Local Injection of Endothelin-1 Produces Pain-Like Behavior and Excitation of Nociceptors in Rats

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Neurobehavioral and neurophysiological actions of the peptide endothelin-1 (ET-1) were investigated after subcutaneous plantar hindpaw injections in adult male Sprague Dawley rats. Hindpaw flinching developed within minutes after ET-1 (8–16 nmol) injection, peaked at 30 min, lasted for 60 min, and was strongly inhibited by the endothelin-A (ET$_A$) receptor antagonist, BQ-123 (3.2 M). In separate experiments, impulse activity of single, physiologically characterized sensory C-, Aδ-, and Aβ-fibers was recorded from the sciatic nerve in anesthetized rats after subcutaneous injections of endothelin-1 (1–20 nmol), alone or together with BQ-123 (3.2 M), into the plantar hindpaw receptive fields of these units. All nociceptive C-fibers (31 of 33 C-fibers studied) were excited by ET-1 (1–20 nmol) in a dose-dependent manner. For doses of 16–20 nmol, the mean latency for afferent activation after injection of ET-1 was 3.16 ± 0.31 min, and the mean and maximum response frequency were 2.02 ± 0.48 impulses (imp)/sec and 14.0 ± 3.2 imp/sec, respectively. All 10 nociceptive Aδ-fibers (of 12 Aδ-fibers studied) also responded to 1–20 nmol of ET-1 in a dose-dependent manner with a mean latency of 3.5 ± 0.12 min and mean response frequency of 3.3 ± 2.3 imp/sec. In contrast, most Aβ-fibers (9 of 12) did not respond to ET-1. BQ-123, when coinjected with ET-1, blocked ET-1-induced activation in all C- and Aδ-fibers tested. These data demonstrate that subcutaneous administration of ET-1 to the rat plantar hindpaw produces pain-like behavior and selective excitation of nociceptive fibers through activation of ET$_A$ receptors.

Key words: excitability; peripheral nerve; algogenic; C-fiber; nociceptor; cancer

Pain is a frequent and disabling consequence of metastatic prostate and breast cancer in humans. The cause of this pain is unknown but may involve mediator-dependent signaling by tumor cells to spinal nerve roots. One candidate mediator, the potent vasoconstrictive peptide and mitogen endothelin-1 (ET-1), is secreted in high concentrations by metastatic prostate and breast cancer cells and is known to induce pain-like behavior in animals and pain in humans (Ferreira et al., 1989; Dahlof et al., 1990; Hammerman et al., 1997; Piovezan et al., 1997, 2000; Carducci et al., 1998; Davar et al., 1998; De-Melo et al., 1998; Graido-Gonzalez et al., 1998; Fareed et al., 2000; Jarvis et al., 2000). ET-1 is also found in high concentration in both dorsal root ganglion neurons (Giaid et al., 1989) and satellite cells (Kar et al., 1991), whereas endothelin-A (ET$_A$) receptors are found on small to medium sized dorsal root ganglion neurons and their axons (Pomonis et al., 2001), which is further evidence supporting a potential role for ET-1 in pain signaling. In rodents, intraperitoneal administration of ET-1 produces pain behavior that is ET receptor-mediated (Raffa et al., 1996a,b), whereas intra-articular administration of ET-1 in dogs produces pain behavior and hy-peralgesia (Ferreira et al., 1989). Similarly, in mice, intraplantar ET-1 potentiates pain states in models of chemical- and inflammation-induced pain (Piovezan et al., 1997; De-Melo et al., 1998). In humans, ET-1 injected into the brachial artery induced severe pain and prolonged, touch-evoked allodynia (Dahlof et al., 1990), and Carducci et al. (1998) have reported that an endothelin-A receptor antagonist can reduce verbal reports of pain in patients with metastatic prostate cancer.

In support of these findings in animals and humans, we have recently described neurobehavioral effects of ET-1 applied extra-neurally or intraneurally to rat sciatic nerve (Davar et al., 1998; Fareed et al., 2000). These experiments showed that ET-1 induces hindpaw flinching behavior in rats that is dose dependent and mediated by ET$_A$ receptors, and that it may be attributable to direct actions on primary afferents.

To establish the selectivity of the actions of ET-1 for excitation of functionally identified peripheral axons, we have now studied whether ET-1 applied subcutaneously to the rat plantar hindpaw can produce hindpaw flinching that is accompanied by spike activity in single primary afferents supplying the plantar hindpaw. We have also determined whether an ET$_A$ receptor antagonist can block ET-1-induced flinching behavior or spike activity in identified primary afferents.

MATERIALS AND METHODS

The use of animals in these experiments was 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 investigations 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).

Neurobehavioral experiments

ET-1 and an ET_A receptor antagonist administration. Synthetic ET-1 (98% pure peptide content; American Peptides, Sunnyvale, CA) dissolved in PBS (Life Technologies-BRL, Grand Island, NY), pH 7.4, was injected in a 40 μl volume into the subcutaneous plantar hindpaw of adult (200–225 gm), male Sprague Dawley rats (Harlan, Indianapolis, IN) anesthetized briefly with sevoflurane. To minimize rapid absorption of ET-1 from the injection site, the hindlimb was cooled with a small amount of a packed ice-water mix in a 15 ml polypropylene centrifuge tube (Corning Inc., Corning, NY) placed beneath the limb; a small amount of wet ice wrapped in cotton gauze was also placed on top of the hindlimb. ET-1 (for 200 μM, $n = 12$; for 300 μM, $n = 11$; for 400 μM, $n = 13$ animals) or vehicle (PBS, $n = 10$) was administered in three divided doses (10, 20, and 10 μl at 0, 5, and 9 min, respectively). After completion of injections, animals were allowed to recover completely from anesthesia for 5 min before behavioral observations began.

To establish that the pain-inducing actions of subcutaneously administered ET-1 was ET_A receptor dependent, a selective antagonist of the ET_A receptor (BQ-123; American Peptides) was injected in a solution containing a final concentration of 3.2 mM BQ-123 alone, 20 min (20 μl) and 10 min (20 μl) before the cessation of anesthesia, and then together with 400 μM ($n = 7$) or 200 μM ET-1 ($n = 5$) in three doses over a 9–10 min period. The concentration chosen for this antagonist was well in excess of its K_I (10 nM), which was determined directly in other tissues (Ishikawa et al., 1992; Marsault et al., 1993).

