British Journal of Pharmacology (2003) 139, 672–680
www.nature.com/bjp

Prostaglandin E_2 increases the expression of the neurokinin$_1$ receptor in adult sensory neurones in culture: a novel role of prostaglandins

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1 Peripheral inflammation causes an increase in the proportion of primary afferent neurones that express neurokinin$_1$ (NK_1) receptors for substance P (SP). This upregulation may contribute to the neuronal mechanisms of inflammatory pain. The aim of this study was to identify endogenous mediators that stimulate upregulation of NK_1 receptors in dorsal root ganglion (DRG) neurones. Cultured DRG neurones from the adult normal rat were exposed for 2 days to media that contained specific mediators, namely potassium in high concentration, prostaglandin E_2 (PGE_2), somatostatin (SRIF), and compounds influencing second messenger cascades. After fixation neurones were labelled with an NK_1 receptor antibody.

2 Repetitive addition of the inflammatory mediator PGE_2 or dibutyryl-cyclic adenosine 3′,5′ monophophate (db-cAMP) to the culture medium enhanced the proportion of neurones with NK_1 receptor-like immunoreactivity from about 12% up to 40%. PGE_2-induced upregulation was prevented by coadministration of PGE_2 and a protein kinase A inhibitor or SRIF to the medium. High potassium concentration, protein kinase C inhibitors and omission of nerve growth factor from the medium had no effect.

3 In calcium-imaging experiments, bath application of SP evoked increases of the intracellular calcium concentration in about 20% of the neurones. This proportion increased to about 40% after PGE_2-pretreatment, but the increase was prevented when PGE_2 and SRIF were coadministered to the medium.

4 These data show that the expression of NK_1 receptor-like immunoreactivity in DRG neurones is regulated by the inflammatory mediator PGE_2. This upregulation depends on the intracellular adenylyl cyclase – protein kinase A pathway.
British Journal of Pharmacology (2003) **139,** 672 – 680. doi:10.1038/sj.bjp.0705278

Keywords: Neurokinin$_1$ receptor; prostaglandin E_2; PKA; PKC; cAMP; SRIF

Abbreviations: cAMP, cyclic adenosine 3′,5′monophosphate; db-cAMP, dibutyryl-cAMP; DRG, dorsal root ganglion; EP, Receptor for PGE_2; IR, immunoreactivity; NK_1 receptor, neurokinin$_1$ receptor; PGE_2, prostaglandin E_2; PKA, protein kinase A; PKC, protein kinase C; SP, substance P; SRIF, somatostatin

Introduction

The substance P (SP) – neurokinin$_1$ (NK_1) receptor system contributes to the generation of pain, particularly under inflammatory conditions. SP is synthetised in a proportion of nociceptive primary afferent neurones and is released upon noxious stimulation. SP release from sensory endings produces neurogenic inflammation through NK_1 receptors in peripheral tissue, and SP release from spinal endings enhances excitability of spinal cord neurones through NK_1 receptors in the dorsal horn (Schaible and Grubb, 1993; Millan, 1999; Harrison and Geppetti, 2001).

While NK_1 receptors are heavily expressed in the spinal cord (Mantyh *et al*., 1995) several approaches have also shown some NK_1 receptor localization in primary afferent fibres and dorsal root ganglion (DRG) neurones (Andoh *et al*., 1996; Hu *et al*., 1997; Brechenmacher *et al*., 1998; Li and Zhao, 1998; Segond von Banchet *et al*., 1999; Szucs *et al*., 1999; but see Yashpal *et al*., 1991; McCarson and Krause, 1994; Brown *et al*., 1995). In cultured DRG neurones, NK_1 receptors are mainly present in small-sized unmyelinated neurones, and they are often colocalized with SP (Segond von Banchet and Schaible, 1999). DRG neurones with NK_1 receptors are usually capsaicin-responsive, indicating that they are nociceptive (Brechenmacher *et al*., 1998). Local application of SP to the knee joint sensitizes articular nociceptors to mechanical stimuli (Herbert and Schmidt, 2001; Pawlak *et al*., 2001), supporting a role of NK_1 receptors in primary afferent neurones *in vivo*. On the other hand, NK_1 autoreceptors on primary afferent neurones may reduce release of SP (Malcangio and Bowery, 1999).

During peripheral inflammation the synthesis of SP in DRG neurones is upregulated (Donnerer *et al*., 1992; Garrett *et al*., 1995). Recently, upregulation has also been shown for NK_1 receptors in rat primary afferent neurones. At least in the first 10 days of antigen-induced arthritis, a higher proportion of DRG neurones show NK_1 receptor-like IR (Segond von Banchet *et al*., 2000), and during complete Freund's adjuvant (CFA) induced inflammation a higher proportion of

