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Modulators of Calcium Influx Regulate Membrane Excitability in Rat Dorsal Root Ganglion Neurons

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Abstract

Background—Chronic neuropathic pain resulting from neuronal damage remains difficult to treat, in part due to incomplete understanding of underlying cellular mechanisms. We have previously shown that inward Ca^{2+} flux (I_{Ca}) across the sensory neuron plasmalemma is decreased in a rodent model of chronic neuropathic pain, but the direct consequence of this loss of I_{Ca} on function of the sensory neuron has not been defined. We therefore examined the extent to which altered membrane properties after nerve injury, especially increased excitability that may contribute to chronic pain, are attributable to diminished Ca^{2+} entry.

Methods—Intracellular microelectrode measurements were obtained from A-type neurons of dorsal root ganglia excised from control rats and those with neuropathic pain behavior following spinal nerve ligation. Recording conditions were varied to suppress or promote I_{Ca} while biophysical parameters and excitability were determined.

Results—Both lowered external bath Ca^{2+} concentration and blockade of I_{Ca} with bath cadmium diminished the duration and area of the afterhyperpolarization (AHP), accompanied by decreased current threshold for action potential (AP) initiation and increased repetitive firing during sustained depolarization. Reciprocally, elevated bath Ca^{2+} increased the AHP and suppressed repetitive firing. Voltage sag during neuronal hyperpolarization, indicative of the cation-nonspecific H-current, diminished with lowered bath Ca^{2+} , cadmium application, or chelation of intracellular Ca^{2+} . Additional recordings with selective blockers of I_{Ca} subtypes showed that N-, P/Q, L-, and R-type currents each contribute to generation of the AHP, and that blockade of any of these as well as the

T-type current slows the AP upstroke, prolongs the AP duration, and (except for L-type current) decreases the current threshold for AP initiation.

Conclusions—Taken together, our findings show that suppression of I_{Ca} decreases the AHP, reduces the hyperpolarization-induced voltage sag, and increases excitability in sensory neurons, replicating changes that follow peripheral nerve trauma. This suggests that the loss of I_{Ca} previously demonstrated in injured sensory neurons contributes to their dysfunction and hyperexcitability, and may lead to neuropathic pain.

Implications Statement—Loss of inward Ca^{2+} current in A-type neurons, such as follows peripheral nerve injury, contributes to increased sensory neuron excitability. Measures that increase inward Ca^{2+} flux may potentially be therapeutic for painful peripheral neuropathy.

Introduction

Activity of primary sensory neurons induced by natural stimulation or by processes such as trauma, inflammation and nerve injury, is the origin of all but a small fraction of painful sensations. Sensory afferents are additionally the afferent source of intraoperative nociceptive reflexes that trigger cardiovascular, ventilatory and neurohumoral efferent pathways. The central role of these neurons in painful conditions and anesthesia makes it critical to understand the regulation of their excitability. The sensory neuron plasmalemma is equipped with a variety of voltage-activated ion channels conducting Na^+ , K^+ or Ca^{2+} , which together determine the biophysical function of the membrane. Currents through voltage-activated Ca^{2+} channels play a critical double role. First, inward Ca^{2+} flux (I_{Ca}) depolarizes the cell, and thus contributes to action potential (AP) formation. Once reaching the intracellular compartment, however, Ca^{2+} is also a key second messenger, controlling a broad range of neuronal functions including kinase activity, neurotransmitter release, cell differentiation and growth, genetic expression, and cell death. Membrane events are regulated by intracellular Ca^{2+} through depression of I_{Ca} (1), stimulation of Ca^{2+} -activated Cl^- channels ($I_{Cl(Ca)}$) (2), and opening of Ca^{2+} -activated K^+ channels ($I_{K(Ca)}$), which are widely distributed among dorsal root ganglion (DRG) neurons of all sizes (3).

Pain resulting from neuronal damage is a special case in which the primary pathology involves disrupted regulation of sensory neuron excitability at the site of injury and in the DRG proximal to the injury (4–6). We and others have reported loss of both high-voltage activated (HVA) (3,7–9) and low voltage-activated (LVA) subtypes (10) of I_{Ca} in DRG neurons following peripheral nerve injury. Although the effects of decreased I_{Ca} upon neuronal membrane properties have been detailed in other cell types, the only studies examining I_{Ca} regulation of DRG neuronal excitability have employed the patch-clamp electrophysiological technique on dissociated neurons (11,12). This is problematic, since dialysis of the cytoplasm by the solution in the patch pipette disrupts natural biophysical events, as demonstrated by substantially prolonged action potential (AP) durations, more frequent afterdepolarizations following the AP, and diminished AHP amplitude, compared to microelectrode recording (2,6,13,14). For instance, although AP durations in control neurons are consistently less than 4mS when recorded extracellularly or by high resistance intracellular microelectrodes (6,15), durations may be as long as 60mS using the patch-clamp method (11), which indicates clearly abnormal recording conditions. Furthermore, intracellular regulation of Ca^{2+} concentrations is disrupted by the patch clamp technique (16), and amplifiers typically used for patch clamp recording are not ideally suited for recording APs (17). Finally, dissociation itself produces an injury-like effect on neuronal excitability (18).