Behavioral measurements. 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 Plexiglas cage. Repetitive and spontaneous flinching of the hindpaw (rapid lifting of the entire hindlimb that begins with hip flexion and includes dorsiflexion of the toes) was measured beginning 5 min after ET-1 administration. The number of these hindpaw flinches occurring every 5 min was measured over the next 60 min.

Neurophysiological experiments

Surgery and anesthesia. Neurophysiological experiments were conducted on 30 adult, male Sprague Dawley rats weighing 250–300 gm (Taconic Laboratories, Germantown, NY, and Harlan). Rats were initially anesthetized with urethane (1.3 gm/kg, i.p.) (Sigma, St. Louis, MO) or sodium pentobarbital (50–60 mg/kg). The left carotid artery and right or left jugular vein were cannulated for continuous monitoring of blood pressure and to permit intravenous administration of additional doses of pentobarbital (10–20 mg/kg, i.v.) or chloralose (15 mg/kg, i.v.) to maintain general anesthesia. Anesthetic dosing was titrated to the absence of corneal reflexes, heart rate, withdrawal reflexes to noxious stimuli, and blood pressure fluctuations. Heart rate was monitored with a Tektronix 498 EKG Monitor (Tektronix, Beaverton, OR). Tracheotomy was performed for artificial respiration. During recordings, rats were immobilized with pancuronium bromide (Sigma; 1 mg $\cdot$ kg^{-1} $\cdot$ hr^{-1}, i.v.) and artificially ventilated via a respirator (RSP1002, Kent Scientific Corporation). 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%. In some experiments, arterial blood gases were sampled and analyzed with a blood gas analyzer. Core body temperature was monitored by a rectal thermometer and maintained at 36–37.5°C with a circulating water heating pad and heat lamps. At the end of an experiment, rats were euthanized with an overdose of sodium pentobarbital (100–200 mg/kg, i.v.).

Recording procedures. Single-unit nerve activity was recorded from the left or right sciatic nerve. 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 Co.) to immobilize the lower spine and pelvis. The hindlimb was not cooled as in neurobehavioral experiments because spike responses (see Results) were easily obtained with subcutaneous injection of ET-1. 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 (Carl 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 on the surrounding tissues.

The action potential of an isolated afferent fiber was amplified 20,000–50,000×, filtered with a bandwidth of 100–300 to 1000–1500 Hz (Grass P511 or WPI DAM8 amplifier), and processed through an audio amplifier and an oscilloscope. Nerve impulse activity and blood pressure were simultaneously recorded on a K2G thermal recorder (Astro-Med, W. Warwick, RI). Nerve activity also was fed into a PC-compatible computer through an analog-to-digital interface board for subsequent off-line quantitative analysis. Discharge frequency was counted by using window discriminator (DataWave Technology, Longmont, CO, or Spike-2, Cambridge Electronics Design, Cambridge, UK), and a histogram was created for each fiber. Accurate counting of the discharge frequency was verified for each fiber by comparing the constructed histogram with the hardcopy recorded simultaneously. The nerve activity is presented here in two forms: as native records and as bin histograms (see, for example, Fig. 3).

Isolation, identification, and classification of fibers. To search for units we used two approaches: (1) electrical stimulation of sciatic nerve fibers at a site between the recording site and the fiber's receptive field (RF) and (2) mechanical stimulation of their hindpaw RFs. Electrical stimulation was performed either with silver electrodes placed beneath the most distal part of the exposed sciatic nerve or with transcutaneous needle electrodes. One pair of transcutaneous electrodes was placed in the heel area to permit multiunit stimulation of plantar and sural nerve fibers. Another pair of fine needle electrodes was placed just proximal to the RFs of identified units. Stimulus duration was 0.2 msec for Aβ- and Aδ-fibers and 0.5–0.75 msec for C-fibers. The amplitude of stimuli was usually set at 1.5 times the fiber response threshold, whereas the frequency of stimuli was usually 1 Hz. Responses to natural stimulation of their RFs and conduction velocity (CV) of fibers were the main criteria used for physiological characterization and classification. Two types of mechanical stimulation of RFs were used: (1) noncalibrated search stimuli such as stroking, tapping, or moderately firm pressure applied with a cotton-tipped swab to plantar surfaces and (2) stimuli using calibrated von Frey hairs (Stoelting, Wood Dale, IL) for more precise physiological characterization of units. In addition, light brushing and pinching with nonserrated forceps were also used for low- and high-intensity mechanical stimulation, respectively (see Fig. 3). Conduction velocities were calculated by dividing the distance between the stimulating and recording electrodes by the latency of the electrically evoked spike. Units with CVs >20 m/sec were identified as Aβ-fibers, units with CVs of 2.2–20 m/sec were identified as Aδ-fibers, and 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). C-fibers were classified as high-threshold mechanoreceptors (HTMs) if they fired in response to a strong pinch of the skin with forceps, perceived as painful when applied to the authors; these HTMs were defined as nociceptors. Units that responded to light stroking by a soft-bristled brush but not to pinch were non-nociceptive units, which were almost never C- or Aδ-fibers but most often Aβ-fibers. To confirm that we were recording from a single (the same) unit, a modification of the methods of Iggo (1958) was used. In this procedure, electrical stimulation is applied to the RF of a unit at the same time as repeated physiologic stimulation. If electrical stimulation occurs during the refractory period for impulses generated by a previous physiologic stimulus, the spike response is blocked.

In some instances, the thermal threshold of recorded afferents was determined. After the receptive field was located on the skin, a 1 cm^2 Peltier-controlled thermode (Yale University, New Haven, CT), maintained at 30°C, was gently placed on the surface of the skin. Thermal stimulation was performed by increasing the probe surface temperature from 38 to 51°C, in 1°C increments for 5 sec every 30 sec. The probe temperature was allowed to return to baseline between successive stimuli. The minimal temperature required to stimulate the afferent was considered as the thermal threshold. This threshold was measured at least twice, separated by 10–12 min, to ensure reproducibility. The thermal responsiveness of several additional units was also determined by applying a heated metal spatula (~52°C) and a piece of ice (cold stimulation) to the cutaneous RF. On the basis of their responses to different stimuli (and their CVs), all units studied were classified as (1) polymodal (mechano-heat) nociceptors (mainly associated with C-fibers: C-PMNs), (2) high-threshold mechanoresponsive C- and Aδ-fibers for which thermal responsiveness was not checked (HTMr), and (3) non-nociceptive mechanoreceptors (mainly Aβ- and a few Aδ- and C-fibers).