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primary afferent fibres in the glabrous skin express NK_1 receptors (Carlton and Coggeshall, 2002). It has not been identified as to which stimuli induce upregulation of NK_1 receptors. Candidates are neurotrophic factors such as the nerve growth factor (NGF) because NGF regulates synthesis of SP (Lindsay and Harmar, 1989; Donnerer *et al*., 1993; Ji *et al*., 1996) and bradykinin receptors (Rueff *et al*., 1996; Petersen *et al*., 1998; Kasai and Mizumura, 1999). Since some mediators in the central nervous system not only increase short-term sensitivity in neurones but also induce neuronal long-term changes, we reasoned whether expression of NK_1 receptors may also be influenced by inflammatory mediators such as prostaglandins (PGs) that activate and sensitize nociceptors (Ferreira *et al*., 1988; Grubb *et al*., 1991; Cunha *et al*., 1992; Nicol *et al*., 1997). To address this question, we cultured DRG neurones from adult normal rats in media containing the inflammatory mediator PGE_2 and other compounds of interest. Then we determined the proportion of neurones with NK_1 receptor-like immunoreactivity (IR), and we used the calcium-imaging technique to assess SP-induced increases of intracellular calcium. This approach is based on our previous observation that lumbar DRG neurones from rats with antigen-induced arthritis show a pronounced up-regulation of NK_1 receptors in the first 10 days of inflammation (Segond von Banchet *et al*., 2000). Here we show that repetitive addition of PGE_2 to the culture medium enhances the expression of NK_1 receptors in DRG neurones. This effect is mimicked by dibutyryl-cyclic adenosine 3′5′ monophosphate (db-cAMP) and prevented by inhibition of protein kinase A or by coadministration of somatostatin (SRIF). Depolarization of neurones with high potassium concentration, inhibition of protein kinase C (PKC) and omission of NGF from the medium had no effect.

Methods

Primary culture

Male Wistar rats, 60 days old, were killed with a lethal dose of ether, and DRGs from all segments of the spinal cord were dissected. Ganglia were incubated at 37°C with 215 U ml^{-1} collagenase type II dissolved in Ham's F-12 medium for 100 min. After washing with Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS), the ganglia were placed in Dulbecco's modified Eagle's medium (DMEM) containing 10,000 U ml^{-1} trypsin for 11 min at 37°C. The cells were dispersed by gentle agitation and aspiration with a fire-polished Pasteur-pipette. The dispersed cells were collected by centrifugation ($500 \times g$, 5 min), washed three times in DMEM and centrifuged. The obtained cell pellets were suspended in Ham's F-12 medium containing 10% heat-inactivated horse serum, 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, and in most cases 100 ng ml^{-1} NGF. Cells were plated on poly-L-lysine (200 μg ml^{-1})-coated glass coverslips (diameter 13 mm) and maintained for 3 days at 37°C in a humidified incubator gassed with 3.5% CO_2 and air. The standard medium was replaced every day.

To examine whether the expression of the NK_1 receptor is influenced by mediators, single or several compounds were added to the standard medium after the initial overnight setting period. The substances were added to the neurones from the second day for 2 days every 2 h (with a break between 8:00 p.m. and 8:00 a.m.). The following compounds were used: (a) 10^{-8} M or 10^{-6} M prostaglandin E_2 (PGE_2), (b) 10^{-6} M somatostatin (SRIF), (c) 10^{-6} M SRIF together with 10^{-8} M PGE_2, (d) 50 mM K^+ for depolarization. In some cases, standard medium without NGF was used. In order to exclude effects of the heat-inactivated horse serum on the expression of NK_1 receptor-like IR, we performed experiments in which we left out the heat-inactivated horse serum from the medium. To test whether the expression of NK_1 receptors depends on the activation of cAMP or protein kinase A (PKA), neurones were cultured in a medium containing 10^{-6} M db-cAMP or the PKA inhibitor H-89 (10^{-6} M). In addition, neurones were cultured together with a PKC inhibitor (Myr-RFARKGALRQKNV, 10^{-6} M). In order to test the importance of these second messenger pathways for the effects of PGE_2, these compounds were also coadministered with 10^{-6} M PGE_2. Finally, some experiments were added in which we made only a single application of PGE_2 (10^{-6} M). Here, the expression of NK_1 receptor-like IR was assessed at either 15, 60 or 120 min after PGE_2 administration to test for rapid effects of PGE_2.

Immunocytochemical experiments

For immunocytochemistry, cells were fixed and then labelled. The coverslips were transferred into 2% paraformaldehyde and 0.05% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) plus 0.3% Triton X-100 for 30 min. After washing with PBS plus 0.3% Triton X-100 (PBS TX-100), they were incubated with 50 mM glycine in PBS TX-100 and thereafter with 5% bovine serum albumin (BSA) and 0.1% gelatine in PBS TX-100 for 30 min. Then the cells were washed with PBS TX-100 and incubated for 30 min in PBS TX-100 containing 2% normal goat serum. Thereafter, the cells were washed with PBS TX-100 containing 0.1% acetylated BSA (BSA-C) and incubated overnight with an anti-NK_1 antibody diluted 1 : 100 in PBS TX-100 plus 1% normal goat serum at 4°C in a moist chamber (the antibody was developed in rabbit, against NK_1 receptor peptide, amino acids 393 – 407, rat). The coverslips were extensively rinsed in PBS TX-100 plus 0.1% BSA-C and thereafter in PBS TX-100. After washing the cells were incubated for 4 hours at 20°C with a gold-labelled (10 nm) anti-rabbit antibody developed in goat, diluted 1 : 100 in PBS TX-100 plus 1% normal goat serum. After washing with PBS TX-100, PBS and ddH_2O, the gold particles were intensified with silver enhancer (R-Gent, pH 5.5) for 20 min at 21°C. The reaction was stopped by washing in ddH_2O. To test for specificity of the detection system, cells were incubated only with the secondary antibody (see Figure 1a).

