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Endothelin-1 raises excitability and reduces potassium currents in sensory neurons

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Abstract

Exposure to endothelin-1 (ET-1, 50nM) of sensory neurons, acutely isolated from rat dorsal root ganglia, results in an increase in the number of action potentials elicited by a linear ramp of stimulating current. The changes are complete in 5 minutes after ET-1 treatment and do not reverse in 5-10 minutes after ET-1's removal. Neither the resting potential, nor the threshold potential for the first or second action potentials, nor their rate of rise or decay, are changed by ET-1 exposure, but the slow depolarizations which occur before the first and second action potentials during the current ramp are increased by ca. 50% by ET-1. The delayed rectifier type of K^+ currents (I_K), measured under whole-cell voltage clamp, are depressed by ~30% during such exposure to ET-1. The voltage-dependent gating of steady-state I_K and the current kinetics are unchanged by ET-1, leaving the sole effect as a drop in the number of available channels. I_K is affected by ET-1 only in Isolectin B_4-positive cells, suggesting that there may be a selective action in enhancing impulse activity on this class of nociceptive neuron. This decrease in I_K will potentiate the excitability-inducing actions of the previously reported negative shift in tetrodotoxin-resistant Na^+ channel gating in such neurons.

Keywords

pain; nociceptor; potassium channel; endothelin-1; excitability

Introduction

Endothelin-1 (ET-1) is an endogenous peptide that contributes strongly to elevated pain from inflammation, incision and cancer [21]. Previous studies have shown that *in vivo* application of ET-1, both on trunk axons of sciatic nerve [6] and injected subcutaneously [12,14], or into joints [8], causes pain and induces impulses to occur spontaneously and highly selectively in peripheral nociceptors [14]. Spontaneous firing in nociceptors *in vivo* appears in a minute or so after ET-1 injection into their cutaneous receptive field and occurs as bursts of action potentials separated by quiet periods, each lasting for several minutes, showing that ET-1 can generate repetitive firing in single axons [14]. Exposure to ET-1 of isolated sensory neuron soma from dorsal root ganglia (DRG) acutely shifts the *activation* gating of tetrodotoxin-resistant Na^+ currents (TTX-R) in the negative voltage direction [43], a change that would

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lower the threshold for impulse generation [15,19]. Although this action is consistent with increased isolated sensory nerve excitation, ET-1-induced changes in their excitability have yet to be directly shown, and voltage-gated ionic channels other than TTX-R have not been investigated.

In the present paper we determine the changes in excitability of acutely isolated small and medium diameter sensory neurons by brief exposure to ET-1, at concentrations near the reported K_D values in other cellular systems [32]. Neurons are categorized as peptide- or non-peptide containing by staining with the lectin isolectin B_4 (IB_4) [35]. We also examined ET-1's influence on the delayed-rectifier type of K^+ currents, since such currents are an important factor in the ability of cells to fire impulses repetitively [4,5,19,36], and from their voltage-dependence and kinetics are likely contributors to the slow depolarizations that are enhanced by ET-1 and have been identified as important parameters for the modulation of excitability by other agents, such as Prostaglandin E_2 (PGE2) and nerve growth factor [20,41]. Potassium channels also have been identified as targets of drugs that relieve elevated pain [2,10,25].

Materials and methods

Preparation of dissociated DRG neurons

The use of animals was approved by the Harvard Medical School Committee on Animals, in accordance with guidelines for the humane treatment of laboratory animals. Dorsal root ganglia (DRG) were isolated from male Sprague-Dawley rats (100-120 gm; Charles River, Wilmington, MA), as previously reported [33,43]. Rats were anesthetized with sevoflurane inhalation anesthetic (Novaplus™; Abbott Laboratories, North Chicago, IL) and their L3- L6 DRG were harvested, after which the animals were decapitated. Dissected DRG were first incubated with 1mg/ml collagenase/dispase (Roche Diagnostics Corp., Indianapolis, IN) for ca. 2hr at 37°C, and then the tissue was removed from this initial digestion medium and exposed to 0.25% (w/v) trypsin (Invitrogen; Carlsbad, CA) at 37°C and triturated several times after 8-10 min. Single cells were decanted from this suspension, washed free on digestive enzymes and placed in culture medium composed of neurobasal media (Invitrogen; Carlsbad, CA) supplemented with 1× B27 supplement, 100U/ml penicillin-streptomycin, 2mM L-glutamine and 50ng/ml nerve growth factor(all from Invitrogen; Carlsbad, CA). Cells were plated and incubated at 37°C with 95% O_2 + 5% CO_2. Cells were recorded from 12 to 24 hours after plating.