Since injury also produces substantial changes in voltage-gated Na^+ and K^+ channels of sensory neurons (19,20), the overall purpose of this study is to clarify the extent to which altered membrane functions after injury, especially increased excitability, are specifically attributable

to diminished Ca^{2+} entry. Therefore, the present study examines the influence of changes in I_{Ca} and intracellular Ca^{2+} upon AP dimensions, threshold for initiation of APs, and repetitive firing. We employed a technique designed to minimize disruption of cellular Ca^{2+} signaling, by using intracellular microelectrode recording from intact DRGs.

Materials and Methods

All procedures used in the study were approved by the Animal Resource Center of the Medical College of Wisconsin. Experiments were performed on male Sprague-Dawley rats (200–300g) obtained from a single vendor (Taconic Inc., Germanville, NY). Our general strategy was to record from intact DRGs, which avoids disruptive effects of cell dissociation, and to employ multiple pharmacologic measures that together will delineate the role of I_{Ca} in sensory neuron activation.

Tissue Preparation

Ganglia were removed 21±4 days after surgery, a time at which hyperalgesia has fully developed (21). Rats were anesthetized with halothane in oxygen, and a laminectomy was performed up to the second thoracic level while the surgical field was perfused with artificial cerebrospinal fluid (aCSF, in mM: NaCl 128, KCl 3.5, MgCl_2 1.2, CaCl_2 2.3, NaH_2PO_4 1.2, NaHCO_3 24.0, glucose 11.0) aerated with 5% CO_2 and 95% O_2 to maintain a pH of 7.35. The fourth and fifth lumbar DRGs and attached dorsal roots were removed and the connective tissue capsule dissected away from the ganglia under 20× magnification. Ganglia were transferred to a glass-bottomed recording chamber and perfused with 35°C aCSF. The proximal cut end of dorsal roots was placed on a pair of platinum wire stimulating electrodes. DRG neurons were viewed using an upright microscope equipped with differential interference contrast optics and infrared illumination. Neuronal soma diameter was determined with the focal plane adjusted to reveal the maximum somatic area using a calibrated video image.

Electrophysiological Recording

Intracellular recordings were performed with microelectrodes fashioned from borosilicate glass (1mmOD, 0.5mmID, with Omega fiber – FHC Bowdoinham, USA) using a programmable micropipette puller (P-97, Sutter Instrument Company, Novato, CA). Microelectrode resistances were 80–120 MΩ when filled with 2M potassium acetate. Neurons were predominantly selected from the two outermost cell layers of the dorsal medial aspect of the DRG, and were impaled under direct vision by minimally indenting the membrane and then applying an oscillating current to the microelectrode. Data were acquired after stable recordings were achieved, typically within 5min of puncture. Membrane potential was recorded using an active bridge amplifier (Axoclamp 2B, Axon Instruments, Foster City, CA), except in protocols requiring simultaneous current injection through the electrode, for which we used discontinuous current clamp recording mode. This allows accurate recording of voltage, although the noise level is increased compared to bridge mode. During discontinuous mode recording, the switching frequency from current injection to recording was 2kHz, and full settling of the electrode charge was confirmed. Currents were filtered at 1 kHz (discontinuous mode) and 10kHz (bridge mode), and then digitized at 10kHz (discontinuous mode) or 40 kHz (bridge mode; Digidata 1322A and Axograph 4.9, Axon Instruments) for data acquisition and analysis. Somatic APs were produced by axonal conduction of APs from a dorsal root site 18–22mm distant, where the root was stimulated with square-wave pulses of up to 90mA lasting 0.06mS *via* bipolar electrodes, except in specified protocols in which current was injected through the recording electrode.

Inclusion criteria were a resting membrane potential (RMP) negative to −50mV, and an AP amplitude greater than 40mV. Together, these excluded approximately 6% of recordings. APs

measures (Fig. 1) were obtained from single traces after consistency of dimensions was confirmed by comparison to 10 sequential APs. The presence of a hump or inflection on the descending limb of the AP was determined by examination of the differentiated trace (Fig. 1B and 1C). Determinations of AP and AHP durations and the area under the curve for the AHP (AHParea) were performed digitally. Input resistance was calculated from the minimum potential achieved during 100ms hyperpolarizing current (0.5nA) injection through the recording electrode (15). Sensory neurons show time-dependent rectification ("sag") in response to hyperpolarization, predominantly attributable to the hyperpolarization-induced H-current (22). If a neuron showed sag with 0.5nA injection, additional traces during injection of lower depolarizing currents were examined to assure that the input resistance calculation was not affected. Sag was quantified as the fractional return from the peak hyperpolarized membrane potential back towards RMP during injection of a 1.2nA hyperpolarization current for 100ms. Rheobase was determined as the minimum current able to elicit an AP during incremental depolarizing current injection of 0.5–10nA for 100ms. The pattern of impulse generation was determined during current injection steps beyond rheobase, at which neurons either produced single APs or fired repetitively. The influence of bath Ca^{2+} level or drug upon AP firing pattern was measured at a depolarizing voltage that first produced a bath Ca^{2+} - or drug-induced difference in the number of APs generated.

