Local Injection of a Selective Endothelin-B Receptor Agonist Inhibits Endothelin-1-Induced Pain-Like Behavior and Excitation of Nociceptors in a Naloxone-Sensitive Manner

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We showed previously that subcutaneous injection of the injury-associated peptide mediator endothelin-1 (ET-1) into the rat plantar hindpaw produces pain behavior and selective excitation of nociceptors, both through activation of ET_A receptors likely on nociceptive terminals. The potential role of ET_B receptor activation in these actions of ET-1-has not been examined. Therefore, in these experiments, we studied the effect of blocking or activating ET_B receptors on ET-1-induced hindpaw flinching and excitation of nociceptors in rats. An ET_B receptor-selective antagonist, BQ-788 (3 mM), coinjected with ET-1 (200 μM) reduced the time-to-peak of flinching and significantly enhanced the average maximal flinch frequency (MFF). In contrast, coinjection of an ET_B receptor selective agonist, IRL-1620 (100 or 200 μM), with ET-1 reduced the average MFF and the average total number of flinches. Interestingly, this unexpected inhibitory effect of IRL-1620 was prevented by the nonselective opioid receptor antagonist naloxone (2.75 mM). To confirm these inhibitory actions, we studied the effects of IRL-1620 on ET-1-induced spike responses in single, physiologically characterized nociceptive C-fibers. IRL-1620 suppressed spike responses to ET-1 in all ($n = 12$) C-units, with mean and maximum response frequencies of 0.08 ± 0.02 and 1.5 ± 0.4 impulses/sec versus 0.32 ± 0.07 and 4.17 ± 0.17 impulses/sec for ET-1 alone. In additional support of the behavioral results, coinjection of naloxone (2.75 mM) completely prevented this inhibitory action of IRL-1620. These results establish that ET_B receptor activation inhibits ET-1-induced pain behavior and nociception in a naloxone-sensitive manner and point to a previously unrecognized dual modulation of acute nociceptive signaling by ET_A and ET_B receptors in cutaneous tissues.

Key words: nociception; analgesia; G-protein; endothelin-1; opioid; potassium channel

Endothelin-1 (ET-1) is a potent, endogenous vasoactive peptide (Hickey at al., 1985; Yanagisawa et al., 1988) whose effects are mediated by two distinct G-protein-coupled receptors, the endothelin-A (ET_A) and the endothelin-B (ET_B) receptor, which usually mediate vasoconstriction and vasodilatation, respectively (Rubanyi and Polokoff, 1994). Interestingly, ET-1 has a role in pain signaling in animals and humans (Hammerman et al., 1997; Davar et al., 1998; De-Melo et al., 1998; Graido-Gonzalez et al., 1998; Jarvis et al., 2000). For example, in rodents, intraperitoneal administration of ET-1 produces an abdominal writhing response that is ET_A receptor mediated and that may be behavioral evidence of acute pain (Raffa et al., 1996a,b). Intra-articular administration of ET-1 also has been shown recently to induce pain in rodents that is ET_A receptor mediated, and ET-1 is known to potentiate pain states in several animal models of acute chemical- or inflammation-induced pain (Ferreira et al., 1989; Piovezan et al., 1997, 1998, 2000). In humans, the intra-arterial administration of ET-1 is reported to induce severe pain that is associated with prolonged touch-evoked allodynia in the injected limb (Dahlof et al., 1990), and, more recently, Carducci et al. (1998) have reported that antagonists of the endothelin-A receptor can reduce pain in patients with metastatic prostate cancer. Consistent with a role in cutaneous injury, ET-1 is also oversecreted after skin damage (Ahn et al., 1998) in which it might contribute to both local inflammation (Griswold et al., 1999) and pain.

Consistent with this evidence of ET-1-induced pain in animals and humans, we described recently ET_A receptor-dependent flinching behavior and selective excitation of nociceptors after subcutaneous injection of ET-1 into the rat plantar hindpaw (Gokin et al., 2001). At a cellular level, we demonstrated ET_A receptor-mediated excitation of nociceptor-like sensory neurons (ND7 cells) and enhancements of tetrodotoxin-resistant sodium currents in acutely isolated rat dorsal root ganglion neurons (Chen et al., 2000; Zhou et al., 2001, 2002). These results, together with anatomic evidence of ET_A (but not ET_B) receptors on small-diameter DRG neurons and their axons (Pomonis et al., 2001), further strengthen the potential importance of ET-1 (and ET_A receptors) in the pathogenesis of pain.

Whereas a role for ET_A receptors in ET-1-induced pain has been established, the importance of ET_B receptors for this pain is not known. Although antihyperalgesic effects of ET_B receptor activation have been described in some models of exogenous ET-1 delivery (Piovezan et al., 2000), the mechanism of this effect has not been determined. Furthermore, despite evidence to support a role for the ET_B receptor in cutaneous inflammation (Griswold et al., 1999), the role of the ET_B receptor in acute pain produced by ET-1 administration remains unknown. Therefore,

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in these experiments, we examined the effects of either a locally administered ET_B receptor antagonist or an ET_B receptor agonist on hindpaw flinching and excitation of nociceptors induced by the acute subcutaneous application of ET-1. The potential role of opioid receptors in ET_B receptor-mediated inhibition of ET-1-induced flinching and nociceptive firing that we observed during the course of these experiments was also examined.

MATERIALS AND METHODS

General. Experiments were performed on 122 adult (175–225 gm) male Sprague Dawley rats (Harlan Sprague Dawley, Indianapolis, IN). All procedures were approved by the Standing Committee on Animals at Harvard Medical School. Animals were treated and cared for according to the ethical standards and guidelines for investigators of experimental pain in animals prescribed by the Committee for Research and Ethical Issues of the International Association for the Study of Pain (Zimmermann, 1983). All rats were housed in a viral antibody-free facility (three per cage) on a 12 hr light/dark cycle with food and water available *ad libitum*. Before beginning experiments, animals were handled for 1–2 d and were thereby acclimated to both the testing environment and the experimenters.

