Tactile Allodynia Initiated by Local Subcutaneous Endothelin-1 Is Prolonged by Activation of TRPV-1 Receptors

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Subcutaneous endothelin-1 (ET-1; 200 μ*M*, 2 nmoles/paw) injected into the rat hind paw, has been shown to cause robust hind paw flinching (HPF) and paw licking, and to induce impulses selectively in primary nociceptors. Here we report that a much lower [ET-1] sensitizes the paw to a nocifensive withdrawal response to tactile stimulation (by von Frey hairs, VFH), a sensitization that involves local TRPV1 receptors. Injection of 10 μ*M* ET-1 (0.1 nmole/paw) causes only marginal HPF but rapidly (20 mins after injection) lowers the force threshold for paw withdrawal (PWT) to VFH, to ~30% of pre-injection baseline. Such *tactile allodynia* persists for 3 hrs. In rats pre-injected with the TRPV1-antagonists capsazepine (CPZ; 1.33 m*M*) or 5′-iodoresiniferatoxin (I-RTX; 0.13 μ*M*), 15 min before ET-1, a fast initial drop in PWT, as with ET-1 alone, occurs (to 40% or to 19% of baseline, respectively), but this earliest reduction then regresses back to the pre-injection PWT value more rapidly than with ET-1 alone. The recovery of allodynia from the maximum value is about two times faster for ET-1+ CPZ and about 4 times faster for ET-1+ I-RTX, compared with that from ET-1 + vehicle ($t_{½}$ = 130, 60, and 250 mins, respectively). In contrast, spontaneous pain indicated by overt HPF from ET-1 is not attenuated by TRPV1 antagonists. Tactile allodynia is similarly abbreviated by antagonists of both ET_A (BQ-123, 32 nmoles/paw) and ET_B (BQ-788, 30 nmoles/paw) receptors, whereas HPF is abolished by this ET_A antagonist but enhanced by the ET_B antagonist. We conclude that low ET-1 causes tactile allodynia, which is characterized by a different time-course and pharmacology than ET-1-induced nociception, and that local TRPV1 receptors are involved in the maintenance of this ET-1-induced allodynia but not in the overt algesic action of ET-1. Exp Biol Med 231:1165–1170, 2006

Key words: allodynia; nociception; endothelin-1; vanilloid receptor

Endothelin-1 (ET-1), an endogenous, potent, peptide vasoconstrictor, whose tissue levels increase under pathological conditions, has been recognized as a pro-algesic mediator involved in the pathogenesis of pain states ranging from trauma to cancer, effects that are independent of its vasoconstrictive actions. Application of exogenous ET-1 locally induces different types of nocifensive behaviors in animals (1–6) and causes pain in humans (1, 8). Direct application of ET-1 excites rat nociceptors *in vivo* (9), stimulates intracellular Ca^{+2} release in cultured sensory neuron-like cells (10) and induces changes in gating of Na^{+} channels that favor excitation in sensory neurons (11). Administration of ET-1 also amplifies responses to other noxious mechanical and thermal stimuli in rodents (1, 7, 12, 13). It potentiates the actions of other pain mediators (e.g., capsaicin, an agonist of the vanilloid (TRPV1) receptor); both nociceptive responses induced by capsaicin *in vivo* (14) and capsaicin-evoked release of substance P (SP) and calcitonin gene-related peptide (CGRP) from dorsal root ganglia (DRG) neurons are amplified by ET-1 (15). More recently it has been shown that ET-1 potentiates capsaicin-evoked electrophysiological effects in DRG neurons (16–18). TRPV1, an excitatory ion channel expressed by nociceptors, which is activated by capsaicin or other vanilloid, protons, or noxious heat (>43°C), is well known for its contributions to acute thermal nociception and inflammation/injury-elicited mechanical and thermal hyperalgesia (19–21).

