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Endothelins induce ET_B receptor-mediated mechanical hypernociception in rat hindpaw: roles of cAMP and protein kinase C

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Abstract

The present study assesses the capacity of endothelins to induce mechanical hypernociception, and characterises the receptors involved and the contribution of cAMP and protein kinases A (PKA) and C (PKC) to this effect. Intraplantar administration of endothelin-1, endothelin-2 or endothelin-3 (3–30 pmol) induced dose- and time-dependent mechanical hypernociception, which was inhibited by BQ-788 (*N*-cys-2,6-dimethylpiperidinocarbonyl-L-γ-methylleucyl-D-1-methoxycarboyl-D-norleucine; endothelin ET_B receptor antagonist), but not BQ-123 (cyclo[D-Trp-D-Asp-Pro-D-Val-Leu]; endothelin ET_A receptor antagonist; each at 30 pmol). The selective endothelin ET_B receptor agonist BQ-3020 (*N*-Ac-Ala^{11,15}-endothelin-1 (6–21)) fully mimicked the hypernociceptive effects of the natural endothelins. Treatments with indomethacin, atenolol or dexamethasone did not inhibit endothelin-1-evoked mechanical hypernociception. However, endothelin-1-induced mechanical hypernociception was potentiated by the cAMP phosphodiesterase inhibitor rolipram (4-[3-(cyclopentyloxy)-4-methoxyphenyl]-2-pyrrolidinone) and inhibited by the PKC inhibitors staurosporine and calphostin C, but was unaffected by the PKA inhibitor H89 (*N*-[2-((*p*-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide). Thus, endothelins, acting through endothelin ET_B receptors, induce mechanical hypernociception in the rat hindpaw via cAMP formation and activation of the PKC-dependent phosphorylation cascade.

Keywords: Endothelin; Mechanical hyperalgesia; Endothelin ET_B receptor; cAMP; Protein kinase

1. Introduction

Endothelin-1, endothelin-2 and endothelin-3 are 21-amino acid residue peptides of the endothelin family produced by many cell types, including several implicated in immune defence systems (for review, see Kedzierski and Yanagisawa, 2001). These potent peptides exert widespread biological actions via activation of two specific G protein-coupled receptors, named endothelin ET_A and ET_B receptors.

Endothelin ET_A receptors have higher affinity for endothelin-1 and endothelin-2 than for endothelin-3, and can be blocked by several selective antagonists, including BQ-123 (for review, see Davenport, 2002). In contrast, endothelin ET_B receptors do not discriminate between the three isopeptides, but can be selectively activated by agonists such as BQ-3020 or blocked by antagonists such as BQ-788. Both receptor types can couple to multiple intracellular signalling mechanisms, depending on cell type, including phospholipases C, D and A2, adenylyl cyclase and guanylyl cyclase (for extensive review, see Sokolovsky, 1995). Furthermore, activation of protein kinases A and C, as well as intracellular calcium increases also can underlie many effects of endothelins mediated via endothelin ET_A and/or ET_B receptors (Wu-Wong et al., 1996; Dulin et al., 2001).

Many of these various signalling pathways contribute importantly to inflammatory pain by sensitising nocicep-

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tors, a process resulting in a state of hypernociception also known as hyperalgesia or allodynia (for review, see Riedel and Neeck, 2001). There are two groups of hyperalgesic mediators that satisfy the experimental and clinical criteria for agents that directly sensitise nociceptors: eicosanoids and sympathetic amines (Ferreira et al., 1978a; Levine et al., 1986, Nakamura and Ferreira, 1987). Nonetheless, the generation and/or release of these final mediators in mechanical hyperalgesia induced by carrageenin or lipopolysaccharide (LPS) depends on prior recruitment of additional mediators, including cytokines tumour necrosis factor α, interleukin-6, interleukin-1β and cytokine-induced neutrophil chemoattractant-1 (CINC-1) (Cunha et al., 1991, 1992; Lorenzetti et al., 2002).

