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Redox Modulation of T-Type Calcium Channels in Rat Peripheral Nociceptors

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Summary

Although T-type calcium channels were first described in sensory neurons, their function in sensory processing remains unclear. In isolated rat sensory neurons, we show that redox agents modulate T currents but not other voltage- and ligand-gated channels thought to mediate pain sensitivity. Similarly, redox agents modulate currents through $Ca_v3.2$ recombinant channels. When injected into peripheral receptive fields, reducing agents, including the endogenous amino acid L-cysteine, induce thermal hyperalgesia. This hyperalgesia is blocked by the oxidizing agent 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB) and the T channel antagonist mibefradil. DTNB alone and in combination with mibefradil induces thermal analgesia. Likewise, L-cysteine induces mechanical DTNB-sensitive hyperalgesia in peripheral receptive fields. These data strongly suggest a role for T channels in peripheral nociception. Redox sites on T channels in peripheral nociceptors could be important targets for agents that modify pain perception.

Introduction

T-type (low-voltage activated [LVA]) Ca^{2+} channels were first described in peripheral sensory neurons of the dorsal root ganglion (DRG) (Carbone and Lux, 1984), where these channels are thought to play a crucial role in the control of cell excitability (White et al., 1989). Subsequently, T channels have been identified in a variety of excitable and nonexcitable cells (Huguenard, 1996; Ertel et al., 1997). Recent cloning of $\alpha 1$ subunits of T channels has revealed the existence of at least three subtypes, named G ($Ca_v3.1$; Perez-Reyes et al., 1998), H ($Ca_v3.2$; Cribbs et al., 1998), and I ($Ca_v3.3$; Lee et al., 1999), that are likely to contribute to the heterogeneity of T-type Ca^{2+} currents observed in native cells (Herrington and Lingle, 1992; Todorovic and Lingle, 1998). While subtypes of high-voltage activated (HVA) Ca^{2+} channels play important roles in synaptic transmission, the function of T channels remains less certain (Llinas, 1988; Huguenard, 1996). Because T channels act over a range of membrane potentials near the resting potential of most cells, these channels are thought to regulate cellular excitability under physiological and pathological conditions. Proposed roles for neuronal T channels include promotion of calcium-dependent burst firing, generation of low-amplitude intrinsic neuronal oscillations, elevation of calcium entry, and boosting of dendritic signals, possibly contributing to pacemaker activity, wakefulness, and seizure susceptibility (Huguenard, 1996; Ertel et al., 1997).

Although their unique biophysical properties make T currents relatively easy to study in vitro, pharmacological tools that allow manipulation of their function are limited. Unlike the family of HVA Ca^{2+} channels, no natural toxins or venom components have been identified that alter T channels selectively. Thus, despite their abundance in nociceptive neurons (Cardens et al., 1995), the function of T-type Ca^{2+} channels in sensory processing remains unknown. In the present study, we describe redox modulation of T-type Ca^{2+} currents in rat primary sensory neurons and directly examine the effects of redox agents on thermal and mechanical sensation following injection into the peripheral receptive fields of these neurons. We provide evidence that reducing agents, including dithiothreitol (DTT) and the endogenous amino acid L-cysteine, promote cutaneous thermal and mechanical hyperalgesia via effects on T channels. Conversely, the oxidizing agent 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB) and T-type calcium channel antagonist mibefradil produce analgesia to cutaneous thermal stimuli. The results demonstrate a novel role for peripheral T-type Ca^{2+} channels in boosting nociceptive signals. In addition, these results suggest a potential role for endogenous or exogenous redox agents in the modulation of pain sensation.

Results

In Vitro Studies

The DRG contains cell bodies of primary afferent (sensory) fibers that originate as pain endings in the periphery and terminate in the dorsal horn of the spinal cord. Whole-cell recordings from dissociated DRG neurons of adult rats are used to study peripheral nociceptive mechanisms because the small size of peripheral nerve endings precludes direct measurement of currents from sensory endings. We limited our experiments to small diameter (<27 μm) acutely dissociated neurons because the majority of these cells are involved in nociceptive processing (Cardens et al., 1995; Coderre et al., 1993; Levine et al., 1993; Snider and McMahon, 1998).

In an effort to identify endogenous modulators of excitability in sensory neurons, we initially examined whether redox agents alter voltage-gated currents that

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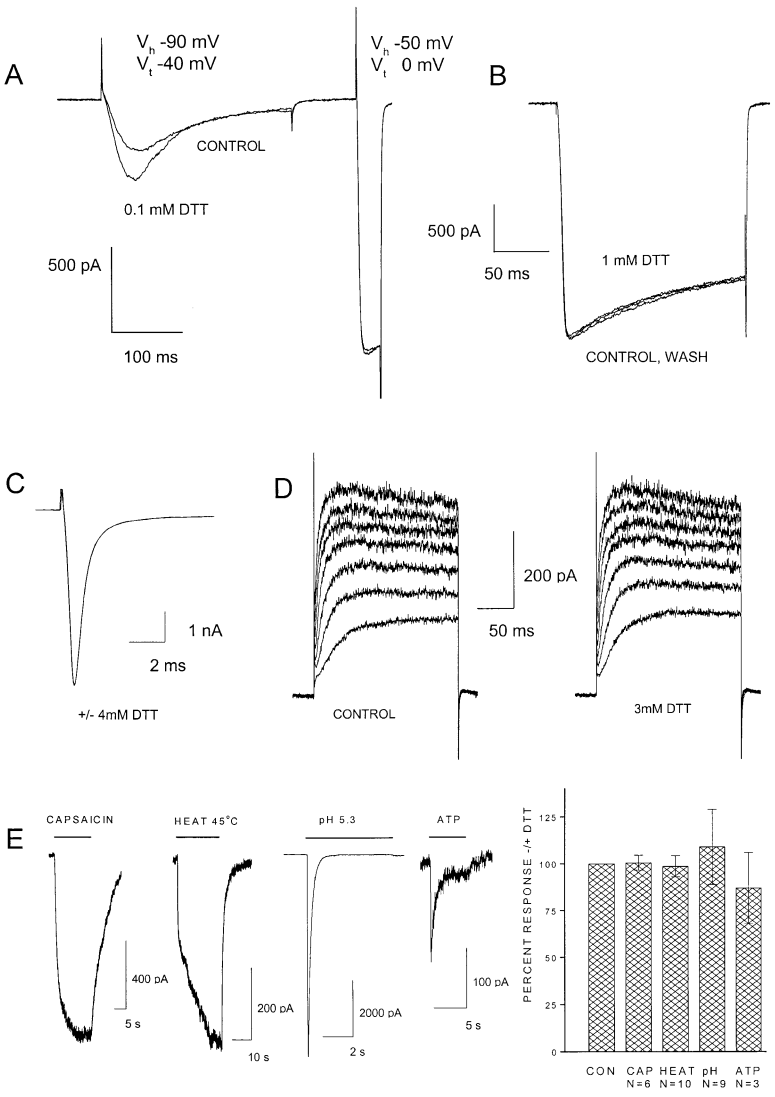

Figure 1. DTT Selectively Modulates T-Type Ca^{2+} Currents but Not Other Voltage- and Ligand-Gated Currents in Acutely Dissociated DRG Neurons

(A) The traces depict results from an experiment in which a double-pulse protocol was used to record T-type (LVA) and HVA Ca^{2+} currents in a DRG cell. Note that 0.1 mM DTT selectively increased the T current about 2-fold, while the HVA current was not affected. The amplitude of T currents was measured as the difference from the peak of the inward current to the current remaining at the end of a 200 ms test step to avoid contamination by a small residual HVA component. Note acceleration of current activation and inactivation kinetics during application of DTT.

(B) The trace shows the lack of effect of 1 mM DTT on isolated HVA Ca^{2+} currents (V_h, −60mV; V_t, 0mV). Bars indicate calibration.

(C) The raw traces show the lack of effect of 4 mM DTT upon voltage-gated Na^+ currents in another DRG cell. These currents were obtained by holding the neuron at −60mV and stepping to 0mV. Traces in the absence and presence of DTT are overlaid.

(D) These panels depict a family of outward potassium currents elicited from a holding potential of −60mV to test potentials of 0 to +60mV under control conditions (left) and in the presence of 3 mM DTT (right). Note the lack of effects on current kinetics and amplitudes.

