FORUM REVIEW ARTICLE

Redox Regulation of Neuronal Voltage-Gated Calcium Channels

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Abstract

Significance: Voltage-gated calcium channels are ubiquitously expressed in neurons and are key regulators of cellular excitability and synaptic transmitter release. There is accumulating evidence that multiple subtypes of voltage-gated calcium channels may be regulated by oxidation and reduction. However, the redox mechanisms involved in the regulation of channel function are not well understood. **Recent Advances:** Several studies have established that both T-type and high-voltage-activated subtypes of voltage-gated calcium channel can be redox-regulated. This article reviews different mechanisms that can be involved in redox regulation of calcium channel function and their implication in neuronal function, particularly in pain pathways and thalamic oscillation. **Critical Issues:** A current critical issue in the field is to decipher precise mechanisms of calcium channel modulation *via* redox reactions. In this review we discuss covalent post-translational modification *via* oxidation of cysteine molecules and chelation of trace metals, and reactions involving nitric oxide-related molecules and free radicals. Improved understanding of the roles of redox-based reactions in regulation of voltage-gated calcium channels may lead to improved understanding of novel redox mechanisms in physiological and pathological processes. **Future Directions:** Identification of redox mechanisms and sites on voltage-gated calcium channel may allow development of novel and specific ion channel therapies for unmet medical needs. Thus, it may be possible to regulate the redox state of these channels in treatment of pathological process such as epilepsy and neuropathic pain. *Antioxid. Redox Signal.* 21, 880–891.

Introduction

FUNCTION OF MANY PROTEINS including ion channels can be regulated by oxidation and reduction (redox) reactions. Traditional redox reactions were understood to involve covalent modification of proteins *via* direct interaction with their sulfhydryl (thiol) groups. In addition, many redox agents can function as chelators of trace metals and produce physiological effects by affecting function of ion channels indirectly *via* regulation of local concentrations of the metals. In this article we will review these and some other mechanisms that can contribute to regulation of the function of voltage-gated calcium channels *via* redox-related reactions. We will focus on mechanisms involving covalent post-translational modification, chelation of trace metals, reactions involving nitric oxide (NO)-related molecules, and reactions involving free radicals. Multiple subtypes of voltage-gated calcium channels in nerve tissue have central functions in sensory, cognitive, and motor pathways, and in controlling cellular excitability and neuro-

transmitter release. Hence, regulation of calcium current in different neurons by redox agents may be important in tuning neuronal excitability and transmitter release in physiological and pathological conditions. Several studies have shown that these channels can be regulated *via* different redox mechanisms. An important issue is that even small changes in function of a particular calcium channel may produce very large physiological effects. Where this has been examined, small changes in calcium influx into presynaptic terminals can result in profound changes in transmitter release and synaptic efficacy (76). Indeed, presynaptic transmitter release is proportional up to the fourth power of calcium entry (12). Thus, even if calcium channels are only weakly modulated by exogenous and/or endogenous redox agents, this can very significantly alter the neuronal signaling. It is reasonable to propose that regulating channel function is more physiological than blocking it with antagonists, which, in many instances, may cause unwanted consequences. Thus, pharmacological targeting of redox sites on voltage-gated

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calcium channels may be a good strategy for development of novel ion channel therapies while minimizing side effects.

Multiple Isoforms of T-Type Calcium Channels

Voltage-gated calcium channels, which are heteromeric complexes in the plasma membrane of virtually all cell types, show a high level of electrophysiological and pharmacological diversity. On the basis of their sensitivity to the voltage at which they gate currents, these channels are subdivided into two major families, namely low-voltage-activated (LVA), which activate with small depolarizations near resting membrane potentials, and high-voltage-activated (HVA) calcium currents, which require stronger depolarization for their activation. LVA channels are also called transient (T-type) currents due to their rapid inactivation when compared with the more sustained calcium currents carried by HVA channels. Figure 1 depicts basic properties of two types of inward calcium currents in acutely dissociated cells from the dorsal root ganglion (DRG). Note that T-current (top gray trace) rapidly inactivates during a depolarizing pulse while

FIG. 1. Different subtypes of calcium currents in sensory neurons. Original traces of T-type current (top gray trace) and total HVA-type calcium current (bottom black trace) in acutely dissociated medium size DRG neuron evoked with voltage steps from -90 to -40 mV and from -40 to 0 mV, respectively. Calibration bars pertain to both graphs. Currents are recorded with 10 mM Ba^{2+} as a charge carrier. DRG, dorsal root ganglion; HVA, high-voltage-activated.

HVA current (bottom black trace) shows minimal inactivation during a depolarizing pulse. The first detailed description of biophysical properties of T-type channels using patch-clamp experiments was done by Carbone and Lux in cultured DRG neurons of small size (10). They found that the conductance was different from previously described HVA calcium currents in that T-type currents exhibit small single channel conductance, faster kinetics of macroscopic voltage-dependent inactivation, slower kinetics of channel closing from fully activated states (deactivation), and finally, lower voltages at which channels become activated. These unique kinetic features are crucial in endowing neurons that possess T-type channels with a lower threshold for spike firing. Soon after these initial biophysical studies, White *et al.* (74) linked these newly discovered T-type calcium channels with increased excitability of DRG neurons. They demonstrated that T-type channels provide the basis for after-depolarizing potentials (ADP) in a subpopulation of medium size DRG neurons in rat that could be crowned with repetitive burst firing of action potentials similar to phenomena described in many central nervous system (CNS) neurons, especially in the thalamus (25). The biophysical properties and large density of T-type currents in medium size DRG cells are very similar to ones later described in a novel subpopulation of small, putative nociceptive DRG neurons termed "T-rich" cells (44), raising the possibility that T-type currents in these cells may play a prominent role in regulation of cellular excitability. Figure 2 shows recordings of an action potential and ADP in a "T-rich" DRG neuron that is evoked by application of L-cysteine that augments T-currents, and its blockade with nickel, a classical T-type channel antagonist.

