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Endothelin-1-induced ET_A receptor-mediated nociception, hyperalgesia and oedema in the mouse hind-paw: modulation by simultaneous ET_B receptor activation

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1 Endothelin-1 causes ET_A receptor-mediated enhancement of capsaicin-induced nociception in mice. We have assessed if this hyperalgesic effect of endothelin-1 is also accompanied by other pro-inflammatory effects, namely nociception and oedema, and characterized the endothelin ET receptors involved.

2 Intraplantar (i.pl.) hind-paw injection of endothelin-1 (0.3–30 pmol) induced graded nociceptive responses (accumulated licking time: vehicle, 20.5 ± 3.3 s; endothelin-1 at 30 pmol, 78.1 ± 9.8 s), largely confined to the first 15 min. Endothelin-1 (1–10 pmol) potentiated ipsilateral capsaicin-induced (0.1 μg, i.pl.; at 30 min) nociception (vehicle, 40.2 ± 2.6 s; endothelin-1 at 10 pmol, 98.4 ± 5.8 s, but 30 pmol was inactive), and caused oedema (increase in paw weight 5 min after capsaicin: vehicle, 46.3 ± 2.3 mg; endothelin-1 at 30 pmol, 100.3 ± 6.1 mg).

3 Selective ET_B receptor agonists sarafotoxin S6c (up to 30 pmol) and IRL 1620 (up to 100 pmol) were inactive, whereas endothelin-3 (up to 30 pmol) induced only modest oedema.

4 ET_A receptor antagonists BQ-123 (1 nmol, i.pl.) or A-127722-5 (6 μmol kg^{-1}, i.v.) prevented all effects of endothelin-1 (10 pmol), but the ET_B receptor antagonist BQ-788 (1 or 10 nmol, i.pl.) was ineffective.

5 BQ-788 (10 nmol, i.pl.) unveiled hyperalgesic effects of 30 pmol endothelin-1 and endothelin-3. Sarafotoxin S6c (30 pmol, i.pl.) did not modify endothelin-1-induced (10 pmol) nociception or oedema, but abolished hyperalgesia.

6 Thus, endothelin-1 triggers ET_A receptor-mediated nociception, hyperalgesia and oedema in the mouse hind-paw. Simultaneous activation of ET_B receptors by endothelin-1 or selective agonists can limit the hyperalgesic, but not the nociceptive or oedematogenic, effects of the peptide.
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Abbreviations: i.pl., intraplantar; DMSO, dimethylsulphoxide; PBS, phosphate-buffered saline

Introduction

Endothelins are a family of potent 21-residue peptides, produced by a vast array of cell types in response to diverse stimuli, which seem to exert important roles by autocrine and paracrine actions mediated through specific ET_A and ET_B receptors (for review, see Webb, 1998). Receptors of the ET_A type display higher affinity for endothelin-1 than endothelin-3 and can be selectively blocked by antagonists such as the peptide BQ-123 and the non-peptide A-127722-5, whereas ET_B receptors show equal affinities for both peptides, can be selectively stimulated by sarafotoxin S6c and IRL 1620 and blocked by antagonists such as the peptide BQ-788, among others (Opgenorth *et al*., 1996; for reviews, see Masaki *et al*., 1994; Webb & Meek, 1997).

Various stimuli which enhance endothelin-1 production and/or release, *in vivo* or *in vitro*, are important inducers or mediators of inflammatory responses, including carrageenan, Gram-negative bacterial lipopolysaccharide, bradykinin and cytokines, such as tumour necrosis factor-α, interleukin-1 and interleukin-6 (Bertelli *et al*., 1992; Sugiura *et al*., 1989; Vemulapalli *et al*., 1994; Klemm *et al*., 1995; for review, see Rae & Henriques, 1998). In this context, it is hardly surprising that endothelin-1 has been implicated in inflammation. Among its several pro-inflammatory properties, endothelin-1 can enhance microvascular permeability (Filep *et al*., 1995), induce leukocyte activation (Ishida *et al*., 1990) and recruitment by upregulating adhesion molecule expression in endothelium and leukocytes (McCarron *et al*., 1993; Ishizuka *et al*., 1999; Zouki *et al*., 1999), trigger cytokine release (Helset *et al*., 1993; Stankova *et al*., 1996; Woods *et al*., 1999), degranulate mast cells (Yamamura *et al*., 1994) and cause fever (Fabrício *et al*., 1998).

Endothelins may also exert potential roles in inflammatory nociception, for endothelin-1 elicits nociceptive behaviour when injected intra-arterially in the human forearm (Dahlof *et al*., 1990), intra-articularly in dogs (articular incapacitation test) and rats, or intraperitoneally in mice (writhing test) (Ferreira *et al*., 1989; De-Melo *et al*., 1998; Raffa & Jacoby, 1991). Given intradermally, endothelin-1 also sensitizes the human forearm or the rat paw to noxious mechanical stimuli (Ferreira *et al*., 1989), and the mouse hind-paw to formalin-induced pain (Piovezan *et al*., 1997). This sensitising effect resembles another typical feature of inflammation, hyperalgesia, a process whereby pain elicited by noxious stimuli applied

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to the inflamed site is enhanced (for reviews, see Carstens, 1995; Millan, 1999).

Recently, we reported that endothelin-1 can exert a dual effect in the model of capsaicin-induced pain in the mouse hind-paw (Piovezan *et al*., 1998). At low doses, the peptide causes ET_A receptor-mediated hyperalgesia (i.e. it potentiates capsaicin-induced nociception), whereas at higher doses it induces an anti-hyperalgesic effect, which we suggested to be mediated *via* a distinct receptor, possibly of the ET_B type. The aims of the present study were to further characterize the pro-inflammatory effects of endothelin-1 in the mouse hind-paw, namely its ability to also cause nociception and oedema, as well as to identify the ET receptors involved. Part of the results of this study have been published recently in abstract form (Rae *et al*., 1999).