(E) Small sensory neurons are sensitive to 1 μM capsaicin, heat, protons, and 100 μM ATP, which induce inward currents at −60mV. Representative control traces are shown for each current on the left; horizontal bars indicate times of application. Baseline pH was 7.4 in experiments with proton-gated currents. The bar graph on the right summarizes the lack of significant effect of DTT on the peak amplitude of these currents (vertical bars indicate ± SEM). For capsaicin-, proton-, and ATP-gated currents, 1–3 mM DTT was coapplied with second application of ligand. In the case of ATP, we waited for 1 min between applications to allow full recovery of responses (Cook and McCleskey, 1997). For heat-gated currents, we measured control responses first and then incubated approximately 1–2 mM DTT from 3 to 15 min in the bath before subsequent application of heat. Both direct application of DTT and bath incubation with DTT result in augmentation of T currents in our experiments.

regulate the excitability of nociceptive cells. We found that the reducing agent DTT (0.1 and 1 mM), at a test potential of −35mV, selectively augmented T-type Ca^{2+} currents by 128% ± 16% (n = 14) and 142% ± 18% (n = 11), respectively, while leaving HVA Ca^{2+} currents in the same cells unaffected (n = 5 for 0.1 mM, and n = 7 for 1 mM; Figures 1A and 1B). In all, DTT enhanced T currents in 34 of 39 cells (86%), and this effect was completely reversible within 2 min after removal of DTT. An apparent steady-state effect was observed with up to 1 min application of DTT (0.1–1 mM), and no change in maximal effect was observed with longer applications up to 5 min (n = 3; data not shown). The effect of DTT on T currents was accompanied by a change in current kinetics. Activation times, measured as the 10%–90% rise times of peak currents, were accelerated in the presence of DTT. At −35mV, the control 10%–90% rise time was 17.2 ± 2.5 ms, and in the presence of DTT it was 10.1 ± 1.4 ms (mean ± SEM, n = 5, $p < 0.05$). At −35mV, the inactivation time constant (τ) was 50 ± 3.7 ms in control and 25.5 ± 1.6 ms in the presence of DTT (n = 5, $p < 0.01$). We also examined whether increasing the temperature of in vitro experiments to 37°C (the normal in vivo temperature) altered responses to reducing agents, since higher temperatures are likely to increase the amplitude of T currents and accelerate activation and inactivation kinetics in the absence of redox modulation. At 37°C, 0.1 mM DTT increased peak T currents about 2-fold, similar to the effect observed at 22°C. However, changes in current kinetics with DTT were less apparent at higher temperatures (n = 3; data not shown).

We also examined the effects of DTT upon voltage-gated K^+ and Na^+ currents in small-size sensory neurons. Figure 1C illustrates the lack of effect of 4 mM DTT on inward Na^+ currents in these cells (n = 7), and Figure 1D shows the lack of effect of 3 mM DTT on a family of outward potassium currents (n = 5).

T channels are heterogeneously expressed in DRG

cells of different sizes but are uniformly present in small-diameter acutely dissociated cells of adult rat (Scroggs and Fox, 1992). Small DRG cells have electrical properties of nociceptors and sensitivity to capsaicin (Todorovic and Anderson, 1992; Cardens et al., 1995), features that identify these cells as chemical and thermal nociceptive sensory neurons (Snider and McMahon, 1998; Reichling and Levine, 2000; Caterina and Julius, 1999). Therefore, we tested the sensitivity of these cells to a variety of noxious stimuli, including capsaicin, heat, acid pH, and ATP. Capsaicin (1 μM) evoked inward currents in 9 of 12 small DRG cells (75%) that had T currents sensitive to DTT, indicating that our in vitro experiments include cells that participate in thermal and chemical nociception. However, up to 3 mM DTT had no effect on capsaicin-induced currents (1% $\pm$ 4% change, n = 6; Figure 1E). To test the sensitivity of heat channels to DTT, we applied heated external solution (45°C $\pm$ 2°C) to these cells. Nineteen small DRG cells were studied, and ten of them (53%) responded with inward currents, as depicted in Figure 1E. All DRG neurons responded to low pH (pH 5.3, n = 9), but only 3 of 15 cells (20%) responded to 100 μM ATP. Currents elicited by heat, low pH, and ATP were not significantly altered by 1–3 mM DTT (Figure 1E). These data strongly suggest that DTT selectively modulates T-type Ca^{2+} currents in capsaicin-sensitive sensory neurons but not other voltage-gated currents or channels thought to mediate heat or chemical sensitivity.

If reducing agents enhance currents through T-type Ca^{2+} channels, we would expect that oxidizing agents should decrease current flow through these channels. Indeed, application of 1 mM DTNB blocked peak T currents by about 50% (46% $\pm$ 2.7%, n = 9) in all tested cells (Figure 2B) and, when applied immediately after DTT, reversed the effects of DTT on current kinetics (n = 5; Figure 2A). In contrast to DTT, DTNB did not significantly affect the time course of activation or inactivation (at $-$35mV, the 10%–90% rise time was 17 $\pm$ 4.5 ms, and the inactivation τ was 50.7 $\pm$ 5.3 ms, n = 4, $p > 0.05$). The half-time for spontaneous recovery from DTNB was 26 $\pm$ 2.5 s (n = 5; Figure 2B). However, if 10 μM DTT was added immediately after DTNB, half recovery was achieved in only 13 $\pm$ 3 s ($p < 0.05$, n = 3). The inhibition of peak currents by DTNB was selective for T-type Ca^{2+} channels because applications of up to 3 mM DTNB, which completely blocked T currents (Figure 2B), did not alter HVA Ca^{2+} (n = 6), voltage-gated Na^{+} (n = 3), or K^{+} (n = 3) currents (data not shown).

The effects of DTT on T currents were mimicked by the endogenous reducing agent L-cysteine (Figure 2C). L-cysteine augmented T currents with a threshold of 10 μM and maximal effects at about 100 μM (130% $\pm$ 11% increase, n = 7). Similar to DTT, L-cysteine also increased rates of current activation and inactivation (Figure 2C). At maximal concentrations, L-cysteine and DTT consistently increased T currents by about 2-fold. A low concentration of DTNB (0.1 mM) had little effect on T currents when administered alone (3.3% $\pm$ 3.3% block) but completely reversed the effects of both L-cysteine and DTT when applied immediately after a reducing agent (Figure 2D). The half-time of spontaneous oxidation in four DRG cells after exposure to reducing agent was 76.6 $\pm$ 12 s, while, in the presence of 0.1 mM DTNB, the recovery half-time was only 30 $\pm$ 3 s ($p < 0.05$).

Effects of Redox Agents on Recombinant T-Type Ca^{2+} Channels

Molecular studies have shown that $Ca_v3.2$ mRNA is the most abundant isoform present in small DRG cells (Talley et al., 1999). This subtype of T channels also has the most similar pharmacological profile to the native DRG currents (Todorovic et al., 2000). Therefore, we examined the redox sensitivity of $Ca_v3.2$ channels expressed in HEK cells as a representative nociceptor T-type current. DTT (0.1 and 1 mM) increased peak T currents by an average of 2.5-fold (157% $\pm$ 50% increase for 0.1 mM DTT, n = 5; Figure 2E). Similar to DRG cells, the increased current was accompanied by acceleration of activation and inactivation kinetics (Figure 2E). The effects of DTT were mimicked by L-cysteine (n = 2). Also, similar to DRG cells, 1 mM DTNB blocked $Ca_v3.2$ currents by 50% $\pm$ 3.4% (n = 7; Figure 2E), without significant effect on current kinetics, while 3 mM DTNB completely blocked these currents in HEK cells (n = 6). On average, the control 10%–90% rise time at a V_t of $-$30mV was 8 $\pm$ 0.9 ms, and the control inactivation τ was 24.7 $\pm$ 2 ms. In the presence of DTT, rise time was 4.9 $\pm$ 0.7 ms ($p < 0.05$), and inactivation τ was 15.8 $\pm$ 2 ms (n = 9, $p < 0.05$). In the presence of 1 mM DTNB, these times were 7.7 $\pm$ 0.9 ms and 28 $\pm$ 5 ms (n = 6, $p > 0.05$), respectively. At lower concentrations, DTNB fully reversed effects of DTT on current kinetics (n = 3; data not shown), peak current amplitude, and time course of recovery (Figure 2F). The average half-time for the fully reduced $Ca_v3.2$ channel to spontaneously return to baseline levels was 83 $\pm$ 12 s in control conditions and 43 $\pm$ 3 s in the presence of 0.1 mM DTNB (n = 3, $p < 0.05$). These results suggest that $Ca_v3.2$ T-type channels, the predominant isoform in sensory neurons (Talley et al., 1999), have sensitivity to redox agents similar to native DRG cells.