Drugs. All drugs were diluted in PBS (Invitrogen, Rockville, MD), pH 7.4, and kept on ice during experiments. Synthetic ET-1 (98% pure peptide content), the ET_B receptor-selective antagonist BQ-788 (*N-cis*-2,6-dimethylpiperidinocarbonyl-L-γ-methylleucyl-D-1-methoxycarbonyl-trptophanyl-D-Nle) (reported IC_{50} of 1.2 nM at the ET_B receptor and 1.3 μM at the ET_A receptor) (Ishikawa et al., 1992), and the ET_B receptor-selective agonist IRL-1620 (Suc-Asp-Glu-Glu-Ala-Val-Tyr-Phe-Ala-His-Leu-Asp-lle-lle-Trp) (reported K_I of 16 pM at the ET_B receptor and 1.9 μM at the ET_A receptor) (Takai et al., 1992) were supplied by American Peptides (Sunnyvale, CA). The concentration of ET-1 used (200 μM) gives less than a saturating response for flinching behavior, as described by Fareed et al. (2000). Therefore, we assume that we are providing an effective concentration of ET-1 to target receptors that is less than four times the K_D (80% occupancy), where the K_D for both the ET_A and ET_B receptors are in the high picomolar to low nanomolar range (Rubanyi and Polokoff, 1994). BQ-788 was used at concentrations well in excess of the K_I (~100 nM) (Webber et al., 1998) to ensure complete blockade of the ET_B receptor. Naloxone was obtained from Sigma (St. Louis, MO) and dissolved in PBS. The dose of naloxone used for local injection was based on previously described reports of efficacy in rat models of cutaneous pain (Stein et al., 1990a; Eisenberg et al., 1996).

Injection procedures. For injections, naive rats were briefly anesthetized with the rapidly reversible inhalational anesthetic sevoflurane (3–4 min of inhalation). The method of ET-1 administration that we described previously (Gokin et al., 2001) was modified as follows to minimize the volume injected and to reduce the number of injections. Control and experimental subcutaneous injections were always performed as either a single 20 μl injection (for the 4 nmol dose of ET-1 alone) or as two sequential 10 μl injections. The first injection always contained one of the following: vehicle (when examining the effects of ET-1 alone), an ET_B receptor agonist or antagonist, or naloxone. The second injection contained ET-1 mixed with one of these agents. Agonists, antagonists, and naloxone were always injected at the same concentration for both injections and were given in the first injection to optimize binding to the target. The first injection was made 40 sec after the onset of limb cooling as described by Gokin et al. (2001). The hindlimb was cooled with a small amount of packed ice in a 15 ml polypropylene centrifuge tube (Corning, Corning, NY) placed beneath the limb; a small amount of ice in a small (~25 ml) plastic bag was also placed on top of the hindlimb. The second injection was performed 1.5–2 min after the first injection. Both injections were delivered subcutaneously into the midplantar paw. After the first injection, the area was lightly outlined with a felt tip marker, and the second injection was made along the same needle track into the marked area, usually ~1 cm distal to the heel.

Behavioral assessments. Behavioral assessments were performed as described previously (Davar et al., 1998), with animals freely moving on a flat surface that was enclosed by an inverted, large clear Plexiglas cage. Repetitive and spontaneous flinching of the ipsilateral hindpaw (rapid lifting of the entire hindlimb that begins with hip flexion and includes dorsiflexion of the toes) and the number of events and duration of biting or licking were counted beginning 5 min after ET-1 injection and continuing every 5 min for 75 min of observation. Blinding was performed in initial studies by providing experimenters with unlabeled tubes containing ET-1 or control solutions. However, the very clear and robust effect of ET-1 when compared with PBS made it difficult for the experimenters to remain blind. Similar results (effect readily discerned) were obtained when comparing ET-1 with BQ-788, IRL-1620, and naloxone, reducing the usefulness of blinding.

Neurophysiological experiments. Using methods we described previously in detail (Gokin et al., 2001), single-unit nerve activity was recorded from the right sciatic nerve before and after injection of ET-1, IRL-1620 together with ET-1, or IRL-1620 together with ET-1 and naloxone. Briefly, 12 adult male Sprague Dawley rats weighing 250–300 gm (Harlan Sprague Dawley) were initially anesthetized with intraperitoneal urethane (1.3 gm/kg; Sigma) or sodium pentobarbital (50–60 mg/kg). The right jugular vein was cannulated to permit intravenous administration of additional doses of sodium pentobarbital to maintain general anesthesia, titrated to the absence of corneal reflexes, accelerated heart rate, and withdrawal reflexes to noxious stimuli. Heart rate was monitored with a Tektronix (Beaverton, OR) 498 EKG monitor. Tracheotomy was performed for artificial respiration. During recordings, rats were immobilized with pancuronium bromide ($1\ mg \cdot kg^{-1} \cdot h^{-1}$, i.v.; Sigma) and artificially ventilated via a respirator (RSP1002; Kent Scientific, Torrington, CT). End-tidal CO_2 was continuously monitored with an end-tidal CO_2 analyzer (IITC Life Science, Woodland Hills, CA) and maintained at 4–4.5%. Core body temperature was monitored by a rectal thermometer and maintained at 36–37.5°C with a circulating water heating pad and heating lamps. At the end of an experiment, rats were killed with an overdose of sodium pentobarbital (100–200 mg/kg, i.v.).

To record single-unit activity, a restricted skin incision was made over the posterior hindlimb, and the skin and muscle were opened to expose the middle and distal part of the sciatic nerve. Rats were then placed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA) to immobilize the lower spine and pelvis. The skin at the incision was sewn to a metal ring to form a pool. The fascia and sheath overlying the sciatic nerve were carefully removed, and the nerve was placed on a platform and covered with warm mineral oil. Small nerve filaments, transected proximally, were teased gently from the sciatic under a dissecting microscope (Zeiss, Thornwood, NY). Isolated fine filaments were then wrapped around a silver wire recording electrode, which was connected to a high-impedance probe. One or two such electrodes were used for recording from one or two separate microfilaments. Reference electrodes were placed in the surrounding tissues.

The action potential of an isolated afferent fiber was amplified 1000×, filtered with a bandwidth of 300–3000 Hz with a DAM8 amplifier (World Precision Instruments, Sarasota FL). Filtered signals were visualized on an oscilloscope (model 5301; Tektronix), with parallel audio monitoring, and recorded and stored on computer disc using the CED1401 Plus interface (Cambridge Electronics Design, Cambridge, UK) coupled to a Pentium processor-based personal computer. Signals were analyzed with Spike-3 software (Cambridge Electronics Design). Discharge frequency was counted by using spike shape discrimination (Spike-2; Cambridge Electronics Design), and a histogram was created for each fiber. The nerve activity is presented here as both native records and bin histograms.