Neurons were classified by the method of Villiere and McLachlan (15). Conduction velocity (CV) was measured by dividing the distance between stimulation and recording sites by the conduction latency. Neurons with dorsal root $\text{CV} < 1.5\text{m/S}$ were considered C-type, neurons with $\text{CV} > 15\text{m/S}$ were considered A α / β -type, and neurons with $\text{CV} > 1.5\text{m/S}$ but $\text{CV} < 10\text{m/S}$ were considered A δ -type. For neurons with CV between 10 and 15m/S, long AP duration was used to categorize the cells as A δ -types (15). Too few C-type neurons were successfully recorded to provide statistical analysis, and are not reported here.

Ca^{2+} current modulation

The influence of I_{Ca} on membrane biophysical events was revealed by various techniques that alter I_{Ca} or intracellular Ca^{2+} concentration. Baseline electrophysiologic parameters were measured in neurons in aCSF, after which they were exposed by bath change to an external solution identical except for a low Ca^{2+} concentration, in which CaCl_2 was substituted with MgCl_2 ($n = 25$ A α / β , 6 A δ). This resulted in a measured Ca^{2+} concentration of $0.35 \pm 0.02\text{mM}$ ($n = 3$ measurements, by Ca^{2+} -sensitive electrode, ABL 515, Radiometer, Copenhagen). Magnesium was added (final concentration 3.5mM) to exclude the possible influence of changed surface charge, which is otherwise a potent source of neuronal stimulation (23), and to maintain a constant divalent cation effect on potassium channels (24). Stable recordings were achieved after a wash-in interval of 3 min. Using conventional patch-clamp recording of whole-cell I_{Ca} (7) in dissociated DRG neurons, we confirmed that only $6 \pm 4\%$ of baseline I_{Ca} remains during these low bath Ca^{2+} conditions ($n = 4$; Fig. 2). In supplementary experiments, I_{Ca} was decreased in other neurons ($n = 13$ A α / β , 7 A δ) by bath exchange to aCSF containing cadmium $200\mu\text{M}$, a widely-used blocker of neuronal voltage-activated Ca^{2+} channels (13, 25).

In other recordings ($n = 6$ A α / β and 1 A δ neurons), ethylenediamine-tetraacetic acid (EDTA) $500\mu\text{M}$ was added to the potassium acetate micropipette solution to chelate intracellular Ca^{2+} upon entry, using a previously established iontophoretic method in which injection is achieved using a 1nA hyperpolarizing current for 60s (26). Preliminary studies showed that current injection of this magnitude alone had no effect on measured parameters. Further studies ($n = 10$ A α / β , 1 A δ neurons) used the fast Ca^{2+} chelator 1,2-Bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid as the cell-permeant acetoxymethyl ester (BAPTA-AM) to lower intracellular concentration (27). This was dissolved in bath solution ($200\mu\text{M}$) and delivered by

a microperfusion technique from a pipette with a 10 μ diameter tip that was positioned 200 μ from the impaled neuron, and ejected continuously by pressure applied to the back end of the pipette (Picospritzer II, General Valve Corp, Fairfield, NJ). Preliminary experiments in which cell depolarization was measured during microperfusion application of solutions of various K⁺ concentrations indicated an effective 5-fold dilution of pipette solution into the bath at the cell membrane, so the BAPTA-AM concentration was approximately 40 μ M at the cell surface.

In order to further test the role of intracellular Ca²⁺ on sensory neuron function, increased I_{Ca} was provoked in other neurons by bath change to a solution with Ca²⁺ concentration elevated to 5mM or 7mM. Since the results of these two concentrations were comparable, the results were pooled for analysis (n = 8 A α / β and 9 A δ neurons). Patch-clamp measurement of I_{Ca} showed a 35 $\pm$ 16% increase of I_{Ca} under these conditions (n=4; Fig. 2).