In Vivo Studies

Because redox agents modulate T currents in isolated neurons that participate in thermal nociception, we examined whether these agents modify in vivo responses to radiant thermal (heat) stimulation (Jevtovic-Todorovic et al., 1998; Hargreaves et al., 1988). In these studies, 100 μl of test compounds were injected directly into peripheral receptive fields of sensory neurons in the hindpaw of adult rats, and the latency to paw withdrawal in the presence of a radiant heat stimulus was measured. Injection of the reducing agent L-cysteine produced a dose-dependent exaggerated thermal response (Figure 3B). L-cysteine (12 μg/100 μl) reversibly decreased paw withdrawal latencies (PWLs) by about 40% 10–20 min ($p < 0.005$) following injection. Injection of the same volume of vehicle (saline) had no effect on PWLs (Figure 3A). Similar but less potent hyperalgesic responses were observed following injection of DTT. Compared with contralateral, noninjected paws in the same animals, 150 μg/100 μl DTT decreased PWLs by >25% 10–20 min after injection ($p < 0.05$; Figure 3C). These changes in thermal PWLs are of similar magnitude to effects observed in animals with neuropathic pain following in-

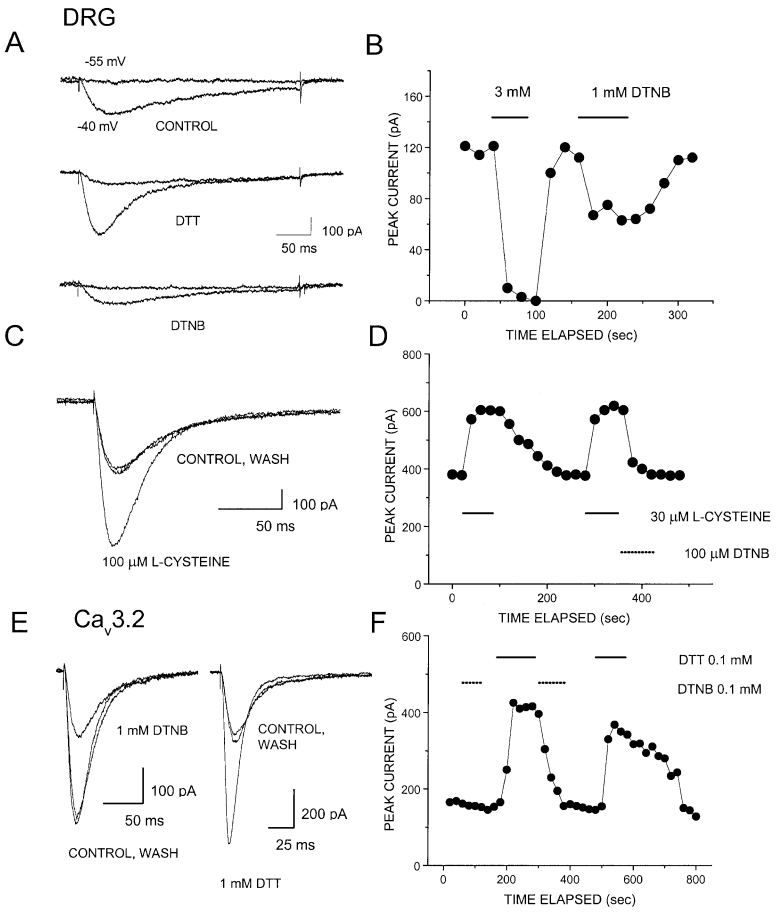

Figure 2. Characterization of Redox Modulation of T-Type Ca^{2+} Currents in Rat Sensory Neurons and $Ca_v3.2$ Currents in HEK Cells

(A) Raw traces from an experiment showing that DTNB blocks T currents in rat DRG cells without affecting current kinetics but reverses DTT-induced kinetic changes of T currents. Currents at V_t of −55mV and −40mV are depicted here (V_h, −90mV). The 10%–90% rise time at −40mV for control was 12.8 ms, 9.6 ms in the presence of 0.1 mM DTT, and 13.6 ms in the presence of 1 mM DTNB, which was applied immediately after DTT. Inactivation τ at −40mV for control was 53.5 ms; for DTT, it was 35.3 ms; and for DTNB, it was 50 ms.

(B) DTNB (1 mM and 3 mM) induced a fully reversible blockade of T currents in DRG cells. Drug applications are denoted by the horizontal bars.

(C) L-cysteine mimicked the effects of DTT in sensory neurons. The traces show results from an experiment in which T currents were evoked from a V_h of −90mV to V_t −35mV. L-cysteine (100 μM) increased the peak T current about 2-fold and increased the speed of current activation and inactivation, as evidenced by crossover of traces prior, during, and after application of L-cysteine. Bars indicate calibration.

(D) This graph displays results from experiments in which 0.1 mM DTNB increased the rate of recovery from the effects of L-cysteine on T currents. Bars indicate times of drug applications.

(E) DTNB (1 mM) blocked about 60% of the peak current in HEK cells expressing $Ca_v3.2$, without obvious changes in current kinetics. DTT (1 mM) in another HEK cell increased the peak $Ca_v3.2$ current 2.5-fold and accelerated both activation and inactivation of current. Rise time (10%–90%) was 9.25 and 6 ms; inactivation τ was 20.5 and 11.7 ms before and during application of DTT, respectively.

(F) The graph shows the time course from another experiment in $Ca_v3.2$-transfected HEK cell, illustrating that DTNB accelerates recovery from the fully reduced state (half-time for recovery was 40 versus 90 s with and without DTNB, respectively).

jury to sensory nerves (Jevtovic-Todorovic et al., 1998). L-cysteine and DTT injections did not cause animals to lick or flinch their paws, suggesting that the reducing agents do not directly produce pain but rather augment transmission of thermal nociceptive signals from peripheral nerve endings.

It has been shown previously that intrathecal administration of redox agents can influence pain perception, putatively by interaction with spinal NMDA receptors (NMDAR) (Laughlin et al., 1998). To explore the potential role of NMDAR in cutaneous thermal hyperalgesia, we injected 25 μg/100 μl of ketamine, a noncompetitive NMDAR antagonist, into hindpaws and found that ketamine neither altered thermal PWLs (n = 8) nor affected the thermal hyperalgesia induced by DTT (n = 8; data not shown). Also, intraplantar injections of a competitive NMDAR antagonist, D-APV (4 μg/100 μl), did not affect baseline thermal nociception (n = 8) or L-cysteine-induced thermal hyperalgesia (n = 6; data not shown). In prior studies, ketamine (2.5 μg/100 μl) and AP-7 (4 μg/100 μl) injected locally attenuated the development of thermal hyperalgesia and allodynia associated with subacute joint inflammation (Lawand et al., 1997). Similarly, another even more potent and selective noncompetitive NMDA antagonist, MK-801 (0.35 μg/100 μl), did not affect baseline thermal PWLs (n = 8) or the hyperalgesic response to L-cysteine when injected into hindpaws (Figure 3D; n = 8). At this dose, MK-801 has been reported to block hyperalgesia to peripherally applied glutamate (Zhou et al., 1996).

If the cutaneous thermal hyperalgesia observed following DTT and L-cysteine results from the reducing properties of these agents, then oxidizing agents should antagonize the hyperalgesia. Indeed, coinjection of 4 μg/100 μl DTNB completely reversed the effects of L-cysteine (Figure 4C) and DTT (Figure 4D), while having no effect on thermal PWLs alone (Figure 4B). Moreover, a higher dose of DTNB (40 μg/100 μl) prolonged PWLs, indicating an analgesic effect (Figure 4B). At 10 min following injection, DTNB increased the latency time by 4.3 s ($p < 0.0005$, n = 12). This analgesic effect was completely reversed by 1.5 μg/100 μl DTT, which had no effect on its own (n = 6; data not shown). Dimethyl sulfoxide (DMSO) (1%), the vehicle used to dissolve DTNB, had no effect on PWLs (Figure 4A) and did not interfere with the effects of the reducing agents (n = 6; data not shown).

Reducing Agents Modulate Mechanical Nociception

The majority of small-size sensory neurons are polymodal nociceptors that respond to a variety of mechanical, thermal, and chemical stimuli. If T-type channels serve

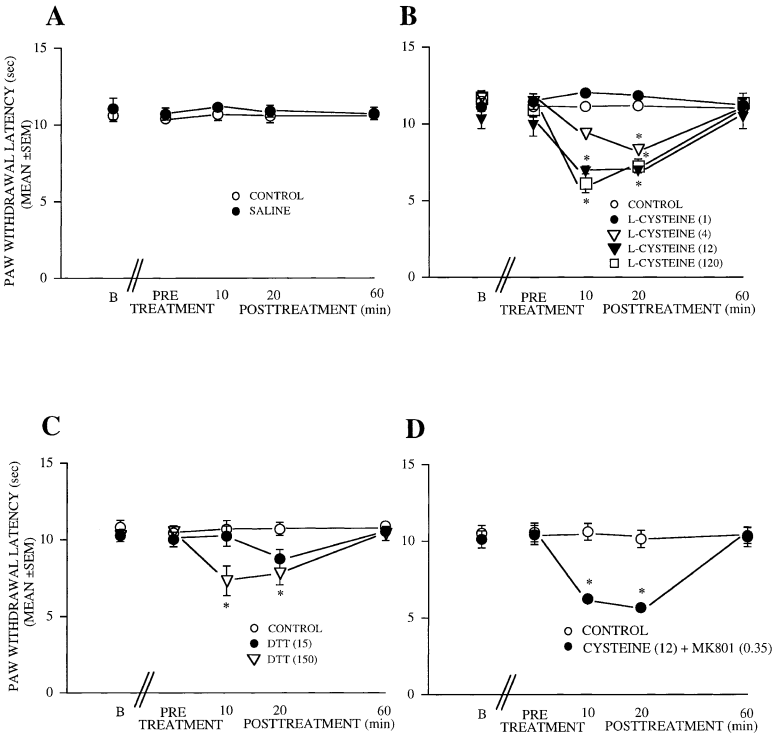

Figure 3. Reducing Agents Induce Thermal Hyperalgesia in Adult Rats

(A) Injections of saline, the vehicle for DTT and L-cysteine, had no effect on PWLs. "B" indicates baseline PWL obtained 2 days before study, and "pretreatment" indicates value just prior to test injection.