Electrophysiology

The whole-cell patch-clamp technique was used to record membrane potentials and K^+ currents [18]. Borosilicate glass electrodes (World Precision Instruments, Sarasota, FL) were pulled by a P-97 Puller (Sutter Instruments, Novato, CA), which had resistances of 1.5-6MΩ. Measurements were made in current- or voltage-clamp mode with an Axopatch 200B amplifier (Axon Instruments, Foster City, CA). Series resistance was compensated by ~80%. Pipette and cell capacitances were partially compensated. A P/4 procedure was used for leak subtraction during voltage-clamp measurements [1]. Data were acquired and analyzed using Clampex 8.2 and Clampfit 8.2 software, respectively (Axon Instruments). In the current-clamp experiments, the neurons were bathed in a solution containing the following (mM): NaCl, 140; KCl, 5; $MgCl_2$, 1; $CaCl_2$, 2; glucose, 10; HEPES, 10 (pH adjusted to 7.4 with 1N NaOH). The pipette intracellular solution contained (in mM): KCl, 140; $MgCl_2$, 5; Na_2ATP, 4; Na_2GTP, 0.3; $CaCl_2$, 2.5; EGTA, 5; HEPES, 10 (adjusted to pH 7.3 with KOH). The diameter of the neuron soma was estimated at 200× magnification. Only neurons with a stable initial resting potential, that drifted by less than 2-3 millivolts during the 10 min of baseline recording, were used in these experiments. Stimulating ramps of linearly increasing current (range 0.1-1.2 nA/sec) were chosen to produce 2-4 action potentials (APs) over a 1 s depolarization for each tested

neuron in a control, "baseline" period. This same ramp was used throughout the remaining tests on that cell, including those during and after ET-1 application. In addition to the number of APs during the ramp, the threshold potential to begin the upswing of those APs and their maximum rate of rise $(dV/dt)_{rise}$ and decay $(dV/dt)_{fall}$, cells were characterized by their resting potential, input resistance, R_m (as the change in membrane potential divided by a step of outward current, hyperpolarizing the cell by 15-20 mV), and by the slopes of the quasi-linear slow membrane depolarizations that occurred in response to the depolarizing current ramps leading up to the first and second APs ($(dV/dt)_{slow1}$ and $(dV/dt)_{slow2}$, respectively).

In most experiments the neurons were characterized by markers for nociceptor sub-type by IB_4 staining [35]. Neurons were incubated for 10 min with IB_4 (10μg/ml) labeled with fluorescein isothiocyanate (FITC-IB4, Sigma-Aldrich, St. Louis, MO), then rinsed once with bath solution. Cells were then visualized by fluorescence using the epi-fluorescence mode of the inverted microscope while the electrode seal was formed.

In the voltage-clamp experiments, K^+ currents were recorded in a bath solution containing (mM): cholineCl, 130; $MgCl_2$, 1; KCl, 5; $CoCl_2$, 2; and HEPES, 10; pH adjusted to 7.4 with hydroxylcholine. Electrodes were filled with intracellular solution containing (in mM): KCl, 120; $MgCl_2$, 2.5; EGTA, 10; Na_2ATP, 2 and Na_2GTP, 0.3; HEPES, 10; at pH 7.3 adjusted with KOH.

The neurons were held at -80 mV; 200 ms pulses were used to activate currents with + 10 mV increments to + 30 mV. Neurons were perfused with external solution for at least 10 minutes to obtain the control baseline response; then exposed to 50nM endothelin-1 (Alexis Biochemicals, San Diego, CA) for 10 minutes. In about 1/3 of the experiments recording was continued for an additional 10 min after washout of ET-1, but the diminished K^+ currents were never restored.

Data analysis

All values are given as means ± standard error of the mean. Instantaneous K^+ conductance (G_K) was calculated according to Ohm's Law: $G_K = I_K / (E_m + E_K)$, where I_K is the K^+ current at a specified time (usually the end of the depolarizing test pulse), E_m is the membrane potential set by the voltage-clamp amplifier and E_K is the calculated Nernst potential of K^+. All G_K were normalized to the maximum G_K, G_{max}, of the particular recorded cell. Conductance data were fitted with the Boltzmann equation:

$$G_k' = G_{max} + (G_{max}\ G_{min})/(1+\exp((E_m - E_{0.5})/k)),$$

Where G_K' is the calculated G_K, G_{min} is G_K's minimum value, E_m is the membrane voltage, $E_{0.5}$ is the mid-point voltage at which half the maximum change in G_K occurs, and k is the voltage-sensitivity of K^+ channel gating. The mean ± standard error for G_K' was calculated from all neurons, before, during and after ET-1 exposure, and the average Boltzmann parameters were determined after fitting G_K vs E_m data for each cell.