Analysis of immunocytochemical data

From each cover slip, 100 structurally intact neurones were examined with a light microscope (Axioplan 2, Zeiss, Germany) coupled to a CCD video camera and an image analysing system (KS 300, Zeiss, Germany). The mean area and mean grey value were determined for each neuronal soma. To take into account differences in the basal grey values on each coated coverslip, a relative grey value of each neurone was calculated by dividing the mean grey value of the neurone through the grey value of the coverslip background. The relative grey value of the neurones had a range from 0 (= white) to 1 (= black). For an unbiased discrimination of

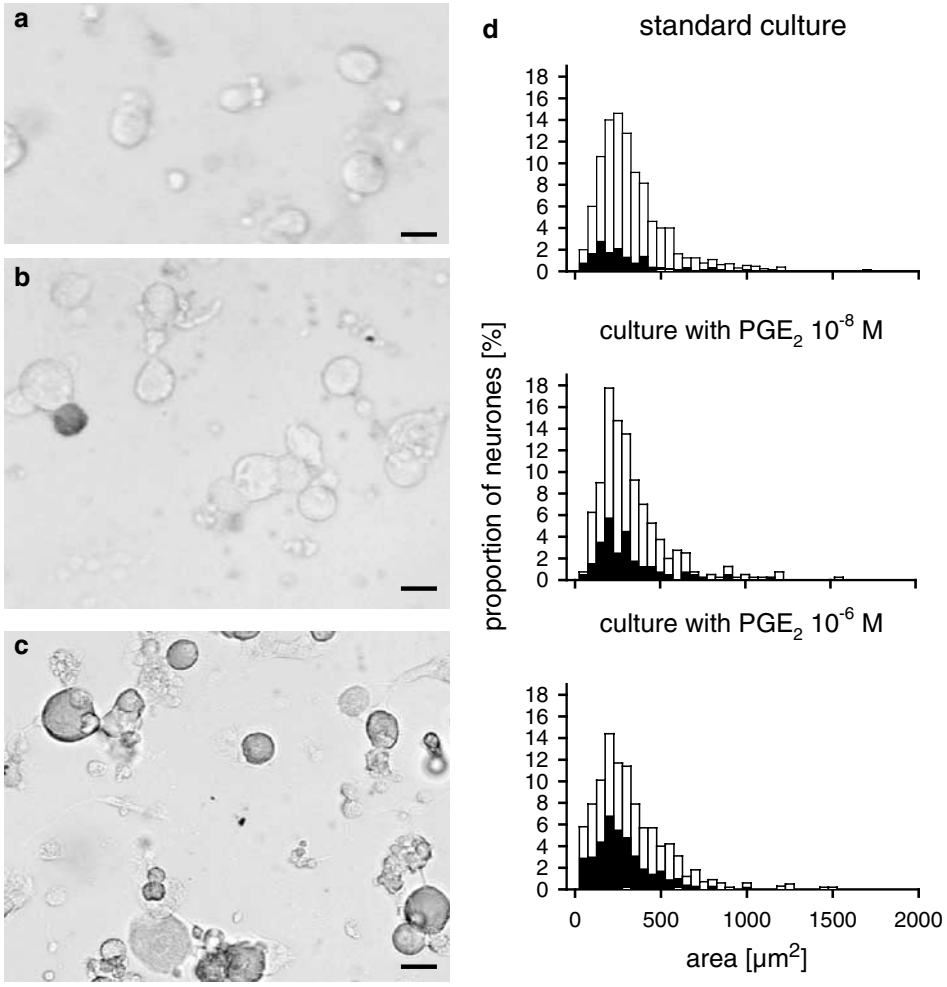

Figure 1 Photomicrographs showing NK_1 receptor-like IR in cultured DRG neurones. (a) Neurones from control incubations without anti-NK_1 receptor antibody. (b) Neurones cultured in a standard medium and then treated with the anti-NK_1 receptor antibody. Just one neurone was labelled for NK_1 receptor-like IR (dark cell). (c) DRG neurones cultured in standard medium plus 10^{-6} M PGE_2. The bar represents 20 μm. (d) Size distribution of neurones sampled (white bars) and of neurones with NK_1 receptor-like IR (black bars) under different conditions.

cells with or without positive labelling with the anti-NK1 antibody, all neurones were considered as positive that showed a relative grey value above that of neurones from the control incubations, which were not treated with the NK_1 receptor antibody. The value was 0.16, and thus all neurones with grey density > 0.16 were considered positive for NK_1 receptor-like IR. Proportions of labelled neurones are expressed as mean $\pm$s.d. Proportions of neurones with NK_1 receptor-like IR in different samples of neurones were compared using the χ^2 test ($P<0.05$ was accepted as significant) taking into account multiple comparisons when necessary.

Ca^{2+}-imaging

Cultured neurones were loaded with 5 μM Fura-2 acetoxymethylester (Fura-2/AM) dissolved in dimethyl sulphoxide (DMSO) and 0.02% pluronic-127 detergent, which remained on the coverslips for 30 min at 20°C. After incubation, the neurones were washed several times with HEPES buffer (150 mM NaCl, 5 mM KCl, 10 mM glucose, 10 mM HEPES, 2 mM $CaCl_2$, 2 mM $MgCl_2(6H_2O)$) and then left in this buffer solution for about 20 min to complete cytoplasmatic dye esterification. The glass coverslips with dye-loaded cells were mounted on the stage of a fluorescence microscope (Nikon) and visualized with an object lens (60 $\times$). The experimental chamber (volume 1 ml) was superfused with HEPES buffer at a rate of about 2 ml min^{-1}. All experiments were performed at 20 – 24°C. The perfusion was stopped before compounds were added to the bath.