The pore-forming α_1 subunit of all known voltage-gated calcium channels consists of four domains (I–IV), each of which is made of six transmembrane segments (S). The second transmembrane segment of each domain forms the walls of the pore; the selectivity filter is formed by the pore loops; and the S4 loop acts as the voltage sensor. Most of the channel is thought to be intracellular, with short extracellular loops, except for a large segment linking IS5 to the pore loop. The cloning of α_1 subunits of T-channels has revealed the existence of at least three subtypes, G ($\text{Ca}_v3.1$) (55), H ($\text{Ca}_v3.2$) (14), and I ($\text{Ca}_v3.3$) (31), which are likely to contribute to the heterogeneity of T-currents in native cells (19, 67). Although all of the

FIG. 2. Current-clamp recordings of action potential waveforms are displayed from an acutely dissociated "T-rich" DRG neuron. The small current injection evoked only sub-threshold depolarization in control condition (left black trace). In the presence of L-cysteine, the same current injection evoked fast action potential followed by after-depolarization (ADP) (middle gray trace). The membrane response to L-cysteine was completely abolished when T-channels are blocked with addition of nickel to the external solution (right black trace). Calibration bars pertain to all three graphs.

isoforms of T-type channels possess similar kinetic features distinct from HVA channels, some unique properties in channel gating, inactivation and deactivation differentiate these related channels [reviewed in Perez-Reyes (54)]. Molecular analysis has indicated that mRNA for $\text{Ca}_v3.2$ ($\alpha 1\text{H}$) is the most abundant of T-type channel isoforms in peripheral sensory DRG neurons of small and medium size, and in cells of the nucleus reticularis thalami (nRT). In contrast, $\text{Ca}_v3.1$ ($\alpha 1\text{G}$) and $\text{Ca}_v3.3$ ($\alpha 1\text{I}$) isoforms are much less present in DRG cells but are highly expressed in thalamocortical (TC) neurons of the ventrobasal thalamus and in nRT cells, respectively (66).

Multiple Subtypes of HVA Calcium Currents

Pharmacological and physiological experiments on native calcium currents (11, 29) support the existence of at least five types of HVA calcium currents (L-, N-, P-, Q-, and R-currents). The $\alpha 1$ subunits that form the pore of the neuronal calcium channels are the products of different gene families: Ca_v1 (former $\alpha 1\text{C}$) encoding L-type, $\text{Ca}_v2.1$ ($\alpha 1\text{A}$) encoding P/Q-type, $\text{Ca}_v2.2$ ($\alpha 1\text{B}$) encoding N-type, and $\text{Ca}_v2.3$ ($\alpha 1\text{E}$) encoding R-type HVA current. Most HVA channels activate over a similar voltage range and are completely blocked with divalent metals such as cadmium. Thus, subtype identification relies on the sensitivity to particular pharmacological agents. L-current is blocked reversibly by $5\ \mu\text{M}$ nifedipine; N-current is blocked irreversibly by ω -conotoxin GVIA; P-current is blocked essentially irreversibly (recovery time constant ~ 50 min) by ω -agatoxin (Aga) GVIA (< 100 nM) (43); Q-current is blocked by ω -conotoxin MVIIC (42). With all the other currents blocked, there remains a cadmium-sensitive inward current, the R- (resistant) $\text{Ca}_v2.3$ current. SNX-482 is a synthetic peptide that selectively and irreversibly blocks $\text{Ca}_v2.3$ currents in mammalian cell lines (5, 71).

Depending on the synaptic system being examined, P-, L-, N-, and Q-subtypes of HVA currents may contribute to the calcium influx, which results in transmitter release (11). Because of the paucity of selective blockers, the role of R-current remains poorly understood; but it recently was shown that this subtype can evoke excitatory transmitter release in brainstem neurons (77) and that has a role in synaptic plasticity (5) and thalamic signaling (26). Unlike T-type channels, the diversity of HVA calcium channels in neurons is also complicated by the presence of ancillary subunits that can modulate the channels' functions. Associated with the pore-forming $\alpha 1$ subunit is the membrane-anchored, largely extracellular $\alpha 2$ - δ , the cytoplasmic β , and sometimes a transmembrane γ subunit; these subunits dramatically influence the properties and surface expression of these channels [reviewed by Khosravani and Zamponi (29)].

Redox Modulation of Neuronal T-Type Voltage-Gated Calcium Currents with L-Cysteine and Related Molecules

Redox modulation of T-type channels in peripheral nociceptors

Peripheral nociceptors encompass several subpopulations of pain-sensing sensory neurons of the DRG. It is somewhat ironic that the first study that clearly documented the existence of T-type channels was done using cultured DRG cells (10) because almost two decades elapsed after this discovery before the role of T-channels in sensory processing, and no-