(B) L-cysteine induced a dose-dependent decrease of thermal PWLs. L-cysteine (4, [open triangles], 12 [filled triangles], and 120 [open squares] μg/100 μl) significantly decreased PWLs (*, $p < 0.005$) 10 and 20 min postinjection when compared with noninjected paws. PWLs return to control values by 60 min following injection.

(C) DTT, at 15 and 150 μg/100 μl, also produced a dose-dependent decrease of PWLs; *, $p < 0.05$, injected versus noninjected paw. Note that the effects of L-cysteine and DTT are fully reversible.

(D) MK-801 (0.35 μg/100 μl) failed to block the hyperalgesia induced by 12 μg/100 μl of L-cysteine. *, $p < 0.005$, injected versus noninjected paw.

as general amplifiers of peripheral nociception, these channels would be expected to be involved in pain sensation generated through other modalities. We therefore tested the possibility that mechanical sensation could also be modulated by redox agents injected in peripheral receptive fields of sensory neurons. We first determined the size of von Frey filament (4.93), which, when applied to the ventral side of the hindpaw, causes nociceptive behavior (measured as the number of paw withdrawals per ten trials). These withdrawal responses were compared in injected and noninjected paws before (baseline and 0 min), 10, and 60 min following injection of 100 μl of redox agents in hindpaws. Figure 5A indicates that injections of saline or 1% DMSO did not cause any

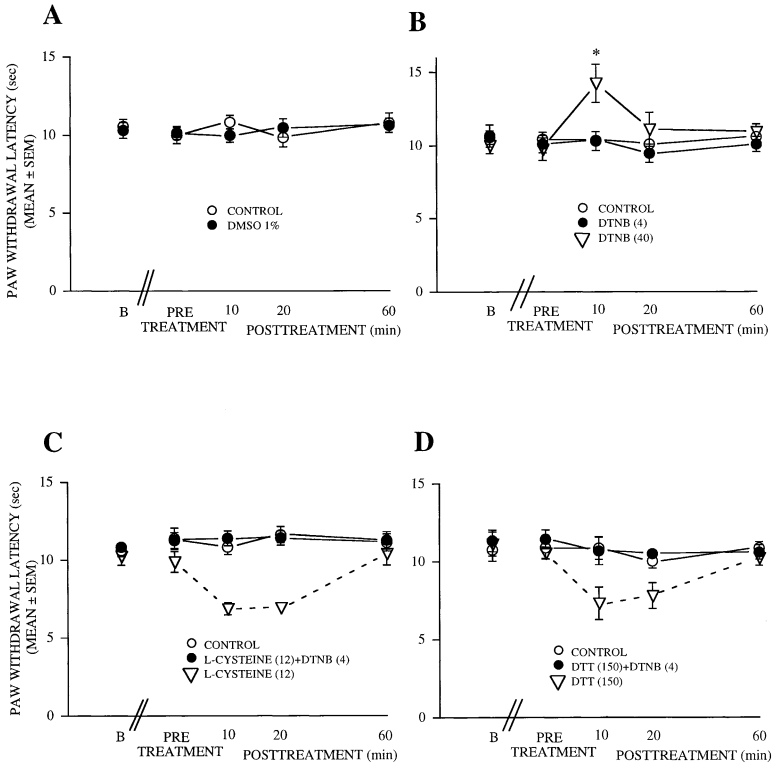

Figure 4. DTNB Induces Analgesia in Thermal PWL Testing and Blocks Hyperalgesia Induced by Reducing Agents

(A) DMSO (1%), the vehicle used for DTNB, has no effect on PWLs when administered alone. All points are averages of at least eight animals, and vertical bars indicate ± SEM.

(B) The oxidizing agent DTNB induced a brief dose-dependent analgesia. *, $p < 0.0005$, injected versus noninjected paws, with full recovery after 20 min from injection.

(C) At 4 μg/100 μl, DTNB had no effect on PWL alone. However, this dose completely reversed L-cysteine-induced thermal hyperalgesia.

(D) Similarly, 4 μg/100 μl DTNB also blocked the effects of DTT on PWLs. There was no statistical difference between injected and noninjected paws in (C) or (D). Dotted lines in (C) and (D) are taken from Figures 3B and 3C, respectively.

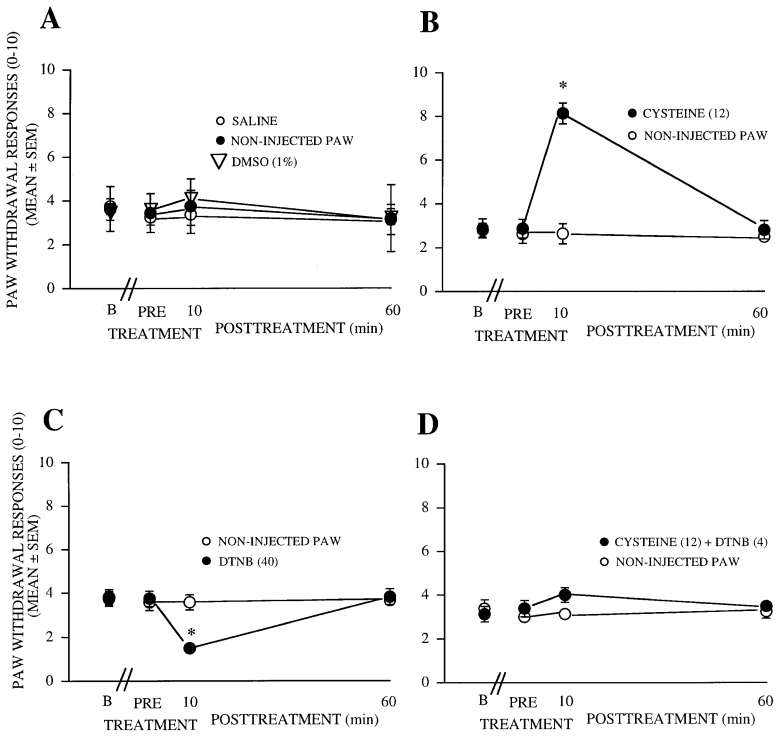

Figure 5. Redox Agents Modulate Peripheral Mechanical Nociception

(A) Injections of saline or 1% DMSO did not change paw withdrawal responses in animals tested with a 4.93 von Frey filament (n = 8 for each condition; control [noninjected] sides are grouped together).

(B) L-cysteine (12 μg/100 μL) induced an increase in the withdrawal responses (injected versus noninjected side) 10 min after injection. Note a full return of the response to baseline at 60 min. *, $p < 0.0001$; n = 8 animals.

(C) When DTNB (40 μg/100 μl) was injected in peripheral receptive fields, fewer paw withdrawals were measured to filament stimulation 10 min after injection, indicating an analgesic response. *, $p < 0.01$; n = 12 animals.

(D) When a lower dose of DTNB (4 μg/100 μL) was coinjected with L-cysteine (12 μg/100 μl), the hyperalgesic response to mechanical stimulation typically observed with L-cysteine was completely blocked.

change in paw withdrawal responses. However, injection of 12 μg/100 μl L-cysteine in saline caused a reversible hyperalgesic response (Figure 5B), increasing the withdrawal responses by 2.6-fold after 10 min ($p < 0.0001$, n = 8 animals). When 40 μg/100 μl of DTNB was injected (Figure 5C), withdrawal responses after 10 min indicated an analgesic effect (60% less than control, $p < 0.01$, n = 12 animals). A lower dose of DTNB (4 μg/100 μl) had little effect on its own but completely reversed the hyperalgesic effect of L-cysteine (Figure 5D; n = 8, $p > 0.05$). Similarly, a lower dose of L-cysteine completely reversed the analgesic effects of DTNB (n = 8; data not shown). These data indicate that redox agents modulate peripheral mechanical nociception as well as thermal nociception.