To search for units, we used electrical stimulation of nerve fibers at a site between the recording site and the receptive field (RF) of the fiber, and we used mechanical stimulation of their hindpaw RFs. Stimuli from a Grass Instruments (Quincy, MA) model 88 stimulator were delivered via bipolar sharp needle electrodes with duration of 0.5–0.75 msec and amplitude of 30–100 V to activate C-fibers. The main criteria used for physiological characterization and classification of fibers were responses to natural stimulation of their RFs and their conduction velocities, as described previously (Gokin et al., 2001). Conduction velocities (CVs) were calculated by dividing the distance between the stimulating and recording electrodes by the latency of the electrically evoked spike. Units with CVs <2 m/sec were identified as C-fibers (Sanders and Zimmermann, 1986; Handwerker et al., 1991; Leem et al., 1993; Huang et al., 1997). Physiological characterization of C-fibers was based on their responses to various mechanical (light and strong) and thermal (heat and cold) stimulation. They were classified as high-threshold mechanoreceptors (HTMs) if they fired predominantly in response to a strong pinch of the skin with forceps, or von Frey monofilaments (15–52 gm). The thermal responsiveness of mechanically activatable units was determined by applying a heated metal spatula (~52°C) and a piece of ice (cold stimulation) to their cutaneous RFs. On the basis of both their responses to these stimuli and their CVs, C-units were classified as either (1)

polymodal (mechano-heat) nociceptors (C-PMNs) or (2) high-threshold mechanoresponsive (HTMr) C-fibers, several of which also responded to thermal stimuli (heat or cold). To confirm that we were recording from the same electrically and physiologically activated unit, a modification of the "blocking" method of Iggo (1958) was used, as described recently by Gokin et al. (2001).

Drug injections were performed in an identical manner to those in the neurobehavioral experiments, except that limb cooling was not used because we showed previously that spike responses are easily obtained with subcutaneous injection of ET-1 without cooling (Gokin et al., 2001). Recording continued in most instances for 40 min to 2 hr after administration of drugs.

Data analysis. The maximal (peak) number of flinches per 5 min epoch that occurred during the observation period (75 min) for each animal was defined as the maximal flinch frequency (MFF) occurring within a 5 min block and was scored independent of the time of occurrence. The MFF, time to reach MFF, total number of flinches occurring within the 75 min observation period, number of biting or licking events, and total duration of biting or licking behavior were also determined for each animal. Data are reported as means ± SEM. To establish significant differences between the effects of different ET-1 doses or between injected agents, an unpaired, two-tailed Student's *t* test was applied (Origin 5.1; Origin Lab, Northampton, MA), with $p < 0.05$ considered significant.

For neurophysiological experiments, a response was defined as firing that occurred after completion of the second of two injections and needle withdrawal. The latency of spike response was measured as the time from the end of ET-1 injection to the onset of the first nonelectrically evoked response. The duration of responses was measured from the onset of response until afferent activity returned to baseline. Mean response frequency (MRF) (in impulses per second) was calculated as the number of spikes divided by the duration of the entire ET-1-induced response. Maximum frequency (MxF) was determined, to characterize responses within bursting patterns, as the number of spikes within a brief (1 sec) interval of rapid firing. Duration and MRF were used as quantitative parameters for comparing the magnitudes of responses to different doses of ET-1. All results are presented as means ± SEM. One-way ANOVA was used to evaluate the significance of the difference of means. Differences were considered statistically significant at $p < 0.05$.

RESULTS

General and behavioral effects of ET-1 injection

Dermatologic effects

Similar to results described recently by Gokin et al. (2001), subcutaneous administration of 2 (10 μl of 200 μM), 4 (20 μl of 200 μM), and 6 (10 μl of 600 μM) nmol of ET-1 into the rat plantar hindpaw induced immediate blanching at the injection site that began 2–5 min after injection and that reached a maximum size of 2.5 mm^2. This blanching was followed 5–15 min later by the development of local erythema of the plantar surface and at 20–50 min by diffuse rubor of the hindpaw below the knee that lasted for 60–70 min before resolving.

ET-1-induced hindpaw flinching

When ET-1 was injected subcutaneously as a single bolus into the rat plantar paw, ipsilateral hindpaw flinching, a behavioral response that indicates pain behavior in the rat, was observed in 100% of animals (Davar et al., 1998; Fareed et al., 2000; Gokin et al., 2001). Biting or licking of the hindpaw was also observed in most animals and is reported at the end of Results. Flinching began 5–10 min after observations began, increasing with time until a MFF was reached at ~30–50 min and resolving to near baseline by 75 min (Gokin et al., 2001). For single injections of ET-1 (4 nmol, 20 μl of 200 μM), the averaged MFF of 40 ± 4 flinches/5 min ($n = 12$) occurred at 41 ± 3 min after observations began, whereas the averaged total number of flinches was 178 ± 29 over the 75 min observation period.

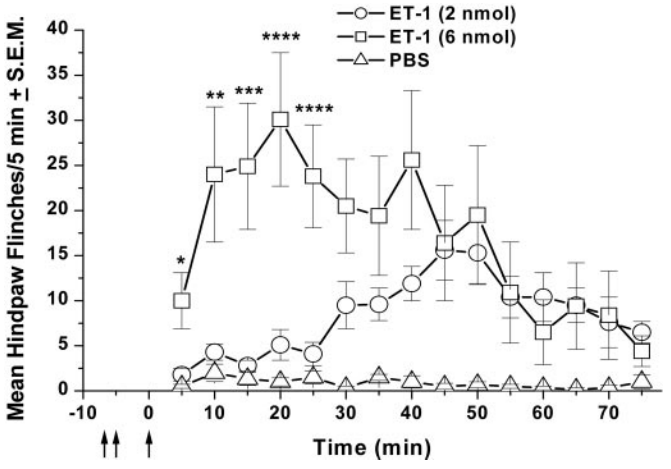

Figure 1. Comparison of the effects of ET-1 injected subcutaneously at concentrations of 200 and 600 μM (2 and 6 nmol doses, respectively) on the mean number of hindpaw flinches per 5 min period of observation. The *three arrows* point to the following: *left arrow*, injection of PBS; *middle arrow*, injection of ET-1 or PBS 1.5–2 min later; and *right arrow*, the beginning of behavioral observations. Significant differences between these doses are present at 5 ($^*p < 0.01$), 10 ($^{**}p < 0.005$), 15 ($^{***}p < 0.002$), 20 ($^{****}p < 0.001$), and 25 ($p < 0.001$) min after observations began.