ception in particular, was firmly established. This was likely due to the fact that selective modulators and/or pharmacological blockers of T-type channels were not available for a long time. The first study that strongly suggested a nociceptive role of T-channels in pain processing was done using *in vitro* and *in vivo* approaches with endogenous and exogenous redox agents (68). That study showed that T-currents in acutely dissociated sensory neurons are significantly enhanced (about twofold) when T-channels are in a reduced state induced either using an endogenous amino acid such as L-cysteine or with dithiothreitol (DTT). Conversely, T-currents in DRG cells are inhibited when T-channels are in an oxidized state induced by application of 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB), suggesting the possible importance of putative redox-sensitive sites in peripheral T-type channels. In contrast, the same study has documented that in DRG cells, neither of these agents affected the amplitudes of HVA calcium currents even at 10-fold higher concentrations. It was shown subsequently that enhancement of T-currents with reducing agents can affect cellular excitability profoundly by enhancing influx of calcium ions during the action potential in at least three different subtypes of peripheral nociceptors (20, 44, 47). Figure 2 shows that application of L-cysteine in T-rich neurons increased membrane response and induced spike firing followed by ADP. Further, a computational modeling study has demonstrated that an increase in the cellular excitability of putative nociceptive "T-rich" cells in DRGs can be achieved with hyperpolarizing gating shifts in T-channels induced by addition of L-cysteine (44). Thus, both an increase in the amplitude of T-current and alterations in the gating parameters of T-channels in nociceptors may increase the probability of spike firing. Further studies using Markov-state models have shown that L-cysteine induces alterations of T-current gating in nociceptors by increasing the transition rate from resting to open and decreasing the transition rate from open to inactivated states (46). These prominent changes in T-channel amplitude and kinetics with redox agents could translate into altered pain perception since pain is directly proportional to measures of cellular excitability of DRG cells. Indeed, some of the most prominent symptoms of pathological pain perception such as hyperalgesia (enhanced pain perception), allodynia (sensation of pain associated with nonpainful stimuli), and spontaneous pain are linked to increased excitability of peripheral nociceptors (8).

Consistent with this notion, L-cysteine was shown to induce potent hyperalgesia when injected into the peripheral receptive fields of sensory neurons of healthy rats *in vivo*—likely due to modulation of the $\text{Ca}_v3.2$ isoform of T-type channels in the periphery (68). This notion was validated by the demonstration that hyperalgesia induced by local peripheral injections of L-cysteine is present only in wild-type mice, not in $\text{Ca}_v3.2$ -knockout mice (47). Further, molecular mutagenesis studies have shown that the extracellular histidine 191 in repeat I unique to $\text{Ca}_v3.2$ is critical for the action of reducing agents that chelate endogenously bound divalent metal ions (e.g., Zn^{2+}), which appears to exert tonic inhibition of T-channels, both *in vitro* and *in vivo* (47, 72). In contrast, cysteine-modifying agents such as N-ethyl-maleimide (NEM) or UV light do not prevent the effects of reducing agents on T-current in DRG cells (47). Figure 3 shows a schematic representation of $\text{Ca}_v3.2$ channel structure with the critical H191 residue. Recent molecular studies have established that the metal-binding site of the $\text{Ca}_v3.2$ channel is composed of

FIG. 3. Critical metal-binding residue of Ca_v3.2 channels. Schematic diagram of Ca_v3.2 shows the position of H191 residues in the extracellular loop between transmembrane segments 3 and 4 of domain 1, and the amino acid sequence of the loop across all T-channel isoforms. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

an Asp-Glu-His motif in repeat IS3-IS4 and a second aspartate residue in IS2 (27). It has been proposed that the metal-binding site stabilizes a closed conformation of the voltage-sensor in repeat I of Ca_v3.2 and consequently inhibits channel opening.

A possible related phenomenon recently was described for the gaseous transmitter hydrogen sulfide (H₂S), an endogenous compound formed from L-cysteine. It was described that H₂S facilitates Ca_v3.2 currents *in vitro*, possibly *via* chelation of zinc, consequently leading to both peripheral and central sensitization of acute somatic (28, 36) and visceral (38, 48) nociception, and maintenance of chronic neuropathic pain (65). However, molecular pathways of H₂S-mediated sensitization of peripheral nociceptors have not been well studied. Importantly, reducing agents and other endogenous metal chelators present in the body (*e.g.*, albumin, histidine, H₂S) may serve as natural modulators for the Ca_v3.2 T-channels, which, in turn, may fine-tune excitability of peripheral nociceptors.

The effects of several other endogenous redox agents on isolated T-currents and cellular excitability of sensory neurons *in vitro*, and on behavioral measures of pain responses *in vivo*, were studied subsequently. Since endogenous thiol-modifying redox agents are normally present in peripheral tissues, *in vivo* studies were designed to evaluate the peripheral nociceptive effects of locally injected reducing cysteine analogues such as L-cysteine, D-cysteine, and D,L-homocysteine, and of oxidizing cysteine analogues such as L-cystine, D-cystine, and D,L-homocystine, using the acute model of thermal peripheral nociception in intact rats (53). It was found that reducing cysteine analogs induced potent dose- and time-dependent hyperalgesia, and conversely that the oxidizing cysteine analogs induced potent dose- and time-dependent analgesia. Importantly, in the presence of (3β,5α,17β)-17-hydroxyestrane-3-carbonitrile (3βOH), a novel and potent voltage-dependent blocker of T-channels, the hyperalgesic effects of reducing agents were diminished, whereas the analgesic effects of the oxidizing agents were enhanced, strongly

suggesting that the observed nociceptive effects were, at least in part, mediated *via* the peripheral T-channels. These findings imply that changes in the redox states of the peripheral nociceptors (favoring either reduced or oxidized forms of cysteine molecules) may function as a local intrinsic mechanism for controlling peripheral pain perception. Figure 4 depicts control of peripheral nociception in rat skin with analogues of cysteine molecules.

