 (selective COX-2 inhibitor) in normal rats, whereas both drugs reduced pain related behaviours in the formalin test (Yamamoto & Nozaki-

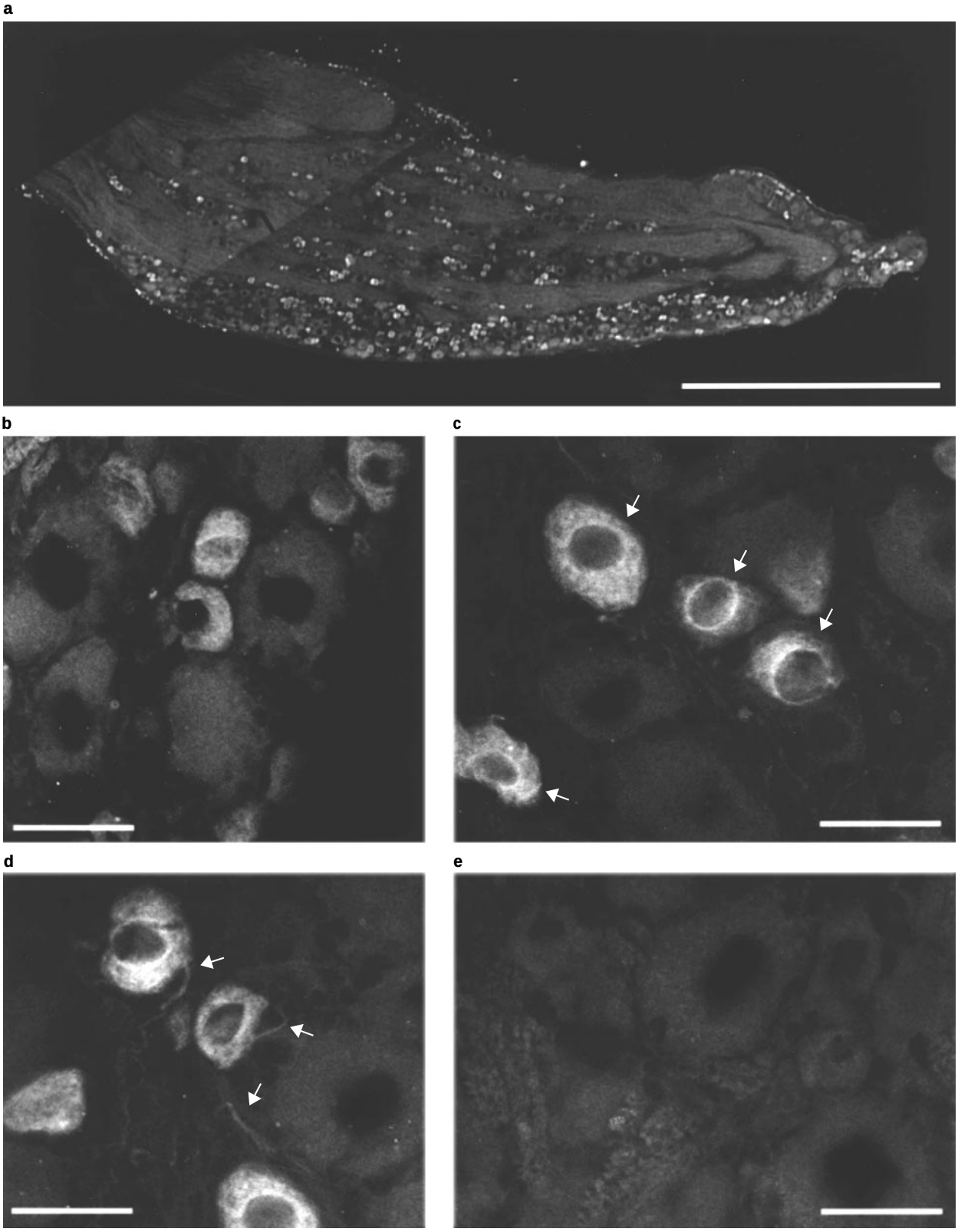

Figure 6 Rat dorsal root ganglia immunocytochemistry for COX-1. Ten-micron sections of L5 rat dorsal root ganglion were incubated with a mouse anti-sheep COX-1 antibody and an FITC-coupled secondary antibody. A montage (a) of an L5 rat dorsal root ganglion, taken with a confocal laser scanning microscope, shows numerous labelled cell bodies throughout the ganglion (scale bar = 1 mm). COX-1-li was restricted primarily to the small and medium sized cell bodies and was almost absent from larger cells (b). COX-1-li was found mainly in the cytoplasm and the perinuclear region (c, arrows) although individual axonal processes were also clearly labelled (d, arrows). Omission of the primary antibody from the incubation medium reduced staining to background levels (e). (Scale bar = 25 μm, b, c, d, e).