Drug doses and injection procedures. As described above in Neurobehavioral experiments, an ET-1 stock solution was prepared to 400 μM in PBS, pH 7.4. BQ-123 was dissolved in PBS to obtain a 6.4 mM stock, which was kept frozen and prepared for injection *ex tempore* by adding the stock to an equal volume of 400 μM ET-1 solution to produce a final concentration of 200 μM of ET-1 and 3.2 mM BQ-123. ET-1 and ET-1 together with BQ-123 were applied via subcutaneous microinjections into or in the immediate vicinity (within 1–2mm) of the RF of a fiber's. ET-1 was initially injected as a single 50 μl bolus of a 400 μM solution (20 nmol) and then, in an effort to better mirror the neurobehavioral experiments, in multiple (4) 10 μl volumes of a 400 μM solution. To establish the concentration dependence of the effects of ET-1, progressively lower concentrations of ET-1 were administered as 2 $\times$ 10 μl of 400 μM solution (8 nmol) and 1 $\times$ 10 μl of 200 μM solution (2 nmol). All injections were performed over a 1–4 min duration. No significant differences in spike response to ET-1 injection were observed between the single and multiple injection procedures at the 400 μM concentration (data not shown). Vehicle controls were also performed as single or multiple injections, either as PBS alone or PBS before ET-1 injection. Recording continued in most instances for 40 min to 2 hr after administration of drugs.

Data analysis. The total number of hindpaw flinches was determined during a 60 min observation period, which was divided into 12 periods of 5 min each, after subcutaneous injection of ET-1 into the plantar hindpaw. To detect interactions between the experimental groups, an ANOVA (StatView 4.5, SAS Institute, Cary, NC) statistical analysis was performed. When significant *F* ratios were found, all possible pairwise comparisons were performed using a multiple *t* statistic (Fisher's protected least significant difference).

A responding unit was defined as one that began to fire after injection was complete and the needle had been withdrawn. The latency of spike response was measured as the time from the end of ET-1 injection (last injection in the case of multiple injections) 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, impulses per second) was calculated as the number of spikes divided by the duration of the entire ET-1-induced response. To characterize responses within bursting patterns, maximum frequency (MxF) was determined 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 of the results are presented as means ± SEM. One-way ANOVA was used to evaluate the significance of the difference of means. The ratio of responding to nonresponding units between different classes of fibers was compared using a χ^2 contingency table. Differences were considered statistically significant at $p < 0.05$.

RESULTS

Dermatologic effects

Subcutaneous administration of ET-1 (200–400 μM) to the rat plantar hindpaw produced an area of blanching within minutes at the site of injection (2.5 mm^2). A larger area (7.5 mm^2) of erythema could be observed beginning 15–20 min after ET-1 administration that progressed to diffuse rubor of the hindpaw that lasted for 60 min and then resolved. Animals occasionally demonstrated signs of stress (red tears) beginning near the end of the 60 min period of observation, which was likely caused by systemic effects of ET-1. Lowering the concentration (200–300 μM) of ET-1 minimized these signs of stress while preserving pain behavior (see below). Animals (*n* = 3) showing significant signs of toxicity at 400 μM ET-1 (red tears and reduced exploration) were not included in the data analysis.

Neurobehavioral effects

Subcutaneous injection of 40 μl of PBS into the subcutaneous plantar hindpaw produced minimal evidence of hindpaw flinching (Fig. 1). In comparison, subcutaneous injection of ET-1 produced dose-dependent ipsilateral hindpaw flinching identical in appearance to that observed previously after application of ET-1 to rat sciatic nerve (Davar et al., 1998; Fareed et al., 2000). Hindpaw
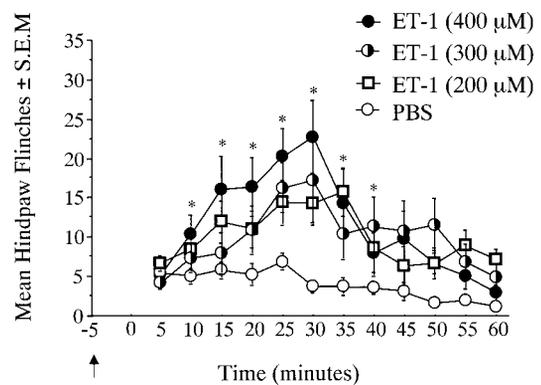

Figure 1. Hindpaw flinching induced by the injection of 8, 12, and 16 nmol (*n* = 10 animals for each dose) ET-1 (equivalent to 200, 300, and 400 μM, respectively) subcutaneously into the rat plantar hindpaw. Termination of ET-1 administration was at *t* = −5 min; animals were allowed 5 min to recover before assessments began. The frequency of hindpaw flinching occurring every 5 min, measured from *t* = 0, over a period of 60 min was determined. Differences between 400 μM ET-1 and PBS were present from 10 to 40 min after observations began (* $p < 0.05$; f-ANOVA, StatView).

flinching was observed from 10 until 40 min after the onset of observations in at least 10 animals receiving 400 μM ET-1 (Fig. 1). Treatment with 300 μM ET-1 resulted in a significant difference with control animals between 25 and 35 min when compared with 200 μM (Fig. 1). When BQ-123 was administered subcutaneously in multiple doses both before and at the time of ET-1 (200 or 400 μM) injection, it completely blocked hindpaw flinching during the entire period of observation (Fig. 2).