The cells were illuminated alternately with light of 340 nm (specific for Fura-2 that has bound Ca^{2+}) and 380 nm (specific for Ca^{2+}-free Fura-2), and light of about 510 nm was collected via a cooled CCD camera. The photomultiplier was coupled to a personal computer for data acquisition. Data were collected every 260 ms and stored in sequential files. The resulting images were analysed using software from T.I.L.L. Photonics. In each experiment, 340 – 380 nm ratio values were calculated after subtraction of the background signal. To monitor the local Ca^{2+} from the ratio images, specific areas of interest were

chosen and the ratio value for the designated areas was averaged and plotted as a function of time.

Each experiment was started by stopping the superfusion. After 10 s, a test volume (100 μl) of the buffer solution was added to the bath, and 20 s later, 100 μl of a solution with SP (final concentration of 10^{-4} M) was applied to the bath. To check the vitality of the cultured neurones, the cells were exposed to 50 mM potassium at the end of the experiment. In another set of experiments the neurones were incubated with the NK_1 receptor antagonist SR 140333 (10^{-6} M) for 15 min before the experiments were started.

To test the influence of PGE_2 or SRIF on the SP-induced rise of $[Ca^{2+}]_i$, neurones were cultured with 10^{-6} M PGE_2 or with 10^{-6} M PGE_2 together with 10^{-6} M SRIF, or with 10^{-6} M PGE_2 together with 10^{-6} M SRIF plus cyclo-SRIF (10^{-6} M) for 2 days, for protocol see above. In total, 26 independent cultures of DRG neurones were used for the Ca^{2+}-imaging experiments, and 1031 neurones were analysed.

The following criteria have been used to identify a response to SP application. The change of $[Ca^{2+}]_i$ had to be well above spontaneous fluctuations. Since these were very small, we defined a change of 5% as a threshold for a positive effect. Further criteria were: latency between application of SP and start of the response $\leqslant$5 s, time to peak $\leqslant$5 s, pronounced reversibility within 50 s, no response to application of buffer alone, and response to application of 50 mM K^+ in buffer.

Drugs

R-Gent, BSA-C and db-cAMP were supplied by BioTrend (50876 Köln, Germany) and PGE_2 by Calbiochem (65796 Bad Soden, Germany). Ham's F-12 medium, DMEM, heat-inactivated horse serum, penicillin and streptomycin were supplied by GibcoBRL (76344 Eggenstein-Leopoldshafen, Germany). Collagenase type A and NGF (NGF 7S, recombinant mouse) were purchased from Paesel, Lorei (63452 Hanau, Germany). The anti-NK_1 receptor antibody, the protein kinase A inhibitor (H-89), the PKC inhibitor (Myr-RFARKGALRQKNV) and trypsin were supplied by Sigma (82024 Taufenkirchen, Germany). SRIF and cyclo-SRIF were purchased from Bachem (69126 Heidelberg, Germany). Fura-2/AM and the pluronic-127 detergent were supplied by Molecular Probes (2333 AA Leiden, The Netherlands). The NK_1 receptor antagonist SR140333 was a gift from Sanofi (75013 Paris, France). All other reagents were supplied by Fluka (89231 Neu-Ulm, Germany) or Sigma.

Results

NK_1 receptor-like IR in the presence or absence of inflammatory mediators

In the standard culture (medium containing 100 ng ml^{-1} NFG), only a small proportion of cultured DRG neurones showed NK_1 receptor-like IR. Figure 1b displays a cover slip with neurones cultured for 3 days. The dark neurone was labelled for NK_1 receptor-like IR. When standard cultures (3 days) from all experiments were pooled, on an average 11.8$\pm$2.3% of the DRG neurones (13 cultures) were labelled with the anti-NK_1 receptor antibody. Figure 1a shows