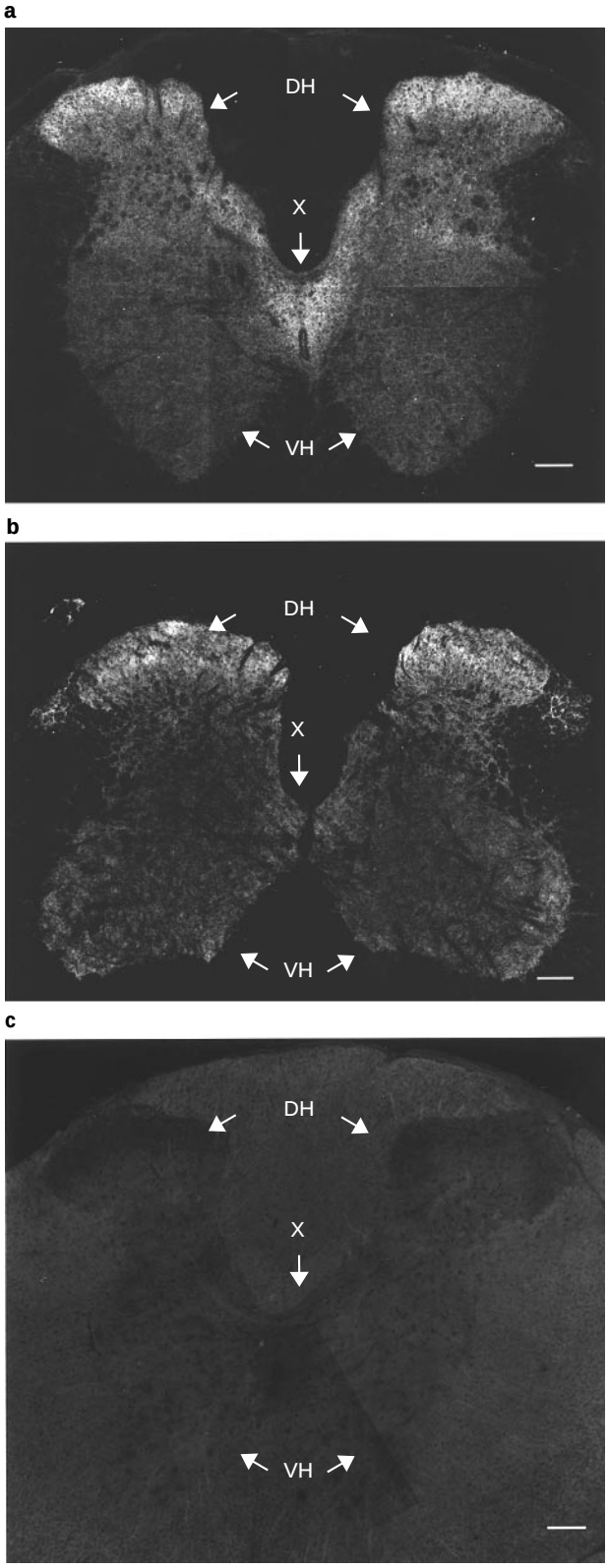

Figure 7 Three composite confocal photomicrographs of rat lumbar spinal cord illustrating COX-2-li immunoreactivity. Fifteen-micron transverse sections at the level of L3 (a) and L5 (b) were incubated in a rabbit anti-mouse COX-2 antibody and an FITC-coupled secondary antibody. COX-2-li was present on both the left and right sides of the superficial dorsal horn (DH) (a, b) and around the central canal in lamina X (a). Diffuse COX-2-li can also be seen in the deep dorsal horn around laminae IV and V (a). (c) Shows a 15 μm control section from L5 spinal cord where primary antibody was omitted from the protocol. (VH, ventral horn; scale bars = 200 μm).