Neurophysiological effects

We recorded impulse activity from 68 single, physiologically characterized afferent fibers of different classes: C-fibers (*n* = 38), Aδ-fibers (*n* = 16), and Aβ-fibers (*n* = 14), before and after subcutaneous injections of ET-1 alone or with BQ 123 into their RFs. The fibers were examined in three series of experiments: first, with the same (16 nmol) or slightly higher (20 nmol) doses of ET-1 than those used in behavioral studies; second, with lower doses (1–8 nmol); and finally, with 16 nmol of ET-1 together with BQ 123. RFs of fibers were primarily located on the glabrous surface of the plantar hindpaw (except in a few instances when
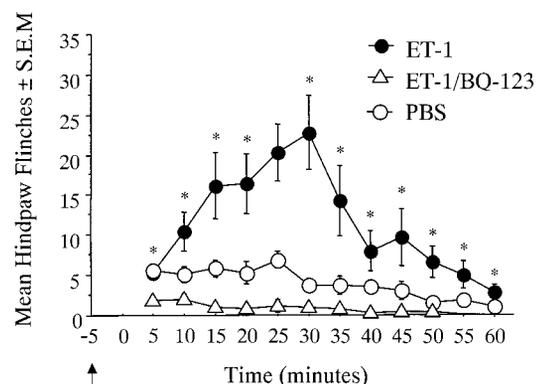
Figure 2. Blockade of ET-1-induced hindpaw flinching by subcutaneous BQ-123 (3.2 mM) applied together with ET-1 (400 μM) over a 9–10 min period. Termination of ET-1 + BQ-123 administration was at *t* = −5 min; animals were allowed 5 min to recover before assessments began. Differences between ET-1/BQ-123-treated and ET-1-treated animals are significant at all time points (* $p < 0.05$; f-ANOVA, StatView).
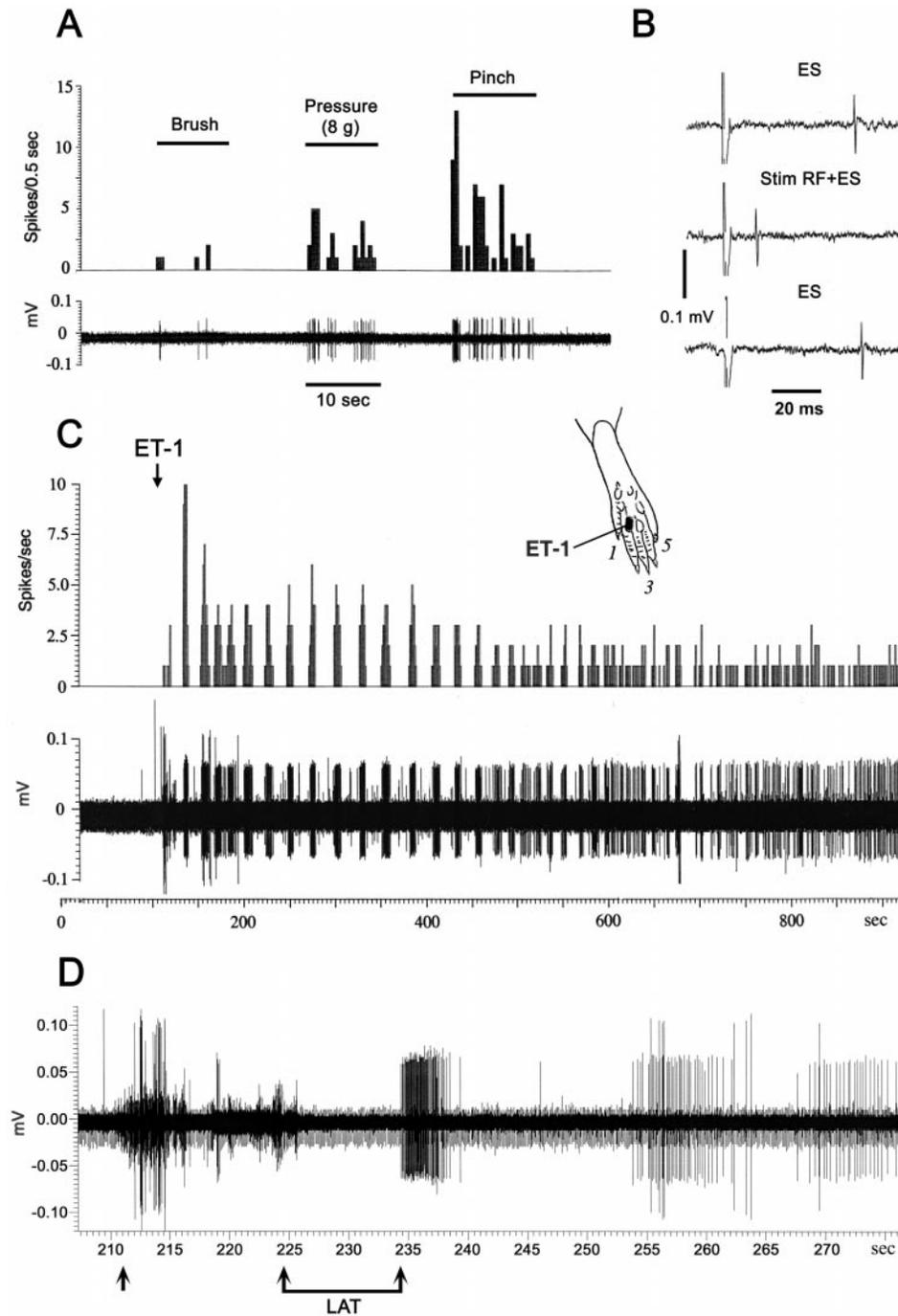
Figure 3. Excitation of an HTMr C-fiber after subcutaneous injection of ET-1 into the rat plantar hindpaw. *A*, Physiological characterization of an HTMr C-fiber (CV 0.72 m/sec) showing the fiber response to graded mechanical stimulation (*below*), presented also as a bin histogram (*above*). *B*, Procedure for confirming single unit activity. *Top* and *bottom traces*, Spikes are evoked by transcutaneous electrical nerve stimulation (*ES*) that has a consistent latency of 63 msec; *middle trace*, the electrically evoked spike fails to appear, presumably because of the refractory period generated by the spike evoked by mechanical stimulation of the receptive field. *C*, Representative C-fiber response (CV 1.8 m/sec) to the subcutaneous injection of 2 nmol (10 μl of 200 μM) of ET-1 (*below*), presented also as a bin histogram (*above*). Note the short latency to onset of spiking, and the "bursting" pattern of this spike activity occurring within the first 10 min of the response. *Inset* shows the location of the receptive field of this unit. *D*, Expanded time scale of the early response to ET-1 (shown in *C*) illustrating the approach to latency measurement. The *arrow* points to the beginning of injection, whereas the *twin arrows* and *intervening line* denote the end of injection (needle withdrawal) and latency (*LAT*) to the first measured spike response.

they were located on the ankle, at the border between glabrous and hairy skin on the plantar hindpaw and, in one instance, on the leg) (Figs. 3, 6, 7, *insets*). These RFs were within regions that are primarily innervated by the plantar and sural nerves, and they ranged in size from 1 × 2 mm to 3 × 4 mm for C- and Aδ-fibers, and from 2 × 3 mm up to 7 × 10 mm for Aβ-fibers. The conduction velocities of C-units ranged from 0.47 to 1.8 msec (mean 0.91 ± 0.05 msec), of Aδ-fibers from 2.2 to 12.8 msec (6.9 ± 1.0 msec), and of Aβ-fibers from 23 to 51 msec (32.5 ± 2.0 msec). The vast majority of C-, Aδ-, and Aβ-fibers (82, 72, and 81%, respectively) had no ongoing spontaneous activity. In those units in which spontaneous activity was observed, the mean frequency was very low (0.04–0.17 imp/sec).