The ratios for a particular parameter before and then after ET-1 treatment, or after washout, were determined for each individual cell, and then the mean of the ratios was taken and the S.E. of that mean of ratios calculated. This is why the listed mean of the ratio measurements is not equal to the ratio of the separately calculated mean parameters.

Statistical differences for comparing the parameters for the control and ET-1 treatment groups were evaluated by ANOVA with significance assigned for $P < 0.05$.

Results

The number of APs in DRG neurons stimulated by a current ramp was increased by ET-1, although neither the resting potential nor the input resistance was affected (Figure 1, Table 1). Action potentials were recorded under stable conditions in normal physiological solution. Stability was established by the requirement that over a 10 min recording session under "Control" conditions, without ET-1, the maximum number of APs elicited by the ramp (4.00 ± 0.52; n=6; Figure 1) was unchanged compared to the average number recorded during ramp stimulation in the preceding 10 min "Baseline" period (3.67 ± 0.42; n=6, $p>0.05$). The average of the ratio of [stimulated APs during Control]: [stimulated APs during baseline] equaled 1.13 ± 0.15; (n=6). In the 10 min following these "Control" recordings, corresponding to the ET-1 washout period (see below), the ramp continued to stimulate the same number of APs (3.50 ± 1.06; n=5). In contrast, as exemplified in Figure 1, when DRG cells (identified by immunofluorescence as IB_4-positive) were exposed to ET-1 (50 nM) the number of APs elicited by the ramp increased to 5.86 ± 0.86 (n=7) from a Baseline value of 3.00 ± 0.44 (n=7, $p<0.05$). When the ratio of [APs during ET-1]: [APs during baseline] for each cell was averaged over the total population it equaled 2.24 ± 0.41 ($p<0.05$). This enhanced excitability persisted during the 10 minute washout period after ET-1's removal (number of stimulated APs = 5.00 ± 2.21; n=7, significantly greater than during baseline).

The properties of these APs were not changed by ET-1. The threshold potential from which the first AP began its rapid rise was changed on average by only -1.3 mV from the threshold potential before ET-1, not significantly different from the average changes in Control (IB_4 – positive) cells (Table 1). Threshold for the second AP in a train was almost the same as for the first, and also was unchanged by ET-1 (data not shown). A detectable *decrease* in the maximum rate-of-rise of the first AP, -8.4 V/s, which reflected a general trend of decline in this parameter over time, was not significantly different in ET-1 and control cells (Table 1). The maximum rate-of-fall of the repolarizing phase of the first AP (Table 1) or the second AP (not listed) was also unaffected by ET-1 (data not shown).

One parameter that was significantly changed by ET-1 was the rate of the slow depolarizations during the stimulating current ramp, before both the first and the second APs (noted by numerals 1 and 2 in Figure 1). (Slopes of depolarizations between the later APs in the trains were not analyzed.) Table 1 shows that this slope was increased by ca. 50% for both first and second slow responses, with the second response being faster than the first in both the period before and during ET-1 exposure. The steepening of the depolarization by ET-1, when a constant current was injected into the cell, implies that the membrane resistance increased in the potential range of -60 to -20mV. Slow changes in membrane resistance in this voltage range are likely to be due to slow K^+ conductances, like those of the heterogeneous delayed rectifier [31].

The delayed rectifier type of K^+ current was depressed by ET-1 exposure of IB_4-positive neurons (Figure 2A). The current amplitudes measured at the end of a 200 msec long depolarization to +10mV were reduced after 10 min in 50 nM ET-1, by 26.3 ± 9.7 % (n=6; $P<0.01$) from the currents in the baseline period. After a subsequent 10 min wash in ET-1-free bathing medium these changes in I_K had not reversed to any significant degree. Currents measured in cells incubated for an equal time in control, ET-1-free conditions showed no change in comparison with their baseline values (+ 4.8 ± 3.8 %; n=6; $P>0.05$).

Neurons that were negative for IB_4 staining showed no significant change in I_K after a 10 min exposure to 50 nM ET-1 (+7.4 ± 16.2%, n=5; $P>0.10$), as illustrated in Figure 2B.

Measurements of the activation times for G_K were estimated from the times to reach half-maximum current, using recordings at both -30mV and +10mV (n=5). For both of these

potentials, the half-times were insignificantly changed by ET-1 (3.56 ±1.04 msec in Control and 4.04 ± 1.15 msec during ET-1, at +10 mV, and 6.16 ± 1.11 msec in Control and 6.3 ± 1.36 msec during ET-1, at -30 mV (P>0.67 for both).