Dose dependence of the effects of ET-1

Flinching frequency was increased significantly over the 5–25 min period (Fig. 1), as was the averaged MFF (42 ± 9 flinches/5 min; $n = 8$) when 6 nmol was injected compared with 2 nmol (24 ± 2 flinches/5 min; $n = 12$; $p < 0.05$). The mean time to reach MFF (22 ± 4 min for 6 nmol vs 52 ± 3 min for 2 nmol) was also different for these two doses ($p < 0.0001$), as was the mean total number of flinches (253 ± 66 vs 123 ± 13 for 6 vs 2 nmol of ET-1, respectively; $p < 0.05$). A dose of 2 nmol of ET-1 was therefore selected as reliably submaximal, to further study the effects of agents modulating the ET_B receptor.

BQ-788 enhances ET-1-induced flinching

To demonstrate a contribution of ET_B receptors to the pain-inducing actions of ET-1, we next examined the behavioral consequences of administration of a selective ET_B receptor antagonist (BQ-788) before and then together with ET-1. As seen in Fig. 2*A*, BQ-788 (3 mM, 60 nmol) accelerated the development of ET-1-induced hindpaw flinching. Flinching frequency was increased significantly in the presence of BQ-788 at the 15, 20, and 25 min time points (Fig. 2*A*), as was the averaged MFF when compared with 2 nmol of ET-1 alone ($p < 0.05$) in 9 of 12 tested rats (Fig. 3*A*). Three of 12 rats treated with BQ-788 plus ET-1 showed signs of toxicity (red tears and reduced exploratory behavior) similar to what we observed previously with high doses of ET-1 (Gokin et al., 2001) and, despite evidence of hindpaw rubor, had practically no flinching behavior. These animals were not included in the data analysis.

The averaged MFF also occurred earlier in BQ-788-treated rats [20 ± 1 ($n = 9$) vs 52 ± 3 min ($n = 12$) for 2 nmol of ET-1 alone; $p < 0.0001$]. Although BQ-788 increased the mean total number of flinches produced by ET-1 by the same proportion (1.27-fold) as was observed for its effects on MFF, this difference did not reach statistical significance. When administered alone in two subsequent injections of 10 μl, BQ-788 did not evoke flinching that was different from PBS for either MFF or total number.

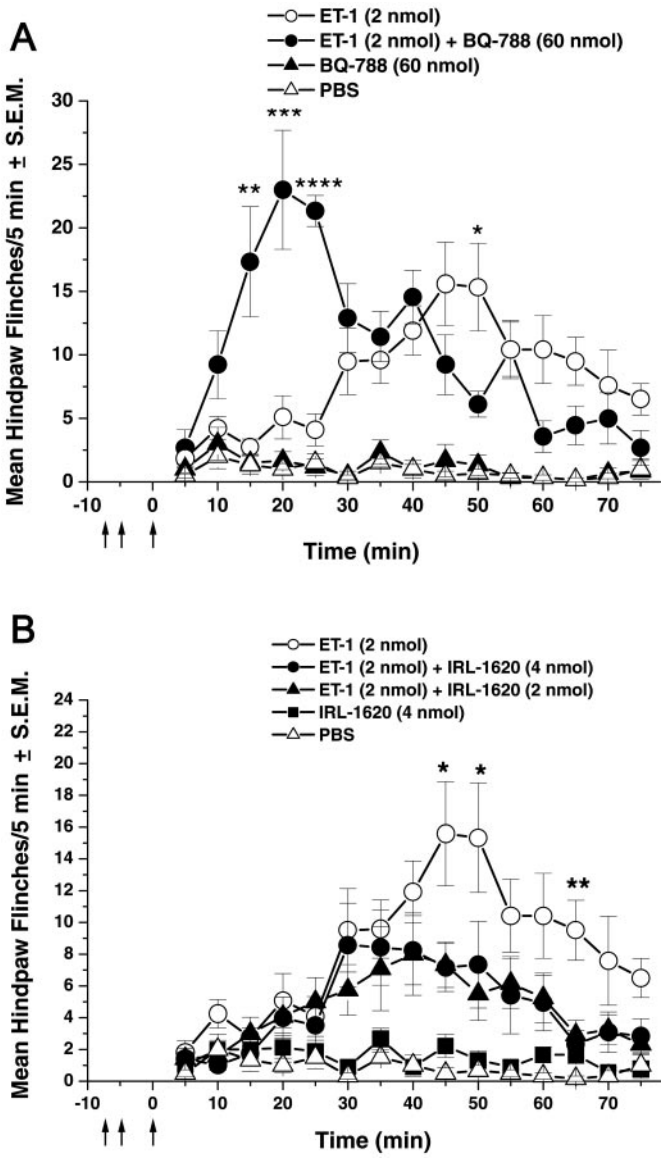

Figure 2. Time course of the effect of ET-1 with BQ-788 (3 mM, total dose of 60 nmol) (*A*) and IRL-1620 (100 or 200 μM, total dose of 2 or 4 nmol) (*B*), the latter two injected before and then together with ET-1 (200 μM, dose of 2 nmol), on the mean number of hindpaw flinches per 5 min period of observation. Effects of BQ-788 (3 mM, 60 nmol) alone, IRL-1620 (200 μM, 4 nmol) alone, and PBS are also presented. The *three arrows* point to the following: *left arrow*, injection of BQ-788 or IRL-1620; *middle arrow*, injection of BQ-788 or IRL-1620 plus ET-1 1.5–2 min later; and *right arrow*, the beginning of behavioral observations. Differences between ET-1-treated and ET-1 plus BQ-788-treated animals are present at 15 (**$p < 0.002$), 20 (***$p < 0.001$), 25 (****$p < 0.0001$), and 50 (*$p < 0.05$) min after observations began. Differences between ET-1 and IRL-1620 (2 nmol) are present at 45 (*$p < 0.05$), 50 (*$p < 0.05$), and 65 (**$p < 0.01$) min after observations began; the 4 nmol dose of IRL-1620 is also significantly different at the 45 (*$p < 0.05$) and 65 (**$p < 0.01$) min time points.