ET-1 administered at low concentrations provokes minimal pain-like behavior in rats (3, 6); however, it induces mechanical hypernociception in rats (12) or mice (13). The acute pain-inducing effects of high concentrations of ET-1 are dependent on activation of ET_A receptors (3, 5, 6, 9), which can be counteracted by ET-1's antinociceptive

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actions mediated via ET_B receptors (5, 22–25) located on keratinocytes (24). However, the mechanisms of nociceptor sensitization by ET-1 underlying its allodynic effects may differ from that of the direct algesic effect. Sensitization's signaling may not only be mediated via ET_B receptors (4, 12, 13, 26) but also may involve activation of TRPV1, viewed as a molecular integrator of several nociceptive stimuli.

Here we begin to explore the possible involvement of TRPV1 in the mechanism of allodynia induced by subcutaneous injections of ET-1 into the rat plantar hind paw.

Materials and Methods

Animals. Sprague-Dawley male rats (220–250 g, Charles River, Kingston, NY), housed (2 per cage) under a 12:12 hr dark:light cycle, were provided with food and water *ad libitum*. Animals were treated according to the ethical standards for investigation of experimental pain in animals of the International Association for the Study of Pain, and using procedures approved by the Harvard Committee on Animals. Rats were placed on an elevated plastic mesh floor (grid 12 × 12 mm) under a Plexiglas box (17 × 28 × 14 cm) and allowed to habituate for 30 min before initial testing. Withdrawal threshold to mechanical stimulation was determined using calibrated von Frey hairs (VFH) applied perpendicular to a hind paw through spacing in the mesh. The animals were tested over 5 days before each treatment to establish a stable, "pretreatment" baseline withdrawal threshold. On the treatment day, the lowest value of the 3 measurements (at 30, 20, and 10 min) before the first injection was considered as the minimal baseline level, against which all acute posttreatment changes were assessed. Each VFH was applied once, starting with a force of 4 mN, and the force was sequentially increased until a withdrawal response occurred or the cutoff value (560 mN) was reached.

Drugs. Two TRPV1 antagonists were used, capsazepine (CPZ) (Sigma Chemical, St. Louis, MO) and 5′-iodoresiniferatoxin (I-RTX) (Sigma). Capsazepine is characterized by relatively low (micromolar) affinity for TRPV1 and by interspecies differences in its *in vivo* pharmacology; in rats and mice it inhibits capsaicin-induced mechanical hyperalgesia but is ineffective against chronic/inflammatory pain (21). The concentrations chosen for CPZ were well in excess of the K_i in the rat, 4–5 μ*M* (27). The affinity of I-RTX on rat TRPV1 is 800-fold higher than that of CPZ ($IC_{50} = 0.7$ and 562 n*M*, respectively) and 10-fold higher on rat versus human receptors ($IC_{50} = 0.7$ and 5.4 n*M*, respectively). Despite I-RTX's high affinity and high antagonist potency on the cellular level and in rat *in vitro* skin-nerve assay, it is ineffective on capsaicin-induced paw flinching in rats (28). The concentrations chosen here for I-RTX were well in excess of its IC_{50} (0.7 n*M*) in the rat, but lower than the concentrations that evoke a TRPV1-independent effect *in vivo* (2 μ*M*) (28). Both, CPZ and I-RTX were dissolved in Dulbecco's modified Eagle's medium (DMSO) as stock solutions (13.3 m*M* and 1.3 m*M*, respectively) and stored at –20°C. Prior to the injection, stock aliquots were diluted with 0.3% Tween 80 (Sigma Chemical) in phosphate buffered saline (PBS, pH = 7.4**,** Invitrogen, Carlsbad, CA). The final concentration of DMSO in the injected solutions was 10%. ET-1 (American Peptide Co., Sunnyvale, CA) was dissolved in PBS to obtain a final concentration 10 μ*M*. ET_A and ET_B receptor antagonists, BQ-123 and BQ-788 (American Peptide) were dissolved in PBS to final concentrations 3.2 and 3 m*M*, respectively. During all experiments, all drug solutions were kept on ice.