Effects of L-cysteine, DTT, and DTNB on T-channels in peripheral nociceptors also were implicated in pathophysiology of neuropathic pain. Using a rat chronic constrictive injury (CCI) model of neuropathic pain, it was observed that peripheral injections of L-cysteine and DTT induced hyperalgesia that was blocked with the classical T-channel blocker mibefradil (70). Conversely, peripheral injections of DTNB completely relieved neuropathic hyperalgesia in CCI rats. However, effects of L-cysteine and DTT were much less pronounced in the CCI rats as compared with sham-operated control rats and the analgesic effect of DTNB was more prominent in neuropathic than control rats. Based on these findings, it was proposed that putative redox sites on peripheral T-type channels could be involved in the mechanisms of sensitization of peripheral nociceptive responses in conditions associated with mechanical injury of peripheral nerves. This suggestion is important since neuropathic pain is difficult to treat with currently available pain killers and redox sites on T-channels may represent a new opportunity for development of much needed pain therapies.

Redox modulation of T-type channels in the thalamus

All three isoforms of T-channels are expressed in the various thalamic nuclei, which are involved in relaying sensory information from the periphery to the cortex. For example, *in situ* hybridization studies have shown that nRT neurons express mostly Ca_v3.2 and Ca_v3.3 isoforms; neurons of the laterodorsal (LD) nucleus express Ca_v3.1 and Ca_v3.3 isoforms; while cells of the ventrobasal (VB) complex are enriched in the Ca_v3.1 isoform (66). Thus, since molecular studies have shown that only recombinant human Ca_v3.2 channels and

FIG. 4. Scheme of proposed local intrinsic mechanism for control of peripheral nociception. Schematic representation of Ca_v3.2 T-type calcium channels in putative nociceptive skin nerve endings and their modulation with reduced and oxidized forms of cysteine molecules. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

native $\text{Ca}_v3.2$ channels in DRG cells are sensitive to L-cysteine, it was reasonable to hypothesize that among thalamic nuclei, reducing agents may modulate only currents in nRT cells. Indeed, Joksovic *et al.* (25) have used whole-cell recordings from thalamic slices to show that L-cysteine and DTT enhance T-currents only in nRT cells, but not in LD and VB nuclei. The use of $\text{Ca}_v3.2$ KO mice confirmed that the effects of L-cysteine and DTT are $\text{Ca}_v3.2$ -specific. In contrast, the oxidizing agent DTNB inhibited T-currents in all three thalamic nuclei. Further, recordings in excised inside out and outside out patches in nRT cells have shown that DTT and L-cysteine are effective only when applied to the external side of the membrane, consistent with molecular studies that established that the $\text{Ca}_v3.2$ -specific H191 residue in the external side of repeat I is critical for these effects (47).

It is well established that prominent T-currents underlie the ability of thalamic neurons to fire low threshold calcium spikes (LTCSs), which in turn allows burst firing of these neurons with small membrane depolarizations (22). Firing of LTCSs in γ -amino-butyric acid (GABA)ergic nRT neurons is the initial critical event leading to synchronization of low-amplitude oscillations in the mutually connected nRT, thalamic relay and cortical neurons, which dominate during sleep states and are crucial in absence seizures (62, 63). Figure 5 depicts corticothalamic loop (Fig. 5A) and original traces of LTCSs in nRT neurons (Fig. 5B). Therefore, the functional effects of redox agents on LTCSs and burst firing in nRT neurons were studied using current-clamp recordings. As expected from the results of voltage-clamp experiments, L-cysteine and DTT enhanced the amplitudes of LTCSs and increased frequency of burst firing in nRT neurons in wild-type mice and rats; this effect was completely absent in nRT cells from $\text{Ca}_v3.2$ knockout mice (25). In contrast, application of DTNB decreased the amplitudes of LTCSs and abolished burst firing in nRT neurons from wild-type rats and mice. Since nRT neurons are critically involved in generation of the low amplitude oscillations of sleep and epilepsy (4), it will be important for future *in vivo* studies to test the notion that redox sites on T-channels may be involved in regulations of these processes.

Molecular Mechanisms of Modulation of $\text{Ca}_v3.2$ T-Current by Lipolic Acid

α -lipoic acid (LA) (thioctic acid, oxidized form) and dihydrolipoic acid (DHLA) (dihydrothioctic acid, reduced form) are endogenous, lipid-soluble, sulfur-containing mitochondrial cofactors that are ubiquitous in living organisms. LA and DHLA are important in the citric-acid cycle and in energy metabolism; in addition, they function as scavengers of free radicals and readily participate in redox reactions that modulate thiol groups in proteins (58). Human and animal studies have clearly documented analgesic properties of LA; however, molecular mechanisms underlying the analgesic properties of LA initially were not understood (7, 39). Lee *et al.* (32) used $\text{Ca}_v3.2$ knockout mice to examine molecular determinants of LA inhibition of T-current in DRG cells and *in vivo* pain responses. The initial voltage-clamp recordings revealed that LA selectively inhibited T-type over HVA currents at physiologically relevant concentrations ($\text{IC}_{50} \sim 3 \mu\text{M}$) and also inhibited the ADP and burst-firing pattern of these DRG cells. But unlike the effect of L-cysteine, the effect of LA was isoform nonspecific: LA induced similar inhibition of recom-

FIG. 5. Thalamocortical loop and T-channels. (A) Schematic representation of corticothalamic circuitry. Sign "+" denotes excitatory and sign "-" denotes inhibitory synapses. **(B)** Original traces of sequence of rebound burst firing pattern in nRT neurons in intact rat brains slices evoked by injections of negative current. Arrows point to the LTCS that are crowned by fast action potentials. Circular arrow indicates that nRT neurons and VB neurons may have direct interaction without involvement of cortical neurons. nRT, nucleus reticularis thalami; IC, internal capsule; VB, ventrobasal nucleus of the thalamus; Ctx, cortex; LTCS, low threshold calcium spike. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

binant $\text{Ca}_v3.1$ and $\text{Ca}_v3.2$ currents. Next, it was found that alkylation of thiol groups on T-channels with NEM almost completely abolished the effects of LA. This result strongly suggested that the effect of LA on T-current is due to alterations of thiol groups on channels. However, since both LA and NEM can readily cross the cell membrane, these experiments could not exclude the possibility that these putative thiol groups were located in the cytoplasm. To address this issue, acutely dissociated DRG cells were dialyzed with strong membrane-impermeant agents such as DTNB or 2-(trimethylammonium) ethyl methanethio sulfonate (MTSET) by including them in the patch-clamp pipette solution. It was found that these agents had little effect on LA inhibition of T-current, strongly suggesting that LA inhibits T-current in DRG cells by oxidizing thiol (cysteine) groups on the extracellular (cytoplasmic) face of the neuronal membrane.