Selective modulation of Ca²⁺ channel subtypes

Sensory neurons express a variety of voltage-gated Ca²⁺ currents, including subtypes with both LVA and HVA activation characteristics (28). Since the methods described above affect all voltage-activated Ca²⁺ currents indiscriminately, we examined the contributions of specific Ca²⁺ channel subtypes to membrane activation before and after microperfusion application of selective blockers. Specifically, the agents and concentrations in the application pipette were as follows: nickel (2mM) and mibefradil (5 μ M) to block the T-type LVA current, nitrendipine (50 μ M) to block the L-type HVA current, BayK 8644 (50 μ M) to amplify L-type current, SNX-111 (2.5 μ M) to block the N-type HVA current, ω -Aga-IVA (2.5 μ M) to block the P/Q-type HVA current, and SNX-482 (2.5 μ M) to block the R-type HVA current. Since the peptide toxins are irreversible, no more than 2 recordings were made from widely separated sites on each ganglion. In other experiments, all the selective blockers (nitrendipine, SNX-111, ω -Aga-IVA, SNX-482) were administered together to examine the effect of complete HVA I_{Ca} blockade. The vital dye fast-green, which is compatible with neuronal electrophysiological recording (29,30), was included at a final concentration of 0.5% in order to permit visual confirmation of drug delivery to the recorded cell during application, and to ensure that new recordings were made from parts of the ganglion that had received no previous drug exposure. Cytochrome C 0.1% was included with the peptide toxins to inhibit nonspecific binding to glass surfaces. The nitrendipine solution included dimethyl sulfoxide at a final concentration of 0.25% in the delivery pipette. Control experiments were performed to determine if pressure ejection of vehicle alone altered recorded parameters. In this portion of the study, dimensions were measured from APs averaged from 10 sequential traces before and 5min after initiation of drug administration, at which time stable changes had evolved.

SNX-111 was the kind gift of Dr. Scott Bowersox from Neurex Corporation (Menlo Park, CA), while ω -Aga-IVA was the gift of Dr. Nicholas Saccomano from Pfizer (Groton, CT), and SNX-482 was purchased from Peptides International (Louisville, KY). All other agents were purchased from Sigma-Aldrich Co. (St. Louis, MO).

Data Analysis and Statistics

The control group included cells from both L4 and L5 ganglia in animals having undergone skin incision only. It was not the purpose of the study to determine differences in biophysical parameters between cell types or injury categories, as has been done by us before (6), so these comparisons were not tested statistically. Data were analyzed using Excel (Microsoft Co. Seattle, USA) and Statistica 6.0 (StatSoft, Tulsa, USA). Averaged results are expressed as means $\pm$ SD. The effect of drug application was evaluated using paired Student's *t*-test, which allows identification of significant drug effects upon measured parameters in the context of natural variability between neurons that results in large SD and differences between group means under baseline conditions. Nonparametric data were evaluated by cross-tabulation with

significance tested by Pearson Chi-square for a main effect and planned *post hoc* paired comparisons by Fisher's exact test. The threshold for statistical significance was accepted at $P < 0.05$.

Results

Electrophysiological data were obtained from a full range of DRG neuronal sizes (average diameter $39.7 \pm 0.6 \mu$ for A α/β , $34.2 \pm 0.8 \mu$ for A δ). As shown in Figure 3, the distributions of neuronal diameters for A α/β and A δ categories extensively overlap. Main results for altered bath Ca^{2+} for are summarized in Table 1. Effects of lowered and raised external bath Ca^{2+} and cadmium administration were reversible on washout (data not shown).

Influence of I_{Ca} modulators on AP parameters

Low bath Ca^{2+} levels depolarized the RMP in A δ neurons and decreased AP amplitude in A α/β and A δ neurons (Fig. 4). Bath Ca^{2+} withdrawal also led to the disappearance of the hump on the descending limb of the AP in 2 of 4 inflected A α/β neurons and in 3 of 5 inflected A δ neurons. Similarly, cadmium application abolished the inflection in both of 2 inflected neurons, so that decreasing I_{Ca} eliminated the inflection in 7 of 11 inflected neurons overall ($P < 0.01$). However, high bath Ca^{2+} did not elicit the appearance of *de novo* inflections. Thus, while I_{Ca} is necessary to produce an inflection, not all cells can do so even if intracellular Ca^{2+} levels are increased. Bath Ca^{2+} withdrawal had no significant effect on AP duration in either A α/β or A δ neurons, and cadmium similarly had no effect on AP duration in A α/β neurons (from 0.85 ± 0.19 to 0.82 ± 0.18 ms; $P = 0.25$, $n = 13$) or in A δ neurons (from 0.96 ± 0.13 to 0.90 ± 0.12 ms; $P = 0.08$, $n = 7$). Contrasting effects on inflection and duration are attributable to our measurement of the duration at the base of the AP. Buffering of intracellular Ca^{2+} with BAPTA-AM did prolong the AP duration (from 0.88 ± 0.08 to 0.96 ± 0.11 ms; $P < 0.05$, $n = 9$). BAPTA-AM also decreased the AP upstroke velocity (from 425 ± 54 to 366 ± 58 mV/ms; $P < 0.05$, $n = 9$), as did cadmium blockade of I_{Ca} in A δ neurons (from 256 ± 51 to 246 ± 52 mV/ms; $P < 0.05$, $n = 7$).