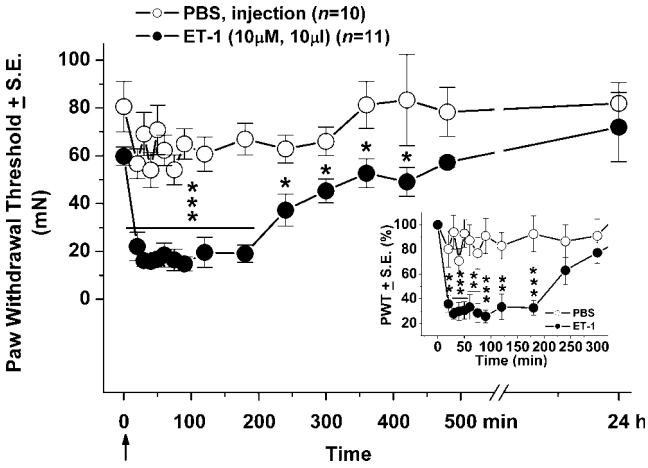

Figure 1. Endothelin-1 injected at a low concentration sensitizes the paw to mechanical stimulation by von Frey hairs. The PWT decreases significantly compared with injected PBS (vehicle for ET-1). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ for ET-1 vs. PBS. Arrow indicates the time of injection. Measurement before the arrow is the minimal baseline PWT value (see Materials and Methods section).

Injection Procedures. ET-1 (10 μ*M*, 10 μl) or its vehicle, PBS, was injected under brief (1.5–3 min) general anesthesia from inhaled sevoflurane (Abbott Labs, North Chicago, IL), using a 30-gauge needle attached to a 10-μl Hamilton microsyringe (Hamilton Co., Reno, NV). Each injection was made into the subcutaneous midplantar hind paw. Capsazepine or I-RTX or their vehicle (10% DMSO in 0.3% Tween 80 in PBS) were injected 15 min before ET-1's injection, in the same volume (10 μl) and into the same area, using the same technique as for ET-1. Subsequent ET-1 was injected during a second brief general anesthesia. Hind paw flinching (number per 5 min epochs) was counted during the first hour after ET-1's injection. Paw withdrawal threshold was measured at 20, 30, 40, 50, 60, 75, 90, 120 mins and then every hour for 6–8 hrs**,** save for ET-1 and related controls, where measurements were extended to 24 hrs (Fig. 1). The observer was blind to the solution administered.

Data analysis. Data for PWT and flinching response are reported as means ± SEM. To establish significant differences between the effects of ET-1 alone and ET-1 injected with other agents, a two-tailed Mann-Whitney *U*