Boltzmann analysis was applied to the steady-state K^+ conductance, G_K, calculated from currents at 200msec of depolarization. This analysis showed that G_{max} was changed, with no change in the mid-point potential, $E_{0.5}$, or in the slope, k, a measure of the voltage sensitivity of gating (Figure 3). In 6 control, buffer-treated cells that were kept in this condition for 10 min and 6 cells treated with ET-1 for 10 min, G_{max}, relative to its value in the pre-treatment baseline period, was 0.83±0.03 for ET-1-treated and 1.07±0.02 for buffer ([ET-1] = 0)-treated neurons (P<0.05); respective $E_{0.5}$ values equaled -18.9 ±1.3 mV and -19.4±0.9 mV, and k values equaled 22.4±1.7 mV and 20.7±1.1mV (P>0.05 for both; Student's *t-test* for both parameters).

Discussion

This investigation has found that a brief exposure to ET-1 can enhance the repetitive firing activity of acutely isolated, small and medium diameter sensory neuron cell bodies, without changing the threshold for firing of the initial impulse in a train, and without affecting the resting membrane potential or resting resistance. The slow depolarization that occurs during the ramp of current that excites these APs is elevated by ET-1, consistent with an increased membrane resistance in this potential range (-60-20 mV). Of the several conductances that subserve the overall membrane resistance at these negative potentials, the delayed rectifier has the appropriate voltage dependence and persistent open state to make an important contribution to these slow depolarizations [4,31]. Consistent with this hypothesis, the same treatment of these cells also reduced the non-inactivating delayed rectifier type of K^+ currents, I_K, a change effected by lowering the available conductance and not by changing the gating kinetics in the relevant voltage range.

ET-1 is known to induce impulse firing, selectively, in peripheral nociceptors in vivo and to cause pain like behavior [14]. Cellular investigations have shown that ET-1 can elevate intracellular [Ca^{2+}] in model sensory neurons [42] and can enhance the activity of TRPV-1 channels [28,39] and modulate the activation of TTX-R Na^+ channels in DRG neurons [43]. The combination of the enhanced activation of TTX-R, whose slow inactivation will lead to a more sustained inward Na^+ current during a long depolarization/stimulus, and the reduction of the non-inactivating outward current through delayed rectifier K^+ channels, which open relatively slowly during depolarization and are important in controlling repetitive firing of action potentials, will act together to enhance excitability, particularly with regard to inducing repetitive firing.

It is noteworthy that neither the threshold potential nor the rates of rise and decay of the individual APs was altered by ET-1. The constancy of the threshold potential is particularly surprising, given that the shifts in TTX-R gating previously observed [43] and the reductions of I_K reported here will both alter currents around the measured threshold, ca. -30mV (Table 1), (but see next paragraph).

The findings in this paper closely parallel those reported by Chi and Nicol [5] in which K^+ currents in sensory neurons shown to be functions of the Kv1.1 channel subunits were specifically blocked by dendrotoxin-K (DTX-K). These K^+ currents, with kinetics much like the ones shown here, were suppressed by ~30% by DTX-K, a treatment that increased the number of APs during a ramp of current by ~ 3-fold, with no change in the resting potential or the AP duration, as also reported here. The averaged threshold was significantly lowered by ~4 mV, somewhat larger than the change seen here (which did not reach significance).

Rheobase current, closely related to the membrane resistance that determines the slow depolarization during the current ramp, was approximately doubled by DTX-K, again very similar to the present findings. The knockdown of Kv1.1 in such neurons using siRNA caused almost identical results as DTX-K, and also rendered the cells insensitive to any actions of DTX-K, strong evidence that the changes in excitability from DTX-K were due to suppression of Kv1.1. Because the changes in excitability and in I_K in the present study are so similar to the effects reported by Chi and Nicol [5], by it seems likely that K^+ channels formed by Kv1.1 subunits are one of the targets for ET-1's pro-excitatory actions.

Sensory neurons stained by IB_4 have functional and anatomical properties different from IB_4 – negative neurons, although neither is a homogeneous population [9,24,27,35]. Only IB_4-positive neurons responded to ET-1 with reduced I_K. At least 6 types of K^+ currents have been detected in sensory neurons; including both rapidly inactivating, A-type K^+ currents (I_A) and delayed rectifier currents [16], and several different isoforms of K^+ currents with different kinetics, and their related protein subunits, have been identified in small, nociceptive-like sensory neurons and often shown to be decreased during injury-induced hyperalgesia [13,17, 25,30,40]. We never observed I_A in any of the cells we studied, although others have reported prominent inactivating currents in IB_4- positive sensory neurons [27,37]. The pattern of IB_4-positive cells expressing only the delayed rectifier type of K^+ current, that characterizes the ET-1 responsive cells here, fits the "Type 1" neuron classified by Petruska et al. [27] although the APs in our cells are briefer than their 7 ms duration. Previously our laboratory reported the selective effect of ET-1 on TTX-R currents in DRG neurons which showed varied sensitivity to capsaicin (CAP) [43], with some cells responding with larger CAP-induced inward currents, some with none, which would be consistent with at least two different cell types in Petruska et al.'s classification scheme [27]. Whether ET-1 responsive cells fir into any existing classification scheme for sensory neurons is not evident. Furthermore, at this time we cannot rule out the possibility that ET-1 modulates other voltage-gated ion channels, including I_A and the various Ca^{+2} channels.