Mibefradil Overcomes the Effects of Reducing Agents Both In Vivo and In Vitro

The results described above suggest that redox agents modulate peripheral thermal nociception via effects on T-type Ca^{2+} channels in primary sensory neurons. To test this further, we used mibefradil, a peripherally acting antihypertensive drug that has been shown to block T-type Ca^{2+} currents preferentially over HVA currents in vascular smooth muscle (Ertel and Clozel, 1997, Ertel et al., 1997) and cerebellar Purkinje cells (McDonough and Bean, 1998). In DRG neurons, we previously found that mibefradil is one of the most potent blockers of T currents yet described, with an IC_{50} of 3 μM (Todorovic and Lingle, 1998). In vivo, injection of 6 μg/100 μl mibefradil into rat hindpaws had no significant effect on thermal PWLs (n = 12 animals; Figure 6A). We were unable to examine doses of mibefradil above 6 μg/100 μl because mibefradil injections caused hyperemia and increases in skin temperature, presumably due to local vasodilating effects at higher doses. However, coinjection of 6 μg/100 μl mibefradil into hindpaws with either L-cysteine or DTT completely blocked the thermal hyperalgesia induced by the reducing agents (Figures 6B and 6C; n = 8 for L-cysteine, and n = 14 animals for DTT).

These results are consistent with the idea that the hyperalgesic responses to reducing agents result from modulation of T channels in nociceptors. However, we were surprised that mibefradil alone had no effect on thermal PWLs yet blocked the effects of L-cysteine and DTT, given that DTNB had analgesic effects at higher doses and also inhibited DRG T currents. This led us to examine interactions between mibefradil and redox agents in vitro. Figure 7A shows data from an experiment in which 100 μM L-cysteine enhanced DRG T currents about 2-fold, and 1 μM mibefradil abolished the cysteine-enhanced portion of the current (55% block of the total T current). Also, 0.3 μM mibefradil alone did not block baseline T currents but, when coapplied with L-cysteine, blocked about 66% of L-cysteine-enhanced and 28% of total T current. This suggests that reduced T channels may have increased affinity for mibefradil. Indeed, we found that L-cysteine shifted mibefradil's IC_{50} for block of T currents from 3 μM (Todorovic and Lingle, 1998) to 0.7 μM (Figure 7C).

We also examined the effect of DTNB on the T channel blocking actions of mibefradil. Figure 7B shows an experiment in which a subthreshold concentration of DTNB (0.2 mM) enhanced the block of T currents by 1 μM mibefradil by about 2-fold. On average, 1 μM mibefradil alone blocked 23.7% ± 1.5%, while the combination of 1 μM mibefradil and 0.2 mM DTNB blocked 49.2% ± 1.1% of the current ($p < 0.001$, n = 4). Consistent with this, 0.2 mM DTNB shifted the mibefradil IC_{50} to 1.1 μM (Figure 7C).

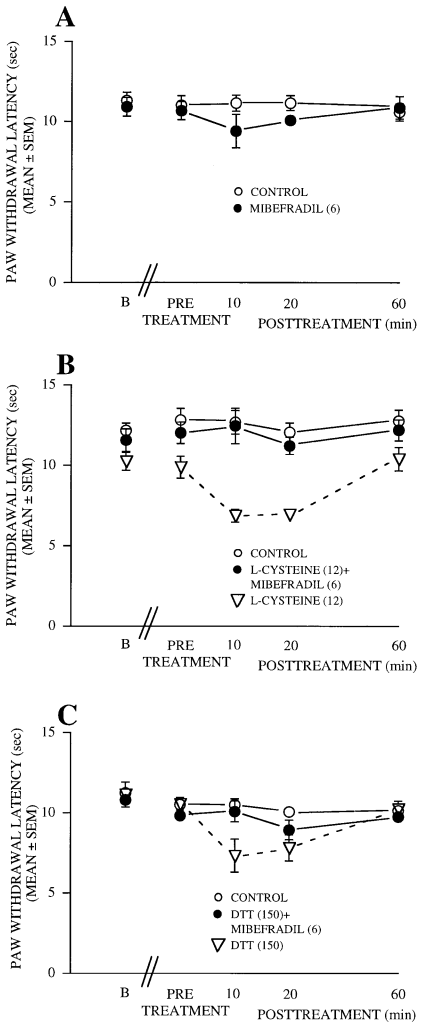

Figure 6. Mibefradil Blocks the Effects of Reducing Agents In Vivo

(A) Mibefradil, at a dose of 6 μg/100 μl (filled circles), had a small but nonsignificant effect on thermal PWLs under control conditions (open circles) (n = 12 animals).

(B) Hyperalgesic responses to 12 μg/100 μl L-cysteine (dashed trace, see Figure 3B) were completely blocked when 6 μg/100 μl mibefradil was coinjected with L-cysteine.

(C) In another experiment in vivo, 6 μg/100 μl mibefradil abolished the hyperalgesic response to 150 μg/100 μl DTT (dashed trace, see Figure 3C). No statistically significant differences were observed between injected paws (solid circles) and noninjected paws (n = 14 animals).

In vivo, we found that a combination of ineffective doses of DTNB and mibefradil induced analgesia, as indicated by a 2.5 s increase in thermal PWLs, measured 10 min after injection (Figure 7D; $n = 12$, $p < 0.001$). This analgesic effect of DTNB plus mibefradil in vivo mirrors the synergistic blocking effect of the two agents on T currents in vitro.

Discussion

Mechanisms of T-Type Ca^{2+} Current Modulation by Redox Agents

We provide evidence that the extracellular reducing agents DTT and L-cysteine augment T currents in rat sensory neurons. The oxidizing agent DTNB, which is also relatively membrane impermeant (Aizenman et al., 1989; Tang and Aizenman, 1993), blocks T currents on its own at higher concentrations and, at lower concentrations, reverses the effects and speeds recovery from the effects of the reducing agents. DTT and L-cysteine produce prominent changes in T current kinetics, as evidenced by acceleration of both activation and inactivation. In contrast, DTNB had little effect on current kinetics. This suggests the existence of putative extracellular redox sites that modulate T channels in rat sensory neurons. Furthermore, spontaneous recovery from the oxidizing effect of DTNB (which results in the formation of relatively stable disulfide bonds or thiobenzoate-proteins) implies the existence of an unknown endogenous reducing factor that has access to peripheral nociceptive pathways.

Since DTT, DTNB, and L-cysteine are all highly selective redox agents for thiol groups on proteins, the redox sites in rat sensory neurons could involve any of the many L-cysteine residues present in putative extracellular regions of T channels (Perez-Reyes et al., 1998; Cribbs et al., 1998). $\alpha 2\delta$ accessory subunits of voltage-gated Ca^{2+} channels also contain extracellular disulfide bonds (De Waard et al., 1996) that could be targets for redox agents. Studies with cloned subunits indicate that $\alpha 2\delta$-1 can increase the expression of $Ca_v3.1$ ($\alpha 1G$) immunoreactivity at the plasma membrane and increase $Ca_v3.1$-mediated currents without affecting biophysical properties of the currents in COS cells and *Xenopus* oocytes (Dolphin et al., 1999). However, no accessory subunits have been documented to coexpress functionally with T channels in DRG cells. Also we have found that currents mediated by $Ca_v3.2$, the most abundant T channel isoform in DRG cells (Talley et al., 1999), are modulated by redox agents in HEK-293 cells in the absence of added accessory subunits. Although these data strongly suggest the existence of redox sites directly on T channels, further biophysical and molecular studies will be necessary to determine the exact nature of these sites.