Methods

Animals

Male Swiss mice (25–30 g), from our own colony, were lodged in a room with controlled temperature ($22 \pm 2^{\circ}C$) and lighting (lights on from 0600 h to 1800 h), with free access to lab chow and tap water. All experiments were conducted between 1000 h and 1700 h, were approved by the institution's Ethical Committee for research on laboratory animals and are in accordance with the ethical guidelines of the International Association for the Study of Pain (Zimmermann, 1983).

Experimental protocols

Each animal received a 20 μl intraplantar (i.pl.) injection of either endothelin-1 (0.3–30 pmol), endothelin-3 (1–30 pmol), sarafotoxin S6c (3–30 pmol), IRL 1620 (10–100 pmol) or vehicle (phosphate-buffered saline, PBS) and was placed, immediately and alone, into a glass jar laying over a mirror (set at an angle of about 60° relative to the table to enable full view of the paws at all times), and the amount of time spent licking the injected hind-paw (in s) was recorded cumulatively over the next 30 min, using a stopwatch chronometer. After this time, capsaicin (0.1 μg in 20 μl of 1% dimethylsulphoxide–DMSO–in saline) was injected into the ipsilateral paw, and the licking time displayed by each animal was recorded for a further 5 min. This dose of capsaicin was chosen as, in preliminary experiments, it induced a reliable and clearly sub-maximal nociceptive response, with no significant oedematogenic effect of its own (results not shown). All animals were sacrificed by cervical dislocation immediately after completion of the observation period, both hind-paws were cut off at the ankle joint and oedema of the injected hind-paw was assessed as the weight difference (in mg) relative to the contra-lateral non-injected hind-paw. A separate group of mice was used to establish the time-course of the oedematogenic effects of endothelin-1 (1–100 pmol) or vehicle, by measuring changes in hind-paw volume plethysmographically relative to baseline, at 5–15-min intervals up to 90 min after injection.

To assess the identity of ET receptors underlying the various effects of endothelin-1, separate groups of mice were pretreated either locally with sarafotoxin S6c (30 pmol; selective ET_B receptor agonist, i.pl., 15 min beforehand), BQ-123 or BQ-788 (1 or 10 nmol in 20 μl of PBS, i.pl., 15 min beforehand; selective peptidic antagonists of endothelin ET_A and ET_B receptors, respectively), or systemically with A-127722-5 (6 μmol kg^{-1}, i.v., 60 min beforehand; selective non-peptidic ET_A receptor antagonist) prior to ipsilateral i.pl. injection of endothelin-1 (10 or 30 pmol). The i.pl. doses of BQ-123 and BQ-788 were selected based on previous studies of our group (De-Melo *et al*., 1998; Piovezan *et al*., 1998). However, given the very slow dissociation rate of endothelins from ET receptors (Wu-Wong *et al*., 1994), as well as the apparent lack of objective pharmacokinetic data on these peptidic antagonists in the literature (at least when administered by i.d., s.c. or i.pl. administration), the short 15 min pre-treatment time was chosen to minimize the possible influence of local enzymatic degradation of the antagonist, as well as to enable its access to the receptors before exposure to the ET receptor agonist. Dosage and pre-treatment time of A-127722-5 were selected based on the study of Opgenorth *et al*. (1996). Control mice were similarly pretreated with an equal volume of the corresponding vehicle used to dissolve each substance. Nociceptive, hyperalgesic and oedematogenic effects of endothelin-1 were recorded as before.

Statistical analysis

In all experiments, responses of drug-treated animals were always assessed in parallel to those of vehicle-treated mice, to minimize the interference of possible fluctuations in responsiveness. Thus, responses of each drug-treated group were compared to those of its corresponding day-matched control group. Results are presented as mean $\pm$ s.e.mean of either the absolute values of accumulated hind-paw licking time or of changes in hind-paw weight or volume. Data were statistically evaluated by analysis of variance followed by Bonferroni's test. The significance level in all cases was set at $P < 0.05$.

Drugs and solutions

Stock solutions (0.1–1 mM) of endothelin-1, endothelin-3, sarafotoxin S6c, BQ-123 (cyclo[DTrp-DAsp-Pro-DVal-Leu]), IRL 1620 (Suc[Glu^9,$Ala^{11,15}$]-endothelin-1_{10-21}) (all from American Peptide Co., Sunnyvale, U.S.A.), BQ-788 (N-*cis*-2,6-dimethylpiperidinocarbonyl-L-γ-methylleucyl-D-1-methoxycarboyl-D-norleucine; Research Biochemicals International, Natick, U.S.A.) were prepared in PBS. All stock solutions were kept at $-18^{\circ}C$ as 50–100 μl aliquots and diluted to the desired concentration in the same vehicle just prior to use. Capsaicin (from Sigma Chemical Company, St. Louis, U.S.A.) was initially dissolved in 50% DMSO in PBS to the concentration of 250 μg ml^{-1}, and diluted thereafter in 1% DMSO in PBS to achieve a final concentration of 5 μg ml^{-1}, which was kept in a refrigerator for up to 1 week. A-127722-5 {[2R-(2α,3β,4α)]-4-(1,3-benzodioxol-5-yl)-1; a kind gift from Abbott Laboratories, Abbott Park, U.S.A.} was dissolved immediately before use in 3% ethanol in water.