However, more than 50 extracellular cysteine molecules are conserved among T-channel isoforms, giving rise to the question of which ones could be critical for LA inhibition of T-current. Cysteine residues are frequently paired and often stabilize structure in proteins by forming covalent disulfide bonds. Therefore, candidate extracellular cysteines were sought that are unique to T-channels *versus* HVA calcium channels and that are in loops with odd numbers of cysteine molecules. The search was narrowed down to two regions: repeat II, where there is a single highly conserved cysteine (C939) in the loop connecting S5 to the pore loop; and repeat I, where there are three conserved cysteine residues in the loop connecting S1 to S2 (C123, C128, C133). Figure 6 depicts these cysteine residues. Ensuing whole-cell recordings from mutated channels determined that in mutants where all four cysteines of $\text{Ca}_v3.2$ were mutated to alanines, LA effects on T-current were completely abolished, while basic kinetic properties of the channel were only minimally affected. Importantly, these mutations also diminished the ability of another thiol-oxidizing agent, DTNB, to inhibit T-currents, while the effects of zinc and reducing agents were spared. These data indicate that different molecular mechanisms mediate the effects of reducing agents such as L-cysteine that can increase cellular excitability of nociceptor T-channels, *versus* oxidizing agents such as LA that decrease, excitability. Ensuing studies tested the idea that inhibition of T-currents in peripheral nociceptors may contribute to analgesic properties of LA. Indeed, LA locally injected into peripheral receptive fields of nociceptors *in vivo* effectively decreased sensitivity to noxious mechanical and thermal stimuli in wild-type but not $\text{Ca}_v3.2$ knockout mice. Overall, this study provided the first mecha-

nistic demonstration of a nociceptive ion channel modulation that may contribute to the documented analgesic properties of LA *in vivo*. Further, the elucidation of the molecular mechanisms underlying the role of modulation of T-channels in peripheral sensitization of pain responses with LA and related compounds suggests new targets for analgesic pharmacotherapy.

Molecular Mechanisms of Modulation of $\text{Ca}_v3.2$ T-Current by Metal-Catalyzed Oxidation of H191

Molecular mechanisms of modulation of $\text{Ca}_v3.2$ T-current by ascorbic acid

Ascorbate (ascorbic acid, vitamin C) is a ubiquitous redox agent present at high concentrations in neurons (56). Ascorbate often functions as an antioxidant, readily scavenging reactive oxygen (ROS) and nitrogen species (RNS). However, ascorbate also can function as a pro-oxidant by catalyzing the formation of several ROS. Ascorbate generates ROS by reducing transition metals *via* a one-electron transfer mechanism, and oxygen *via* a two-electron transfer mechanism; the products of these reactions subsequently can interact to create several powerful ROS, including hydroxyl radicals ($\cdot\text{OH}$). Collectively, this process is termed metal-catalyzed oxidation (MCO) (60). A unique feature of proteinaceous MCO is that only one, or at most a select number of amino acids, are modified. This site-specificity has been attributed to the formation of ROS directly at metal-binding sites, where they attack the functional groups of nearby residues. Histidine residues are particularly prone to MCO, in part because they are often structural components of metal-binding sites (60). It was found that ascorbate potently inhibits isolated DRG T-currents ($\text{IC}_{50} \sim 6 \mu\text{M}$) and recombinant human $\text{Ca}_v3.2$ channels ($\text{IC}_{50} \sim 10 \text{ nM}$) (45). The same study showed that, similar to effects of L-cysteine, ascorbate did not affect recombinant $\text{Ca}_v3.1$, recombinant $\text{Ca}_v3.3$ channels or native HVA calcium channels in DRG cells. Given that extracellular concentrations of ascorbate in the brain are in the range up to 1 mM (56), it is reasonable to infer that concentrations of ascorbate that inhibit T-currents are well within physiological concentrations. To determine the molecular substrate for ascorbate inhibition of $\text{Ca}_v3.2$, chimeras between $\text{Ca}_v3.1$ ($\alpha 1\text{G}$, ascorbate-insensitive) and $\text{Ca}_v3.2$ ($\alpha 1\text{H}$, ascorbate-sensitive) were constructed and screened for sensitivity to ascorbate (45). Based on these experiments, it was found that, similar to L-cysteine, domain I of $\text{Ca}_v3.2$ is required for ascorbate inhibition. Further, ensuing molecular studies revealed that ascorbate inhibits $\text{Ca}_v3.2$ currents by initiating the MCO of a unique, metal-binding histidine residue in domain I of the channel (H191). The proposed mechanism of ascorbate modulation of $\text{Ca}_v3.2$ *via* MCO is depicted in Figure 7. Interestingly, as described earlier, this same residue (H191) is critical for the effects of reducing agents, Zn^{2+} and chelators on T-currents (47). Thus, H191 is an important site for fine tuning of neuronal excitability in cells that express T-channels. However, because highly redox reactive metals such as copper (Cu^{2+}) and iron (Fe^{3+}) are much more likely to play a role in MCO than relatively redox inactive metals such as Zn^{2+} , it was examined whether the Cu^{2+} sensitivity of $\text{Ca}_v3.2$ also is disrupted by the H191Q mutation. It was found that $\text{Ca}_v3.2$ (H191Q) was over 40-fold less sensitive to Cu^{2+} than wild