Endothelin-1 elicits overt nociceptive behaviour when injected into the human forearm, the knee joint of dogs or rats, as well as the peritoneal cavity and hindpaw foot pad of mice (Ferreira et al., 1989; Dahlof et al., 1990; Raffa and Jacoby, 1991; De-Melo et al., 1998; Piovezan et al., 2000). The peptide also sensitises the human forearm and the rat hindpaw to noxious mechanical stimuli (Ferreira et al., 1989), and the mouse hindpaw to nociception induced by formalin or capsaicin (Piovezan et al., 1997, 1998). However, the identity of the receptors implicated in these nociceptive actions of endothelin-1 is still unsettled. Thus, endothelin ET_A receptors appear to mediate endothelin-1-induced nociception in the rat hindpaw and knee joint (Davar et al., 1998; De-Melo et al., 1998; Gokin et al., 2001) and both nociception and hyperalgesia to capsaicin and heat in the mouse hindpaw (Piovezan et al., 2000; Menendez et al., 2003). However, both endothelin ET_A and ET_B receptors mediate endothelin-1-induced hindpaw mechanical hyperalgesia in mice (Baamonde et al., 2004). Abdominal constrictions (i.e. writhes) triggered by endothelins in this species also appear to depend on both endothelin ET_A and ET_B receptors (Raffa et al., 1996), but writhes induced by i.p. phenylbenzoquinone injection in wild-type mice are suppressed following endothelin ET_B (but not ET_A) receptor blockade, and are virtually absent in endothelin ET_B receptor knockout animals (Griswold et al., 1999). In sharp contrast, other studies have reported that ET_B receptors play an antihyperalgesic or antinociceptive role in the hindpaw of mice and rats (Piovezan et al., 2000; Khodorova et al., 2003). On the other hand, the intracellular pathways involved in the nociceptive and hyperalgesic actions of endothelins have not yet been adequately investigated.

In light of these considerations, the present study aimed to further characterise the ability of endothelins to cause mechanical hypernociception in the rat hind paw, by determining which endothelin receptor type(s) is responsible for this effect, as well as the putative contributions of cAMP and of PKA and PKC in the signalling mechanisms involved.

2. Materials and methods

2.1. Animals

Male adult Wistar rats (180–200 g) were housed in a temperature-controlled room (22±1 °C), with access to water and food ad libitum, until use. All experiments were conducted in accordance with NIH guidelines on the welfare of experimental animals and with the approval of the Ethics Committee of the Faculty of Medicine of Ribeirão Preto of the University of São Paulo, where the study was undertaken.

2.2. Nociceptive test: mechanical hypernociception

Mechanical hypernociception was tested in rats as previously described (Ferreira et al., 1978b). Briefly, a constant pressure of 20 mm Hg (measured using a sphygmomanometer) was applied to a 15-mm^2 area on the dorsal surface of the hindpaw, via a syringe piston moved by compressed air, which was discontinued when the rat presented a typical "freezing reaction". This reaction is comprised of brief apnoea, concomitant with retraction of the head and forepaws and a reduction in the escape movements that the animal normally makes in an attempt to free itself from the position imposed by the experimental situation. The apnoea response is also rapidly followed by successive waves of muscular tremor. For each animal, the latency to the onset of the freezing reaction (i.e. reaction time, in s) was measured first before any treatment and then again at different times after administration of hyperalgesic agents. The intensity of mechanical hypernociception was quantified as decreases in reaction time, at the various time points after treatment, relative to baseline responsiveness (usually 33±1 s), calculated by subtracting each of these values from that of the first baseline measurement (Ferreira et al., 1978b).