IRL-1620 inhibits ET-1-induced flinching

To further investigate the role of ET_B receptors, we next studied the effect of the selective ET_B receptor agonist IRL-1620 on ET-1-induced flinching. IRL-1620 (200 μM, 4 nmol) decreased the frequency of flinching at later times (significance reached at 45 and 65 min; $p < 0.05$) (Fig. 2*B*) and reduced the averaged MFF ($n = 12$) when compared with ET-1 alone ($n = 12$; $p < 0.05$)

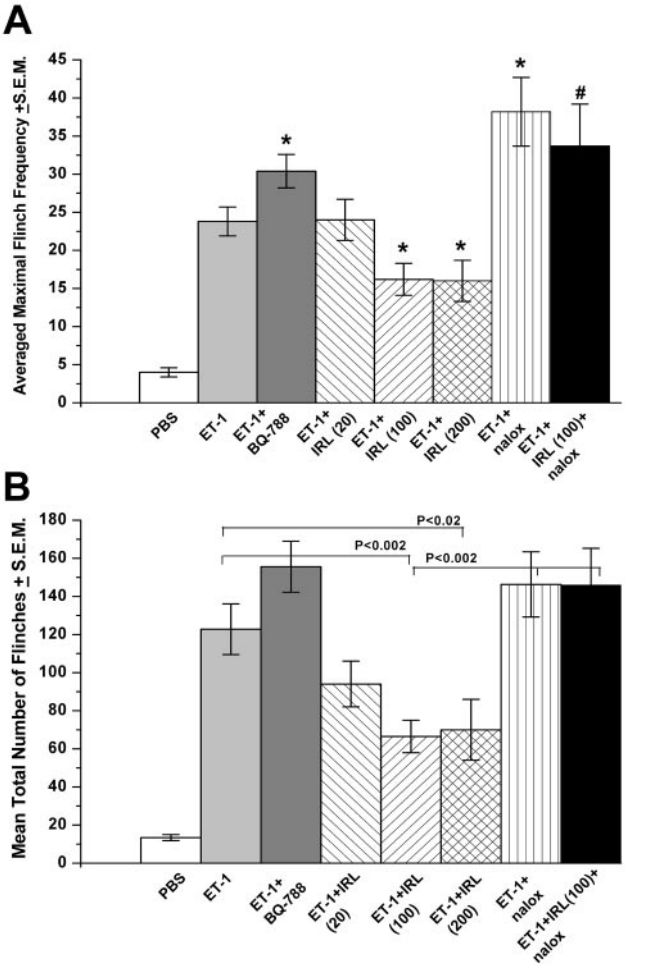

Figure 3. *A*, Averaged MFF in response to ET-1 (200 μM, 2 nmol) injected together with PBS, BQ-788 (3 mM, 60 nmol), IRL-1620 (20, 100, and 200 μM; total dose of 0.2, 2, or 4 nmol, respectively), naloxone (2.75 mM, 55 nmol), or IRL-1620 (100 μM, 2 nmol) plus naloxone. BQ-788 and naloxone enhanced MFF when compared with ET-1 plus PBS ($p < 0.05$), whereas IRL-1620 (100 and 200 μM) reduced MFF ($p < 0.05$); 20 μM IRL-1620 had no effect on ET-1-induced flinching. Naloxone also fully prevented the inhibitory effects of IRL-1620 on MFF (#$p < 0.05$). *B*, Mean total number of flinches in response to ET-1 (200 μM, 2 nmol) injected together with PBS, BQ-788 (3 mM, total dose of 60 nmol), IRL-1620 (20, 100, and 200 μM), naloxone (2.75 mM, 55 nmol), or IRL-1620 (100 μM) plus naloxone. IRL-1620 (100 and 200 μM) inhibits flinching produced by ET-1, whereas naloxone abolished IRL-1620 (100 μM) inhibition of flinching.

(Fig. 3*A*). The mean total number of flinches was also decreased by IRL-1620 ($p < 0.02$) (Fig. 3*B*). Although IRL-1620 administered alone in two 10 μl injections also induced flinching behavior (22 ± 3 flinches; $n = 9$) that was slightly higher than observed for PBS alone (13 ± 2; $n = 8$; $p < 0.05$), this was not unexpected because some activation of the ET_A receptor may occur at this concentration of the agonist (Takai et al., 1992).

To help determine whether the actions of IRL-1620 were mediated at a single class of receptors, we next determined the relationship between IRL-1620 dose and flinching behavior. Coadministration of 2 nmol (20 μl total volume, 100 μM) of IRL-1620 with ET-1 had similar effects to 4 nmol of IRL-1620 on ET-1-induced hindpaw flinching (Fig. 2*B*). The averaged MFF was reduced ($n = 12$; $p < 0.05$) (Fig. 3*A*), as was the mean total number of flinches ($p < 0.002$) (Fig. 3*B*) when compared with

ET-1 alone. At the lowest concentration of IRL-1620 (20 μM, total dose of 0.4 nmol) that we used, neither MFF nor total flinches were different from ET-1 alone (Fig. 3). However, MFF did occur earlier (30.4 ± 5.8 vs 51.7 ± 3.1 min for ET-1 alone; $p < 0.000005$), at the same time point observed with higher concentrations of IRL-1620. To reduce any possible actions of IRL-1620 at the ET_A receptor, while maintaining its inhibitory effects, IRL-1620 was used at the 100 μM concentration in subsequent experiments.

Naloxone enhances ET-1-induced flinching

Opioids are known to have analgesic actions in both the CNS and peripheral nervous system. To examine the potential role of endogenous opioids in the expression of ET-1-induced pain behavior and in ET_B receptor-mediated antinociception (see below), we next administered the nonselective opioid receptor antagonist naloxone (2.75 mM, total dose of 55 nmol) before and then together with ET-1 (2 nmol). The averaged MFF was ~50% higher for naloxone plus ET-1 than for ET-1 alone ($p < 0.01$) (Fig. 3*A*). Averaged MFF also occurred earlier in naloxone-treated rats (41 ± 2 vs 52 ± 3 min for ET-1 alone; $p < 0.01$). However, naloxone did not alter the mean total number of flinches observed (Fig. 3*B*), nor did naloxone alone induce flinching behavior that was different from PBS ($p > 0.05$).

Naloxone prevents the inhibition of ET-1-induced flinching by IRL-1620

To more specifically examine the role of opioids in ET_B receptor-induced antinociception, naloxone was injected together with IRL-1620 (100 μM, 2 nmol) before and then together with ET-1 (2 nmol). The averaged MFF for IRL-1620 plus ET-1 plus naloxone ($n = 12$) was twice that for IRL-1620 plus ET-1 without naloxone ($n = 12$; $p < 0.05$), which is evidence that IRL-1620 has no effect in the presence of naloxone (Fig. 3*A*). Averaged MFF in this group occurred at 40 ± 3 min and was not different from the averaged MFF for ET-1 plus naloxone ($p > 0.05$). The mean total number of flinches evoked by IRL-1620 plus ET-1 plus naloxone was identical to that evoked by ET-1 plus naloxone (Fig. 3*B*) and, in both cases, was higher than that evoked by IRL-1620 plus ET-1 ($p < 0.002$). In support of these actions of naloxone at opioid receptors, we have recently observed, in a preliminary manner, that a μ-opioid receptor-selective antagonist (CTOP) can also prevent the actions of IRL-1620 (data not shown).