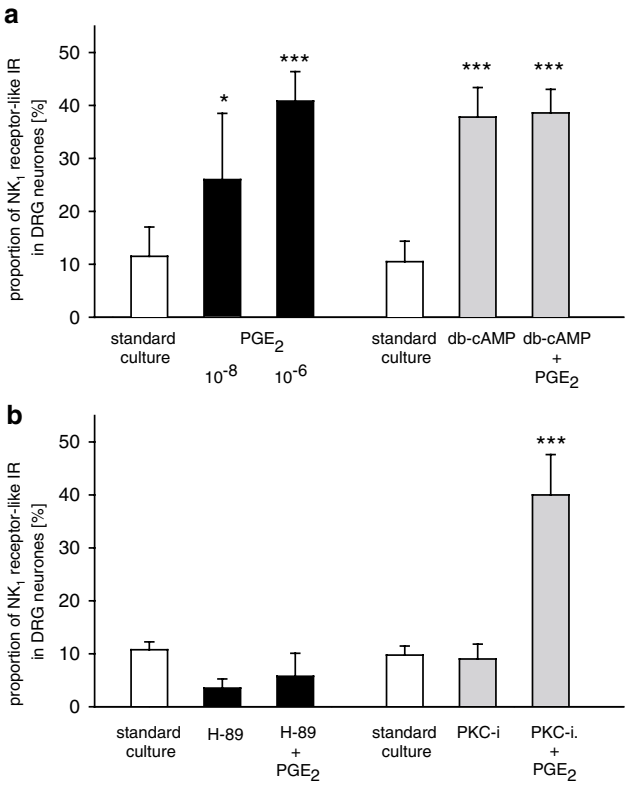

Figure 2 (a) Influence of PGE_2, db-cAMP, and coadministration of db-cAMP and PGE_2 on the proportion of DRG neurones with NK_1 receptor-like IR. (b) Influence of the PKA inhibitor H-89 and the PKC inhibitor (PKC-i) Myr-RFARKGALRQKNV and coadministration of either substance with PGE_2 on the proportions of DRG neurones with NK_1 receptor-like IR. Error bars indicate s.d. $^*P<0.05$, $^{***}P<0.001$.

neurones from control incubations without anti-NK_1 receptor antibody. In these cultures, no cells were labelled.

To test the effect of depolarization, 50 mM potassium was added to four cultures (Domann *et al*., 1997; Nicolson *et al*., 2002). This treatment did not change the proportion of neurones with NK_1 receptor-like IR (11.5$\pm$3.4% *versus* 11.5$\pm$1.9 % in four control cultures). Culturing of neurones without NGF did not alter the proportion of positive neurones either (11.3$\pm$2.6%, $n=4$ cultures, *versus* 11.3$\pm$2.9% in four control cultures). In contrast, administration of PGE_2 significantly increased the proportion of neurones with NK_1 receptor-like IR. Figure 1c illustrates DRG neurones with NK_1 receptor-like IR after culturing with 10^{-6} M PGE_2. While in the control cultures ($n=6$) 11.5$\pm$5.5% of the neurones were labelled, 26.0$\pm$12.5% of the neurones were positive after administration of 10^{-8} M PGE_2 (four cultures), and 40.8$\pm$5.6% of the neurones were labelled with the anti-NK_1 receptor antibody after administration of 10^{-6} M PGE_2 (six cultures) (Figure 2a). The increase was significant for both concentrations of PGE_2 (χ^2 test). Figure 1d shows the size distribution of the neurones cultured with or without PGE_2. In each sample of neurones mainly small- and medium-sized neurones ($<500\ \mu m^2$) were labelled with the anti-NK_1 receptor antibody (black bars). Thus, the upregulation of the NK_1 receptor in response to PGE_2 was induced in a population of small- and medium-sized DRG neurones.

Further experiments were carried out to exclude any effect of the heat-inactivated horse serum on the effect of PGE_2 on NK_1 receptor expression. When DRG neurones were cultured for 3 days without serum, $10.5 \pm 1.9\%$ of the neurones were labelled with the anti-NK_1 receptor antibody (four cultures). After application of PGE_2 (10^{-6} M) for two days, $38.8 \pm 2.5\%$ of the DRG neurones were labelled (four cultures). Thus serum had no effect. In order to reveal rapid effects of a single application of PGE_2, DRG neurones were fixed and labelled for NK_1 receptor-like IR at either 15, 60 or 120 min after a single application of PGE_2 (10^{-6} M). In these experiments, the proportions of neurones with positive labelling for NK_1 receptors were $11.8 \pm 2.5\%$ in four control cultures, $8.8 \pm 2.5\%$ after 15 min PGE_2 incubation (four cultures), $11.3 \pm 2.6\%$ after 60 min PGE_2 incubation (four cultures) and $9.8 \pm 1.9\%$ after 120 min PGE_2 incubation (four cultures). Thus, these experiments did not reveal a rapid effect of a single PGE_2 application.

Involvement of second messengers in the upregulation of NK_1 receptor-like IR

To test whether the expression of NK_1 receptor-like IR depends on activation of the adenylyl cyclase – PKA pathway, neurones were cultured in a medium containing db-cAMP (10^{-6} M) or the PKA inhibitor H-89 (10^{-6} M). The influence of both substances on the expression of NK_1 receptors was also tested in the presence of 10^{-6} M PGE_2. In five cultures grown in a medium with db-cAMP, $37.8 \pm 5.6\%$ of the neurones showed NK_1 receptor-like IR, and in five cultures grown in a medium with db-cAMP plus PGE_2 (10^{-6} M), $38.6 \pm 4.4\%$ of the neurones were positive (*versus* $10.5 \pm 3.9\%$ in four control cultures) (Figure 2a). Thus, elevation of cAMP had the same effect as PGE_2, and PGE_2 did not further increase this effect.

When neurones were cultured in the presence of H-89, only $3.5 \pm 1.7\%$ of the neurones showed NK_1 receptor-like IR ($n = 4$ cultures, *versus* $10.8 \pm 1.5\%$ in four control cultures), and after coadministration of H-89 plus PGE_2, $5.6 \pm 4.3\%$ of the neurones were labelled ($n = 4$ cultures) (Figure 2b). Thus inhibition of PKA slightly reduced expression of NK_1 receptor-like IR, and it inhibited the effect of PGE_2. In contrast, the PKC inhibitor (PKC-i) had no effect, and PGE_2 in the presence of the PKC- still induced an upregulation of NK_1 receptor-like IR (to $40.0 \pm 7.6\%$, four cultures, *versus* $9.8 \pm 1.7\%$ in four control cultures) (Figure 2b).