Selective Redox Modulation of T Currents in Rat Sensory Neurons

We tested the selectivity of the reducing agents by examining other DRG voltage-gated (HVA, Na^+, K^+) currents and found that only T currents were affected. Thus, it seems unlikely that the in vivo effects of the reducing agents result from effects on other voltage-gated conductances. Modulation of other currents by redox agents has been reported. For example, redox agents modulate L-type Ca^{2+} currents in ferret ventricular myocytes but in the opposite direction from T currents in DRG cells with DTT blocking and DTNB enhancing currents (Campbell et al., 1996). Redox agents are also known to modulate NMDAR (Aizenman et al., 1989; Tang and Aizenman, 1993). However, in our in vivo experiments, the hyperalgesic effects of DTT and L-cysteine were not blocked by the NMDAR antagonists ketamine, MK-801, or D-APV but were blocked by the T-type Ca^{2+} channel antagonist mibefradil. Additionally, D-APV, ketamine, and MK-801 injected into peripheral receptive fields alone in our study had no effect on baseline ther-

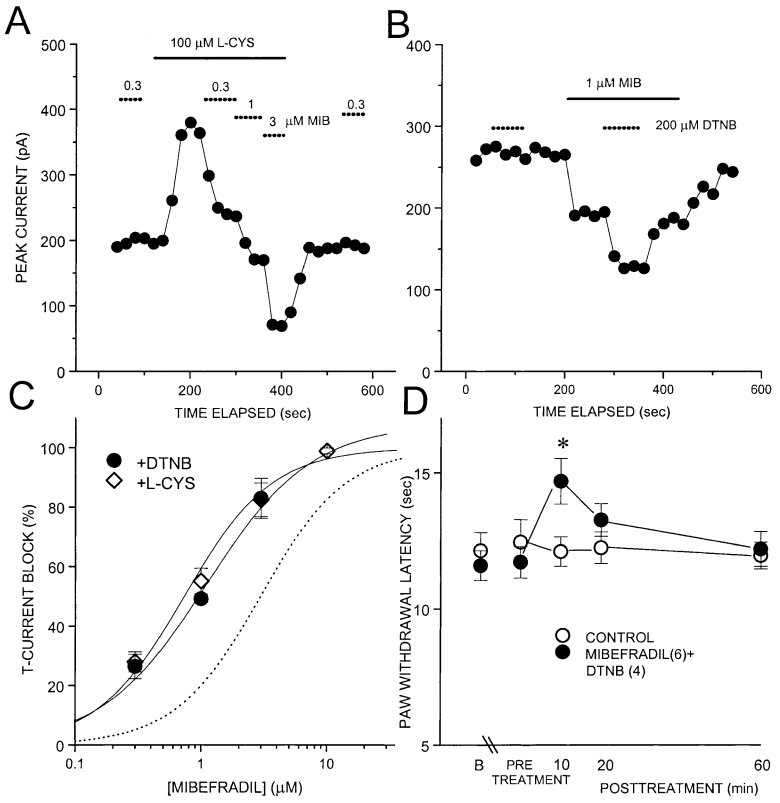

Figure 7. Redox Agents Augment the Effects of Mibefradil In Vitro and In Vivo

(A) The graph shows the time course of mibefradil's blocking effect on L-cysteine-modulated current from a DRG cell. The horizontal solid bar indicates the time of L-cysteine application; dotted bars indicate the times of mibefradil applications. L-cysteine (100 μM) increased the peak T current by about 2-fold. Mibefradil (1 μM) completely blocked the L-cysteine-potentiated current, and 3 μM mibefradil blocked the total T current by >80%. Note that 0.3 μM mibefradil applied before and after L-cysteine did not produce any inhibition of peak T current. However, when coapplied with L-cysteine, 0.3 μM mibefradil blocked about one third of the total current.

(B) DTNB potentiates the blocking effect of mibefradil. The graph shows the time course of an experiment in which DTNB alone (dotted horizontal bar) had little effect on the baseline T current. Mibefradil (1 μM) (solid horizontal bar) blocked about 23% of the current. When DTNB (dotted bar) was coapplied with mibefradil, the magnitude of block increased to about 50%.

(C) DTNB induces a 3-fold shift and L-cysteine induces a 4-fold shift to the left of the mibefradil concentration-response curve in acutely dissociated DRG neurons. The dotted line is a concentration-response curve to mibefradil, obtained with identical recording conditions in these cells with an IC_{50} of 3 μM and Hill n of 1.28 (Todorovic and Lingle, 1998). All points are averages of at least four different cells. Solid lines represent best fits of the Hill equation, giving an IC_{50} of 1.1 ± 0.14 μM and n of 1.04 ± 0.2 in the presence of 200 μM DTNB. Mibefradil had an IC_{50} of 0.7 ± 0.07 μM and n of 1.24 ± 0.14 for T current blockade in the presence of 100 μM L-cysteine.

(D) Mibefradil (6 μg/100 μl), in combination with an ineffective dose of DTNB (4 μg/100 μl; filled circles), produced an analgesic effect. Thermal PWLs were significantly prolonged 10 min after injection from 12.1 ± 0.5 s to 14.7 ± 0.8 s (n = 12 animals, $p < 0.005$).

mal PWLs. Although NMDAR have been suggested to mediate some peripheral nociceptive processes (Lawand et al., 1997; Jackson et al., 1995; Zhou et al., 1996), our results are consistent with studies suggesting that NMDA receptors do not contribute to nociception at peripheral nerve endings under physiological conditions. These receptors may contribute to pathological pain states associated with peripheral tissue inflammation (Lawand et al., 1997; Jackson et al., 1995).

We find that redox agents modulate both thermal and mechanical peripheral nociception and that DTT is ineffective in modulating capsaicin-gated and heat-gated currents in sensory neurons even at several-fold higher concentrations than affecting T currents. This suggests that reducing agents do not augment thermal nociception via effects on the family of heat-sensitive ion channels in sensory neurons (Caterina and Julius, 1999; Reichling and Levine, 2000).

It is possible that in some pathological conditions there is cross-talk between redox modulation of T channels and channels that are thought to be peripheral nociceptive transducers. However, we found no effect of reducing agents on currents elicited by heat, acidic solutions, or ATP. Our results suggest that tissue conditions that favor a more reducing extracellular environment enhance T channel function and boost signals generated from putative nociceptive endings to the spinal cord. Thus, T channels may contribute to neuronal plasticity and responses to noxious peripheral stimuli. A similar role for T channels in augmenting subthreshold dendritic signals has been described in CNS neurons (Magee and Johnston, 1995; Markram and Sakmann, 1994).

Putative Endogenous Redox Modulators in Rat Sensory Neurons

The present study demonstrates that the endogenous amino acid L-cysteine acts as a potent redox modulator of T channels in DRG neurons. In the plasma of healthy human subjects, free L-cysteine concentrations are reported to be as high as 140 μM (Suliman et al., 1997). Because enhancement of T currents in sensory neurons occurs with L-cysteine concentrations in the range 10–100 μM, it appears that L-cysteine could be an important endogenous modulator of T currents. Although the concentrations of amino acids in the extracellular milieu of nociceptive endings is unknown, it is possible that inflammation, burns, tissue hematoma, or trauma result in plasma extravasation and local accumulation of L-cysteine and other thiol-containing amino acids in proximity to putative nociceptive endings. We show here that direct injection of L-cysteine into peripheral receptive fields induces hyperalgesia to thermal and mechanical stimuli. It is possible that other thiol- containing compounds like L-homocysteine or glutathione could also be endogenous redox modulators of T channels

and that these agents could work in concert to upregulate T channel function.

The Effects of Mibefradil on Redox Modulation of T-Type Ca^{2+} Channels

In the present study, we show that concentrations of mibefradil thought to be selective for T currents completely block L-cysteine and DTT-enhanced T currents. The present results also demonstrate that, among the calcium channels in sensory neurons, T channels are selectively and reversibly augmented by reducing agents and that reducing agents produce hyperalgesia in response to thermal stimulation. Because the effects both in vivo and in vitro are reversed by mibefradil, we postulate that T currents in peripheral nerve terminals modulate thermal nociceptive transmission. Mibefradil alone blocked T currents in isolated neurons but did not alter basal pain sensitivity. However, when animals were rendered hyperalgesic by application of reducing agents, mibefradil blocked the effect. This suggests that T current blockers may be useful analgesic agents under conditions in which T channel function is upregulated.

When low concentrations of mibefradil are combined with subthreshold concentrations of the oxidizing agent DTNB, potentiation of block is observed in vitro, and analgesic effects are produced in vivo. This suggests that some degree of T channel function is also necessary for baseline transmission of thermal nociceptive signals.

T-Type Ca^{2+} Channels May Be Targets for Novel Analgesic Agents

Neurotransmitters and neuromodulators released in response to noxious stimulation are known to alter the influx of Ca^{2+} through ligand- and voltage-gated channels in nociceptors (Coderre et al., 1993; Levine et al., 1993). In turn, Ca^{2+} influences the excitability of sensory neurons and triggers release of vesicles from neuronal synaptic terminals (Miller, 1998). This may be particularly important in persistent pain models in which central sensitization and synaptic plasticity seem to be contributing mechanisms (Coderre et al., 1993; Levine et al., 1993). Our results indicate that modulation of peripheral T-type Ca^{2+} channels influences thermal and mechanical nociceptive inputs. Since many pathological pain states are associated with exaggerated thermal and mechanical nociception (Coderre et al., 1993; Reichling and Levine, 2000), peripherally applied T channel antagonists and/or oxidizing agents could represent new classes of drugs to treat these forms of chronic pain.