2.3. Experimental protocols

2.3.1. Mechanical hypernociception induced by endothelins and the influence of endothelin ET_A or ET_B receptor antagonists

Each animal received an intraplantar (i.pl.) injection of either vehicle (100 μl of sterile 0.9% saline), endothelin-1, endothelin-2, endothelin-3 or the selective endothelin ET_B receptor agonist, BQ-3020 (Davenport, 2002; each at 3, 10 or 30 pmol), into the right hindpaw. To investigate the influence of endothelin ET_A and ET_B receptor antagonists on mechanical hypersensitivity induced by endothelins, other rats had their right hindpaws pretreated with either saline (100 μl; control rats), BQ-123 or BQ-788 (selective ET_A or ET_B receptor antagonists, respectively; each at 3, 10 or 30 pmol, i.pl.), 30 min prior to ipsilateral i.pl. injection of endothelin-1, endothelin-2 or endothelin-3 (each at 10 pmol). Some rats were given a combination of BQ-123

plus BQ-788 (each co-injected simultaneously at 10 pmol). In all experiments, reaction time to the mechanical stimulus was measured before treatment, and then repeatedly at 1, 3, 5 and 24 h after treatment, and mechanical hypernociception was calculated as described in Section 2.2. The various doses of endothelin receptor agonists and antagonists were chosen on the basis of previous studies (Piovezan et al., 2000; Fabricio et al., 1998).

2.3.2. Possible mechanisms for endothelin-1-induced mechanical hypernociception

To assess the importance of prostaglandins, sympathetic amines and glucocorticoid-sensitive pathways to mechanical hypernociception induced by endothelin-1, rats were treated initially with the non-selective cyclooxygenase inhibitor indomethacin (5 mg kg^{-1}, i.p.), the selective β_2 adrenoceptor antagonist atenolol (25 μg, i.pl.), a combination of indomethacin plus atenolol (at these same doses and administration routes) or the glucocorticoid dexamethasone (1 mg kg^{-1}, i.p.). Alternatively, to investigate the intracellular signalling mechanisms underlying endothelin-1-induced mechanical hypernociception, rats were given i.pl. injections of either the selective cAMP phosphodiesterase inhibitor rolipram (9 μg), the PKA inhibitor H89 (27 μg) or the PKC inhibitors staurosporine and calphostin C (1 μg or 300 ng, respectively). In all cases, the corresponding control groups were treated identically with the vehicle (sterile saline). Thirty minutes after each treatment (or 1 h in the case of dexamethasone), the rats were challenged with i.pl. injection of endothelin-1 (3 pmol in the experiments involving rolipram, 10 pmol all other groups) and mechanical hypernociception evoked by the peptide was evaluated as before, but at 3 h only, i.e. at the peak of the hypernociceptive effect. The doses of indomethacin, atenonol, dexamethasone, rolipram, H89, staurosporine and calphostin C were selected based on their effectiveness in previous studies (Tonussi and Ferreira, 1994; Ferreira et al., 1997; Lorenzetti et al., 2002, Cunha et al., 1999; Sachs et al., unpublished data). All behavioural observations concerning the experiments described in this and the previous sections were carried out by an observer that was unaware of the drug treatments given to each animal.

2.4. Drugs

The following materials were obtained from the sources indicated. Endothelin-1, endothelin-2, endothelin-3, BQ-123 (cyclo[D-Trp-D-Asp-Pro-D-Val-Leu]) and BQ-788 (*N*-cys-2,6-dimethylpiperidinocarbonyl-L-(-methylleucyl-D-1-methoxycarboyl-D-norleucine)) were purchased from American Peptide (Sunnyvale, CA, USA). BQ-3020 (*N*-Ac, $Ala^{11,15}$-Endothelin-1 (6–21)), a selective endothelin