Effects of ET-1 on C-fibers

In control experiments, seven C-units (all HTMr) were injected with PBS (2 with 50 μl and 5 with 10 μl) (Table 1). Two responded to PBS, one (50 μl volume) very weakly (MRF = 0.12 imp/sec) and briefly (<2 min) and the other (10 μl volume) with MRF significantly lower (0.34 imp/sec) than that observed after subsequent injection of a low concentration of ET-1 (1 nmol in 10 μl PBS) into the same unit (2.25 imp/sec) (data not shown).

Most C-fibers in which the effects of ET-1 were examined (20 of 33) responded to strong (18–52 gm von Frey hair) mechanical stimulation of their RFs, whereas 11 of 33 began to fire with weak initial stimulation (2.26–5.25 gm) but increased their responses

Table 1. Parameters of spike responses of afferent fibers to subcutaneous ET-1 and PBS

	ET-1					PBS
	Number of units responding/ total units studied		Response to ET-1 (16–20 nmol)			Number of units responding/total units studied
Fiber class (range of CV)	1–8 nmol	16–20 nmol	MRF (imp/sec)	Maximum response frequency (imp/sec[1])	Mean duration of response (min)	
C-fibers (0.47–1.8 m/sec)	14 of 16[a]	17 of 17	2.02 ± 0.48	5–25	30 ± 3	2 of 7
Aδ-fibers (2.2–12.8 m/sec)	4 of 6[b]	6 of 6	3.3 ± 2.3	5–45	18 ± 4	2 of 7
Aβ-fibers (23–56 m/sec)	0 of 3	3 of 9[c]	NA	NA	28 ± 3	2 of 13

[a]The two C-fibers that did not respond to ET-1 were low-threshold mechanoreceptors.

[b]Of two Aδ-fibers that did not respond to ET-1, one was a low-threshold mechanoreceptor, and the other was a cold Aδ-fiber.

[c]Of three Aβ-fibers that responded to ET-1, two also responded to PBS; one of these units responded with very high frequency (MRF = 8 imp/sec) to ET-1, whereas the other two responded with very low frequencies (averaged MRF = 0.25 ± 0.15 imp/sec).

Table 2. Concentration dependence of the average maximum frequency of ET-1-induced spike responses during bursts in C-fibers (MxF), and the latency to reach MxF

Dose of ET-1 (nmol)	Average MxF (imp/sec, 1 sec bins)	Time to reach average MxF (min)
16–20 ($n = 7$)	14.0 ± 3.2	7.2 ± 0.6*
8 ($n = 2$)	8.0 ± 2.0	3.1 ± 2.8
2 ($n = 8$)	10.0 ± 3.0	0.26 ± 0.06

*Significantly different from 2 nmol ($F = 29.7$; $p = 0.0004$).

Figure 4. Dose dependence of excitation of C-fibers induced by subcutaneous ET-1 2 nmol ($n = 10$), 8 nmol ($n = 5$), and 16–20 nmol ($n = 17$). Shown are the averaged MRF (*A*), mean duration of responses (*B*), and mean latency of responses (*C*). *$p < 0.05$ when compared with 16–20 nmol and †$p < 0.05$ when compared with 2 nmol.

with progressively stronger stimuli; noxious pinch gave a maximum transient response for both populations of C-fibers of 3–36 imp/sec (mean 14.1 ± 3.7 imp/sec) (Fig. 3*A*). These 31 of 33 units can be classified as HTMrs (Handwerker et al., 1991). Two remaining C-units responded equally to both weak and strong mechanical stimulation and can be classified as low-threshold mechanoresponsive units. Thermal responsiveness was also tested for 11 fibers of the HTMr population (31 of 33), and all responded to thermal stimulation with heat thresholds of between 48° and 50°C. These 11 units were also considered as C-PMNs.

All 17 C-units studied after injection of 16–20 nmol of ET-1 into their RFs were activated with latencies to spike response varying from 1.22 to 5.6 min (mean 3.16 ± 0.31 min) and the duration of responses ranging from 15 to 40 min (mean 30 ± 3 min) (Table 1). The temporal pattern of firing induced by ET-1 in these units was usually an early oscillatory bursting pattern, with bursts 10–15 sec in duration, peaking at ∼3 Hz, and occurring at ∼50 sec intervals. After 5–10 min, the regularity of these burst oscillations was replaced by a more uniform firing of 1–2 Hz average frequency (Fig. 3*C*). The average MRF for all C-fibers tested at these concentrations was 2.02 ± 0.48 imp/sec. The average MxF (calculated on the basis of maximum frequency peaks during bursts for individual C-fibers) was 14.0 ± 3.2 imp/sec (Table 2).

Similarly, injection of 1–8 nmol of ET-1 ($n = 16$) provoked spike responses in 14 of 16 units (all HTMr). The two remaining units, both LTMr, did not respond to ET-1 at doses of 1 and 8 nmol, respectively. Although lower average MRF and duration of spike responses were apparent with these lower doses, these differences were only significant when doses of 2 nmol were compared with 16–20 nmol of ET-1 (Fig. 4). Interestingly, the
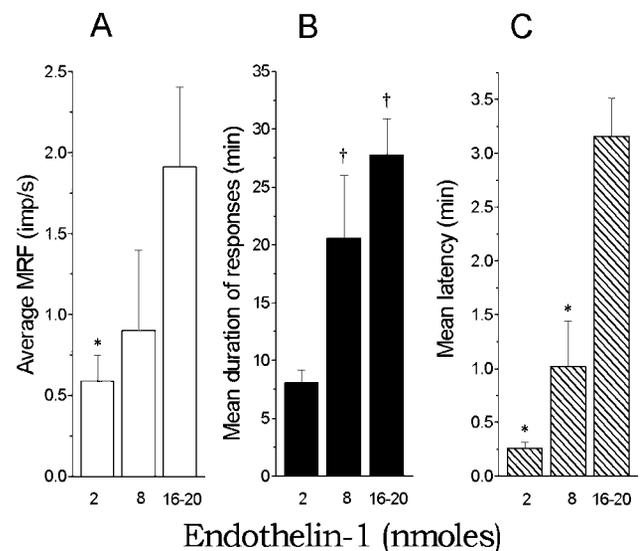
latency of responses diminished with dose and at 2 nmol was theshortest among all doses tested, with a mean latency (0.27 ± 0.06 min) that was significantly less than that found at 20 nmol of ET-1 (Fig. 4). Of two C-units injected with 1 nmol of ET-1, one (an HTMr) responded with an MRF of 2.25 imp/sec and duration of 18 min. The pattern of spike response for doses from 1 to 8 nmol was similar to that observed for 16–20 nmol and was characterized by an initial bursting in regularly spaced episodes that was gradually transformed into sustained firing of decreasing frequency (Fig. 3*C*).