In earlier experiments investigating ET-1's effects on TTX-R Na^+ currents in DRG, the observed changes did not return to their pre-ET-1 settings during the 10-15 minutes after washout of ET-1 from the bathing medium [43]. Such persistent actions are indications of long-lasting chemical modifications, like covalent bonding, that is not readily reversed by intracellular enzymes, e.g. phosphorylation of a channel protein by cellular kinases in the absence of active phosphatases. Impulse firing of primary nociceptors after ET-1's injection into the footpad also lasts for ~30 minutes [14] and behavioral effects from peripherally administered ET-1 can last for hours [6,12] although the many tissues are known to contain an abundance of endothelin-converting enzymes that rapidly degrade ET-1 [7]. Therefore, the persistence of the reductions in I_K and increases in cellular excitability caused by ET-1, acting through GPCRs, are probably due to physiologically relevant chemical reactions and not to some non-physiological process.

Endothelin-1 exerts its actions through two different G protein-coupled receptors (GPCRs), called ET_A and ET_B. Of these, ET_A receptors have been localized in sensory neuron soma by immunocytochemistry [29], are responsible for the intracellular Ca^{+2} transients in model sensory neurons [42] and for the shifts in TTX-R gating in DRG neurons [43] as well the excitability that is induced by peripheral ET-1 acutely applied in vivo [6,14]. ET_B receptors, in contrast, have been localized on satellite cells within DRG [29], on Schwann cells that myelinate larger peripheral axons [29] and in keratinocytes of the epidermis [22] and in several other types of cells in the nervous and cardiovascular systems [see review, ref. 21]. Activation of ET_B receptors can also be pro-algesic, particularly under inflammatory conditions [12], but can also have a potent analgesic action, one that involves release of peripheral opioids and their

activation of mu opiate receptors on distal terminals of nociceptors [22]. It is very likely, therefore, that the effects on I_K reported here are mediated via ET_A receptors.

Delayed rectifier K^+ currents in sensory neurons are modulated by several different receptors and pathways. Their suppression by NGF involves the $p75^{NTR}$ receptor, acting through a ceramide-activated kinase [41]. In these same types of cells PGE_2 suppresses I_K through a cAMP transduction pathway, likely involving PKA [11,20]. The inflammatory mediator TNF-α similarly acutely depresses non-inactivating I_K in isolated sensory neurons, an effect blocked by the COX inhibitor indomethacin, suggesting a coupling between TNF-α receptors and prostaglandin synthesis or release [23]. Interestingly, both NGF and PGE2 also act on sensory neurons to elevate TTX-R Na^+ and TRPV1 currents [26,27,34,41]. Brief exposure to NGF also increases excitability of DRG neurons, increasing the number of action potentials during a ramp by ~2-fold, as in the present study, and also not changing the threshold potential for the first AP in the train [41].

The effects of prolonged inflammation of the DRG in vivo are not identical to those from brief exposure to single agents, however. Three to five days of such exposure approximately doubles the amplitude of TTX-R currents (also only in IB_4-positive cells), but increases rather than decreases the K^+ currents [38]. Such differences between acute exposure in vitro and prolonged treatment in vivo could be due to several factors, including the influence of surrounding cells and the likelihood that brief exposure relies on modifications of existing channels whereas prolonged treatment can affect transcriptional regulation of the numbers of channels and their regulatory proteins.

These combined changes in the activities of different ion channels will have a strong, possibly synergistic effect in enhancing excitability, particularly for inducing repetitive firing. Endothelin-1 now joins this set of agents that enhance excitability; it too enhances TTX-R Na^+ currents and suppresses I_K. Although we do not know the intracellular pathway(s) for these effects of ET-1, the amplification of TRPV1 currents by ET-1 occurs via PKC [28,39], and if this is also the pathway for ET-1's modulation of TTX-R and I_K, then these three agents, NGF, PGE2 and ET-1, act on different receptors to activate separate intracellular pathways that converge on common targets. Their combined actions in the setting of peripheral tissue injury or inflammation probably ensures a strong, acute and prolonged response in nociceptor firing. When added to the amplified response of TRPV1 caused by these agents, and possibly others, e.g., bradykinin, 5-HT, the net effect will integrate a heightened transduction of nociceptive stimuli with reduced threshold and elevated repetitive firing to produce an powerful sensitization of primary nociceptors.