Taguchi, 1996). These data are compatible with the results obtained in the present experiments where indomethacin (i.v. and i.t.) and SC58125 (i.v.) had no effect on the baseline C-fibre evoked reflex response, nor on the responses of individual dorsal horn neurones to noxious mechanical stimulation applied to the ankle or knee joint, but did reduce the wind-up of C-fibre evoked reflexes, i.e. a situation where spinal hyperexcitability had been intentionally induced. Taken together, these data suggest that the processing of nociceptive inputs is not regulated by eicosanoids except in circumstances where neuronal hyperexcitability has been induced, e.g. C-fibre induced reflex wind-up, inflammation-induced hyperexcitability (Malmberg & Yaksh, 1992; Chapman & Dickenson, 1992; Neugebauer *et al*., 1994). However, it should be noted that one of these studies found the wind-up of individual dorsal horn neurones in normal rats to be unaffected by intrathecally applied indomethacin at concentrations comparable to, or higher than, those used in this study (Chapman & Dickenson, 1992). One might speculate that this discrepancy arises from either different recording configurations used, i.e. larger effects of indomethacin may be seen in the reflex than on individual dorsal horn neurones, or the different anaesthetics used.

The IC_{50} of indomethacin in COX inhibition assays is approximately 10 times lower for COX-1 than COX-2 (Gierse *et al*., 1996). This relative lack of selectivity means that indomethacin will inhibit both the COX-1 and COX-2 isoforms which were found to be present in the spinal cord of normal rats. SC58125, which selectively inhibits the COX-2 isoform in cox inhibition assays *in vitro* (IC_{50}: COX-1 = >100 μM, COX-2 = 0.04 μM, Gierse *et al*., 1996) and *in vivo* (ED_{50}: COX-1 = >10 mg kg^{-1}, COX-2 = 10 mg kg^{-1}, Seibert *et al*., 1994), was also very effective in reducing wind-up, and had no effect on the baseline reflex, indicating that it also had a significant central action. These data are consistent with the effects of another selective COX-2 inhibitor, NS398, on pain-related behaviours in the formalin test (Yamamoto & Nozaki-Taguchi, 1996). Unfortunately, low solubility and problems with vehicle toxicity prevented us from testing this compound intrathecally. Taken together these results strongly suggest that the development of wind-up can be inhibited by spinal COX inhibition, a component of which may be due to inhibition of the COX-2 isoform.

The lack of effect of any NSAIDs on the baseline reflex indicates that the surgery involved in the experimental paradigms described here did not significantly affect the excitability of spinal cord neurones, since the magnitude of the baseline reflex remained unchanged during an experiment and was unaffected by intravenously applied indomethacin. These are important observations since several factors, including the amount of surgical intervention and the depth of anaesthesia, can have profound effects in this type of experiment due to activation of peripheral nociceptors and influences on the excitability of spinal cord neurones (Hartell & Headley, 1989, 1991; Clarke & Matthews, 1990).

Presence and localization of COX isoforms

COX-1 isoform Northern blot analysis has shown that mRNAs encoding COX-1 and COX-2 are present in normal spinal cord (Beiche *et al*., 1996; Hay *et al*., 1997) and the present study now indicates that the respective proteins are also present. COX-1 expression occurs in brain (O'Neill and Ford-Hutchinson, 1993; Seibert *et al*., 1994), but only at very low levels, which may explain the long exposure times required to demonstrate the protein in spinal cord samples in Western blotting experiments. This may also explain why relatively little specific COX-1-li was identified in tissue sections of spinal cord incubated with the COX-1 antibody although this may have been hampered by the fact that the sections remained hydrated and uncleared for immunofluorescence microscopy. However, interestingly, the same antibody revealed very clear COX-1-li in small to medium sized cell bodies in the DRG from normal animals. Many neurones of this size (below