Experimental Procedures

Electrophysiological Methods

Acutely Dissociated DRG Neurons

Dissociated DRG cells were prepared from adult rats (100–420 gm) and used within 4 hr for whole-cell recordings as described elsewhere (Todorovic and Lingle, 1998; Todorovic et al., 2000). In brief, eight to ten DRGs from thoracic and lumbar regions were dissected and incubated at 36°C for 60–100 min in Tyrode's solution containing (in mM) NaCl, 140; KCl, 4; $MgCl_2$, 2; glucose, 10; and N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES), 10; adjusted to pH 7.4 with NaOH. This solution was supplemented with 5 mg/ml collagenase (Sigma type I) and 5 mg/ml dispase II (Boehringer-Manheim). The duration of enzymatic treatment did not alter the effects of redox agents on T-type calcium currents. Single neuronal somata were obtained by trituration in Tyrode's solution at room temperature. For recordings, cells were plated onto glass coverslip and placed in a culture dish that was perfused with external solution. All data were obtained from small-diameter neurons (21–27 μm) without visible processes.

Transfected Cells

HEK cells were stably transfected with human $Ca_v3.2$ constructs (cell lines AH-13 or Q31) as described previously (Lee et al., 1999; Todorovic et al., 2000). Cells were typically used 1–3 days after plating. Because these cells are routinely grown with 10% fetal bovine serum (FBS), which may contain endogenous redox agents, we routinely incubated these cells in our external solution for at least 30 min at room temperature before experiments.

Electrophysiological Recordings

Recordings were made with standard whole-cell voltage-clamp techniques (Hamill et al., 1981). Electrodes were fabricated from microcapillary tubes and fire polished to resistances of 1–4 MΩ. Voltage commands and digitization of membrane currents were done with Clampex 6.0 of the pClamp software package (Axon Instruments, Foster City, CA) running on an IBM-compatible computer. Neurons were typically held at −90mV and depolarized to −35mV every 20 s to evoke inward T currents. Membrane currents were recorded with an EPC 7 patch-clamp amplifier (List Instruments). All T currents are measured as peak response relative to the end of the depolarizing pulse to avoid small contaminating HVA currents that could be present in some cells. Data were analyzed using Clampfit (Axon Instruments) and Origin 4.0. Currents were filtered at 5 kHz. Reported series resistance values were taken directly from the reading of the amplifier. Average uncompensated series resistance for DRG cells was 7.9 ± 4.5 MΩ (mean ± SD), and the average capacitance was 14 ± 5 pF (mean ± SD, n = 107 neurons). For HEK cells, average capacitance (C_m) was 19.5 ± 6 pF and average series resistance (R_s) was 4.5 ± 2 MΩ (n = 14). Series resistance typically was compensated 50%–70% during experiments. In most experiments, a P/5 protocol was used for online leakage subtractions.

Solutions

A glass syringe served as a reservoir for a gravity-driven local perfusion system that consisted of multiple, independently controlled glass capillary tubes. Switching between solutions was accomplished by manually controlled valves, except for applications of capsaicin, heat, acid, and ATP, when computer-driven electronic valves designed for fast drug applications were used. Most experiments were done at room temperature (20°C–24°C), except when the effect of higher temperature on redox modulation of T currents and heat-gated currents was studied. In this case, extracellular solution was heated in the syringe and perfusion tube by a thermoresistive device. The temperature in the bath was measured with a microprobe positioned close to the recorded cell. All drugs were prepared as stock solutions (ATP, MK-801, and ketamine as 10 mM; L-cysteine, DTT, and D-APV as 100 mM stocks in water) and freshly diluted to appropriate concentrations at the time of the experiment. Final L-cysteine dilutions were done at the time of the experiment and used within 1 hr because of instability resulting from spontaneous oxidation in the presence of trace metal ions. Stock solutions of DTNB (600 mM), capsaicin (10 mM), and mibefradil (100 mM) were prepared in DMSO. The maximal concentration of DMSO was 0.5% for in vitro experiments. At these concentrations, DMSO had no effect on either T currents or holding potential (data not shown). Mibefradil (Ro 40-5967) was a kind gift of F. Hoffman-La Roche, Basel, to Dr. Christopher Lingle. All other chemicals were obtained from Sigma (St. Louis, MO) and/or Aldrich Chemicals (Milwaukee, WI).

The standard external solution used to isolate Ca^{2+} currents contained (in mM) $BaCl_2$, 5–10; tetraethylammonium (TEA) chloride, 160; N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES), 10; with pH adjusted to 7.4 with TEAOH. To isolate T currents, we blocked most HVA currents in these cells with intracellular F^- and with application of 5 μM nifedipine (L-type HVA channel blocker) and 1 μM ω-CgTx-GVIA (ω-conotoxin-GVIA, N-type HVA blocker) in the external solution (Todorovic and Lingle, 1998). The standard external solution for recording voltage-gated K^+ and Na^+ currents and capsaicin-, heat-, proton-, and ATP-gated currents contained (in mM) NaCl, 140; KCl, 5; $MgCl_2$, 2; $CaCl_2$, 0.5; glucose 10; and HEPES, 10; pH 7.4 with NaOH. The standard pipette solution used

to record T and HVA Ca^{2+} currents, capsaicin-, heat-, proton-, and ATP-gated currents and voltage-gated Na^{+} currents contained (in mM) Cs-methane sulfonate, 110; phosphocreatine, 14; HEPES, 10; EGTA, 9; Mg-ATP 5; and Tris-GTP, 0.3; pH adjusted to 7.2 with CsOH. For recording voltage-gated potassium currents, KCl replaced Cs-methane sulfonate, and 2 mM QX 314 was added to pipette solution to block voltage-gated Na^{+} currents. For recording T-type Ca^{2+} currents in isolation, we used the following F^{-}-based intracellular solution (in mM): TEAOH, 135–140; EGTA, 10; HEPES, 40; and $MgCl_2$, 2; titrated to pH 7.15–7.25 with HF.

Current Analysis

Concentration-response data were fit to the function PB([MIBEFRADIL]) = $PB_{max}/(1 + (IC_{50}/[MIBEFRADIL])^{n})$, where PB_{max} is the maximal percent block by mibefradil of peak T currents, the IC_{50} is the concentration that produces half-maximal block, and n is the apparent Hill coefficient. Fitted values are reported with 95% linear confidence limits. The time course of T current inactivation was examined using a single-exponential fit to current decay. Fitting was done either with Origin 4.0 (Microcal Software, Northhampton, MA) or Clampfit 6.0 (Axon Instruments).

Behavioral Studies

All experimental protocols were approved by the Washington University Animal Studies Committee. The nociceptive response to thermal (heat) stimulation was tested in a commercially available paw thermal stimulation system as described elsewhere (Jevtovic-Todorovic et al., 1998). In brief, the device consists of a clear plastic chamber (10 × 20 × 24 cm) that sits on a clear elevated glass floor and is temperature regulated at 30°C. Adult female Sprague-Dawley rats were placed in the plastic chamber and given 10–15 min to accommodate. A radiant heat source mounted on a portable holder beneath the glass floor was positioned to deliver a thermal stimulus to the plantar side of the hindpaw. When an animal withdraws its paw, a photocell detects interruption of a light reflection, and an automatic timer shuts off. This provides an accurate record of PWL. The PWL can be measured with this apparatus with a precision of 0.1 s. To prevent injury, the thermal source is automatically discontinued after 20 s if the rat fails to withdraw its paw.

For measurements of mechanical nociception, rats were placed in a clear plastic, wire mesh-bottomed cage divided into four individual compartments, permitting free movement of animals while allowing access to the paws. Von Frey Filaments (Stoelting, Wood Dale, IL) were used to assess the mechanical threshold for paw withdrawal. These filaments are designated by the manufacturer as the $\log_{10}$ (milligram weight required to cause buckling ×10). Ten trials per filament were used for each test period, and the number of paw withdrawals was recorded (Sluka and Willis, 1997). We identified the filament 4.93 (corresponding to 8.50 gm buckling weight) that applied to the plantar surface of the foot causes a noxious response resulting in an average of three to four paw withdrawals in ten trials. Baseline was determined first in both paws 1–2 days prior to actual testing and compared to time (just prior to injection, marked as "0" time on our graphs). If these two values differed by more than two withdrawals, animals were not used for further experiments.

To test the effects of drugs in peripheral receptive fields, we injected 100 μl of test compounds intradermally in the ventral side of the left hindpaw. The noninjected side (right hindpaw) was used as a control in each animal. All solutions were pH balanced to 7.4 to avoid skin irritation. No signs of skin inflammation, discoloration, or irritation were noted at the sites of injection with test compounds. All doses are expressed in micrograms per 100 μL. For animals included in this study, baseline values were compared to thermal PWLs or withdrawal responses on noninjected and injected paws at various time points during the testing, as indicated in the figures (posttreatment values). In the data displayed, every point is an average of at least eight animals, and values represent mean ± SEM. Statistical analysis was performed using an ANOVA comparing within-subject variables: paw condition (injected versus noninjected) and test session (prior to drug administration or 10, 20, or 60 min posttreatment).