Results

Nociception induced by ET receptor agonists

Local injection of endothelin-1 into the hind-paw induced dose-dependent nociception *per se*, leading to significant increases in accumulated licking time at doses greater than 3 pmol (Figure 1a). Licking responses directed to hind-paws injected with endothelin-1 at 10 and 30 pmol were far greater than those elicited by a similar injection of PBS. Although the onset of such endothelin-1-induced licking was somewhat variable, the response was usually short-lived ($\sim$5 min) in each animal and largely confined to the first 15 min post-injection. In a sample of 20 mice injected with 10 pmol of endothelin-1,

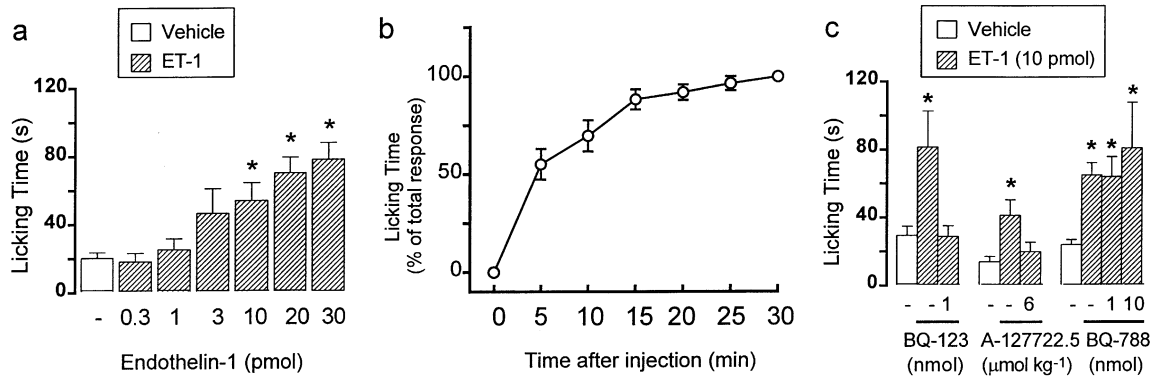

Figure 1 Nociceptive effects of endothelin-1 in the mouse hind-paw, as evaluated by the time spent licking the injected paw (in s). Accumulated licking time (a) over the first 30 min following intraplantar injection of either vehicle (PBS) or endothelin-1 (ET-1). Time-course (b) of endothelin-1-induced (10 pmol; i.pl.) nociceptive effect, presented as accumulated percentages (in 5-min bins) of the total licking time displayed over the 30-min session. Influence of prior treatment (c) with BQ-123 (1 nmol, i.pl.), A-127722-5 (6 μmol kg^{-1}; i.v.), BQ-788 (1 or 10 nmol; i.pl.) or vehicle on nociception induced by endothelin-1 (10 pmol, i.pl.). Mean$\pm$s.e.mean values of 6–12 (a,c) or 20 animals (b). *$P<0.05$ when compared to respective control value (ANOVA followed by Bonferroni's test).

Table 1 Influence of ET receptor antagonists on nociceptive, hyperalgesic and oedematogenic effects of endothelin-3 in the mouse hind-paw

Treatment		*Pre-treatment*		*Nociception*	*Hyperalgesia*	*Oedema*
Agonist	pmol	*Antagonist*	*Dose*	*Licking* (s)	*Licking* (s)	Δ (mg)
PBS (control)	–	–	–	17.3$\pm$3.9	47.6$\pm$4.0	50.5$\pm$1.5
Endothelin-3	1	–	–	19.3$\pm$4.8	56.8$\pm$5.8	52.3$\pm$2.1
	10	–	–	18.8$\pm$3.2	59.0$\pm$3.4	60.1$\pm$1.8*
	30	–	–	27.0$\pm$3.3	58.7$\pm$7.2	62.3$\pm$1.9*
PBS (control)	–	PBS	–	N.D.	N.D.	45.7$\pm$0.9
Endothelin-3	30	PBS	–	N.D.	N.D.	59.8$\pm$1.6*
	30	BQ-123	1 nmol	N.D.	N.D.	42.7$\pm$1.8
PBS (control)	–	Vehicle[#]	–	N.D.	N.D.	45.8$\pm$2.1
Endothelin-3	30	Vehicle[#]	–	N.D.	N.D.	58.7$\pm$3.2*
	30	A-127722-5[#]	6 μmol kg^{-1}	N.D.	N.D.	41.5$\pm$3.4
PBS (control)	–	PBS	–	13.1$\pm$3.9	40.1$\pm$5.8	50.7$\pm$2.1
Endothelin-3	30	PBS	–	24.4$\pm$4.3	54.5$\pm$10.0	62.2$\pm$1.5*
	30	BQ-788	10 nmol	34.7$\pm$12.6	76.1$\pm$11.2*	74.2$\pm$6.3*
PBS (control)	–	PBS	–	14.1$\pm$3.5	44.0$\pm$6.0	53.7$\pm$1.9
PBS	–	BQ-123	1 nmol	19.7$\pm$8.7	41.3$\pm$6.2	54.4$\pm$2.7
PBS	–	A-127722-5[#]	6 μmol kg^{-1}	26.0$\pm$11.1	47.8$\pm$13.2	50.0$\pm$3.5
PBS	–	BQ-788	10 nmol	29.6$\pm$7.2	58.4$\pm$7.3	71.7$\pm$3.8*

Nociception and hyperalgesia were quantified as total licking time (in s) measured over first 30 min after i.pl. agonist injection or first 5 min following capsaicin injection (0.1 μg, i.pl.), respectively. Oedema was assessed as the difference in weight between injected and non-injected hind-paws (Δ , in mg), immediately after hyperalgesia measurement. Mean$\pm$s.e.mean values of 6–12 experiments. [#]Intravenous administration. All other pre-treatments were given by i.pl. injection. N.D. denotes values not determined. *$P<0.05$ when compared to respective control value (ANOVA followed by Bonferroni's test).

in which individual licking times were measured cumulatively in 5-min bins over 30 min, 88.3$\pm$5.1% of the total nociceptive response occurred within 15 min of injection, and only one animal had not displayed its full nociceptive response up to 25 min (Figure 1b). On the other hand, and in sharp contrast to endothelin-1, neither endothelin-3 (up to 30 pmol; Table 1) nor the selective ET_B receptor agonists sarafotoxin S6c and IRL 1620 (1–100 pmol; Table 2) induced significant nociceptive responses.