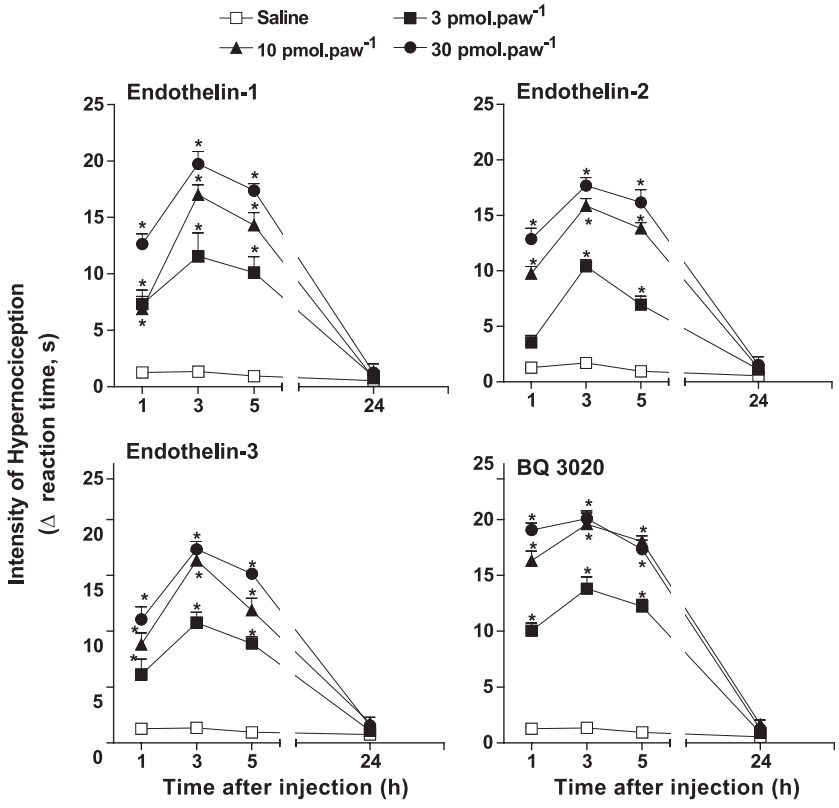

Fig. 1. Mechanical hypernociception induced by endothelin-1, endothelin-2, endothelin-3 or the selective endothelin ET_B receptor agonist BQ-3020. Each peptide was injected via i.pl. injection (at the doses indicated) into the right hindpaw and the intensity of hypernociception was measured at 1, 3, 5 and 24 h later. Control rats were similarly treated with saline (100 μl). Each value represents the mean±S.E.M. for five animals and is representative of two different experiments. *P<0.05 compared with the saline-treated group (ANOVA followed by Bonferroni t-test).

ET_B receptor agonist, was purchased from Novabiochem (San Diego, CA, USA). Indomethacin was from Merck, Sharp and Dohme (São Paulo, SP, Brazil), whereas dexamethasone, atenolol, rolipram (4-[3-(cyclopentyloxy)-4-methoxyphenyl]-2-pyrrolidinone; a phosphodiesterase inhibitor), staurosporine, calphostin C and H89 (*N*-[2-((*p*-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide) were all purchased from Sigma (St. Louis, MO, USA).

2.5. Data analysis

Results are presented as mean±standard error of the mean (S.E.M.) for groups of five animals and are always representative of at least two experiments. The differences between the experimental groups were compared by analysis of variance (ANOVA). In the case of significance, individual comparisons were subsequently made with the Bonferroni test. The level of significance was set at $P<0.05$.

3. Results

3.1. Mechanical hypernociception induced by intraplantar injection of endothelin-1, endothelin-2, endothelin-3 or BQ-3020

Intraplantar injection of endothelin-1, endothelin-2, endothelin-3 or of the selective ET_B receptor agonist BQ-3020 (3, 10 or 30 pmol, in 100 μl) into the hind paws of rats evoked dose- and time-dependent mechanical hypernociception. In all cases, except at doses of 30 pmol of BQ3020, the hypernociceptive responses were already significant 1 h after injection, reached a peak at 3 h and fully returned to control levels within 24 h. The peak of the hypernociceptive response induced by 30 pmol of BQ 3020 was between 1 and 3 h. In contrast, similar injection of saline into the hind paws of control rats did not alter the threshold for mechanical nociceptive responsiveness at all time points evaluated (Fig. 1).