FIG. 6. Putative cysteine residues that can be oxidized in $\text{Ca}_v3.2$ channels. Schematic diagram of $\text{Ca}_v3.2$ shows the positions of conserved mutated cysteine residues in the extracellular face of the channel in domains I and II. Designated cysteine residues (in green) were mutated to alanine residues (in red). To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

FIG. 7. A mechanistic summary of a proposed model for the potent inhibition of Ca_v3.2 by ascorbate, which occurs by MCO. (A) At rest, Cu²⁺, or perhaps another redox-sensitive transition metal such as Fe³⁺, is bound to H191 and possibly to other residues [Kang *et al.* (27)] that comprise the high-affinity metal-binding site on the external surface of Ca_v3.2. **(B)** After the addition of ascorbate (AH⁻), Cu²⁺ is reduced by a one-electron transfer mechanism and O₂ is reduced by a two-electron transfer mechanism; **(C, D)** The products of these reactions, Cu⁺ and H₂O₂, subsequently react to produce [•]OH in a site-specific manner. **(E)** The C-2 position of the imidazole ring of H191 is modified by [•]OH, most likely resulting in 2-oxo-H191. This modification causes gating changes and ultimately reduces T-currents *via* a presumed allosteric mechanism. **(F)** The structure of histidine and 2-oxo-histidine. MCO, metal-catalyzed oxidation. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

type Ca_v3.2, supporting the hypothesis that H191 is part of a general high-affinity metal-binding site located on the external surface of Ca_v3.2 (47). Isoform-specific inhibition of Ca_v3.2 current also was evident in native thalamic cells where ascorbate inhibited only T-currents in nRT cells but not in VB and LD neurons (45). Finally, in this study it was found that in intact brain slices, ascorbate inhibited LTCs and associated burst firing in nRT neurons. Hence, ascorbate may modulate Ca_v3.2-dependent neuronal excitability in physiological and pathological conditions such as pain and absence epilepsy where Ca_v3.2 channels are critically involved. To address this possibility, Okubo *et al.* (50), in an excellent *in vivo* pain study, have shown that a skin-permeable formulation of ointment containing ascorbic acid applied to peripheral receptive fields of nociceptors suppressed neuropathic hyperalgesia induced by application of NaHS (donor of H₂S in tissues) or by me-

chanical injury of the spinal nerve or by systemic administration of the neurotoxic anti-cancer drug paclitaxel. In this study, the analgesic effects of ascorbate were mimicked fully by the T-type channel blocker NNC 55-0396. It is tempting to speculate based on this animal study that analgesic effects of ascorbate reported in humans (13, 79) may be mediated, at least partly, by the ascorbate inhibition of Ca_v3.2 channels in pain pathways.

Molecular mechanisms of modulation of Ca_v3.2 T-current by nitrous oxide

Nitrous oxide (N₂O; laughing gas) has been used in clinical practice as an anesthetic and excellent analgesic for close to two centuries. It is also a common drug of abuse. Despite its long use, the molecular mechanisms underlying its clinical

effects are poorly understood, partly because of difficulties in working with this agent *in vitro*. The first articles describing specific interactions between N_2O and *N*-methyl-D-aspartate (NMDA) receptors in the CNS have begun to explore this mechanism (21, 40). The $Ca_v3.2$ T-channel isoform is abundantly expressed in the major pain processing regions of the peripheral and CNS, namely in the peripheral sensory neurons and superficial layers of the dorsal horn in the spinal cord, respectively (66); and the direct implication of $Ca_v3.2$ channels in pain, strongly suggesting that at least part of N_2O 's potent analgesia effect could be mediated by blockade of $Ca_v3.2$ T-channels. However, a mechanistic link between these two phenomena had not been established until recently. The first study of the effects of N_2O on ion channels in pain pathways showed that N_2O inhibition of T-channels is specific for the $Ca_v3.2$ isoform (69). HVA calcium channels and other isoforms of T-channels like $Ca_v3.1$ (69) and $Ca_v3.3$ (23) were not inhibited by N_2O . The interaction between N_2O and $Ca_v3.2$ channels *in vivo* was explored in a commonly used model for inflammatory pain with injections of formalin into the hind paws of wild-type mice and $Ca_v3.2$ knockout mice. The analgesic effect of N_2O in the formalin test was greatly diminished in $Ca_v3.2$ knockout mice compared with wild-type mice. Orestes *et al.* (51) used metal chelators to provide evidence of the dependence of N_2O on bound redox-reactive metal ions such as iron or possibly copper and application of catalase to demonstrate the necessity of H_2O_2 and hydroxyl radicals. They showed that intraperitoneal application of an SOD/catalase mimetic, EUK-134, is able to reduce pain and inflammation in the formalin test, and consequently abolish the analgesic effects of N_2O in this paradigm, suggesting that H_2O_2 and N_2O inhibit T-currents by a common pathway. They also used oxidation of epinephrine to adrenochrome to model the generation of ROS by N_2O and iron in a cell-free system. Thus, this study presented novel evidence that N_2O inhibits T-channels in pain pathways *via* ROS in a cell-free solution, individual neurons, and whole animals. Collectively, this study supports the hypothesis that N_2O , similar to ascorbate, indirectly inhibits T-currents by oxidizing the channel-bound metal and producing ROS, which then participate in MCO of the unique H191 residue of $Ca_v3.2$ T-channels. These findings demonstrated a novel mechanism of interaction between N_2O and ion channels, furthering our understanding of this widely used analgesic in pain processing. Although description of $Ca_v3.2$ modulation by MCO is intriguing and certainly novel among ion channels, the functional implications of this mechanism for pain perception *in vivo* will need to be studied in more detail in future experiments. Further research also will be needed to investigate whether other ion channels (*e.g.*, NR1/NR2 subunits of NMDA receptors) that, like $Ca_v3.2$ channels, are also known to contain high-affinity metal-binding sites and can be modified by ROS (3, 18) are subject to similar regulation by N_2O .