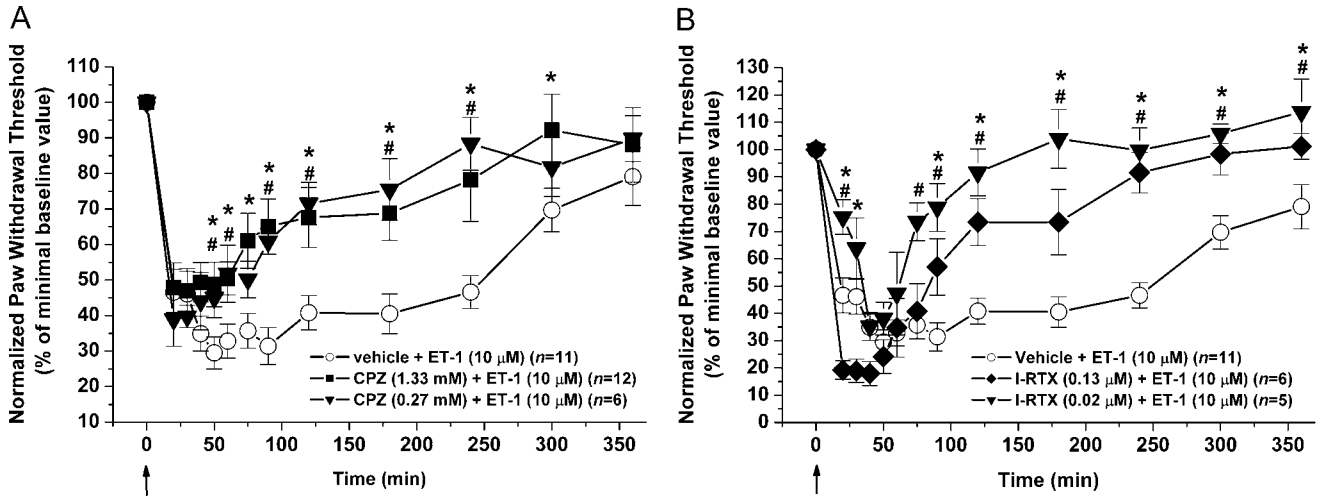

Figure 2. Comparison of the effects of TRPV1 receptor antagonists, CPZ and 5′- I-RTX, on ET-1-induced allodynia. (A) Attenuation of the late phase of the ET-1-induced allodynic response by CPZ, injected subcutaneously into the rat plantar hind paw 15 mins before ET-1. $^{*}P < 0.05$ for 1.33 m*M* CPZ (dose: 13.3 nmoles/paw) + ET-1 vs. vehicle + ET-1; $^{\#}P < 0.05$ for 0.27 m*M* CPZ (dose: 2.7 nmoles/paw) + ET-1 vs. vehicle + ET-1. (B) I-RTX injected locally 15 mins before ET-1 (dose: 0.1 nmole/paw) fully reverses ET-1-induced allodynia by 240–300 mins after injection. $^{*}P < 0.05$ for 0.13 μ*M* I-RTX (dose: 1.3 pmole/paw) + ET-1 vs. vehicle + ET-1; $^{\#}P < 0.05$ for 0.02 μ*M* I-RTX (dose: 0.2 pmole/paw) + ET-1 vs. vehicle + ET-1.

test was applied with $P < 0.05$ considered significant. The PWTs were normalized in percentage to minimal baseline values for each rat, and then averaged. Half-times for recovery from maximum allodynia were calculated as compared with pre-injection PWT values and are given as means and the range.

Results

Subcutaneous ET-1 at 10 μ*M* (dose: 0.1 nmole/paw) induces a significant drop in PWT to mechanical stimulation with VFH, compared with subcutaneous PBS (Fig. 1). The maximum decrease in PWT, to ~30% of the pre-injection baseline for normalized values (see inset in Fig. 1), develops within 20–30 mins and remains relatively constant for another 2 hrs, being significantly different from the PBS effect for 3 hrs after injection.

To evaluate the involvement of TRPV1 receptors in this allodynic response to low [ET-1], we applied different doses of CPZ or I-RTX locally 15 mins before ET-1. Neither dose of CPZ affected the earliest measured drop in PWT induced by ET-1 (Fig. 2A). However, the constant phase of allodynia from ET-1 + CPZ is shorter, and all the later allodynia (4–5 hrs) is less than that from ET-1 alone. This phenomenon of faster recovery can be quantitated by the rate of regression; half-times for recovery from maximum allodynia are 250 mins (range 210–280 mins) for ET-1 (+vehicle for TRPV1 antagonists), 130 mins (90–210 mins) for ET-1 + 1.33 m*M* CPZ (dose: 13.3 nmoles/paw) and 100 mins (70–140 mins) for ET-1 + 0.27 m*M* CPZ (dose: 2.7 nmoles/paw). Vehicle itself administered before and then together with ET-1 did not produce any significant changes in ET-1-dependent allodynia ($P > 0.05$).

Unlike the delayed antihyperalgesic effect of CPZ, effects of I-RTX first appear at the beginning of allodynia (Fig. 2B). At the lower concentration (0.02 μ*M*, dose 0.2 pmol/paw), I-RTX reduces the initial drop in PWT and also accelerates its regression back to the baseline value. At the higher concentration (0.13 μ*M*, dose 1.3 pmol/paw), I-RTX significantly enhances the initial drop in PWT but still speeds its reversal. For both doses, the constant allodynic phase is shortened compared with ET-1 + vehicle. The respective half-times for recovery of allodynia from the maximum value are ET-1 (+ vehicle for the antagonist), 250 mins (range 210–280 mins); ET-1 + 0.13 μ*M* I-RTX, 60 mins (45–90 mins); ET-1 + 0.02 μ*M* I-RTX, 35 mins (25–50 mins).