When ET-1 was coinjected with BQ-123, three units (two C-PMNs) of five tested did not respond. In the other two units, very weak spike activity (MRF = 0.05–0.09 imp/sec) occurred, of long latency (3–5 min) and short duration (5–12 min). Interestingly, in three of five fibers, spike activity developed 48–64 min after coinjection, possibly caused by a waning of the effects of BQ-123, but well beyond the duration of responses to 8 nmol of ET-1 alone (∼20 min) (Fig. 5, Table 1).
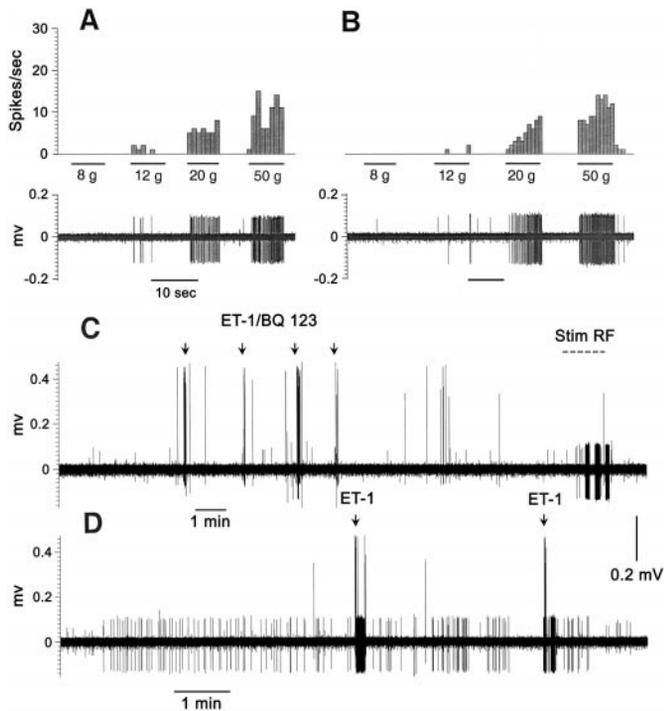
Figure 5. Blockade of HTMr C-fiber (CV 1.18 m/sec) activation by coinjection of BQ 123 (3.2 mM) together with 8 nmol of ET-1. The physiological responses of this unit to graded mechanical stimulation (von Frey hairs) before (*A*) and after (*B*) ET-1/BQ-123 are shown. *C*, Four successive injections of ET-1/BQ 123 (10 μl each) failed to induce spike responses for a total period of 60 min), although the unit maintained its responsiveness to graded mechanical stimulation (*Stim RF*). *D*, Spike activity appeared at ~50 min after the coinjection of ET-1/BQ-123 and was augmented by successive injections of ET-1 (2 nmol) alone.

Effects of ET-1 on Aδ-fibers

Only two of seven Aδ-fibers with RFs that were injected with PBS responded to this vehicle (Table 1). The first, an LTMr, responded to a 50 μl injection with 3 sec latency, MRF = 0.22 imp/sec, and duration <2 min. Another Aδ-fiber (HTMr) responded to a 40 μl injection of PBS with a much shorter period (9.1 min) of much lower MRF (0.6 imp/sec) than the responses to subsequent ET-1 injection (16 nmol) into the RF of this same unit (23 min and 1.0 imp/sec, respectively). The remaining five Aδ-units did not respond to 10 μl injections of PBS.

Of the 12 Aδ-fibers investigated for the effects of ET-1, 11 had their RFs on the plantar hindpaw, whereas 1 had its RF on the leg. Five of 12 units responded only to intense mechanical stimulation (13–50 gm) and were classified as HTMr, whereas 5 of 12 began to fire with weak mechanical stimuli (0.72–5.25 gm) but increased their responses with increasing stimulus intensity. Of the remaining two Aδ-fibers studied, one responded only to weak mechanical stimulation (LTMr), and another responded only to cold stimuli (cold unit). Thermal thresholds for 4 of these 12 fibers were examined and in 3 of 4 fibers were found to range from 42 to 44°C (non-noxious); one additional fiber had a thermal threshold of 48°C (mechanoheat nociceptor).

Ten of 12 Aδ-fibers with RFs that were injected with ET-1 began to fire impulses. Doses of 16–20 nmol of ET-1 produced spike responses in all six Aδ-units exposed to these doses (Fig. 6, Table 1), similar to those observed for C-fibers. The latency to

onset of activity ranged from 0.96 to 7.2 min (mean 3.5 ± 0.12 min) with response duration varying from 13 to 20 min (mean = 18 ± 4). The MRF for most (5 of 6) units ranged from 0.96 to 1.44 imp/sec and, in a single instance, reached a maximum of 12.4 imp/sec. The averaged MRF for all six Aδ-units was 3.3 ± 2.3 imp/sec, whereas the MxF during bursts (see below) ranged from 7 to 45 imp/sec. These Aδ-units had an irregular response pattern (Fig. 6A) that in most cases resembled the burst-like pattern observed in C-fibers (see above), but were usually of longer duration and had a characteristic shape consisting of a slow crescendo to very high frequency (29–45 imp/sec) followed by relatively steep frequency decay (Fig. 6B). The total duration of individual bursts ranged from 0.5 to 0.8 min, and their periodicity was 0.92 ± 0.12/min.