Different Mechanisms of Modulation of T-Type and HVA Voltage-Gated Calcium Channels by NO

In the presence of redox-reactive trace metals, byproducts of cellular respiration may include ROS. ROS have been implicated in a number of pathological conditions, including chronic and inflammatory pain (15), and in neurodegenerative diseases of the CNS (33, 61). Intraplantar and intrathecal

applications of free radical scavengers have been shown to reduce oxidative stress, inflammation, and pain (49, 57). By minimizing the ROS level, these agents are believed to prevent the induction of stress-related gene responses, and to reduce molecular damage *via* lipid peroxidation and protein oxidation. Affected proteins may include potassium channels and NMDA receptors, which are known to have altered responses after exposure to ROS (3).

Cells with high metabolic demands such as neurons also can generate RNS. One such molecule, NO or endothelium-derived relaxing factor is an important signaling molecule. The multiple effects of NO could be mediated *via* widely studied G-protein signaling cascades or *via* more recently discovered NO-related redox species in neurons (17, 33, 61). At least three NO-related redox forms exist, including $NO^\bullet$ and the nitrosonium ion equivalents (NO^+) with one fewer electrons than $NO^\bullet$, and nitroxyl anion (NO^-), which has one additional electron compared with $NO^\bullet$. The balance among these NO species is governed by the redox milieu of tissue, pH, temperature, and the presence of catalytic amounts of transitional metals (33, 61). The latter two forms of NO can be transferred readily to endogenous thiol groups on proteins to form nitrosothiols. This reaction, termed S-nitrosylation (transnitrosylation) is a covalent post-translational modification that may play an important role in the regulation of function of many proteins including ion channels (17). In the case of the well-studied NMDA receptor redox site, S-nitrosylation decreases NMDA receptor activity, presumably by favoring formation of disulfide bonds between critical cysteine residues, similar to oxidation with DTNB (33).

Even though there is good evidence that NO is produced in peripheral nociceptors and is upregulated in sensory neurons after nerve injury (34), its function in peripheral nociception is uncertain. For example, some studies suggest that NO can act as a pro-algesic agent, facilitating prostaglandin-induced hyperalgesia (1), while other studies have shown that NO may have peripheral antinociceptive effects (52, 59). These contrasting effects of NO donors may result from different metabolic conditions in tissue that can favor generation of different forms of NO. There is strong evidence that NO is produced endogenously in cholinergic projection neurons in the thalamus (73). These fibers are implicated in the regulation of oscillatory behavior of thalamic neurons associated with arousal and sleep (41); so we focus here on the modulation of voltage-gated calcium channels in the thalamus and DRG cells.

Unlike other forms of redox-based reactions that are reported mostly for T-type channels, NO-related molecules have been reported to modulate both T-type and HVA voltage-gated calcium currents. For example, Yoshimura *et al.* (78) have reported that N-type HVA calcium currents in DRG cells are modulated by S-nitroso-acetyl-penicillamin (SNAP) *via* a G-protein-dependent pathway. This conclusion was based on the fact that inhibition of calcium current was abolished in the presence of specific soluble guanylate cyclase inhibitor 1H-(1,2,4) oxadiazolo (4,3-a) quinoxalin-1-one (ODQ) and was mimicked by the membrane-permeant cyclic guanosine monophosphate (cGMP) analogue 8-bromo-guanosine-3',5'-cyclomonophosphate. Similarly, SNAP inhibited in L-type calcium channels of bovine chromaffin cells in a cGMP-dependent manner (9).

Based on the fact that T-type channels are modulated by ROS, it was reasonable to hypothesize that RNS and

S-nitrosothiols (SNOs) also may modulate T-currents in thalamic and DRG neurons. It was found in one study that extracellular application of several SNOs, including S-nitrosoglutathione (SNOG), S-nitrosocysteine (SNOC), and SNAP, similarly inhibited T-type currents in whole-cell recordings from intact nRT (Ca_v3.2 and Ca_v3.3 currents) and TC (Ca_v3.1 currents) neurons in brain slices (24). The effects of SNOs were strongly stereoselective given that L-S-nitrosocysteine (L-SNOC) was five- to sixfold more effective in inhibiting T-type current than was D-S-nitrosocysteine (D-SNOC). The effects of SNOG were abolished if cells had been treated with NEM, an irreversible and relatively specific alkylating agent, and were minimally affected by 8-Br cGMP. In addition, bath applications of SNOG inhibited T-type currents in excised outside-out patches and whole-cell recordings to a similar extent, with minimal effect on cell-attached recordings, suggesting a direct effect on the external side of neuronal membrane. Biophysical studies indicated that SNOG decreased the availability of T-type channels at physiological potentials by modifying gating and stabilizing inactive states of the channel. In current-clamp experiments, SNOG diminished the amplitude of both LTCs and resulting burst firing with minimal effects on the passive membrane properties of nRT neurons. Taken together, the results suggested that SNOs may be a class of endogenous agents that control the functional states of the thalamus by inhibiting T-type channels, likely *via* S-nitrosylation. However, the study did not address molecular mechanisms for effects of SNOs on T-type currents. Thus, in the following study the same group examined the effects of SNOG on T-currents in acutely dissociated DRG cells and recombinant T-channel currents in human embryonic kidney (HEK) cells (30) and found that SNOG had biphasic effects on DRG T-current, with reversible transient increase in the amplitudes of T-currents followed by inhibition that was very slowly and incompletely reversible. The enhancing effect of SNOG on DRG T-currents, but not the inhibitory effect, was eliminated if cells were incubated with the nonspecific metal chelator diethylenetriaminepentaacetic acid (DTPA), suggesting different mechanisms for T-current modulation. The inhibitory effect of SNOG on T-current in DRG cells was abolished with pretreatment of cells with NEM, similar to thalamic T-current, but was not affected with co-applications of ODQ. These results suggested that, as in thalamus, inhibitory effect of SNOG upon DRG T-currents is likely *via* direct S-nitrosylation and not *via* G-protein-dependent processes. Further, in HEK cells stably transfected with human variants of T-channel isoforms, SNOG similarly inhibited recombinant Ca_v3.1, Ca_v3.2, and Ca_v3.3 currents. Further molecular studies using mutated channels in which four conserved cysteine residues in repeat I and II of the Ca_v3.2 channels were replaced with alanines (Fig. 6) confirmed that, as for effects of LA, these four cysteines are critical for the inhibitory effects of SNOG on Ca_v3.2 currents (30). Thus, it appears that these cysteine residues are covalently modified by oxidation with multiple endogenous redox agents.