Effect of SRIF on the PGE_2-induced upregulation of NK_1 receptor-like IR

While PGE_2 activates and sensitizes primary afferent neurones and is thus pronociceptive, the peptide SRIF inhibits primary afferent neurones (Heppelmann and Pawlak, 1997, 1999; Carlton *et al*., 2001b). As SRIF actions are also mediated by G-protein-coupled receptors, we tested whether SRIF is able to influence the expression of NK_1 receptors in DRG neurones. For this purpose, DRG neurones were grown in a medium containing SRIF or SRIF together with PGE_2. As shown in Figure 3, the addition of SRIF to the medium slightly decreased the proportion of neurones with NK_1 receptor-like IR to $4.3 \pm 4.6\%$ (eight cultures, *versus* $11.0 \pm 7.7\%$ in seven control cultures). In addition, SRIF completely blocked the PGE_2-induced upregulation of NK_1 receptor-like IR in DRG neurones. In cultures grown in a medium containing SRIF together with PGE_2, only $6.4 \pm 5.0\%$ (seven cultures) of all neurones expressed NK_1 receptor-like IR (*versus* $8.3 \pm 4.3\%$ in six control cultures and $38.9 \pm 6.2\%$ in seven cultures grown in a medium containing 10^{-6} M PGE_2).

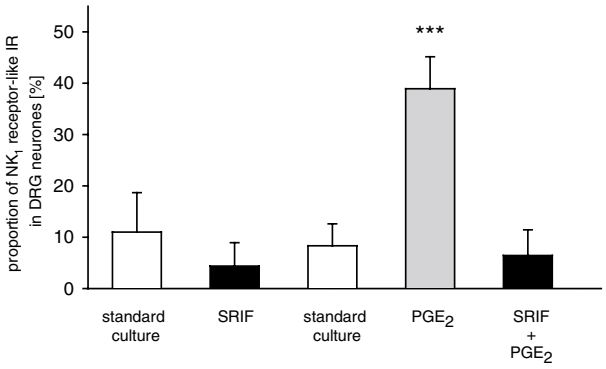

Figure 3 Influence of SRIF on the PGE_2-induced upregulation of the proportion of NK_1 receptor-like IR in DRG neurones. Same type of display as in Figure 2, $***P < 0.001$.

Ca^{2+}-imaging studies

In order to further substantiate the observation of a PGE_2-induced upregulation of NK_1 receptor-like IR in DRG neurones, we added functional studies using the Ca^{2+}-imaging technique. Figure 4a shows a typical SP-induced rise of $[Ca^{2+}]_i$ of an individual neurone. The addition of buffer had no effect, but SP caused a transient rise in $[Ca^{2+}]_i$. The neurone also showed a typical potassium (K^+)-induced rise of $[Ca^{2+}]_i$. Figure 4b summarizes all data. In neurones grown in the standard culture (six cultures) 50 of 248 (20%) analysed cells showed an SP-induced rise of $[Ca^{2+}]_i$. Preincubation of the cells with the NK_1 receptor-specific antagonist SR 140333 inhibited the SP-induced rise of $[Ca^{2+}]_i$. In these three cultures, only six of 150 neurones (4%) showed an effect of SP. In neurones that were cultured with 10^{-6} M PGE_2 for 2 days before the Ca^{2+}-imaging experiments (nine cultures), 134 of 316 neurones (42%) showed an SP-induced rise of $[Ca^{2+}]_i$. After preincubation of neurones with both SRIF and PGE_2, only nine of 180 neurones (5%, five cultures) showed an SP-induced rise of $[Ca^{2+}]_i$. To confirm that the effect of SRIF is mediated by binding of SRIF to somatostatin (sst) receptors, DRG neurones were cultured with SRIF together with the sst receptor antagonist cyclo-SRIF and PGE_2. Under these conditions, 49 of 137 neurones (35%, three cultures) showed an SP-induced rise of the $[Ca^{2+}]_i$. Collectively, these data confirm the immunocytochemical data that show an upregulation of NK_1 receptor-like IR after PGE_2 and an inhibition of this effect by SRIF.

Discussion

The present results demonstrate for the first time that long-term exposure of DRG neurones to PGE_2 *in vitro* significantly enhances the expression of NK_1 receptor-like IR in DRG neurones. This upregulation depends on the activation of the adenylyl cyclase – PKA cascade in the cells. Blockade of PKC,