3.2. Influence of BQ-123 and BQ-788 on mechanical hypernociception induced by natural endothelins

Prior i.pl. treatment with the selective endothelin ET_B receptor antagonist BQ-788 (at 3, 10 or 30 pmol; 30 min beforehand) inhibited the mechanical hypernociception induced by i.pl. challenge of the ipsilateral hindpaw with either endothelin-1, endothelin-2 or endothelin-3. The effects of BQ-788 were clearly dose-dependent and evident at 1, 3 or 5 h after challenge (Fig. 2, panels A, B and C show the results for 3 h post-challenge only). In sharp contrast, pretreatment of the hindpaw with similar doses of the selective ET_A receptor antagonist BQ-123 failed to modify mechanical hypernociception triggered by any of the three natural endothelins. Moreover, the inhibition of hypernociception afforded by i.pl. co-injection of BQ-123 plus BQ-788 (each at 10 pmol) was not different from that caused by BQ-788 alone.

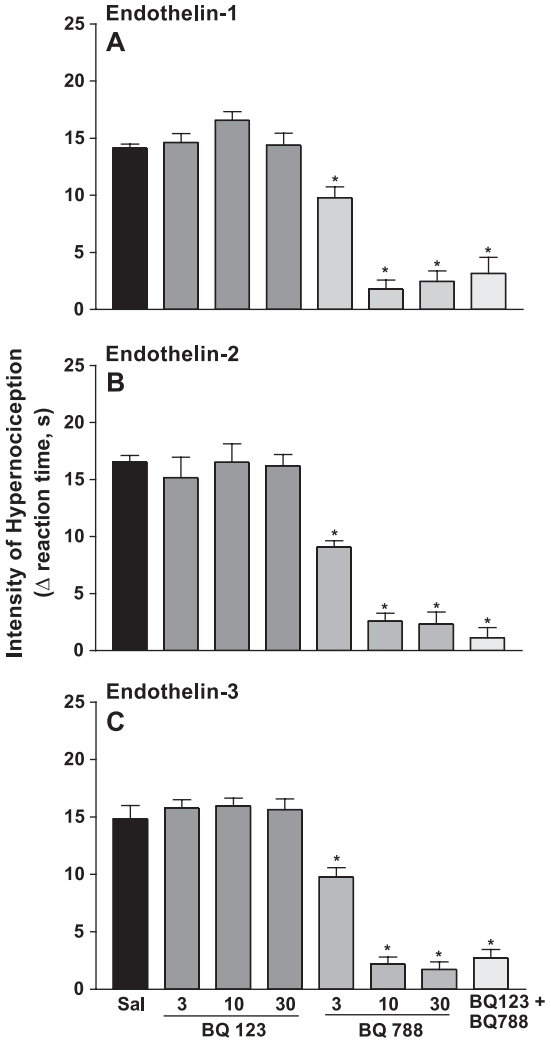

Fig. 2. Effects of BQ-123 and/or BQ-788 (selective endothelin ET_A and ET_B receptors antagonists, respectively) on mechanical hypernociception induced by i.pl. injection of endothelin-1 (panel A), endothelin-2 (panel B) or endothelin-3 (panel C). Saline (100 μl), BQ-788, BQ-123 (each at 3, 10 or 30 pmol paw^{-1}) or the combination of BQ-123 plus BQ-788 (both at 10 pmol paw^{-1}) were injected 30 min prior to each endothelin (each at 10 pmol paw^{-1}), and the intensity of hypernociception was measured at 3 later. Each value represents the mean±S.E.M. for five animals and is representative of two different experiments. *$P<0.05$ compared with the saline-pretreated group (ANOVA followed by Bonferroni *t*-test).