Influence of I_{Ca} modulators upon AHP

Low bath Ca^{2+} levels caused a decrease in AHP duration and area for both A α/β and A δ neurons (Fig. 4, Table 1). This observation was supported by decreased AHP area when I_{Ca} was blocked by cadmium in A α/β neurons (from 238 ± 269 to 147 ± 152 mV·mS; $P = 0.01$, $n = 13$), although a change was not confirmed in A δ neurons (from 227 ± 234 to 147 ± 161 mV·mS; $P = 0.11$, $n = 7$). Combined application of all HVA I_{Ca} blockers to neurons ($n = 7$ A α/β and 5 A δ neurons) similarly reduced AHP duration (from 21.1 ± 22.0 to 13.0 ± 15.2 mS; $P < 0.01$) and AHP area (from 397 ± 570 to 204 ± 344 mV·mS; $P < 0.05$). The importance of Ca^{2+} influx for generating the AHP was further substantiated by chelating intracellular Ca^{2+} with EDTA iontophoresis, which similarly decreased AHP area (from 103 ± 76 to 79 ± 67 mV·mS; $P = 0.05$, $n = 7$; Fig. 4), as did BAPTA-AM application (from 187 ± 163 to 134 ± 111 mV·mS; $P < 0.05$, $n = 9$). Reciprocally, bath change to high Ca^{2+} concentration increased the AHP duration and area in A α/β neurons (Fig. 5), providing confirmation of the dependence of the AHP on I_{Ca} . Variability in baseline AHP dimensions between groups reflects natural heterogeneity of $\text{I}_{\text{K(Ca)}}$ ion sensory neurons (31).

Influence of I_{Ca} modulators upon excitability

Low bath Ca^{2+} solution decreased rheobase in A α/β neurons (Fig. 6, Table 1), which also followed blocking I_{Ca} using cadmium (from 1.77 ± 1.6 nA to 1.45 ± 1.27 nA; $P = 0.02$, $n = 13$), and by application of BAPTA-AM (from 3.10 ± 1.7 nA to 2.39 ± 1.72 nA; $P < 0.01$, $n = 7$). Three of 38 A α/β and 1 of 10 A δ neurons fired repetitively during sustained depolarization under baseline conditions, but 9 additional A α/β neurons fired repetitively in low Ca^{2+} solution (P

<0.05). The average number of APs in A α / β neurons during these sustained (100ms) depolarizations followed a similar pattern, increasing from 1.3 ± 0.9 to 10.9 ± 13.4 ($P = 0.01$) with lowered bath Ca^{2+} . Cadmium similarly provoked novel repetitive firing in A α / β neurons (from 3 to 6 of 13 cells), and A δ neurons (from 2 to 4 of 6 cells, Fig. 7), as did BAPTA-AM (from 2 to 5 of 7 cells; average rate increased from 1.1 ± 0.8 to 13.1 ± 5.6 , $P < 0.05$, $n = 7$). During combined application of HVA blockers, 4 of 4 nonaccommodating neurons fired more rapidly.

Changing bath to high Ca^{2+} solution had the opposite effect on the pattern of neuronal firing, causing 2 of 7 repetitively firing A α / β neurons (Fig. 6) and 1 of 2 repetitively firing A δ neurons to become adapting and fire only once during sustained depolarization.

Hyperpolarization-induced voltage rectification

The depolarizing sag in membrane potential during hyperpolarization was diminished by low Ca^{2+} bath solution in both A α / β and A δ neurons (Fig. 7A, Table 1), as did combined application of HVA I_{Ca} blockers (from $21.8 \pm 18.0\%$ to $12.4 \pm 18.0\%$; $P < 0.05$, $n = 7$ A α / β and 5 A δ neurons), cadmium application (from $37.6 \pm 17.6\%$ to $23.2 \pm 11.4\%$; $P < 0.05$, $n = 5$ A δ neurons), and chelation of intracellular Ca^{2+} with either EDTA (from $19.23 \pm 17.2\%$ to $14.9 \pm 16.8\%$; $P < 0.05$, $n = 5$; Fig. 7B) or BAPTA-AM (from $28.0.6 \pm 19.5\%$ to $13.9 \pm 17.6\%$; $P < 0.05$, $n = 8$). Together, these observations indicate a Ca^{2+} dependency for the hyperpolarization-induced inward current.

Response to selective blockers of Ca^{2+} channel subtypes

The regulation of AP dimensions and neuronal excitability by individual I_{Ca} subtypes was further examined using selective blockers on 59 A α / β neurons (Table 3, Fig. 8). Mibefradil, in contrast to low Ca^{2+} bath solution, hyperpolarized the RMP, which may be attributable to nonspecific effects of this agent (32,33). AP amplitude was increased by nickel and SNX-482 but decreased by nitrendipine. All agents prolonged AP duration, and all agents except nickel and nitrendipine slowed the CV. AHP area was decreased by SNX-111, nitrendipine, ω -Aga-IVA, and less so by SNX-482. All blockers except nitrendipine decreased rheobase, indicating an excitatory influence, although repetitive firing during sustained depolarization was not affected by individual application of the selective blockers. Voltage sag after membrane hyperpolarization was decreased only by mibefradil, ω -Aga-IVA, and SNX-482. None of the agents produced a consistent change in the presence or absence of inflection. The only effect of pressure ejection of vehicle alone ($n = 9$ A α / β neurons and 1 A δ neuron) was to decrease the slope of the ascending limb of the AP (dV/dt), which was decreased to a greater extent by all agents.