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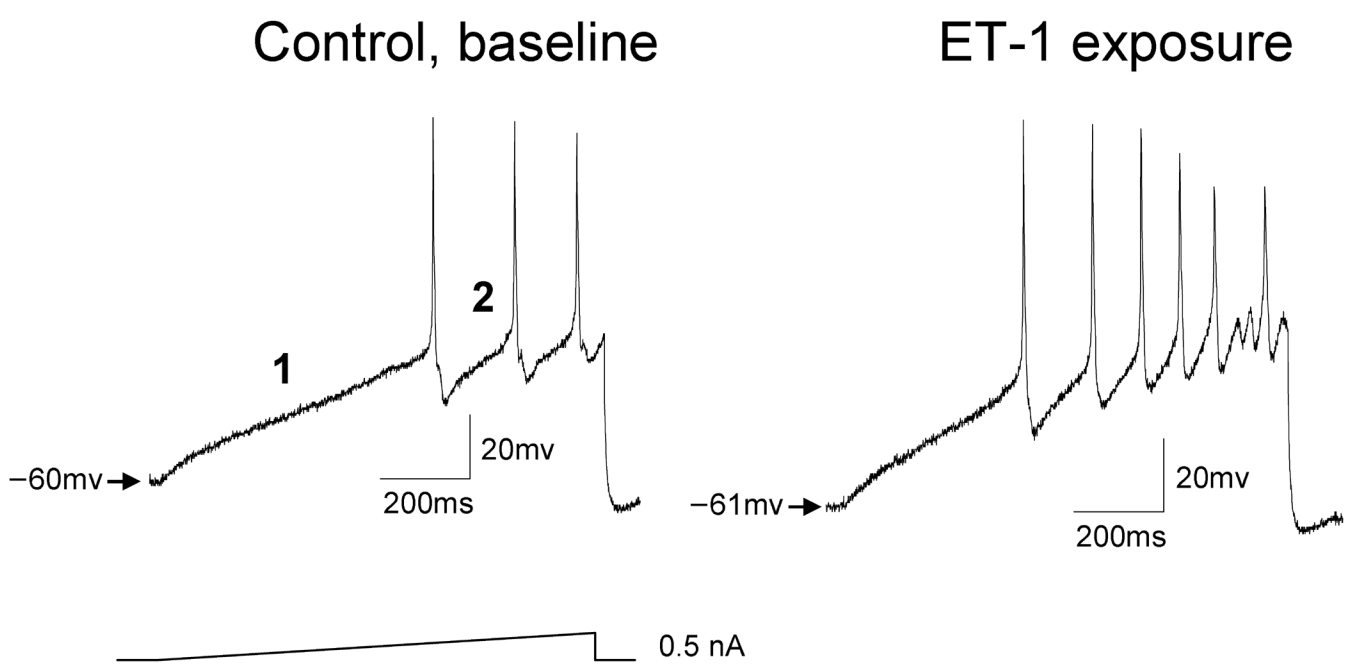

Figure 1.
Membrane depolarization and action potentials (upper traces) in a current-clamped medium diameter rat DRG soma in response to a ramp of linearly increasing inward current (lower trace). The ramp's slope was adjusted, to 0.5 nA/sec, so that the 1 sec long depolarization triggered 3 APs in control conditions (left side). When the same current ramp was applied to the same cell after 10min in 50nM ET-1, 6 APs were evoked (right side). S1 and S2 indicate the slow depolarizations during ramp stimulation leading to the first and second action potential.