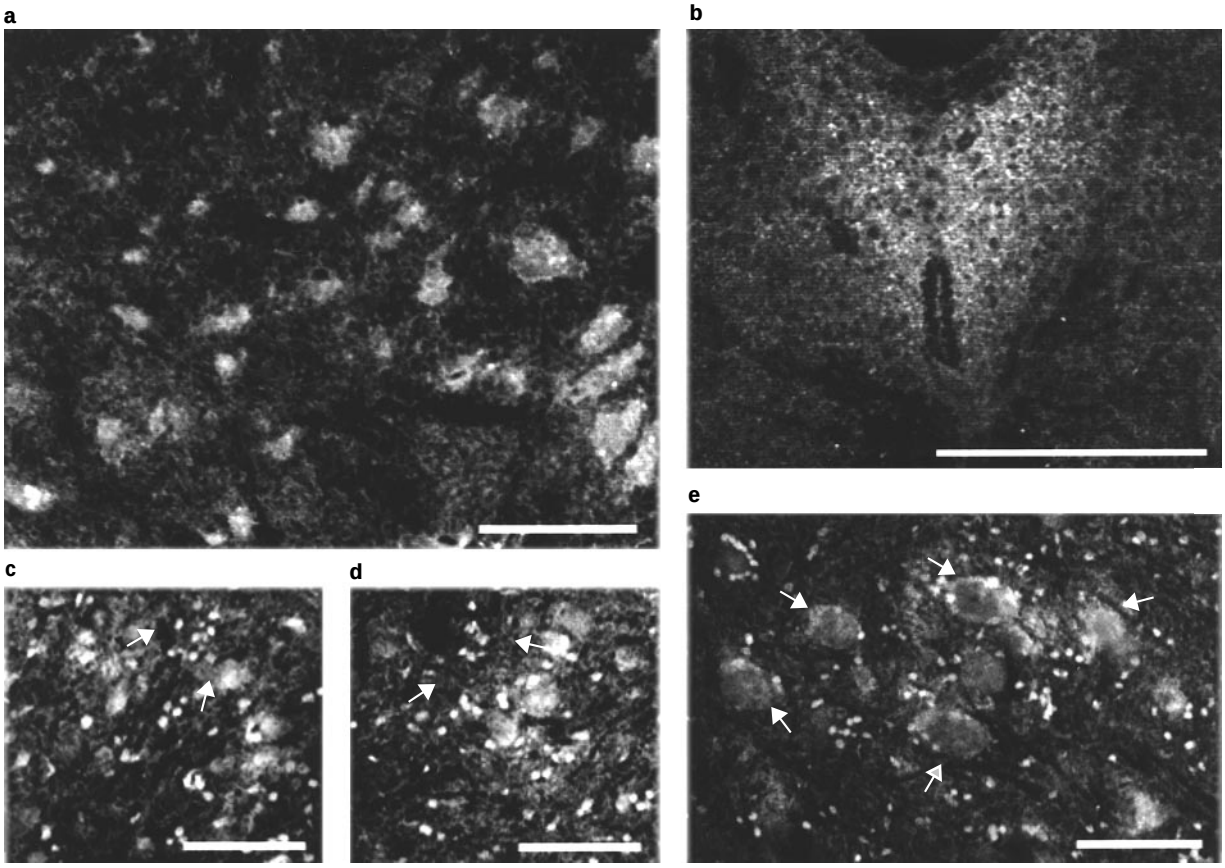

Figure 8 Five confocal photomicrographs of rat lumbar spinal cord illustrating COX-2-li immunoreactivity in different regions of the spinal cord. Fifteen-micron transverse sections of L3 spinal cord were incubated in a rabbit anti-mouse COX-2 antibody and an FITC-coupled secondary antibody. (a) and (e) Regions of ventral horn of spinal cord which show clear COX-2-li in individual neurones. (b) The regions around the central canal which were often shown to have strong COX-2-li. A number of neurones around the central canal (c) and in the deep dorsal horn (d) were also labelled. (c), (d) and (e) have been counterstained with the nuclear stain propidium iodide which is responsible for the punctate staining observed in these photographs. (Scale bars: a, c, d, e = 25 μm; b = 75 μm).

Table 1 Basal prostaglandin release from rat spinal cord

	Assay sensitivity (pg/tube)	*Mean concentration* (pg/tube)	*Mean basal release* (pg ml^{-1} min^{-1})
PGD_2	12.0 ± 1.5	142.9 ± 18.2	294.1 ± 22.1 (n = 4)
PGE_2	3.7 ± 0.9	79.9 ± 9.9	160.0 ± 17.7 (n = 13)
6-keto-$PGF_{1\alpha}$	3.6 ± 0.7	22.5 ± 4.4	47.6 ± 6.7 (n = 6)
$PGF_{2\alpha}$	4.7 ± 0.9	12.1 ± 0.9	23.3 ± 2.1 (n = 14)

The values (mean ± s.e.mean) for the basal presence of PGE_2, PGD_2, 6-keto-$PGF_{1\alpha}$ (the stable metabolite of PGI_2) and $PGF_{2\alpha}$ in spinal cord superfusate samples are shown in pg ml^{-1} min^{-1}. Numbers in parentheses represent the number of animals from which the samples were obtained.