As shown in Figure 1c, nociception triggered by endothelin-1 (10 pmol) was fully prevented by prior treatment with the selective ET_A receptor antagonists BQ-123 (1 nmol, i.pl.) or A-127722-5 (6 μmol kg^{-1}, i.v.), but was unaffected by the selective ET_B receptor antagonist BQ-788 (1 or 10 nmol, i.pl.). Furthermore, BQ-788 (10 nmol) also failed to modify endothelin-3-induced nociception, and none of the antagonists alone affected basal nociceptive responses of control PBS-treated mice or nociception triggered by capsaicin (0.1 μg; Table 1).

Hyperalgesia induced by ET receptor agonists

Fully confirming previous findings (Piovezan *et al*., 1998), endothelin-1 significantly potentiated nociceptive responses to capsaicin (0.1 μg, i.pl.), i.e. caused hyperalgesia, when given i.pl. at doses between 1 and 20 pmol 30 min beforehand (Figure 2a). The dose-response curve to this effect of the peptide was bell-shaped, with a maximum at 10 pmol and no significant effect at all at a slightly higher dose (30 pmol). Unlike endothelin-1, neither endothelin-3 (up to 30 pmol; Table 1) nor the selective ET_B receptor agonists sarafotoxin S6c (1, 3, 10 or 30 pmol) or IRL 1620 (10, 30 or 100 pmol) modified capsaicin-induced nociceptive responses (Table 2).

As depicted in Figure 2b, hyperalgesia induced by endothelin-1 (10 pmol) was fully blocked by prior treatment with either BQ-123 (1 nmol, i.pl.) or A-127722-5 (6 μmol kg^{-1}, i.v.), but was not altered by the selective ET_B receptor antagonist BQ-788 (1 or 10 nmol, i.pl.). On the other hand, pre-treatment with BQ-788 (10 nmol)

uncovered a significant hyperalgesic effect of endothelin-3 (30 pmol; Table 1).

Oedema induced by ET receptor agonists

Local injection of endothelin-1, at doses greater than 3 pmol, into the hind-paw induced dose-dependent oedema, leading to significant increases in weight above those seen in vehicle-treated animals at the time of sacrifice (i.e. 35 min after its injection and recording nociceptive responses to capsaicin 0.1 μg) (Figure 3a). Capsaicin alone, at this small dose, did not increase hind-paw weight any more than did PBS ($n=6$, results not shown). As shown in Figure 3b, when hind-paw volume was assessed directly by plethysmography in a separate group of mice, the oedematogenic actions of endothelin-1 (3, 10, 30 or 100 pmol) displayed a rapid onset, starting 5 min after injection, reached peak values at 15–30 min and subsided thereafter. Injection of PBS induced a much smaller and transient increase in paw volume which was virtually absent by 15 min. In this regard, it should be noted that the increase in hind-paw weight detected in PBS-treated controls at 35 min (Figure 3a) can be ascribed to the volume of the capsaicin injection (20 μl), given only 5 min prior to sacrifice. Endothelin-3, at 10 or 30 pmol, also caused hind-paw oedema, but this effect was much more modest than that observed with endothelin-1 (Table 1). Sarafotoxin S6c (1, 3, 10 or 30 pmol) or IRL 1620 (10, 30 or 100 pmol) each failed to cause oedematogenic effects (Table 2).

Oedema triggered by endothelin-1 (10 pmol) was fully prevented by prior treatment with the selective ET_A receptor antagonists BQ-123 (1 nmol, i.pl.) or A-127722-5 (6 μmol kg^{-1}, i.v.), but was unaffected by the selective ET_B receptor antagonist BQ-788 (1 or 10 nmol, i.pl.; Figure 3c). Likewise, BQ-788 (10 nmol) also failed to modify the oedematogenic effect of endothelin-3 (30 pmol; Table 1). Neither of the ET_A receptor antagonists alone influenced hind-paw weight in PBS-treated mice, but the highest dose of BQ-788 displayed a modest, yet significant, oedematogenic effect (Table 1).

Modulatory role of ET_B receptors on effects of endothelin-1

Pre-treatment with the selective ET_B receptor antagonist BQ-788 (at 10 nmol, i.pl.) was found to unmask a significant hyperalgesic response to a normally ineffective dose of endothelin-1 (30 pmol), without modifying the peptide's nociceptive or oedematogenic effects (Figure 4). On the other hand, as is also shown in Figure 4, prior i.pl. treatment with the selective ET_B receptor agonist sarafotoxin S6c (30 pmol) selectively inhibited the hyperalgesic, but not the nociceptive or oedematogenic, effect of endothelin-1 (10 pmol).