Biting and licking behavior induced by ET-1 injection

Biting or licking events were also observed after ET-1 administration in 92% (2 nmol) and 75% (6 nmol) of rats compared with 13% for PBS injections and ranged from 1 to 39 events per rat. Neither the mean number nor the duration of biting or licking events were different between different doses of ET-1 or when ET-1 was compared with BQ-788, IRL-1620, or naloxone administered together with ET-1.

Neurophysiological effects

To obtain more direct evidence of a naloxone-sensitive inhibitory effect of IRL-1620 on ET-1 induced pain, impulse activity was recorded from 21 physiologically characterized nociceptive C-fibers. Recordings were made before and after subcutaneous injection of ET-1 (200 μM, 2 nmol; $n = 6$), IRL-1620 (100 μM, 1 nmol) followed 1–2 min later by ET-1 (200 μM) plus IRL-1620 ($n = 12$), and before and after subcutaneous injection of IRL-1620 (100 μM) plus naloxone (2.75 mM) followed 1–2 min later by ET-1 (200 μM) plus IRL-1620 plus naloxone ($n = 3$) into the cutaneous RFs of these units. The RFs of these units were located on the glabrous hindpaw within territory innervated by the plantar and sural nerves and were 1–2 mm in size (Fig. 4). The conduction velocities of C-units ranged from 0.63 to 1.1 (mean of 0.86 ± 0.03) m/sec and were not significantly different between experiments. Most fibers had no ongoing spontaneous activity.

ET-1 induces spike responses in C-nociceptors

Four of the six units studied here responded only to strong (15–52 gm von Frey hair, numbers 5.18–5.88) mechanical stimulation of their RFs (HTMr units). Noxious pinch gave a maximal response of 11–29 impulses/sec (mean of 17.5 ± 5.4). The remaining two units also responded to heat (C-PMNs). All six fibers responded to ET-1 (200 μM). Five responded in a nearly identical manner, showing the typical bursting discharge pattern (Fig. 4*A*) that we described previously (Gokin et al., 2001). The latency (0.315 ± 0.08 min), MRF (0.318 ± 0.07 impulses/sec), and MxF (4.14 ± 0.17 impulses/sec) of these six units were also similar to those reported previously by us (Gokin et al., 2001). The duration of responses for these units was 24.13 ± 3.76 min; most of these units were not observed beyond 40 min because we never detected a resumption of spiking, under these conditions, once the activity of a unit had returned to baseline.

IRL-1620 inhibits ET-1-induced spike responses

Twelve C-units were studied. Six were classified as HTMr, two as C-PMNs, two as HTMr that also responded to cold, and two remaining units, one an HTMr that also responded to low-intensity stimuli and the other an HTMr that also responded to low-intensity stimuli and cold. Overall, IRL-1620 (100 μM) suppressed ET-1 (200 μM) induced spike responses in all units (Table 1). Complete suppression was observed in four of 12 units, whereas late responses of reduced maximal and mean frequency were observed in two of 12 units, and weak responses, showing similar latency to onset to the results with ET-1 alone, were observed in the remaining six units. A typical example of this inhibitory effect of IRL 1620 is seen in Figure 4*B*. Insignificant, or occasionally very short duration (≤20 sec), responses were observed in the injection of IRL-1620 alone, which preceded the injection of IRL-1620 plus ET-1 (Fig. 4*B*).

Naloxone reverses IRL-1620 inhibition of ET-1-induced spike responses

Three units were recorded (two C-PMNs and one HTMr) while injecting ET-1 and IRL-1620 together with naloxone to their RFs. The typical bursting pattern seen with ET-1 alone was observed in all three units (Fig. 4*C*). MRF and MxF of responses determined for these three units were not different from those obtained for ET-1 alone but higher than those obtained with ET-1 plus IRL-1620-treated units (Table 1).

DISCUSSION

These results are evidence that ET_B receptors are important modulators of ET_A receptor-mediated pain in cutaneous tissues. ET-1 delivered as a single bolus subcutaneous injection to the rat plantar hindpaw induces hindpaw flinching that is greater and occurs earlier when it is coadministered with a selective ET_B receptor antagonist, similar to the pattern observed with increas-