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References

Aizenman, E., Lipton, S.A., and Loring, R.H. (1989). Selective modulation of NMDA responses by reduction and oxidation. Neuron *2*, 1257–1263.

Campbell, D.L., Stamler, J.S., and Strauss, H.C. (1996). Redox modulation of L-type calcium channels in ferret ventricular myocytes. J. Gen. Physiol. *108*, 277–293.

Carbone, E., and Lux, H.D. (1984). A low-voltage activated, fully inactivating Ca^{2+} channel in vertebrate sensory neurons. Nature *310*, 501–502.

Cardens, C.G., Del Mar, L.P., and Scroggs, R.S. (1995). Variation in serotonergic inhibition of calcium channel currents in four types of rat sensory neurons differentiated by membrane properties. J. Neurophysiol. *74*, 1870–1879.

Caterina, M.J., and Julius, D. (1999). Sense and specificity: a molecular identity for nociceptors. Curr. Opin. Neurobiol. *9*, 525–530.

Coderre, T.J., Katz, J., Vaccarino, A.L., and Melzack, R. (1993). Contribution of central neuroplasticity to pathological pain: review of clinical and experimental evidence. Pain *52*, 259–285.

Cook, S.P., and McCleskey, E.W. (1997). Desensitization, recovery and Ca^{2+}-dependent modulation of ATP-gated P2X receptors in nociceptors. Neuropharmacology *36*, 1303–1308.

Cribbs, L.L., Lee, J., Yang, J., Satin, J., Zhang, Y., Daud, A., Barcley, J., Williamson, M.P., Fox, M., Rees, M., and Perez-Reyes, E. (1998). Cloning and characterization of α1H from human heart, a member of the T-type Ca^{2+} channel gene family. Circ. Res. *83*, 103–109.

De Waard, M., Gurnett, C.A., and Campbell, K.P. (1996). Structural and functional diversity of voltage-activated calcium channels. In Ion Channels, T. Narahashi, ed. (New York: Plenum Press), pp. 41–87.

Dolphin, A.C., Wyatt, C.N., Richards, J., Beattie, R.E., Craig, P., Lee, J-H., Cribbs, L.L., Volsen, S.G., and Perez-Reyes, E. (1999). The effects of α2δ and other accessory subunits on expression and properties of the calcium channel α1G. J. Physiol. *519*, 35–45.

Ertel, S.I., and Clozel, J.-P. (1997). Mibefradil (Ro 40-5967): the first selective T-type Ca^{2+} channel blocker. Exp. Opin. Invest. Drugs *6*, 569–582.

Ertel, S.I., Ertel, E.A., and Clozel, J. (1997). T-type Ca^{2+} channels and pharmacological blockade: potential pathophysiological relevance. Cardiovasc. Drugs Ther. *11*, 723–739.

Hamill, O.P., Marty, E., Neher, E., Sakmann, B., and Sigworth, F.J. (1981). Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. Pfluegers Arch. *381*, 85–100.

Hargreaves, K., Dubner, R., Brown, F., Flores, C., and Joris, J. (1988). A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. Pain *32*, 77–88.

Herrington, J., and Lingle, C.J. (1992). Kinetic and pharmacological properties of low voltage-activated Ca^{2+} current in rat clonal (GH_3) pituitary cells. J. Neurophysiol. *68*, 213–232.

Huguenard, J.R. (1996). Low-threshold calcium currents in central nervous system neurons. Annu. Rev. Physiol *58*, 329–358.

Jackson, D.L., Graff, C.B., Richardson, J.D., and Hargreaves, K.M. (1995). Glutamate participates in the peripheral modulation of thermal hyperalgesia in rats. Eur. J. Pharmacol. *284*, 321–325.

Jevtovic-Todorovic, V., Wozniak, D.F., Powell, S., Nardi, A., and Olney, J.W. (1998). Clonidine potentiates the neuropathic pain-

relieving action of MK-801 while preventing its neurotoxic and hyperactivity side effects. Brain. Res. *781*, 202–211.

Laughlin, T.M., Kitto, K.F., and Wilcox, G.L. (1998). Redox manipulation of NMDA receptors in vivo: alteration of acute pain transmission and dynorphin-induced allodynia. Pain *80*, 37–43.

Lawand, N.B., Willis, W.D., and Westlund, K.N. (1997). Excitatory amino acid receptor involvement in peripheral nociceptive transmission in rats. Eur. J. Pharmacol. *324*, 169–177.

Lee, J.H., Daud, A.N., Cribbs, L.L., Lacerda, A.E., Pereverzev, A., Klockner, U., Schneider, T., and Perez-Reyes, E. (1999). Cloning and expression of a novel member of the low voltage-activated T-type calcium channel family. J. Neurosci. *19*, 1912–1921.

Levine, J.D., Fields, H.L., and Basbaum, A.I. (1993). Peptides and the primary afferent nociceptors. J. Neurosci. *13*, 2273–2286.

Llinas, R. (1988). The intrinsic electrophysiological properties of mammalian neurons: insight into central nervous system function. Science *242*, 1654–1664.

Magee, J.C., and Johnston, D. (1995). Synaptic activation of voltage-gated channels in the dendrites of hippocampal pyramidal neurons. Science *268*, 301–304.

Markram, H., and Sakmann, B. (1994). Calcium transients in dendrites of neocortical neurons evoked by single subthreshold excitatory postsynaptic potentials via low-voltage-activated calcium channels. Proc. Natl. Acad. Sci. USA *91*, 5207–5211.

McDonough, S.I., and Bean, B.P. (1998). Mibefradil inhibition of T-type calcium channels in cerebellar purkinje neurons. Mol. Pharmacol. *54*, 1080–1087.

Miller, R.J. (1998). Presynaptic receptors. Ann. Rev. Pharmacol.Toxicol. *38*, 201–227.

Perez-Reyes, E., Cribbs, L.L., Daud, A., Lacerda, A.E., Barclay, J., Wiliamson, M.P., Fox, M., Rees, M., and Lee, J. (1998). Molecular characterization of a neuronal low-voltage-activated T-type calcium channel. Nature *391*, 896–900.

Reichling, D.B., and Levine, J.D. (2000). In hot pursuit of the elusive heat transducers. Neuron *26*, 555–558.

Scroggs, R.S., and Fox, A.P. (1992). Calcium current variation between acutely isolated rat dorsal root ganglion neurons of different size. J. Physiol. *445*, 639–658.

Sluka, K.A., and Willis, W.D. (1997). The effects of G-protein and protein kinase inhibitors on the behavioral responses of rats to intradermal injection of capsaicin. Pain *71*, 165–178.

Snider, W.D., and McMahon, S.B. (1998). Tackling pain at the source: new ideas about nociceptors. Neuron *20*, 629–632.

Suliman, M.E., Anderstam, B., Lindholm, B., and Bergstrom, J. (1997). Total, free, and protein-bound sulfur amino acids in uremic patients. Nephrol. Dial. Transplant. *12*, 2332–2338.

Talley, E.M., Cribbs, L.L., Lee, J.H., Daud, A., Perez-Reyes, E., and Bayliss, D.A. (1999). Differential distribution of three members of a gene family encoding low voltage-activated (T-type) calcium channels. J. Neurosci. *19*, 1895–1911.

Tang, L., and Aizenman, E. (1993). The modulation of N-methyl-d-aspartate receptors by redox and alkylating reagents in rat cortical neurons in vitro. J. Physiol. *465*, 303–323.

Todorovic, S.M., and Anderson, E.G. (1992). Serotonin preferentially hyperpolarizes capsaicin-sensitive C type sensory neurons by activating 5-HT1A receptors. Brain Res. *585*, 212–218.

Todorovic, S.M., and Lingle, C.J. (1998). Pharmacological properties of T-type Ca^{2+} current in adult rat sensory neurons: effects of anticonvulsants and anesthetic agents. J. Neurophysiol. *79*, 240–252.

Todorovic, S.M., Perez-Reyes, E., and Lingle, C.J. (2000). Anticonvulsants but not general anesthetics have differential blocking effects on different T-type current variants. Mol. Pharmacol. *58*, 98–108.

White, G., Lovinger, D.M., and Weight, F.F. (1989). Transient low-threshold Ca^{2+} current triggers burst firing through an afterdepolarizing potential in an adult mammalian neuron. Proc. Natl. Acad. Sci. USA *86*, 6802–6806.

Zhou, S., Bonasera, L., and Carlton, S.M. (1996). Peripheral administration of NMDA, AMPA or KA results in pain behaviors in rats. Neuroreport *7*, 895–900.