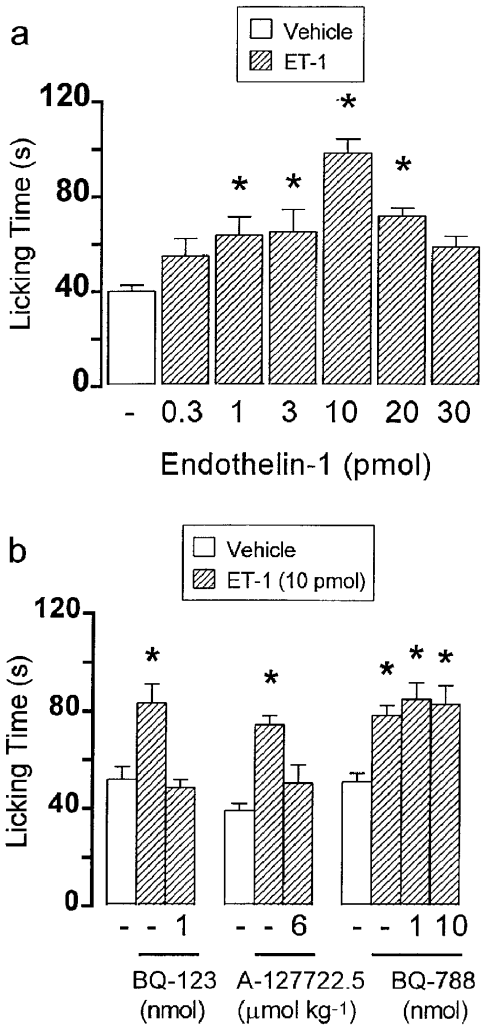

Figure 2 Hyperalgesic effects of endothelin-1 on capsaicin-induced nociception in the mouse hind-paw. Accumulated licking times (a) measured in first 5 min following injection of capsaicin (0.1 μg, i.pl.), 30 min after similar ipsilateral injection of endothelin-1 (ET-1) or vehicle (PBS). Influence of prior treatment (b) with BQ-123 (1 nmol, i.pl.), A-127722-5 (6 μmol kg^{-1}; i.v.), BQ-788 (1 or 10 nmol; i.pl.) or vehicle on hyperalgesia induced by endothelin-1 (10 pmol, i.pl.). Mean $\pm$ s.e.mean values of 6–12 animals. *$P<0.05$ when compared to respective control value (ANOVA followed by Bonferroni's test).

Discussion

The present study clearly corroborates our previous report (Piovezan *et al*., 1998) that endothelin-1 causes pronounced ET_A receptor-mediated hyperalgesia in the model of capsaicin-induced nociception in the mouse hind-paw. However, more importantly, it also demonstrates that this peptide can induce two additional pro-inflammatory effects in this model, nociception and oedema, both of which are dose-related and likewise mediated *via* ET_A receptors. All three effects of endothelin-1 appear to reflect a local action as they are clearly restricted to the injected hind-paw (i.e. no changes can be detected in the contralateral non-injected paw; results not shown).

Nociception induced by endothelin-1 was clearly dose-dependent and similar, at the highest dose tested (30 pmol), to that elicited by 0.1 μg of capsaicin. It should be pointed out, however, that the nociceptive response triggered by this dose of capsaicin is clearly sub-maximal (Sakurada *et al*., 1992; Piovezan *et al*., 1998). Furthermore, the onset of endothelin-1-induced nociception was more variable than that triggered by capsaicin. Whereas capsaicin consistently induces licking within the first 5 min following injection, the onset of licking responses to endothelin-1 quite often occurred at somewhat longer periods after administration, but was largely complete within the first 15 min and could not be observed at all beyond 30 min. Thus, it seems very unlikely that the potentiation of capsaicin-induced nociception by endothelin-1, as demonstrated using the present protocol, was merely the consequence of additive nociceptive effects of both agents. This conclusion is important because it points to a true hyperalgesic effect of endothelin-1 in this model, even though we cannot rule out the possibility of a

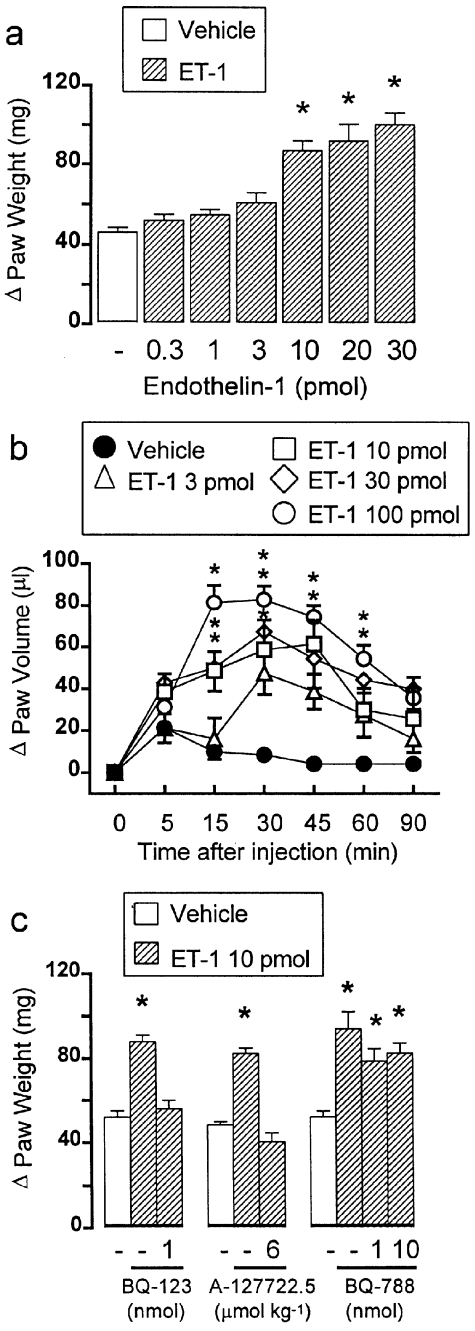

Figure 3 Oedematogenic effects of endothelin-1 in the mouse hind-paw. Oedema is expressed either as increases in hind-paw weight (in a and c, in mg) or volume (in b, in μl), relative to the contra-lateral non-injected paw. Increases in hind-paw weight (a) 35 min after either vehicle (PBS) or endothelin-1. Time-course (b) of the increase in hind-paw volume over the first 90 min after injection of either vehicle or endothelin-1 (3, 10, 30 or 100 pmol, i.pl.). Influence of prior treatment (c) with BQ-123 (1 nmol, i.pl.), A-127722-5 (6 μmol kg^{-1}; i.v.), BQ-788 (1 or 10 nmol; i.pl.) or vehicle on oedema induced by endothelin-1 (10 pmol, i.pl.). All mice in 'a' and 'c', but not in 'b', received capsaicin (0.1 μg, i.pl.) 5 min prior to sacrifice. Mean$\pm$s.e.mean values of 6–12 animals. *$P<0.05$ when compared to respective control value (ANOVA followed by Bonferroni's test).