Doses of 1–8 nmol of ET-1 produced spike responses in four of six Aδ-fibers tested (all HTMr) with latencies to onset of spiking, duration of response, and MRF comparable to that observed for C-fibers. Of the two Aδ-units that did not respond to this dose of ET-1, one was an LTMr and the other was a cold Aδ-fiber.

None of four Aδ-fibers tested (three HTMr and one cold unit) responded to ET-1/BQ 123 coinjections or to additional ET-1 (2 nmol) administration during the 70 min period after the initial injection.

Effects of ET-1 on Aβ fibers

Injections of 40 μl PBS (in 10 μl aliquots) provoked firing in 2 of 13 units, both of which also responded to injections of ET-1 (Table 1 and see below). One of these units responded with very short latency to the first of four PBS (10 μl) injections (0.43 sec) and with MxF of 65 imp/sec and MRF = 40 imp/sec, possibly consistent with an injury-induced discharge. None of four Aβ-units injected with 10 μl PBS revealed spiking activity.

The 12 Aβ-fibers tested for effects of ET-1 were of three main functional types: (1) hair follicle (HF) units (*n* = 3) responding to hair bending at the border between glabrous and hairy skin (Fig. 7, inset), (2) rapidly adapting (RA) units (*n* = 6) responding to touch or light pressure (5–7 gm), and (3) slowly adapting (SA) fibers (*n* = 3) responding with sustained impulse activity to more intense mechanical stimulation (10–20 gm von Frey hair).

In sharp contrast to small-diameter fibers, most Aβ-fibers (9 of 12) did not respond to any dose of ET-1 (Fig. 7C, Table 1), and in particular, none of the three HF units that responded with high-frequency discharge to mechanical stimulation responded to ET-1. Two of three Aβ-units that did respond to 16 nmol of ET-1 were of the SA type. Both of these units had long (~12 min) latencies to onset of spiking, with one unit demonstrating a very low frequency response (MRF = 0.1 imp/sec), whereas the other fired more intensely (MxF = 25 imp/sec, MRF = 8 imp/sec). The third unit that showed a response to ET-1 was a RA receptor that responded with short latency (1 min) and low MRF (0.4 imp/sec) to both ET-1 (16 nmol) and PBS (40 μl) applied to the fleshy pad of the plantar hindpaw.

DISCUSSION

The results of these experiments demonstrate that subcutaneous injection of ET-1 into the rat plantar hindpaw induces flinching behavior that is accompanied by spike activity in single, nociceptive primary afferents. These effects may be mediated by endothelin receptors on pain fibers because coadministration of an endothelin-A receptor antagonist together with ET-1 blocks both the flinching behavior and spike responses in nociceptors.

Blanching of the skin that immediately follows injection of

Figure 6. Spike responses in two different HTMr Aδ-fibers after subcutaneous injection of 16 nmol of ET-1. *A*, In the first unit (CV 3.08 m/sec), a bin histogram illustrates the increasing frequency of spiking within the first 5 min after completion of injections, followed by a gradual decline in spike frequency. Noxious mechanical stimulation performed 9 min after ET-1 injection demonstrates continued responsiveness of the RF of this unit. *Inset* shows the location of the receptive field of this unit. *B*, In the second unit (CV 5.7 m/sec), the characteristic bursting pattern of the spike response is shown in a single episode extracted from the full response after injection with 16 nmol of ET-1.
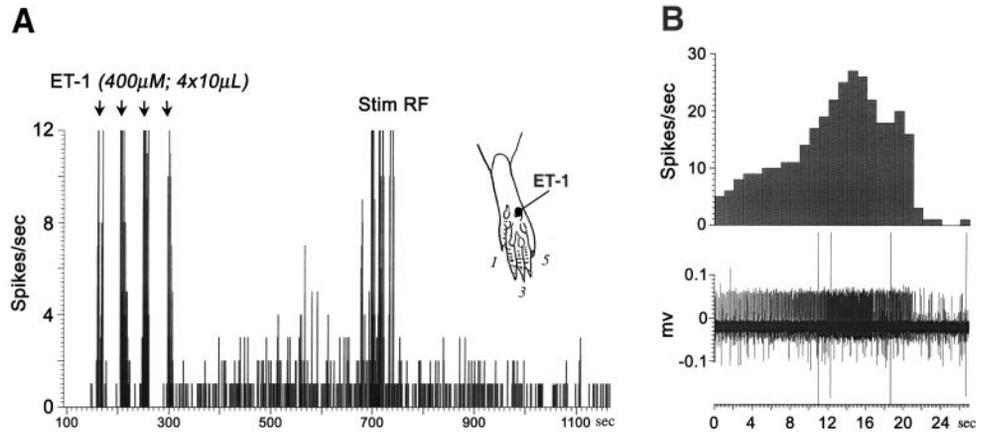

ET-1 into the plantar hindpaw is almost certainly a direct vasoconstrictive effect of ET-1. This vasoconstriction is likely mediated by ET_A receptors on cutaneous blood vessels, but a possible role for endothelin-B (ET_B) receptors cannot be ruled out (Lawrence et al., 1995). In contrast, the more slowly developing erythema and rubor probably reflect a combination of local effects (e.g., limb cooling) and ET-1-mediated release of vasoactive substances either from primary afferent terminals or from other cutaneous cells (e.g., mast cells) as a consequence of primary afferent activation (Crossman et al., 1991). Such proinflammatory, vasoactive substances might contribute directly to, or sustain, the pain behavior and activation of nociceptors (see below).

Although we have previously reported behavioral effects of ET-1 applied to the rat sciatic nerve (Davar et al., 1998; Fareed et al., 2000), this is the first description of an excitatory effect of ET-1 on primary afferent terminals. Although actions on axons cannot be ruled out, we presume that afferent terminals were activated because ET-1 was injected directly into the receptive fields of identified afferents. These effects are likely mediated by

endothelin receptors located immunocytochemically on primary afferents (Pomonis et al., 2001) or, alternatively, by endothelin receptors located on other supportive cells or vascular cells. More significantly, the majority of C- and Aδ-fibers, which were predominantly nociceptive, were excited by ET-1, whereas most Aβ-fibers did not respond to ET-1. The most important implication of these findings is the confirmation of the nocifensive nature of hindpaw flinching induced by subcutaneous administration of ET-1. Furthermore, and consistent with our previous hypotheses, these results suggest a neural basis for this pain behavior through actions on nociceptive afferents. Although ischemic effects of ET-1 might also contribute to nociceptor activation, acute ischemia usually produces paresthesia rather than pain, presumably as a result of ectopic firing in cutaneous afferents (Mogyoros et al., 1997). In addition, ischemia of visceral C-afferents produces activation with much longer latencies and later peak responses than we have observed here with cutaneous C-fibers injected with ET-1 (Fu et al., 1996; Pan et al., 1997).