To elucidate if TRPV1 is involved in the spontaneous nocifensive flinching response induced by ET-1 we counted the total number of flinches that occurred during the 60 mins following injection of low [ET-1]. The marginal number of flinches evoked by 10 μ*M* ET-1 remains unaffected by either 0.27 m*M* CPZ (22 ± 7, $n = 6$) or 1.33 m*M* CPZ (11 ± 3, $n = 10$) compared with controls (vs. 15 ± 1, $n = 4$) ($P > 0.05$), despite the significant attenuation of prolonged allodynia by CPZ. Flinching induced by low [ET-1] is even increased superadditively in the presence of 0.13 μ*M* I-RTX: 54 ± 13 ($n = 6$) compared with 15 ± 1 ($n = 4$) from ET-1 (+ vehicle), and 9 ± 1 ($n = 3$) for I-RTX (+ PBS) ($P < 0.05$). Since ET-1 at higher concentrations induces a more pronounced pain behavior in rats, we administered 1.33 m*M* CPZ before 200 μ*M* ET-1. (PWT was not examined in these experiments.) As shown in Table 1, neither the total number of flinches nor the maximal flinch frequency (MFF) nor its timing after ET-1 are changed by CPZ. Similarly, CPZ has no effect on the biting/licking response to ET-1 in rats.

Table 1. ET-1-induced Pain Behavior in Rats After Capsazepine

Treatment	Flinching			Biting/Licking	
	Total Flinches (No./60 mins)	MFF[a] (No./5 mins)	Time of MFF[b] (mins)	Events (No./60 mins)	Time[c] (sec)
Vehicle + ET-1 (200 μ*M*) (*n* = 9)	121 ± 18	38 ± 7	32.4 ± 2.7	15 ± 3	191 ± 38
Capsazepine (1.33 m*M*) + ET-1 (200 μM) (*n* = 9)	106 ± 12	35 ± 6	27.2 ± 2.9	17 ± 4	193 ± 51

[a] MFF, maximal flinch frequency (maximal number of flinches measured per 5-min epoch).
[b] timed from injection of ET-1.
[c] total time spent biting or licking hind paw in a 60-min period after ET-1 injection.

To determine the role of ET_A receptors in this tactile allodynia, we injected the selective ET_A receptor antagonist BQ-123 (3.2 m*M*, dose 32 nmoles/paw) 15 mins before 10 nmoles ET-1. BQ-123 does not affect the earliest measured response but does reduce peak allodynia at 30 mins after injection, and fully reverses it by 3 hrs after injection (Fig. 3A). The regression of allodynia is accelerated; the half-time being 70 mins (55–100 mins, range) compared with 200 mins (140–260 mins) for ET-1 + PBS (vehicle for the antagonist).

When injected before ET-1, the selective ET_B receptor antagonist BQ-788 (3 m*M*, dose 30 nmoles/paw) does not significantly attenuate the initial drop in PWT induced by ET-1 but does reduce the later phase of allodynia by speeding its reversal (Fig. 3B). Significant differences are present at 75 min and 3 hrs after ET-1 ($P < 0.05$) and the half-time for recovery is 40 mins (compared with 200 mins for ET-1 + PBS).

Discussion

Subcutaneous ET-1 causes tactile allodynia for which the TRPV1 receptor appears to be an important modulator. Conversely, TRPV1 does not affect the overt pain behavior from ET-1 that is shown by flinching and biting/licking responses. Both ET_A and ET_B receptors participate in the slowly regressing phase of ET-1-induced allodynia, although they appear to be uninvolved in its earliest appearance.