3.3. Influence of indomethacin, atenolol and dexamethasone on mechanical hypernociception induced by endothelin-1

We next evaluated the possible participation of prostaglandins, sympathetic amines and glucocorticoid-sensitive cytokines in the genesis of endothelin-1-induced mechanical hypernociception. To this effect, it was found that the responsiveness of rats to mechanical stimulation at 1, 3 or 5 h after challenge with endothelin-1 (10 pmol, i.pl.) was fully

Table 1
Absence of effect of indomethacin, atenolol or dexamethasone on mechanical hypernociception induced by i.pl. endothelin-1 (10 pmol paw^{-1})

Pre-treatment	Mean±S.E.M. reaction time (in s)[a]
Saline (Control rats, equivalent volume)	17.22±0.49
Indomethacin (5 mg kg^{-1}, i.p.)	14.80±1.43
Atenolol (25 μg paw^{-1})	14.32±1.25
Indomethacin+Atenonol (same doses and routes)	13.84±0.67
Dexamethasone (1 mg kg^{-1}, i.p.)	13.26±0.78

[a] Mechanical hypernociception was evaluated 3 h after endothelin-1 treatment in all groups. Results are expressed as means±S.E.M. of reaction time for five animals per group and are representative of two different experiments.

resistant to inhibition by prior treatment with indomethacin (5 mg kg^{-1}, i.p.), atenolol (25 μg, i.pl.), a combination of atenolol plus indomethacin (given simultaneously at these same doses and administration routes) or dexamethasone (1 mg kg^{-1}, i.p.). Table 1 shows the results obtained for challenge at 3 h only. At the doses employed, these drugs effectively reduce mechanical hypernociception triggered by carrageenan, without affecting basal responsiveness to the mechanical stimulus (Tonussi and Ferreira, 1994; Ferreira et al., 1997; Lorenzetti et al., 2002).

3.4. Roles of cAMP, PKA and PKC intracellular signalling mechanisms in mechanical hypernociception induced by endothelin-1

In order to investigate some of the intracellular signalling mechanisms activated by endothelin-1 to cause mechanical hypernociception, we tested the influence of inhibitors of cAMP phosphodiesterase (rolipram), PKA (H89) and PKC (staurosporine and calphostin C). Pretreatment of the rats with rolipram (9 μg, i.pl.) significantly potentiated mechanical hypernociception induced by endothelin-1 (3 pmol; 63% potentiation at 3 h, Fig. 3A). On the other hand, mechanical hypernociception induced by i.pl. endothelin-1 (10 pmol) was significantly inhibited by prior ipsilateral i.pl. treatment with staurosporine (1 μg; 81% inhibition) or calphostin C (0.3 μg; 46% inhibition), but was not affected by H89 (27 μg). These results are shown in Fig. 3B. The responsiveness of rats to mechanical stimulation of hindpaws challenged with saline, instead of endothelin-1, was not changed by any of these pretreatments (results not shown).

4. Discussion

The results of the current study extend the original finding by Ferreira et al. (1989), by showing that not only endothelin-1, but also endothelin-2 and endothelin-3 are potent inducers of mechanical hypernociception in the rat hindpaw. This study also characterises, to our knowledge for the first time, the endothelin receptor type and some of the putative intracellular signalling mechanisms underlying the nociceptor sensitisation by endothelin peptides.

Intraplantar injection of endothelin-1, endothelin-2, endothelin-3 each evoked dose- and time-dependent mechanical hypernociception. The fact that the three natural endothelin isopeptides were equipotent and equieffective in causing long-lasting mechanical hypernociception, allied to the finding that their actions were closely mimicked by BQ-3020, a selective endothelin ET_B receptor agonist (Davenport, 2002), strongly suggests that endothelin ET_B receptors play a major role in mediating this effect. This view was fully substantiated by the results using BQ-788, a selective antagonist of endothelin ET_B receptors (Davenport, 2002), which caused similar dose-dependent inhibitions of mechanical hypernociception induced by all three natural isopeptides. In sharp contrast, the selective endothelin ET_A receptor antagonist BQ-123 (Davenport, 2002) failed to exert any influence when given alone. It also did not modify the degree of inhibition caused by BQ-788 when both antagonists were co-injected. Collectively, these results clearly indicate that the mechanical hypernociception induced by endothelins in the rat hindpaw are mediated solely via endothelin ET_B receptors.