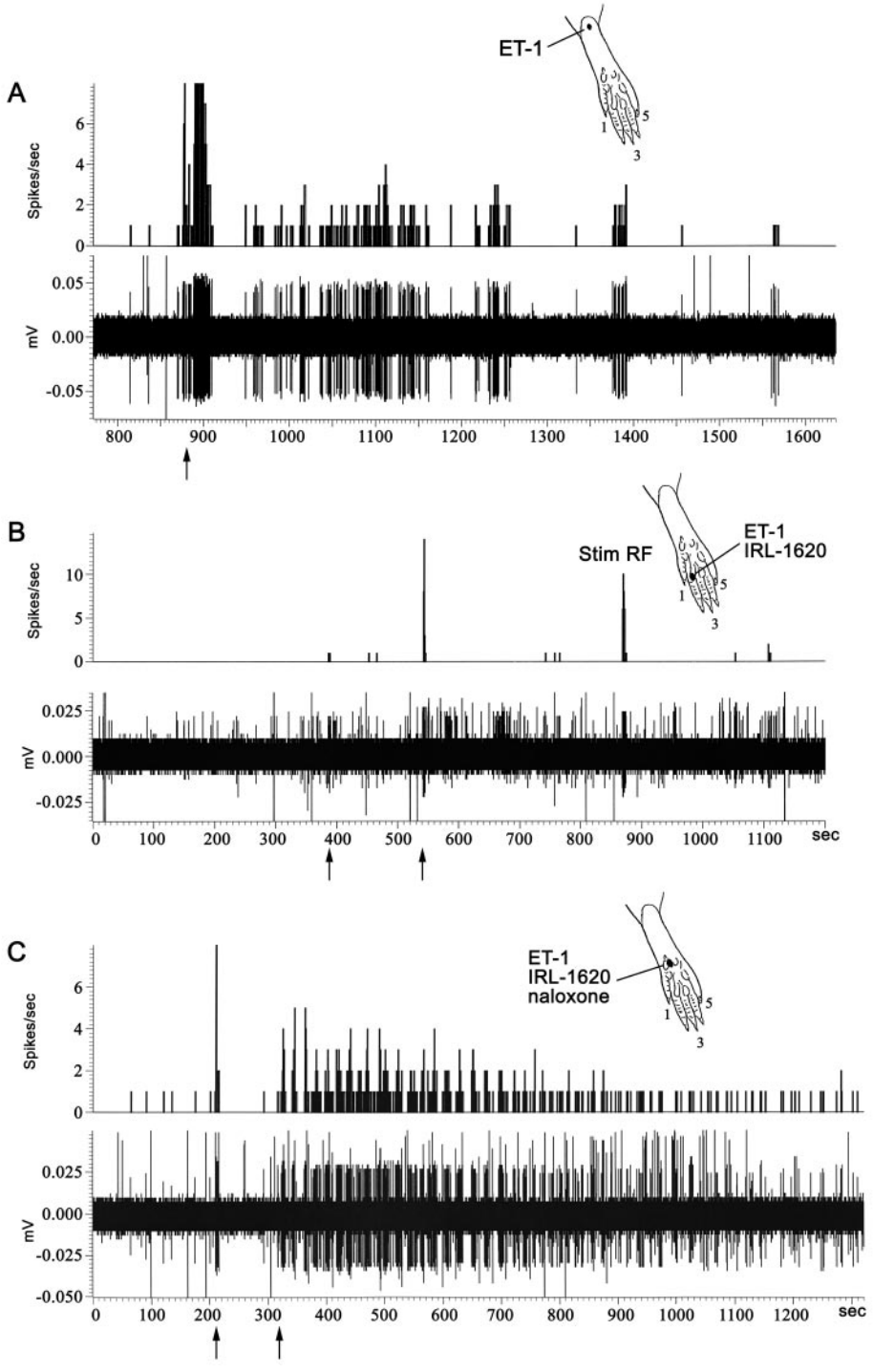

Figure 4. Representative records of spike activity in three C-fibers from three animals after subcutaneous plantar hindpaw injections of ET-1 (*A*), ET-1 plus IRL-1620 (*B*), or ET-1 plus IRL 1620 plus naloxone (*C*), presented as impulse records (*bottom traces*) and bin histograms (*top traces*). *Insets* show the locations of the receptive field of the recorded units on the plantar surface. The volume of each injection was 10 μl. *A*, Injection of ET-1 (2 nmol, 200 μM, at the *upward arrow*) rapidly induces the characteristic "bursting" pattern of a long-lasting spike discharge in a C-PMN fiber. *B*, The same dose of ET-1, in the presence of IRL-1620 (2 nmol total dose, 100 μM), fails to provoke a spike response in an HTMr fiber that lasts beyond the injection discharge (*upward arrows*). Noxious pinch (*Stim RF*) performed 5.5 min after injections demonstrates continued mechanoresponsiveness of the RF of the unit. Impulse activity that is recorded as primarily upward spikes belongs to an Aδ-fiber whose RF overlapped this C-unit. The spikes of the C-unit are almost symmetrically biphasic, and its response is summarized in the accompanying bin histogram. *C*, Injection of naloxone (2.75 mM) together with ET-1 (200 μM) and IRL-1620 (100 μM) induces a spike response in a C-PMN unit with rapid onset and bursting pattern that lasts for >17 min. The *arrow* in *A* indicates the time of ET-1 injection, and those in *B* and *C* show the first (IRL-1620 or IRL-1620 plus naloxone) and the second (ET-1 plus IRL-1620 or ET-1 plus IRL-1620 plus naloxone) injections, respectively.

ing ET-1 dose. Conversely, coadministration of a selective ET_B receptor agonist reduces hindpaw flinching and inhibits ET-1-induced spike responses in nociceptors, both of which are prevented by local injection of the nonselective opioid receptor antagonist naloxone.

Although relatively high concentrations of ET-1 (and BQ-788 and IRL-1620) were used in these experiments, the local concentrations of these agents at the receptor sites are unknown, and their diffusion to the receptors is limited by closely opposed dermal and epidermal cells, the vascular flow that rapidly removes many small molecules, and by the actions of many degradative enzymes. Analogously high concentrations of relatively hydrophobic compounds, such as local anesthetics (e.g., 30–50× the IC_{50} on isolated neurons) are often needed to obtain *in vivo* efficacy from subcutaneous delivery (Khodorova and Strichartz, 2000). The endogenous concentration of ET-1 released by cutaneous tissue injury might indeed be low (Hara et al., 1995; Tsuboi et al., 1995) yet sufficient to induce flinching behavior in rats (Gokin et al., 2001). Increasing the concentration of ET-1 increased flinching, which peaked earlier. This enhanced effect may be the result of increased actions at the ET_A receptor as a consequence of increased ET-1 dose (Fareed et al., 2000). How-

Table 1. Characteristics of spike responses of afferent fibers after subcutaneous injection of ET-1, ET-1 plus IRL-1620, and ET-1 plus IRL-1620 plus naloxone

Drug	Number of units	Mean response frequency (impulses/sec)	Maximum response frequency (impulses/sec)	Mean duration of response (min)	Mean latency of response (min)
ET-1 alone	6	0.32 ± 0.07	4.17 ± 0.17	24.13 ± 3.76	0.32 ± 0.08
ET-1 plus IRL-1620	12	0.08 ± 0.02*	1.5 ± 0.4*	NA[a]	NA[a]
ET-1 plus IRL-1620 plus naloxone	3	0.21 ± 0.10	3.66 ± 0.33	25.5 ± 6.7	0.28 ± 0.14

[a] In four units, there were no responses to IRL-1620 plus ET-1, evidence of complete suppression of the ET-1-induced spike response. The remaining eight units responded either very late or with a minimal spike response, with a mean duration for these responding units of 32.8 ± 3.51 min and mean latency of 3.44 ± 2.29 min. The mean and maximum response frequencies reported are for all 12 units treated with ET-1 plus IRL-1620. NA, Not applicable.

*$p < 0.001$, Fisher's PLSD, when compared with ET-1 alone.