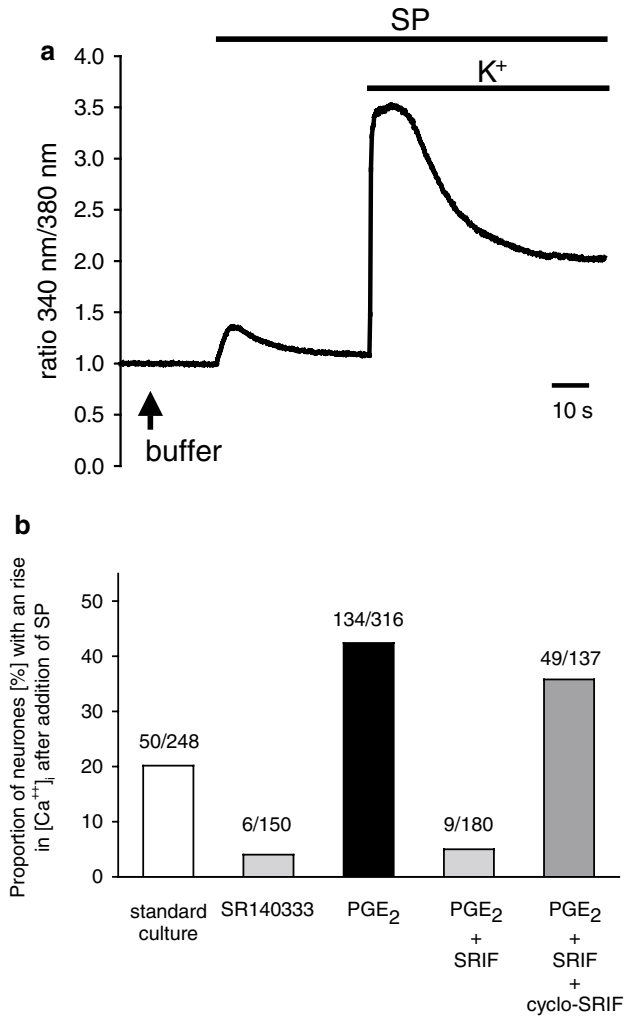

Figure 4 (a) SP-induced transient rise of $[Ca^{2+}]_i$ in an individual DRG neurone. The neurone also shows a typical potassium- (K^+)-induced rise of $[Ca^{2+}]_i$ whereas the addition of buffer had no effect. The ratio value (340 – 380 nm) was plotted as a function of time. (b) Proportion of DRG neurones with a rise in $[Ca^{2+}]_i$ after addition of SP to standard cultures, after preincubation of the neurones with the NK_1 receptor antagonist SR 140333 for 15 min., after preincubation of the neurones for 24 h with PGE_2, after preincubation with SRIF together with PGE_2 or with SRIF together with cyclo-SRIF and PGE_2.

depolarization with high potassium concentration, or the absence of NGF from the medium had no effect on the expression of NK_1 receptor-like IR of the neurones. The coadministration of SRIF prevented the effect of PGE_2. Additionally, Ca^{2+}-imaging studies showed that the incubation with PGE_2 enhanced the proportion of neurones that exhibit an SP-induced rise in $[Ca^{2+}]_i$. This effect is also counteracted by coadministration of SRIF. These data suggest, therefore, that PGE_2 (and possibly other inflammatory mediators) are involved in the upregulation of NK_1 receptors in primary afferent neurones during inflammation. The role of PGE_2 in receptor regulation is a novel observation.

In this study, we used cultured DRG neurones because this approach allowed us to expose the neurones to single mediators. The aim and design of the study were derived from our previous experiments. In lumbar DRG neurones that are taken from rats at 1, 3 and 10 days after induction of antigen-induced arthritis in one knee joint and cultured for 1 day, the proportion of DRG neurones with NK_1 receptor-like IR was enhanced from about 10% up to 50% (Segond von Banchet *et al*., 2000). Thus, we were primarily interested whether the expression of NK_1 receptor-like IR in DRG neurones is altered after exposure to mediators for 2 days because this time point is within the period of maximal upregulation of the NK_1 receptor in lumbar DRG neurones in antigen-induced arthritis.

Effects of PGE_2

We were particularly interested in the effect of PGE_2 because PGs levels are enhanced in inflammatory exudates and because PGs sensitize sensory neurones (Schaible and Grubb, 1993). PGE_2 increases the excitability of primary afferent neurones to depolarizing stimuli (Cui and Nicol, 1995; Lopshire and Nicol, 1998), lowers the threshold for neuronal firing (Handwerker, 1976; Grubb *et al*., 1991; Nicol *et al*., 1997; Gold *et al*., 1998), augments the evoked release of neurotransmitters (Geppetti *et al*., 1991; Vasko *et al*., 1994; Hingtgen *et al*., 1995; Goodis *et al*., 2000) and sensitizes primary afferent neurones *in vivo* to the action of other mediators such as bradykinin within a minute after application of PGE_2 (Schaible and Schmidt, 1988). While these effects are produced on a short-term timescale, the present study reveals in addition long-term effects of PGE_2, namely an influence on receptor expression in DRG neurones. Through this crosseffect to another mediator-receptor system PGE_2 can exert long-lasting effects, although its binding to PG receptors is downregulated during long-term exposure (Southall *et al*., 2002).