In agreement with our results, abdominal writhes induced by i.p. phenylbenzoquinone in wild-type mice are markedly blocked by prior treatment with a selective antagonist of endothelin ET_B (but not ET_A) receptors, whereas endothelin ET_B receptor knockout mice fail to respond altogether to this algogen (Griswold et al., 1999). On the other hand, other studies have shown that stimulation of endothelin ET_B

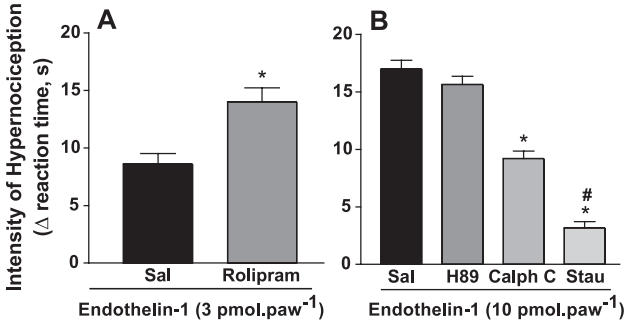

Fig. 3. Influence of the cAMP phosphodiesterase inhibitor rolipram, the PKA inhibitor H89 or the PKC inhibitors staurosporine and calphostin C on hypernociception induced by endothelin-1. Hypernociception was induced by i.pl. injection of endothelin-1 (3 or 10 pmol paw^{-1} in panels A and B, respectively) into the right hindpaw. Saline (100 μl paw^{-1}), rolipram (9 μg paw^{-1}), H89 (27 μg paw^{-1}), staurosporine (stau, 1 μg paw^{-1}) or calphostin C (Calph C, 300 ng paw^{-1}) were given 30 min before endothelin-1 and mechanical hypernociception was evaluated 3 h later. None of these treatments induced significant changes in basal mechanical nociceptive threshold per se (data not shown in the figure). Each value represents the mean±S.E.M. for five animals and is representative of two different experiments. *$P<0.05$ compared with the saline-pretreated group (ANOVA followed by Bonferroni t-test).

receptors in the hindpaw skin of rats causes opioid-dependent antinociceptive effects, which limit the ability of endothelin-1 to cause ET_A receptor-mediated hindpaw flinching behaviour (Khodorova et al., 2002, 2003). The apparent discrepancy between the roles played by ET_B receptors in those studies and the present one (i.e. antinociceptive versus pronociceptive influences) could be due to the significant differences in experimental nociceptive models, protocols and/or doses of endothelins employed in both studies. In fact, the doses of endothelin-1 employed in the present study (up to 30 pmol, i.pl.) did not induce flinches in rats, but using the higher dose of endothelin-1 (2 nmol, i.pl.) and same experimental design described by Khodorova et al. (2003), we confirmed that endothelin-1-induced hindpaw flinches in rats are evoked via endothelin ET_A receptors, whereas endothelin ET_B receptor exert an antinociceptive action (results not shown). Thus, the opposing roles played by endothelin ET_B receptors, as revealed under different experimental conditions, may well reflect merely stimulation of receptors located on distinct cell types, such that ET_B receptors on sensory neurones mediate mechanical hypernociception (a view suggested by present study and Pomonis et al., 2001), whereas those on keratinocytes trigger release of antinociceptive β-endorphin (as shown by Khodorova et al., 2003). Alternatively, these discrepancies could also be attributable to the presence of different subtypes (or splice variants) of endothelin ET_B receptor (Shyamala et al., 1994).