Although the time to onset and peak of the spike responses in C- and Aδ-fibers occurred earlier than the onset and peak of ET-1-induced hindpaw flinching, this is most likely because a critical number of afferents need to be activated to induce flinching behavior. These afferents are probably successively activated by the gradual spread of ET-1, a moderately hydrophobic peptide (Rubanyi and Polokoff, 1994), from subcutaneous sites to the dermis. Another, and coexisting, possibility that was not examined in these experiments, is that the initial spike discharge induces central sensitization to subsequent sensory inputs, thereby prolonging the time course of behavior when compared with the spike responses. Although the latency to spike responses at higher doses might be considered long, for a direct action on nociceptors, several factors argue against this. First, the diffusion of ET-1 from the subcutaneous space to terminals in the dermis may be rate limiting, and second, we have recently observed that ET_B receptor blockade in the presence of ET-1 evokes significant increases in flinching and immediate spike responses (<30 sec), consistent with inhibitory effects of the ET_B receptor on pain behavior and nociceptor activation (Khodorova et al., 2001; our unpublished results). The reduction in latency to onset of spike responses with reduced dose may have been a consequence of ET-1-mediated venous pooling and edema that occurs as dose is increased, reducing access of ET-1 to terminals (Piovezan et al., 2000). Although the mean and maximum frequency of ET-1-induced spike responses in C- and Aδ-fibers is lower than that observed with noxious pinch, spatial integration of fibers acti-
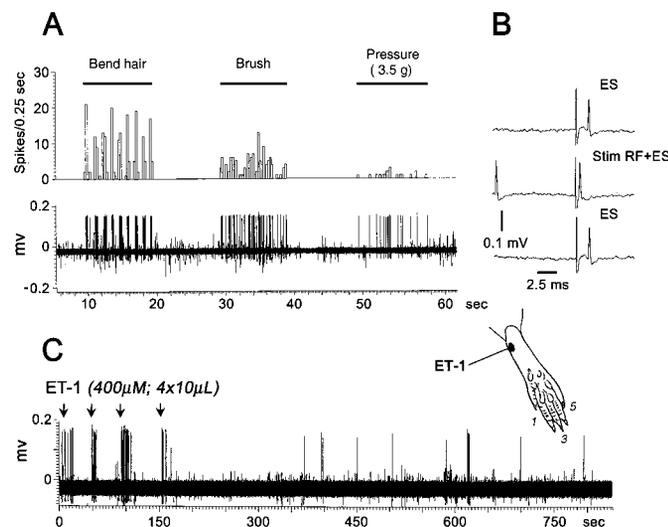
Figure 7. Lack of response of an Aβ-fiber (hair follicle afferent, CV 36.4 m/sec) to subcutaneous injection of 16 nmol of ET-1. *A*, Record of the response of this fiber to hair bending, brush, and von Frey hair (3.5 gm) stimulation of its RF located at the border between hairy and glabrous plantar skin (see *inset*). *B*, Identification of the unit with the collision method (see Fig. 3*B*). *C*, Failure of this same unit to respond to 16 nmol of ET-1 (total dose) into its RF.

vated by ET-1 spreading subcutaneously after injection, and a pattern of firing (bursting) that is known to sensitize second order spinal neurons (Schouenborg and Dickenson, 1985), may have contributed to the observed nocifensive response. Despite the relatively high concentrations of ET-1 used in these studies, the local concentrations at receptor-containing tissues are unknown, and importantly, the ET-1 concentration reached in diseased tissue (e.g., in vertebral tissues at the site of prostate or breast cancer metastasis) that is required to induce pain is also unknown. The effective concentration might indeed be low (<100 μM), yet within the range that can induce spiking in C-fibers. In humans with cancer metastases, small amounts of ET-1 might also sensitize fibers to subsequent noxious or non-noxious stimuli, as has been described in animals (Piovezan et al., 1998, 2000; Ferreira et al., 1989).

Nociceptive C- and Aδ-fibers had very similar spike response characteristics when exposed to ET-1 injection. Although thermal thresholds appeared to be somewhat reduced in Aδ- when compared with C-fibers, the small number of Aδ-units in which thermal thresholds were determined was insufficient to establish any significance to this difference.

In contrast with the effects of ET-1 on nociceptors, Aβ-fibers failed to respond to ET-1 injected into their receptive fields. Although spike responses were observed in a few units, they developed with much longer latencies and had markedly lower mean and maximum frequencies than were observed for C- and Aδ-fibers. Two of the three Aβ-units that did respond to ET-1 also responded to PBS injection, suggesting an injury-related or mechanical, (e.g., volume of injection, needle damage) effect of the procedure per se in those instances. This lack of response to ET-1 in Aβ-fibers is consistent with an absence or low abundance of "excitatory" ET$_A$ receptors on Aβ-fibers. Of course this does not exclude the possibility that other, nonactivating endothelin receptor subtypes (e.g., endothelin-C) might be found on Aβ-fibers. The failure of Aβ-fibers to respond to ET-1 supports the idea that the actions of ET-1 on primary afferents are selective for nociceptors and that the observed behavior (hindpaw flinching) is pain related.

Finally, the blockade of ET-1-induced pain behavior, and spike responses in nociceptors, by an ET$_A$ receptor antagonist clearly establishes a specific action of ET-1 for these phenomena. Although concentrations well in excess of the K_I that might lead to actions at the ET$_B$ receptor were used to study the effects of BQ-123, our recent results suggest that activation of the ET$_B$ receptor would enhance rather than inhibit flinching and spike responses (Khodorova et al., 2001; our unpublished data). Taken together with recent studies demonstrating the presence of ET$_A$ receptors on primary afferents (Pomonis et al., 2001), these results and our previous reports of the actions of ET-1 on sciatic nerve suggest that ET-1 is acting through ET$_A$ receptors found on nociceptive primary afferents to produce pain behavior. The observations that C- and Aδ-fibers that did not respond to ET-1 were non-nociceptive lends further support to the modal specificity of receptor action.

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