1000 μm^2) are known to contain calcitonin gene-related peptide (CGRP) (Lawson *et al*., 1993; Hanesch *et al*., 1993) and substance P (Lawson *et al*., 1993), and this is one method used to classify them as nociceptors. Further studies are needed to reveal the extent of co-localization of COX-1 with other neuropeptides known to be present in nociceptors. The subcellular distribution of COX-1-li observed in the present study is identical to that observed in other cells, i.e. mainly in cytoplasm (probably endoplasmic reticulum), nuclear membrane and some axonal processes (Breder *et al*., 1992; Morita *et al*., 1995), indicating the highly specific binding of this antibody.

The function of the COX-1 protein identified in DRG cell bodies and in some axons of putative nociceptors is unclear, although one might speculate that these cells could synthesize prostaglandins at their axon terminals. The evidence for this at central terminals is relatively weak since we found very few COX-1 immunopositive fibres in the superficial dorsal horn (although many fibres in the dorsal roots appeared labelled). Whether prostaglandins synthesized at the peripheral terminals of the same cells could release prostaglandins as part of the inflammatory process is an intriguing proposition, since eicosanoids are generally thought to originate from damaged cells in tissues or from immunocompetent cells which invade the tissue after damage.

Technical considerations The monoclonal mouse anti-sheep antibody used in this study was highly selective for sheep COX-1 and did not crossreact with sheep COX-2. Technical data for this antibody indicated that the antibody crossreacted with COX-1 in several species including rat but showed little or no crossreactivity with COX-2 in any species tested. The anatomical distribution of COX-1 was very different from that of COX-2 and the subcellular distribution is entirely consistent with existing studies in other tissues indicating that the COX-1-li demonstrated in this study most likely reflects the distribution of the COX-1 isoform.

Cox 2 isoform In normal brain, it is somewhat inexact to term COX-2 an inducible enzyme since it is the predominant isoform in this tissue (Feng *et al*., 1993; Seibert *et al*., 1994). The identification of COX-2 protein in normal rat spinal cord in the present study reflects the situation in brain and confirms

previous studies which have shown COX-2 mRNA to be present in normal rat spinal cord (Beiche *et al*., 1996; Hay *et al*., 1997). Furthermore, the brief time required for tissue removal (3–5 min) is much too short for *de novo* COX-2 protein synthesis (Gardiner *et al*., 1997).

The distribution of COX-2-li in the superficial (and to a lesser extent the deep) layers of the dorsal horn of spinal cord is consistent with COX products being involved in the processing of nociceptive input. Within the superficial dorsal horn, it was difficult to identify individual neuronal cell bodies due to the dense packing but in other regions, e.g. laminae V/VI, VIII, X, most of the COX-2-li was neuronal and restricted to the cell cytoplasm, a subcellular distribution that is consistent with previous studies in brain (Breder *et al*., 1995; Morita *et al*., 1995) and cultured cells (Kaufmann *et al*., 1996). The more diffuse COX-2-li observed throughout the grey matter may be due to labelling of glial cells which would be consistent with other studies, which have shown that both glial cells and neurones within the brain (Bishai & Coceani, 1992) are able to synthesise a variety of eicosanoids including PGE_2 and that cultured rat hippocampal astrocytes (Amruthesh *et al*., 1993) and microglia (Bauer *et al*., 1997) have the ability to metabolise arachidonic acid through the COX pathway. Further subcellular analysis of COX distribution using electron microscopy will have to be performed to examine these points further.