As outlined in "Introduction", many studies have provided evidence that nociceptive effects of endothelins in different experimental models are mediated solely via a single receptor type or via both ET_A and ET_B receptors. Most studies describing overt nociceptive effects of exogenous endothelins have ascribed such actions exclusively to activation of endothelin ET_A receptors, which can be counteracted by antinociceptive ET_B receptors (Piovezan et al., 2000; Gokin et al., 2001; Khodorova et al., 2002, 2003). Conversely, studies using nociceptive models which present a significant nociceptor sensitising component, such as abdominal writhing test (Raffa et al., 1996; Griswold et al., 1999), mechanical nociception (Baamonde et al., 2004; present study) or articular incapacitation in carrageenan-primed joints (De-Melo et al., 1998), have demonstrated that ET_B receptors, either alone or alongside ET_A receptors, promote pronociceptive effects. It is important to point out, however, that overt nociceptive tests do not allow the dissociation between nociceptor sensitisation and activation, two distinct components of the inflammatory pain (Dubner and Hargreaves, 1989). In this regard, it is noteworthy that although endothelin-1 enhances both the first (neurogenic) and second (inflammatory) phases of nociceptive responses to formalin in mice, the selective endothelin ET_B receptor agonist sarafotoxin S6c increases only the latter phase, i.e. that which depends markedly on inflammation-induced sensitisation of nociceptive pathways (Piovezan et al., 1997).

Endothelin-1 can induce firing in peripheral nociceptors in vivo (Gokin et al., 2001) and directly activate isolated dorsal root ganglion neurones. Unlike hindpaw mechanical hypernociception induced by carrageenan (Tonussi and Ferreira, 1994; Ferreira et al., 1997; Lorenzetti et al., 2002), we observed that the response to endothelin-1 was unaffected by prior treatment with the cyclooxygenase inhibitor indomethacin, the β_2 adrenoceptor blocker atenolol or the glucocorticoid dexamethasone (which down-regulates synthesis of eicosanoids and pro-inflammatory cytokines; Goulding, 1998). Thus, this effect of endothelin-1 is independent of the release of endogenous prostaglandins, sympathetic amines or cytokines. It is possible that endothelins induce mechanical hypernociception via direct actions on the nociceptors themselves, but it is equally feasible that these peptides might induce the release of other yet unidentified pronociceptive mediators, which in turn sensitise the nociceptor.

Studies on the molecular events associated with mechanical hypernociception triggered by prostaglandin and sympathetic amines have shown that these final nociceptive mediators sensitise primary sensory neurones by increasing intracellular Ca^{2+} and cAMP levels (Ferreira and Nakamura, 1979; Taiwo et al., 1989). Depending on the cell type, endothelins can either stimulate or inhibit cAMP formation (for review, see Sokolovsky, 1995). In the present study, the cAMP phosphodiesterase inhibitor rolipram potentiated endothelin-1-evoked endothelin ET_B receptor-mediated mechanical hypernociception, suggesting that this process depends on intracellular cAMP formation. There is evidence implicating both PKA and PKC in the biochemical events that take place downstream from cAMP formation to cause nociceptor sensitisation (Gold et al., 1996, 1998; Cunha et al., 1999; Khasar et al., 1999). In our study, we found that two inhibitors of PKC, staurosporine and calphostin C, significantly inhibited mechanical hypernociception evoked by endothelin-1, whereas the PKA inhibitor H89 was ineffective. As none of these agents caused mechanical hypernociception per se (data not shown), the cAMP formation triggered by endothelin-1 appears to activate PKC-dependent pathways to cause mechanical hypernociception. The fact that staurosporine, which is unspecific inhibitor of PKCs (Toledo and Lydon, 1997, was significantly more effective than calphostin C ($P<0.001$) to inhibit the endothelin-induced hypernociception suggest that others kinases, differently of PKA, could also be involved in this process.

In conclusion, the results of the present study demonstrate that endothelins constitute potent inducers of mechanical hypernociception in the rat hindpaw. This effect does not seem to require formation of eicosanoids, proinflammatory cytokines or release of sympathetic amines, and is mediated exclusively via endothelin ET_B receptors positively coupled to cAMP formation by adenylyl-cyclase and subsequent stimulation of nociceptor PKC-dependent (but PKA-independent) signalling pathways.

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