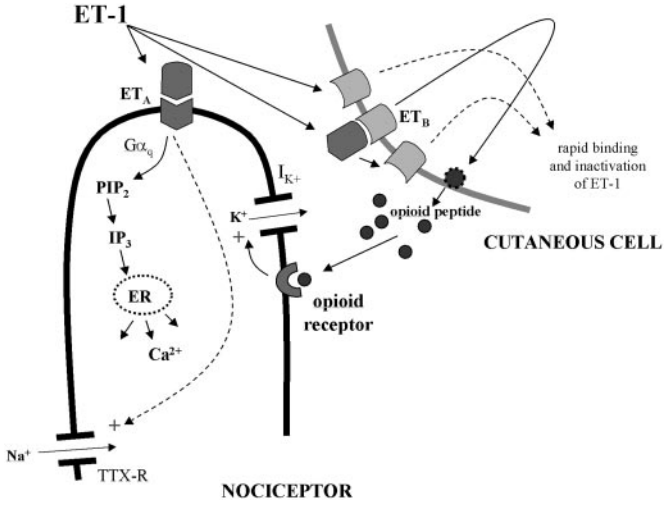

Figure 5. Proposed model for the mechanisms of ET_B receptor-induced inhibition of the effects of ET-1. Activation of ET_A receptors on nociceptors by ET-1 (exogenous or locally released) induces release of calcium from intracellular stores (Zhou et al., 2001) and enhancements of tetrodotoxin-resistant (*TTX-R*) sodium currents (Chen et al., 2000; Zhou et al., 2002), which may contribute to ET-1-induced spike responses and pain (Gokin et al., 2001). In contrast, binding of ET-1 to ET_B receptors on cutaneous cells (e.g., keratinocytes) leads to both the rapid internalization and inactivation of bound ET-1 (Bremnes et al., 2000; Paasche et al., 2001) and to the release of opioid peptides, which activate opioid receptors on nociceptors (Stein et al., 1990b). These opioid receptors are in turn likely coupled to ion channels that could hyperpolarize the nociceptor (e.g., I_K^+), thereby suppressing ET_A receptor-induced nociceptive firing.

ever, increased availability of ET-1 that may occur secondary to its reduced internalization and inactivation by the ET_B receptor, a process described in heterologous expression systems (Bremnes et al., 2000; Paasche et al., 2001), might also contribute to this result (Fig. 5). The earlier onset of flinching is also consistent with reduced actions at the ET_B receptor, as we observed with ET_B receptor blockade, which may lead to enhanced and accelerated actions of ET-1 at the ET_A receptor (Rubanyi and Polokoff, 1994).

Consistent with the proposed role for ET_B receptors in the modulation of the ET_A receptor-mediated response, blockade of the ET_B receptor also increases flinching and accelerates its onset. This suggests the possibility that ET_B receptor activation inhibits and delays the effects of ET_A receptor activation. The substantially reduced (50%) flinching observed with an ET_B receptor agonist further supports the idea that ET_B receptors can suppress ET_A receptor-mediated pain in cutaneous tissues. Lowering the dose of IRL-1620 by a factor of 10 eliminated its inhibition of flinching behavior, pointing to a moderately steep dose–response relationship that is consistent with actions at a single class of receptors.

The inhibitory effect of ET_B receptor activation is unlikely to be through sensory fibers themselves, whereon ET_B receptors have not been detected (Pomonis et al., 2001), and instead is more likely the result of an indirect action mediated by another cell that leads to the suppression of spike activity in nociceptors. The prevention of IRL-1620-induced inhibition of flinching by locally injected naloxone supports this hypothesis and suggests the possibility that ET_B receptor activation on adjacent cutaneous cells leads to the release of an endogenous opioid peptide. The source of this peptide could either be a supportive glial cell [e.g., a non-ensheathing Schwann cell (Pomonis et al., 2001)] found adjacent to cutaneous sensory axons or a resident cutaneous cell (Tada et al., 1998). Among cutaneous cells, keratinocytes are closely associated with sensory axons (Haberberger and Bodenbenner, 2000) and modulate the activity of cutaneous nociceptors (Lewin and Mendell, 1993). They also possess ET_B receptors and express and secrete opioid peptides in a regulated manner (Wintzen et al., 1996, 2000; Zanello et al., 1999), making them reasonable candidates to mediate the ET_B receptor effects we observed here. Other cutaneous cells that possess ET_B receptors and express pro-opiomelanocortin, such as fibroblasts, might also be candidates for a cellular source of ET_B receptor-induced opioid secretion in plantar hindpaw (Teofoli et al., 1999; Shraga-Levine and Sokolovsky, 2000). In addition, inflammatory cells such as lymphocytes recruited to sites of tissue injury could release opioid peptides (Cabot et al., 1997) and might respond to ET-1, but the rapid inhibitory effects we observed here do not support this slower process that is dependent on cellular migration.

Impulse activity in nociceptors after the subcutaneous injection of ET-1, identical in pattern and intensity to what was reported previously (Gokin et al., 2001), was inhibited by coinjection of the ET_B receptor agonist. Spike responses in identified C-nociceptors were completely abolished in several units, whereas the response latency and the duration of response was prolonged in the remaining units. These results provide a mechanism for the inhibitory actions of ET_B receptor activation on ET-1-induced pain behavior. More importantly, the prevention of this effect by locally administered naloxone supports the possibility of an opioid receptor-mediated action of IRL-1620 and helps validate our model of ET_B receptor-induced modulation of ET_A receptor actions on nociceptors in skin (Fig. 5). Incomplete suppression of spike responses in some units might be the result of partial actions of

IRL-1620 at the ET_A receptor, as described above, or secondary to competition with ET-1 for ET_B receptor binding sites, leading to increased actions at ET_A receptors. The prolonged latency with low-level firing in responding units is consistent with the selective actions of this potent ET_B receptor agonist, which would be expected to delay the onset of ET_A receptor-mediated firing.

These results suggest a dual level of control over the pain-related actions of ET-1 in cutaneous tissues, much like its divergent actions of vasoconstriction and vasodilatation in vascular tissue, respectively, mediated by ET_A and ET_B receptors found on different vascular cells (Rubanyi and Polokoff, 1994). Thus, in cutaneous tissues, ET_A receptors on nociceptors can directly activate pain responses, whereas ET_B receptors on supportive cells inhibit these effects of ET-1 in a naloxone-sensitive manner. The source and type of opioid peptide that mediates this effect is unknown but likely originates locally from cutaneous or supportive cells. Based on these results, we surmise that ET_B receptor activation in cutaneous tissues leads to the local release of an endogenous opioid peptide that hyperpolarizes nociceptors, in the face of ET_A receptor-dependent excitation, thereby suppressing impulse generation and inhibiting the effects of ET-1.

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