direct causal relationship between the nociceptive and hyperalgesic effects of the peptide.

The finding that endothelin-1 causes nociception in the mouse hind-paw is in good agreement with previous reports that the peptide causes deep muscular pain in humans, when injected intra-arterially in the forearm (Dalhoff *et al*., 1990),

Table 2 Selective ET_B receptor agonists sarafotoxin S6c and IRL 1620 fail to cause nociceptive, hyperalgesic and oedematogenic effects in the mouse hind-paw

Treatment		*Nociception*	*Hyperalgesia*	*Oedema*
Agonist	pmol	*Licking* (s)	*Licking* (s)	Δ (mg)
PBS (control)	–	22.5±5.9	43.5±5.9	44.7±2.4
Sarafotoxin S6c	3	31.9±4.3	52.5±4.7	46.4±3.5
	10	15.2±2.2	51.8±4.8	41.9±4.0
	30	15.4±4.5	55.4±6.4	45.3±3.2
PBS (control)	–	21.9±5.2	50.9±8.8	44.8±2.5
IRL 1620	10	17.6±6.4	39.1±4.5	44.8±5.6
	30	29.4±7.1	39.0±3.7	41.2±1.9
	100	12.9±8.1	41.9±10.6	44.8±1.9

Nociception and hyperalgesia were quantified as total licking time (in s) measured over first 30 min after i.pl. agonist injection or first 5 min following capsaicin injection (0.1 μg, i.pl.), respectively. Oedema was assessed as the difference in weight between injected and non-injected hind-paws (Δ, in mg), immediately after hyperalgesia measurement. Mean $\pm$s.e. mean values of 6–8 experiments. All values from agonist-treated animals were not statistically different from their respective PBS-treated control values ($P>0.05$; ANOVA followed by Bonferroni's test).

articular incapacitation in dogs (Ferreira *et al*., 1989) and rats (De-Melo *et al*., 1998) and writhing (abdominal constriction) in mice (Ferreira *et al*., 1989; Raffa & Jacoby, 1991). Only the latter two studies attempted to address, to any significant extent, the possible cellular mechanisms and/or mediators involved in endothelin-1-induced nociception, but obtained conflicting evidence as to the possible participation of prostanoids in mediating endothelin-1-induced writhing. Whereas Ferreira *et al*. (1989) found that responses elicited by 1 μg kg^{-1} of endothelin-1 where abolished by indomethacin, Raffa & Jacoby (1991) reported that writhing triggered by a 100 μg kg^{-1} of the peptide was fully resistant to inhibition by indomethacin or another two cyclo-oxygenase blockers. On the other hand, endothelin-1 has recently been found to elicit pain-related paw-flinching behaviour in the rat, when applied (albeit at high concentrations) to the epineural surface of the sciatic nerve (Davar *et al*., 1998), which could indicate that it may directly activate sensory neurones. This view is further substantiated by evidence that the peptide causes substance P-mediated depolarization of ventral roots in the rat newborn spinal cord (Yoshizawa *et al*., 1989), markedly augments substance P release from rat isolated sensory neurones (Dymshitz & Vasko, 1994), interacts with specific binding sites in rat dorsal root ganglia (Kar *et al*., 1991) and causes a histamine-dependent axon-reflex (sensory neuron-mediated) flare reaction in human skin, without directly causing human mast-cell degranulation *in vitro* (Brain *et al*., 1992; Wenzel *et al*., 1998).

The present study also provides considerable evidence that endothelin-1-induced nociception, hyperalgesia and oedema in the mouse hind-paw are all the consequence of ET_A receptor activation. The selective endothelin ET_B receptor agonists sarafotoxin S6c and IRL 1620 (up to 30 and 100 pmol, respectively) each failed to mimic any of the three actions of endothelin-1. Prior local injection of BQ-123 (1 nmol), a selective ET_A receptor antagonist (Ihara *et al*., 1992), fully inhibited the development of nociception, hyperalgesia and oedema triggered by endothelin-1 (10 pmol), whereas these parameters were unaffected by similar treatment with BQ-788 (1 or 10 nmol), a selective ET_B receptor antagonist. Furthermore, systemic pre-treatment with A-127722-5, a potent and highly selective non-peptidic ET_A receptor antagonist, mimicked the actions of locally administered BQ-