We extended our examination of the role of Ca^{2+} entry by amplifying I_{Ca} with the L-current agonist BayK 8644 ($n = 10$ A α / β neurons and 1 A δ neuron; Table 3), which slowed the CV, increased the AP amplitude, and substantially prolonged the AP. The effect on the AHP showed divergent patterns. Maximum AHP amplitude was decreased in 10 neurons (Figs. 9A and 9B), but increased in another (Fig. 9C). Expansion of the AHP area was also seen in two other neurons that developed new AHPs following the afterdepolarization, which were more delayed than the AHP present at baseline (Fig. 11B). Although there were no afterdepolarizations under baseline conditions, these appeared in 7 of 11 neurons during application of BayK 8644, either with loss (Fig. 9A) or expansion (Fig. 9B) of the AHP. In contrast, only 2 small afterdepolarizations developed during application of the selective blockers to 59 neurons (one cell each for SNX-111 and ω -Aga-IVA, $P < 0.001$). BayK 8644 decreased rheobase in all neurons, and 9 of 10 neurons either initiated repetitive firing during application of BayK 8644 or increased firing rate.

Discussion

This study was designed to identify changes in membrane electrical properties of sensory neurons attributable to the loss of inward I_{Ca} , and thereby indicate the consequences of injury-related I_{Ca} loss that we have previously identified. To this end, we manipulated ionic conditions and applied a variety of pharmacologic agents to DRG neurons in their non-dissociated state, while recording membrane events in a manner least likely to disrupt intracellular Ca^{2+} handling. This approach lacks the ability to identify effects on isolated specific currents that patch-clamp membrane control provides, but it allows characterization of the natural interactions of the cell's full complement of membrane voltage-gated and Ca^{2+} -activated channels. Moreover, processes that sequester and extrude intracellular Ca^{2+} remain largely intact. Therefore, the role of I_{Ca} can be put into a physiologically relevant context in the sensory neuron soma. Our observations collectively indicate that loss of I_{Ca} and depression of the intracellular Ca^{2+} level are accompanied by elevated excitability of DRG neurons. Although similar events are likely in C-type neurons, this cannot be proved as those were not examined here.

At any moment, the neuronal transmembrane potential is the result of ionic conductance across the membrane through multiple channel types. The function of these channels is linked, since membrane voltage and intracellular Ca^{2+} regulate opening of these channels, but levels of both voltage and Ca^{2+} concentration are simultaneously controlled by currents through the various channels. Thus, it is impossible *a priori* to predict the final effect on AP dynamics of a change in a single channel. Central to this study is the specific case of divergent electrical effects that may arise from a loss of I_{Ca} . On the one hand, inward Ca^{2+} flux depolarizes the membrane such that decreasing I_{Ca} should inhibit generation of APs and diminish axonal conduction success. However, the entry of a diminished amount of Ca^{2+} results in less recruitment of $I_{K(Ca)}$, which in turn will lead to the excitatory effects of membrane depolarization and elevated membrane resistance (34). In cerebellar Purkinje neurons, for instance, block of I_{Ca} enhances firing since the diminished outward K^+ flux outweighs the decreased inward Ca^{2+} flux (25). Further complexity is added by participation of $I_{Cl(Ca)}$ (2), a depolarizing current that competes with inward $I_{K(Ca)}$. Even membrane hyperpolarization may either directly stabilize the neuron or create conditions for repetitive firing by phasically reactivating Na^+ currents and LVA Ca^{2+} currents. As a result of the intricate interplay of these various currents, experimental observation is necessary to resolve the balance of effects in a natural system.

To determine the end result of decreased I_{Ca} , we modulated inward I_{Ca} using techniques each of which has imperfections. Bath Ca^{2+} withdrawal dependably decreases the driving force for ionic flux through the Ca^{2+} channels but may also change the surface potential and thus disrupt voltage gradients in the immediate microdomain of the channel (23). We compensated for this by reciprocal Mg^{2+} addition, although divalent cations may differ in extent of effects on various channels (24). A factor that further complicates interpretation of Ca^{2+} withdrawal experiments is the presence in neurons of a nonselective cation channel that is suppressed by external Ca^{2+} (35,36), particularly in nociceptive sensory neurons (37). Calcium withdrawal may thus initiate a separate excitatory inward current, which potentially accounts for differences between findings with bath Ca^{2+} withdrawal and I_{Ca} blockade. Also, a direct interaction of Ca^{2+} with the Na^+ channel pore has been demonstrated in which Na^+ channels that are not Ca^{2+} -occupied fail to close (38). We elevated bath Ca^{2+} to predictably increase the concentration gradient driving Ca^{2+} entry. This, however, does not necessarily produce opposite electrophysiological effects compared to Ca^{2+} withdrawal, for instance on AP duration (39), as may be the case if Ca^{2+} -dependent processes are saturated in the baseline condition. Cadmium inhibits I_{Ca} globally, but lacks perfect selectivity since it may also minimally modulate Na^+ currents (25) and K^+ currents (24). Although peptide toxins for HVA I_{Ca} subtypes are highly specific, there is no comparably specific toxin for T-type currents. We therefore used both nickel and