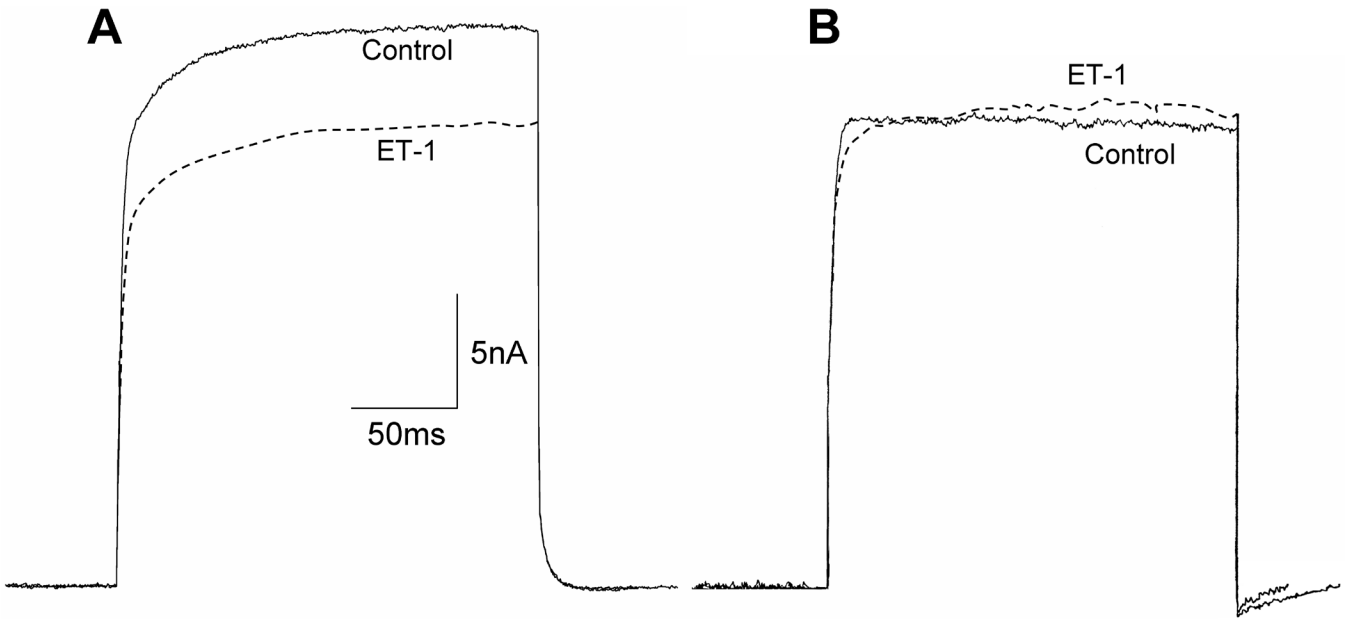

Figure 2.

Delayed rectifier K^+ currents in response to a depolarizing step (+10mV, 200 msec) in voltage clamped small diameter rat DRG neurons. Potassium currents measured in a Na^+-free bathing solution (see Methods) before (Control) and during exposure to 50 nM ET-1. **A**. Currents are reduced in an IB_4-positive cell after 7 min exposure to ET-1. **B**. Curents are unaffected by exposure to 50nM ET-1 in an IB_4 – negative neuron.