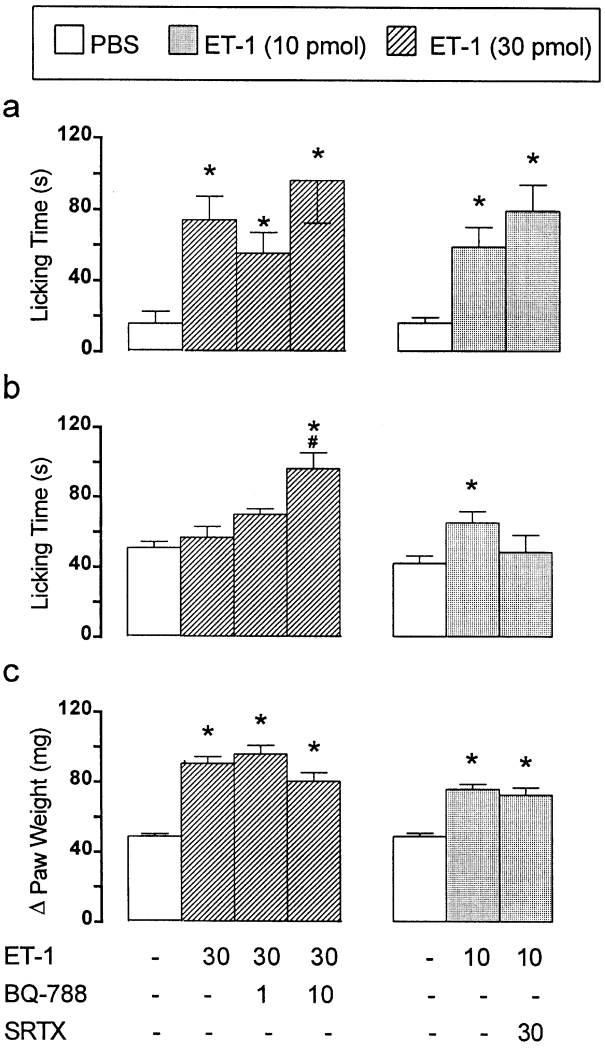

Figure 4 Influence of pre-treatment with BQ-788 (1 or 10 nmol, i.pl.) or sarafotoxin S6c (30 pmol, i.pl.) on nociceptive (a), hyperalgesic (b) and oedematogenic (c) effects of ipsilateral injection of endothelin-1 (10 or 30 pmol; i.pl.) into the mouse hind-paw. BQ-788, sarafotoxin S6c or vehicle (PBS) were injected 15 min prior to endothelin-1. Mean ± s.e.mean values of 6–10 animals. * and #$P<0.05$ when compared to respective vehicle-pretreated/vehicle-treated control value and vehicle-pretreated/endothelin-1-treated value, respectively (ANOVA followed by Bonferroni's test).

123 very closely. Finally, endothelin-3, which displays far lower affinity for ET_A receptors than endothelin-1 (for review, see Masaki *et al.*, 1994) and failed to induce nociceptive behaviour up to 30 pmol, effectively induced a modest oedematogenic effect which was prevented by either BQ-123 or A-127722-5, but slightly potentiated by BQ-788 (10 nmol). However, it should be pointed out that, for reasons that are still unclear, this dose of BQ-788 caused *per se* a similar discrete increase in paw weight in PBS-treated control mice. On the other hand, the finding that BQ-788, at 10 pmol, uncovered a hyperalgesic effect of endothelin-3 and of a high dose of endothelin-1 (30 pmol), without modifying responsiveness to capsaicin alone in PBS-treated mice, attests to its effectiveness and specificity in blocking local ET_B receptors at this dose.

Selective ET receptor antagonists have been instrumental to the identification of the receptors subserving exogenous endothelin-1-induced nociception, hyperalgesia and oedema in other models. This approach has implicated both ET_A and ET_B receptors in the nociception induced by endothelins in the mouse abdominal writhing test (Raffa *et al.*, 1996), or by endothelin-1 injected intra-articularly into the carrageenan-primed joint (De-Melo *et al.*, 1998). However, in agreement with our current findings, the latter study also found that articular nociception triggered by the peptide in the rat naive knee joint appears to be mediated solely *via* ET_A receptors. On the other hand, as regards hyperalgesia, the present results fully substantiate the only previous report on this aspect, which also attributed an exclusive role for ET_A receptors in mediating this potentiating effect of exogenous endothelin-1 on nociception triggered by capsaicin in the mouse hind-paw (Piovezan *et al.*, 1998). The studies which specifically assessed which receptor type(s) mediate increases in microvascular permeability triggered *in vivo* by exogenous ET receptor agonists have proposed that enhancement of plasma extravasation is mediated solely (or almost exclusively) by ET_A receptors in the rat coronary circulation, stomach and duodenum (Filep *et al.*, 1992; 1993) and in rabbit eye (Haque *et al.*, 1996), by ET_B receptors in rat venous mesenteric vasculature (Kurose *et al.*, 1993) and dura mater (Brändli *et al.*, 1996), and by both ET_A and ET_B receptors in rat airways and kidney (Filep *et al.*, 1992; 1993) and guinea-pig airways (Filep *et al.*, 1995).

A few studies have also used ET receptor antagonists to detect the contribution of endogenous endothelins towards nociception, hyperalgesia and oedema (or enhancement of microvascular permeability) in different models of inflammation. De-Melo *et al.* (1998) demonstrated a major role for ET_B (but not ET_A) receptor activation in articular nociception triggered by *E.coli* lipopolysaccharide in rat carrageenan-primed (but not in naive) knee joints. More recently, Griswold *et al.* (1999) reported that pain elicited by phenylbenzoquinone in mice is also markedly inhibited by the selective ET_B receptor antagonist A-192621, but is not affected by SB 234551, a selective ET_A receptor antagonist. Furthermore, the nociceptive effects of phenylbenzoquinone are abolished in ET_B receptor knockout homozygous ($ET_B^{(-/-)}$) mice and substantially blunted in heterozygous ($ET_B^{(+/-)}$) animals. Due to the limitations of either model, it remains to be adequately determined if the ET_B receptor-mediated mechanisms detected by De-Melo *et al.* (1998) and Griswold *et al.* (1999) are the result of nociceptive and/or hyperalgesic actions of endogenous endothelins. As to the possible receptor types implicated in oedema or enhancement of microvascular permeability by endogenous endothelins, mouse hind-paw oedema induced by antigen or carrageenan is significantly attenuated by pre-treatment with a selective ET_A receptor antagonist (Sampaio *et al.*, 1995). However, solely ET_B receptors appear to be involved in neurogenic plasma extravasation in rat dura mater induced by intravenous capsaicin or unilateral electrical stimulation of the trigeminal ganglion (Brändli *et al.*, 1996) or in arachidonic acid-induced ear oedema in mice (Griswold *et al.*, 1999). Altogether, the current results, allied to the studies mentioned in this and the previous paragraph, seem to point to significant species- and tissue-related differences in the ET receptors mediating the nociceptive, hyperalgesic and oedematogenic effects of exogenous and endogenous endothelins.