Hence, it seems that T-channels are mainly modulated *via* direct interactions of SNOs with the conserved cysteine residues on the outer pore of repeats I and II of the T-channels (24, 30). In contrast, literature suggests that DRG HVA calcium channel subtypes are modulated by SNOs *via* G-protein-dependent processes (78). Figure 8 represents schematically these different mechanisms of T-type and HVA calcium

FIG. 8. Scheme depicts dual modulation of T-type and HVA-type voltage-gated calcium channels by SNOs-related molecules in DRG cells. T-type current is likely directly modulated *via* covalent modification of putative thiol groups on the channel, while HVA-type currents are indirectly modulated *via* increase in intracellular concentration of cGMP. SNOG, S-nitrosoglutathione; SNAP, S-nitrosoacetyl-penicillamine; SNAC, S-nitrosocysteine; cGMP, cyclic guanosine mono phosphate; SNOs, S-nitrosothiols.

channel modulation with NO-related molecules in DRG cells. However, at least one study reported that neuronal HVA calcium currents in vestibular hair cells display a dual regulation by NO involving direct S-nitrosylation reactions and indirect stimulation of a cGMP-dependent signaling pathway (2). It also has been shown that recombinant T-channels can be modulated by G-protein-dependent mechanisms (16, 75). Thus, it remains to be determined whether NO may modulate some native T-currents *via* indirect pathways involving G-proteins.

Concluding Remarks

Several studies have identified many endogenous and exogenous redox agents that can regulate function of neuronal voltage-gated calcium channels and consequently fine-tune neuronal excitability and synaptic transmitter release. There is particularly good evidence for the involvement of redox sites on T-type channels in pain signaling. This is important since targeting channel regulation rather than producing channel inhibition may represent a new approach to treating various chronic pain conditions. Further, alterations in neuronal redox potential, concentrations of trace metal ions, and free radicals may play a role in a host of other diseases like neurodegenerative disease including Parkinson's disease, neuronal ischemia, epilepsy, Alzheimer's disease, Menke's disease, and Wilson's disease (6, 33, 37, 64). Further, it has been well documented that among other T-channel isoforms Ca_v3.2 channels are selectively upregulated in conditions associated with subacute and chronic hypoxia (35). Since hypoxic tissue conditions may favor generation of ROS, it is also possible that endogenously generated ROS may modulate Ca_v3.2 currents *via* H191 residue. Future studies would benefit from the use of transgenic animals lacking H191 and other cysteine-containing sites of Ca_v3.2 to address *in vivo* the potential roles of redox modulation of T-channels in physiological and pathological conditions.

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Abbreviations Used

$3\beta\text{OH}$ = (3 β ,5 α ,17 β)-17-hydroxyestrane-3-carbonitrile
ADP = after-depolarizing potential
CCI = chronic constrictive injury
cGMP = cyclic guanosine monophosphate
CNS = central nervous system
DHLE = dihydrolipoic acid
DRG = dorsal root ganglion
DTNB = 5,5'-dithio-bis(2-nitrobenzoic acid)
DTPA = diethylenetriaminepentaacetic acid
DTT = dithiothreitol
GABA = γ -amino-butyric acid
 H_2S = hydrogen sulfide
HEK = human embryonic kidney
HVA = high-voltage-activated
LA = α -lipoic acid
LD = laterodorsal
LTCS = low threshold calcium spike
LVA = low-voltage-activated
MCO = metal-catalyzed oxidation
MTSET = 2-(trimethylammonium) ethyl methanethio sulfonate
 N_2O = nitrous oxide, laughing gas
NEM = N-ethyl-maleimide
NMDA = N-methyl-D-aspartate
NNC 55-0396 = [(1S,2S)-2-(2-(N-[(3-benzimidazol-2-yl)propyl]-N-methylamino)ethyl)-6-fluoro-1,2,3,4-tetrahydro-1-isopropyl-2-naphthyl cyclopropanecarboxylate dihydrochloride
NO = nitric oxide
nRT = nucleus reticularis thalami
ODQ = 1H-(1,2,4) oxadiazolo (4,3-a) quinoxalin-1-one
RNS = reactive nitrogen species
ROS = reactive oxygen species
SNAP = S-nitroso-acetyl-penicillamin
SNOC = S-nitrosocysteine
SNOG = S-nitrosoglutathione
SNOs = S-nitrosothiols
VB = ventrobasal