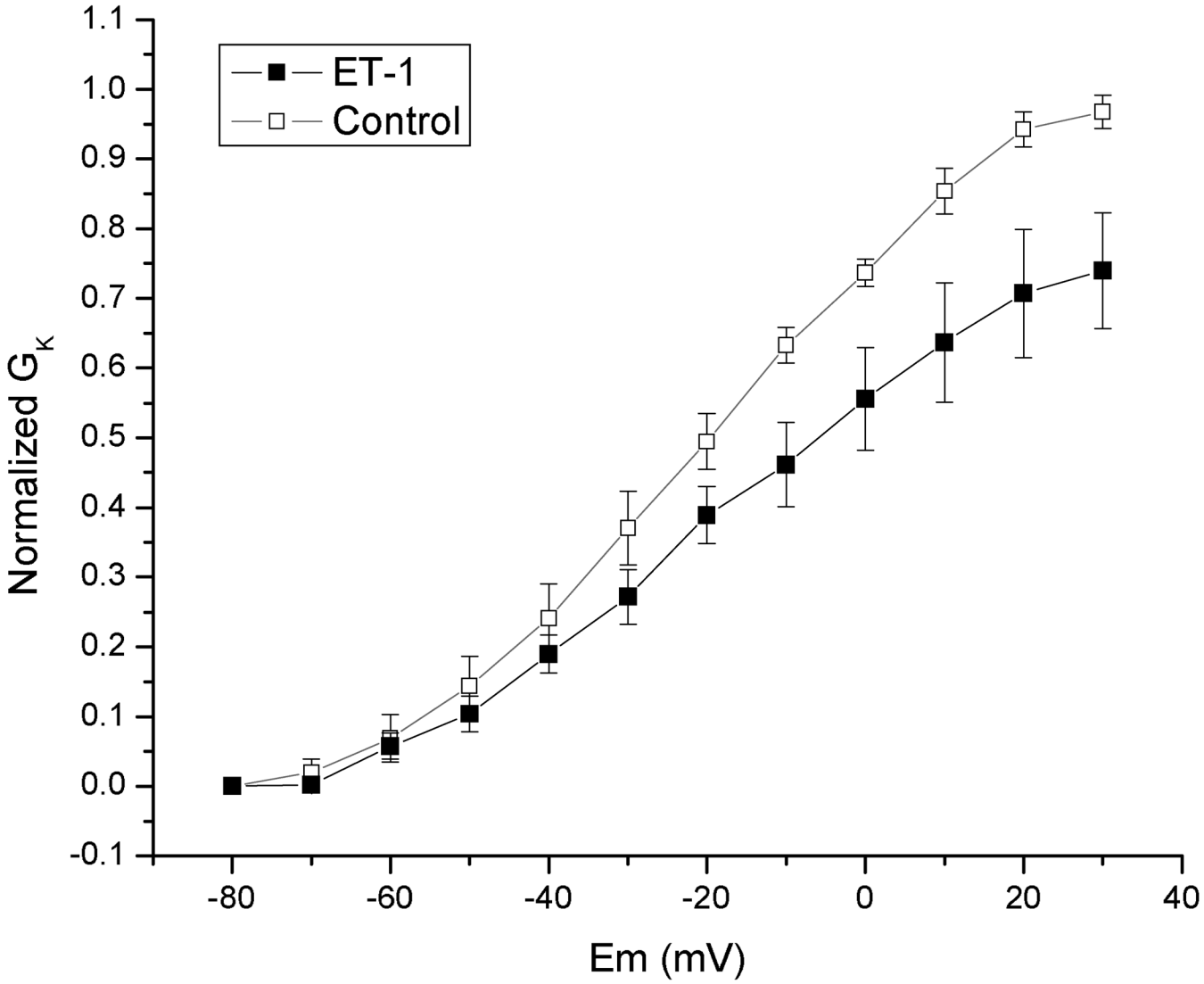

Figure 3.

Normalized K^+ conductance vs test potential calculated from I_K measured at the very end of a 200msec depolarization, in Control media and then at 7-10 min after exposure to 50nM ET-1. n=6 for each condition. Conductance was calculated using an Ohmic assumption and the G vs E data fit by a Boltzmann equation (see methods).

Table 1

Membrane parameters during ET-1 and in control conditions.

Parameter	Baseline	During ET-1	After ET-1	Ratio of during/before
RP [mV] (ET-1) (n=10)	-59.5 ± 3.4	-54.7 ± 3.31	-48.4 ± 6.9	0.92 ± 0.03
RP [mV] (C) (n=6)	-59.3 ± 4.3	-59.0 ± 4.4	-63.7 ± 5.8	0.99 ± 0.03
R_m [MΩ] (ET-1)	274 ± 46	282 ± 47	243 ± 50	1.03 ± 0.05
R_m [MΩ] (C)	240 ± 36	257 ± 55	272 ± 73	1.03 ± 0.10
#AP (ET-1)	3.0 ± 0.4*	5.9 ± 0.9*	5.0 ± 2.2	2.24 ± 0.41
#AP (C)	3.7 ± 0.4	4.0 ± 0.5	3.5 ± 1.1	1.13 ± 0.15
V_{th} AP_1 [mV] (ET-1)	-30.5 ± 3.4	-31.2 ± 2.8	-24.6 ± 2.3	Δ = -1.3 ± 3.3 mV**
V_{th} AP_1 [mV] (C)	-24.2 ± 6.1	-28.3 ± 3.7	-29.8 ± 5.4	Δ = -5.0 ± 8.4 mV
dV/dt_{rise} [V/s] AP_1 (ET-1)	54.8 ± 5.7	46.4 ± 5.00	35.4 ± 7.4	0.87 ± 0.08
dV/dt_{rise} [V/s] AP_1 (C)	47.2 ± 7.5	46.6 ±8.7	36.4 ± 5.1	1.01 ± 0.08
dV/dt_{fall} [V/s] (ET-1)	-21.8 ± 4.1	-20.4 ± 3.7	-15.4 ± 1.4	0.95 ± 0.03
dV/dt_{fall} [V/s] (C)	-22.4 ± 6.1	-21.2 ± 5.4	-20.3 ± 7.2	0.97 ± 0.03
dV/dt_{slow1} [V/s] (ET-1)	0.062 ± 0.019 *	0.084 ± 0.017 *	0.086±0.025	1.56 ± 0.24
dV/dt_{slow1} [V/s] (C)	0.058 ± 0.015	0.061 ± 0.023	0.057±0.020	1.06 ± 0.37
dV/dt_{slow2} [V/s] (ET-1)	0.100 ± 0.012 *	0.145 ± 0.012 *	0.148±0.015	1.52 ± 0.23
dV/dt_{slow2} [V/s] (C)	0.097 ± 0.016	0.088 ± 0.023	0.091±0.024	0.94 ± 0.19

* Significant difference between parameter during baseline period and after 10 min in ET-1 (50nM). ANOVA.

** Average of the difference between threshold voltage for the 1st AP before ET-1 and during ET-1 exposure.

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