It should be noted that the curve for endothelin-1-induced hyperalgesia was clearly bell-shaped, unlike the monophasic appearance of the curves obtained for either its nociceptive or oedematogenic effects, at least in the dose-range studied here. We have suggested previously that the abrupt downward deflection of the curve for the hyperalgesic effect of endothelin-1, at doses only slightly greater than that which is maximally effective (10 pmol), possibly reflects activation of anti-hyperalgesic ET_B receptors as, at 30 pmol, the peptide

abolishes hyperalgesia induced by serotonin, an action mimicked by the selective ET_B receptor agonist IRL-1620 (Piovezan *et al*., 1998). We have now found that prior treatment with the ET_B receptor antagonist BQ-788 (1 and 10 nmol, i.pl.) clearly unveiled, in dose-dependent fashion, a hyperalgesic effect of 30 pmol endothelin-1. Indeed, in mice treated with 10 nmol of BQ-788, the magnitude of hyperalgesia induced by 30 pmol of endothelin-1 was not different from that usually produced by the maximally effective 10 pmol dose of the peptide. Since BQ-788 failed to amplify nociceptive or oedematogenic effects of 30 pmol of endothelin-1, the depressor role of ET_B receptors seems to be restricted to the hyperalgesic component of endothelin-1 action. It could be argued that the failure of BQ-788 to potentiate ET_A receptor-mediated nociception caused by endothelin-1 simply reflects a slow onset of an ET_B receptor-mediated analgesic mechanism. However, this appears unlikely for three reasons. Local injection of sarafotoxin S6c, 30 min prior to endothelin-1, did not influence the degree of nociception (or oedema) triggered by the later peptide, yet inhibited its hyperalgesic effect. As already mentioned, sarafotoxin S6c and IRL 1620 each failed to inhibit nociception induced by capsaicin, indicating that the ET_B receptors are truly anti-hyperalgesic, not analgesic. Finally, endothelin-3, which at any dose would activate a larger proportion of ET_B receptors over ET_A receptors when compared to endothelin-1, only caused significant hyperalgesia when animals were pre-treated with BQ-788. Therefore, in addition to activating ET_A receptors coupled to nociception, hyperalgesia and oedema in the mouse hind-paw, at higher doses endothelin-1 can also trigger a selective anti-hyperalgesic ET_B receptor-mediated mechanism. On the other hand, since none of the ET receptor antagonists influenced *per se* nociception in PBS-treated control mice, it would appear that this process is not normally subject to tonic modulation by endogenous endothelins.

In our view, there is an important difference concerning the role played by ET_B receptors in capsaicin- (current study) and in formalin-induced pain in the mouse hind-paw (Piovezan *et al*., 1997). Unlike capsaicin, formalin induces two clear cut phases of nociceptive response, the first of which has been suggested to reflect non-inflammatory pain, whereas the second seems more related to inflammatory pain (Hunskaar & Hole, 1987). As seen in the current study using the capsaicin model, Piovezan *et al*. (1997) found that endothelin-1 potentiated the first phase of formalin-induced nociception, but sarafotoxin S6c did not. In sharp contrast, both peptides clearly potentiated the second phase of formalin-induced pain. This may indicate that the anti-hyperalgesic role played by ET_B receptors in non-inflammatory pain, as detected in the capsaicin model in the current study, but not evaluated by Piovezan *et al*. (1997), is shifted (or indeed reversed) towards a hyperalgesic role in inflammatory nociception. This hypothetical inflammation-dependent switch in ET_B receptor coupling, which we are currently investigating in the capsaicin model, may help explain why sarafotoxin S6c only induces long-lasting articular incapacitation (nociception) in the rat when injected into a carrageenan-primed, but not into a naive, knee joint, whereas endothelin-1 induces incapacitation in both cases (De-Melo *et al*., 1998), as well as the pivotal role for ET_B receptors in mediating phenylbenzoquinone-induced writhing and, to a lesser extent, arachidonic acid-induced oedema in mice (Griswold *et al*., 1999).

In conclusion, we have shown that endothelin-1 induces nociception, hyperalgesia (to nociception triggered by capsaicin) and oedema in the mouse hind-paw by activating ET_A receptors. Furthermore, the peptide can also activate ET_B receptors locally which selectively counteract its hyperalgesic effects, without influencing nociception or oedema. The intrinsic mechanisms underlying all four effects of endothelin-1 (including anti-hyperalgesia) in this model remain to be fully determined. Considering that several inflammatory stimuli, such as carrageenan, lipopolysaccharide and various cytokines can trigger endothelin release (see Introduction for references), endogenous endothelins may contribute importantly towards the onset of various components of inflammation. If this possibility proves true for humans, ET_A receptor antagonists may well constitute an interesting new anti-inflammatory therapeutic strategy. However, further studies are still needed to clarify the potential anti-hyperalgesic usefulness of selective ET_B receptor agonists and/or antagonists.

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