mibefradil (40), which provided largely congruent results. Nitrendipine was chosen to block L-type current because of its greater specificity in DRG neurons compared to other dihydropyridine blockers (41). Despite these precautions, overlapping effects are noted even with the most selective toxins (42). Finally, modulation of excitability may conceivably also result through I_{Ca} regulation of release of regulatory peptides from glia and neurons in the DRG (43). Because of these various limitations, we employed multiple complementary techniques for decreasing and increasing Ca^{2+} entry to resolve the role of I_{Ca} .

AP Inflection is regulated by I_{Ca} modulators

Withdrawal of bath Ca^{2+} and application of cadmium both reduced the incidence of inflection of the descending limb of sensory neuron APs, presumably through the depression of I_{Ca} . Blockade of individual I_{Ca} subtypes had no effect on AP inflection, perhaps indicating the partial contribution of several subtypes to this feature. Our findings confirm observation on cranial nerve sensory neurons that identified a contribution of I_{Ca} to the inflection (39,44). This contrasts with the effect of nerve injury, which is accompanied by an increase in the frequency of AP inflection (6) despite the development of a concurrent loss of high-voltage and low-voltage activated Ca^{2+} currents (7,10). Since $I_{K(Ca)}$ participates in repolarization of the AP and competes with I_{Ca} in the formation of the inflection (31,45), a possible explanation is that injury induces a proportionately greater loss of $I_{K(Ca)}$ than I_{Ca} , through a decrease of Ca^{2+} -activated K^+ channel number or function. Indeed, we have recently identified such a post-injury deficit (46). Loss of voltage-gated K^+ current (20) might also contribute to more frequent inflections after injury. Although slow TTX-resistant Na^+ current also contributes to the AP inflection (39,47), injury is associated with a decrease of this current (48).

Regulation of AP parameters by I_{Ca} modulators

The RMP is depolarized and the AP amplitude is diminished by low bath Ca^{2+} levels but not by cadmium administration. This may be due to activation of an inward current during low extracellular Ca^{2+} conditions as noted above, although combined administration of HVA blockers also diminishes AP amplitude. Individual selective blockers have a divergent effects on AP amplitude, perhaps indicative of their variable contributions of I_{Ca} versus $I_{K(Ca)}$ during AP depolarization and repolarization. The decrease of AP amplitude following nerve injury (6) may thus be attributed either to loss of L-type current or simultaneous changes in Na^+ channels. Selective blockers of I_{Ca} consistently prolong AP duration, presumably by a dominant indirect inhibition of inward $I_{K(Ca)}$ (25), and thus duplicate the effect of nerve injury (6). The lack of effect of low bath Ca^{2+} and cadmium on AP duration may result from a less complete loss of I_{Ca} . Alternatively, the difference may be due to secondary effects of low extracellular Ca^{2+} noted above. Increased AP duration may contribute to neuropathic pain by enhancing synaptic neurotransmitter release (49).

Decrease of inward Ca^{2+} flux diminishes AHP and increases excitability

Although Ca^{2+} -independent K^+ currents may contribute to the formation of the AHP (50,51), previous studies have shown a critical dependence of the AHP upon I_{Ca} (52–54), which our observations now confirm in adult DRG neurons. AHP regulates a critical aspect of neuronal excitability by determining spike frequency adaptation and the ability of sensory neurons to maintain rapidly firing pulse trains (15,55,56). In this study, repetitive firing was amplified by lowering extracellular Ca^{2+} , while elevated Ca^{2+} had the opposite effect, indicating that the elevated excitability found in neurons after axonal injury may be the result of diminished I_{Ca} .

In various neuronal types, the source of Ca^{2+} that activates $I_{K(Ca)}$ is not uniformly distributed among Ca^{2+} channel subtypes, due to colocalization of specific isoforms of Ca^{2+} channels and Ca^{2+} -activated K^+ channels (57,58). In contrast, we found in sensory neurons that N-type, P/

Q-type and L-type currents all contribute to the generation of the AHP, with a lesser role for R-type current, and that LVA current (T-type) contributes minimally to activation of channels producing the AHP. This may explain why selective blockade of I_{Ca} subtypes minimally contributed to repetitive firing (2 cells of 43 became nonaccommodating), since burst firing is regulated by the combined contributions of more than a single channel subtype alone.