Many effects of PGE_2 are mediated by cAMP, and in fact three out of four PGE_2 receptors, namely the EP2, the EP3C (C is a splice variant) and the EP4 receptor are coupled to the adenylyl pathway and increase the concentration of cAMP (Sugimoto *et al*., 1994; Oida *et al*., 1995). All of these receptors, and in addition the EP_1 receptor that induces Ca^{2+} mobilization via the phosphoinositide pathway, have been identified in DRG neurones (Southall and Vasko, 2001; Vanegas and Schaible, 2001). In the present experiments, the upregulation of the expression of NK_1 receptors by PGE_2 was mimicked by db-cAMP and blocked by H89, an inhibitor of PKA, but it was not inhibited by blockade of PKC. Thus, the adenylyl cyclase – cAMP – PKA pathway is crucial for this PGE_2 effect. We have not identified the further steps that lead to upregulation of the NK_1 receptor expression. While a single PGE_2 application produces neuronal effects (see the last paragraph) within minutes, we have not seen a change of the expression of NK_1 receptor-like IR within 2 h after a single PGE_2 application. This shows, therefore, that either longer or repetitive exposure to PGE_2 is necessary to produce this PGE_2 effect or that processes are involved that need more than 2 h to become manifest. Our data show, however, that repetitive PGE_2 applications can produce an upregulation of NK_1 receptor expression, similar to that seen in the early phase of antigen-induced arthritis.

In calcium-imaging experiments, DRG neurones were activated by bath application of SP, and this effect was blocked by an NK_1 receptor antagonist. The activation of the cells by SP strongly suggests that at least part of the NK_1 receptors are located in the cell membrane. Indeed, in DRG

neurones SP produces an inward current through a Ca^{2+}-permeable nonselective cation channel (Li and Zhao, 1998). Therefore, an influx through the membrane might be the source of elevated $[Ca^{2+}]_i$. In neurones cultured under standard conditions, about 20% of the DRG neurones showed an increase of intracellular Ca^{2+} after SP application, and this proportion is close to the percentage of neurones with NK_1 receptor-like IR. Slight differences may have resulted from setting the threshold for a positive neurone in the densitometric analysis. Importantly, a much higher proportion of neurones showed elevated $[Ca^{2+}]_i$ after SP administration following pretreatment of the cells with PGE_2 for 2 days, confirming the higher expression rate of NK_1 receptor-like IR following treatment with PGE_2.

Effects of SRIF

A possible effect of SRIF on NK_1 receptor expression was assessed because SRIF has an analgesic action (for review, see Selmer *et al*., 2000) and because primary afferent neurones of normal animals are under the tonic inhibitory influence of SRIF (Heppelmann and Pawlak, 1999; Carlton *et al*., 2001a, b). Clinically, SRIF analogues have been successfully used to treat cancer pain (Mollenholt *et al*., 1994), bone pain (Burgess *et al*., 1996) and also inflammatory pain (Fioravanti *et al*., 1995). Furthermore, all known SRIF receptors (sst1 – sst5) are G-protein coupled, and they inhibit the adenylyl cyclase – cAMP – PKA pathway (Patel, 1999; Csaba and Dournaud, 2001). SRIF inhibited the effect of PGE_2 on expression of NK_1 receptor-like IR, and this SRIF effect was inhibited by cyclo-SRIF, an antagonist at sst receptors. We assume, therefore, that SRIF inhibits the PGE_2-induced upregulation of the NK_1 receptor by interfering with the adenylyl cyclase – cAMP – PKA pathway.

Lack of effect of depolarization and NGF

Depolarization of the neurones with 50 mM KCl did not alter NK_1 receptor-like IR, suggesting that depolarization of the neurones is not sufficient to trigger receptor regulation. Interestingly, leaving out NGF from the culture medium did not alter NK_1 receptor-like IR either, although NGF stimulates SP synthesis in DRG neurones (Lindsay and Harmar 1989; Donnerer *et al*., 1992) and induces upregulation of bradykinin 1 receptors in DRG neurones (Petersen *et al*., 1998). Thus, regulation is different for different peptide receptors. Indeed, in rats with antigen-induced arthritis, the upregulation of NK_1 receptor-like IR lasted about 10 days, whereas upregulation of bradykinin receptors in the same ganglia was present up to 42 days (Segond von Banchet *et al*., 2000). PGE_2 was not able to influence the expression of bradykinin receptors in DRG neurones (unpublished observations).

Functional significance

Long-term changes in the receptor expression in primary afferent neurones could be an important basis for long-lasting inflammatory pain. After upregulation of NK_1 receptors in primary afferents (Segond von Banchet *et al*., 2000; Carlton and Coggeshall, 2002; see also Xu and Zhao, 2001) SP is likely to have pronounced effects on these neurones under inflammatory conditions. The present data suggest that mediators in inflamed tissue may determine the level of upregulation. Increase of cAMP and the protein kinase A activity in the neurones seem to be important steps, and different mediators such as PGE_2 and SRIF may exert their mutual effects by adjusting the cAMP level in the cells. The dominant presence of proinflammatory PGE_2 may increase expression of NK_1 receptors, and increased SRIF concentrations may counteract this effect. In the spinal dorsal horn, NK_1 receptors are also upregulated during inflammation (Schäfer *et al*., 1993; McCarson and Krause 1994; Krause *et al*., 1995; Abbadie *et al*., 1997). Since PGE_2 is released in the spinal cord upon noxious stimulation and peripheral inflammation (Ebersberger *et al*., 1999), spinal PGE_2 could also be involved in the regulation of spinal NK_1 receptor expression.

The technical assistance of Mrs A. Wallner and Mrs F. Diebel is greatly appreciated. The work was funded by the Deutsche Forschungsgemeinschaft (Scha 404/9-2). We thank Dr Emonds-Alt (Sanofi, Paris, France) for the donation of the NK_1 receptor antagonist SR140333.

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(Received December 4, 2002
Revised February 14, 2003
Accepted March 13, 2003)