The TRPV1 receptor is important for the development of hyperalgesia from strong noxious-stimuli or inflammation (19–21). Activation of TRPV1 causes nociceptor depolarization and Ca^{+2}/Na^{+} entry, generating action potentials and at the same time evoking a variety of local tissue responses through the release of neuropeptides and glutamate from the distal terminals (29). In addition to the acidosis resulting from inflammation, ischemia, or infection, various endogenously produced substances, such as anandamide, N-arachidonyl dopamine, and the lipoxygenase metabolites of arachidonic acid activate TRPV1 (30, 31). Bradykinin and histamine released during inflammation also appear to use the lipoxygenase/TRPV1 pathway to affect sensory neurons (32, 33). The current study shows that two different TRPV1 antagonists accelerate reversal of ET-1-induced allodynia. The fact that both of these TRPV1-selective blockers attenuate the later phase of allodynia, and at very different concentrations yet consistent with their differing potencies for the rat TRPV1 receptor (21, 28), strongly suggests that the capsaicin receptor contributes to the

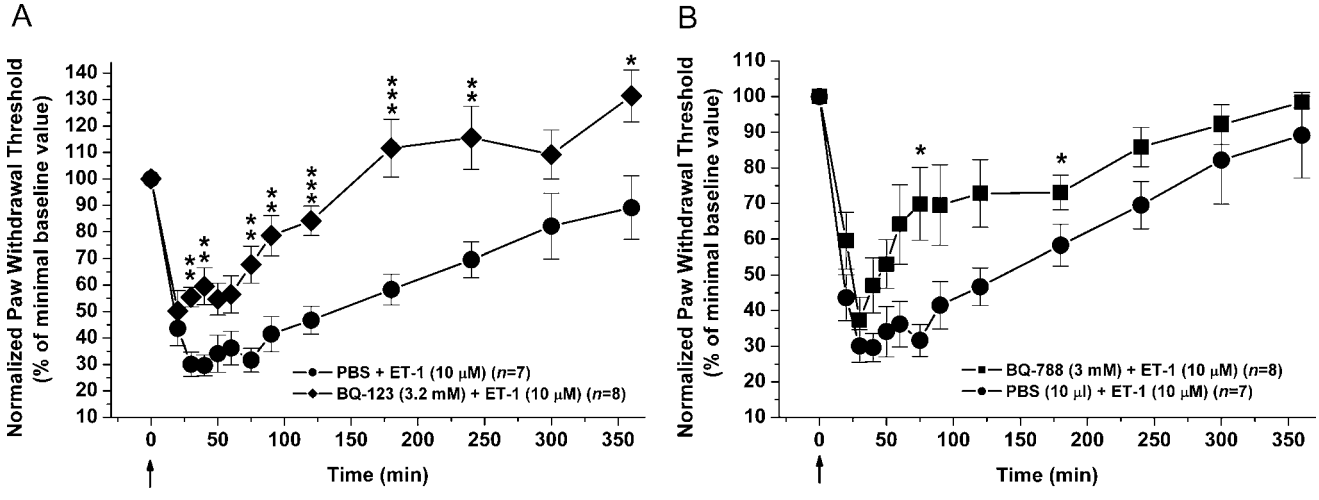

Figure 3. Antagonists to both ET_A and ET_B receptors (BQ-123 and BQ-788, respectively) reduce the later phase of allodynia induced by low concentration of ET-1 (dose: 0.1 nmole/paw). (A) BQ-123 (dose: 32 nmoles/paw) or (B) BQ-788 (dose: 30 nmoles/paw) were injected subcutaneously into the rat plantar hind paw 15 mins before ET-1. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ for the antagonist + ET-1 vs. PBS + ET-1.

maintenance of ET-1-dependent tactile allodynia. The earliest measured allodynia (20 mins), however, is neither prevented nor attenuated by CPZ nor by the pre-injection of antagonists for ET_A or ET_B receptors, implying that the mechanism for the initiation of allodynia may differ from the one responsible for its maintenance. (Although injection of PBS also reduces PWT, it is a much smaller reduction than that from ET-1.) In support of the notion of separate mechanisms, I-RTX actually enhances the initial drop in PWT at the higher concentration (to 19% of baseline) but reduces the initial drop (to 74% of baseline) at the lower concentration compared with the effect of ET-1 (to 30%), whereas both concentrations accelerate allodynic recovery. Injection of this higher dose of I-RTX produced definitive allodynia, accounting for the enhanced drop in early PWT from this pretreatment. Of importance, these concentrations of I-RTX have been reported to exhibit no agonist activity on TRPV1 receptors *in vitro* (28), suggesting that modulation of the initial allodynic response bypasses TRPV1 and rather originates from other actions of I-RTX.