Technical considerations The rabbit anti-mouse COX-2 antibody detected sheep COX-2 but not sheep COX-1 in Western blotting experiments and given that there is 60% amino acid homology between these isoforms, the antibody shows high specificity for the COX-2 isoform. When used to determine COX-2 presence in Western blotting experiments two bands were observed, one at 75 kD, which ran to the same molecular weight as purified sheep COX-2, and a second one at 68 kD. Unpurified COX-2 proteins derived from cell lysates or tissue typically produce a double or triple band in Western blots which are found between 68–75 kD. The explanation for this multiple banding may be partial degradation resulting from sample preparation or, more likely, deglycosylation, which occurs naturally (O'Bannion *et al*., 1991; Feng *et al*., 1993; Habib *et al*., 1993; Percival *et al*., 1994; Kaufmann *et al*., 1996) or may also occur during sample preparation. The subcellular distribution of COX-2-li observed in these experiments is similar to that observed in other neuroanatomical studies of COX-2 distribution (Breder *et al*., 1992, 1995), strongly suggesting that the COX-2-li observed in these experiments reflects the distribution of COX-2.

Superfusion experiments

The presence of both COX isoforms and measurable concentrations of PGE_2 and PGD_2 suggest that eicosanoids may play a role in information processing. These data agree with other studies which have shown PGE_2 and PGD_2 to be the predominant species in brain (Abdel-Halim *et al*., 1977; Abdel-Halim & Änggård, 1979; Bishai & Coceani, 1992; Pandey *et al*., 1995) and further studies are in progress to examine the stimulus-dependency of release for each prostaglandin species in normal rats. Several studies have implicated PGE_2 as the major prostaglandin involved in spinal nociceptive processing and there is evidence that PGE_2 is released from rat spinal cord in response to a number of stimuli (Malmberg & Yaksh, 1994; Sorkin & Moore, 1996) and that PGE_2 can release neurotransmitters, including substance P, from cultured dorsal root ganglion cells (Nicol *et al*., 1992; Vasko *et al*., 1993). However, there is no reason to suppose that PGE_2 is the only prostaglandin metabolite produced by spinal cord neurones and the production of individual species will depend on the relative activities of the different enzymes involved in the conversion of PGH_2 into active metabolites. In agreement with our superfusion experiments, immunocytochemical studies (Urade *et al*., 1989; Vesin *et al*., 1995) have shown prostaglandin D synthase-li in perikarya of lamina II and III neurones of rat and chick spinal cord and prostaglandin D synthase activity in chick spinal cord (Vesin *et al*., 1995). The role of PGD_2 in the spinal cord is unknown, although there has been a suggestion that it might be involved in the control of local blood flow (Vesin *et al*., 1995). More interesting perhaps is the overlapping distribution of prostaglandin D synthase and COX-2 in the superficial dorsal horn of the spinal cord, which may suggest a role for both enzymes in spinal nociceptive processing.

In experiments where the brain and spinal cord are superfused, it is important to avoid damage to ensure that eicosanoids are not released from ischaemic (Gaudet *et al*., 1980; Petroni *et al*., 1989) or haemorrhaging (Rodriguez y Baena *et al*., 1988) CNS tissue since this could contaminate the samples. This is a potential problem in experiments where prostaglandin measurements are made with microdialysis catheters which, due to their mode of action, must be inserted into the neuronal tissue (Marsala *et al*., 1995; Lazarewicz & Saliñska, 1995; Sorkin & Moore, 1996). In our experiments we were careful to ensure that no blood products came into contact with the superfusate and that no damage occurred to the spinal cord or any of the superficial blood vessels. We can also be reasonably confident that prostaglandins released from the surgical site into the circulation would not cross into the CSF since the blood–brain barrier largely prevents this (Jones *et al*., 1994).

In summary, the data presented here show that the biosynthetic machinery required for prostaglandin synthesis is present in the spinal cord of normal rats, in locations consistent with an involvement in spinal nociception. The reduction in C-fibre evoked reflex wind-up, but not of the responses of spinal cord neurones to noxious mechanical stimuli, by NSAIDs indicates that prostaglandins may play a role in the development of spinal hyperexcitability. This hypothesis indicates a special role for prostaglandins in spinal nociceptive processing in situations where spinal hyperexcitability is particularly evident, e.g. following the development of peripheral inflammatory lesions, and explains why the effects of prostaglandins have been most widely described under these circumstances.

This work was supported by The Wellcome Trust. N.J.G. was in receipt of a University of Leicester PhD studentship.

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(Received July 17, 1997
Revised September 8, 1997
Accepted September 15, 1997)