The pain-like spontaneous behavior from ET-1 may also use different mechanisms/pathways than those for allodynia. As noted, I-RTX can evoke a superadditive increase in hind paw flinching, above the sum of HPFs from low [ET-1] plus that from I-RTX alone, while negatively modulating allodynia over the same period. An even higher [I-RTX], 2 μM, is reported to evoke spontaneous nocifensive paw flinching behavior in TRPV1-knockout mice (28), demonstrating an action of I-RTX independent of the vanilloid receptor. In contrast, capsazepine, which substantially truncates ET-1-induced allodynia, leaves ET-1 induced pain-like flinching behavior intact. In addition, ET-1 at 200 μM induces pain-like flinching and biting/licking in rats, with a much briefer duration than that for allodynia (see Table 1), virtually over within 60–75 mins after ET-1's injection (9, 23, 24). In short, separate mechanisms may account for hind paw flinching and for tactile allodynia, the latter involving TRPV1, and the initiation of allodynia may recruit different receptors and cellular pathways than those that contribute to its persistence.

Previous reports provide potentially conflicting evidence that nociceptive effects of ET-1 are mediated either solely by one receptor type or via both ET_A and ET_B receptors. Of importance, whereas the ET_A receptor always causes pronociceptive actions (3, 5, 6), the ET_B receptor may exert an anti- or a pronociceptive influence, depending on the conditions (4, 5, 12, 13, 22, 26). ET_B receptors appear to mediate mechanical hyper-nociception when exogenous ET-1 is administered locally at low concentrations (8–10 μM), or in models of inflammatory pain (12, 13) as tested by ET_B antagonists, while ET_B receptor activation by selective agonists (sarafotoxin S6c or IRL-1620) delivered preemptively has a hypo-nociceptive effect on behavior evoked by inflammatory agents (25).

Picomolar doses of injected ET-1 that yield very low local ET-1 concentrations are probably closer to the levels of endogenous ET-1 occurring during pathological responses, injury, or tumor metastases (34–36). Because ET-1 contributes to both acute and persistent cancer pain, it is important to understand the roles of the different endothelin receptors. Here we administered selective blockers of ET_A and ET_B receptors locally, before ET-1, at concentrations previously shown to be effective on spontaneous pain-like flinching behavior evoked by 200–400 μM ET-1 in rats (9, 23, 24). Although we used concentrations of BQ-123 and BQ-788 far in excess of their K_i values, 10 nM and 100 nM, respectively (see also Refs. 9, 23), the local concentrations of the antagonists at the receptor sites deep in the skin are unknown. Blockade of either receptor sped the reversal of the late phase of ET-1-induced allodynia, while sparing the earliest decrease in PWT after ET-1 injection (see Fig. 3). Thus, traditional endothelin receptors appear important for maintaining of ET-1-induced allodynia, but not for its primary initiation. This process, the initial rise of tactile allodynia, remains unexplained by the current experimental results.

Overall, these data demonstrate that ET-1 delivered at 10 μM locally causes tactile allodynia, which has a different time-course than flinching behavior induced in rats by ET-1 at 20- to 50-fold higher concentrations. TRPV1 receptors participate in the maintenance of allodynic responses in the rat hind paw, although apparently do not contribute to the overt pain behavior caused by exogenous ET-1. Both ET_A and ET_B receptors appear to be involved in the slowly regressing phase of ET-1-induced allodynia. We conclude that the allodynia caused by local, subdermal ET-1 acts by a mechanism different than the one that accounts for pain caused directly by higher ET-1 concentrations.

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