Enhancement of L-type current with the agonist BayK 8644 is associated with substantial prolongation of the AP and generation of afterdepolarizations, which may result directly from sustained inward Ca^{2+} flux or from activation of Cl^{-} channels. This latter option is supported by previous observations of large afterdepolarizations through activation of $I_{Cl(Ca)}$ (2), and BayK 8644 has previously been reported to activate Ca^{2+} -sensitive Cl^{-} current in rat DRG neurons (59). Repetitive firing was induced or enhanced by BayK 8644, and can be attributed to the influence of afterdepolarizations (60). Heterogeneity is evident, however, since increased Ca^{2+} influx from BayK 8644 initiates a new, sustained AHP in a subset of neurons, which highlights the complexity of competing Ca^{2+} effects.

The rheobase current, which is the depolarizing current just adequate to initiate an AP, is decreased in $A\alpha/\beta$ neurons during low bath Ca^{2+} and application of cadmium and most selective I_{Ca} blockers. We cannot explain the decrease in rheobase subsequent to lowering of I_{Ca} , but we note that it is not due in all cases to an increase in input resistance, such as might occur with decreased $I_{K(Ca)}$ or I_h . Therefore, loss of I_{Ca} must lead to an elevated intrinsic excitability of the neuronal membrane, making it easier for generator currents at natural and pathologic sensory transduction sites to initiate neuronal activity. The ubiquitous actions of Ca^{2+} /calmodulin (61) and Ca^{2+} -sensitive kinases (62–65) on a variety of membrane channels may contribute to membrane hyperexcitability during disruption of intracellular Ca^{2+} levels. Increased Ca^{2+} loading during Bay K 8644 application also destabilizes the sensory neuron, however, which points to a system in which normal membrane excitability is maintained only within a certain range of intracellular Ca^{2+} concentrations.

Blockers of I_{Ca} reduce hyperpolarization-induced voltage sag

Voltage sag as recorded in this study in sensory neurons is mostly due to the hyperpolarization-induced current I_h (15), which aids in setting the RMP and may generate excitability by contributing to the pacemaker potential of spontaneously active cells. However, I_h can also suppress excitation by decreasing input resistance in the resting neuron. In our study, depression of I_{Ca} decreased hyperpolarization-induced voltage sag. The loss of I_h in sensory neurons after bath Ca^{2+} withdrawal, application of I_{Ca} blockers, and intracellular Ca^{2+} buffering with EDTA and BAPTA-AM is similar in this respect to the effect of peripheral nerve injury (6). These findings indicate that cytoplasmic Ca^{2+} level regulates the hyperpolarization-induced inwardly rectifying current in sensory neurons, as has been reported for cortical and brainstem neurons (66,67), possibly by acting through the Ca^{2+} -calmodulin signaling pathway and Ca^{2+} /calmodulin-dependent protein kinase II activation (68). Nerve injury may additionally decrease expression of the channel underlying the I_h current (69).

Implications for neuropathic pain

The findings of this study reveal that suppression of I_{Ca} increases DRG neuron excitability. Therefore, the loss of I_{Ca} associated with peripheral nerve injury may in part account for the hyperexcitability of the sensory pathway in neuropathic pain states. Furthermore, processes that lower extracellular Ca^{2+} concentration may be expected to result in neuronal excitation. Both neural trauma (70) and intense neuronal activity, such as happens at the moment of peripheral nerve injury, depress extracellular Ca^{2+} concentrations (71,72), and therefore may initiate processes that amplify sensory processing even in the absence of changes in Ca^{2+} channels.

These findings may appear to contradict reports of analgesia after intrathecal administration of Ca^{2+} channel blockers in experimental nerve injury (73) or clinical neuropathic pain (74). However, neurophysiological processes, including Ca^{2+} signaling, are spatially distinct. For instance, relief of pain by intrathecal I_{Ca} blockade may result from interference with neurotransmitter release in the spinal cord, which is not applicable at peripheral sites. Block of I_{Ca} at the SNL injury site has no analgesic effect (73). Our findings point to the possibility that medications anatomically and pharmacologically targeted at elevating inward Ca^{2+} flux of sensory neurons in the DRG may provide novel treatments for neuropathic pain.

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Figure 1.

A. Measurements determined from action potential (AP) trace. RMP, resting membrane potential; APamp., amplitude of AP; AP95%, duration of AP at 95% of amplitude; t, latency following axonal stimulation; AHPamp., amplitude of afterhyperpolarization; AHP80%, duration of afterhyperpolarization until 80% recovery to baseline. B. AP trace (above) and differentiated wave (below) from an Aα/β-type neuron that lacks an inflection on the descending limb of the AP. C. AP trace (above) and differentiated wave (below) from an Aδ-type neuron, showing an inflection on the descending limb of the AP, as confirmed in the differentiated trace with an interval of decreased negative slope (arrows). Note the different voltage and time scales compared to B.

Figure 2.

Inward Ca^{2+} current under different bath Ca^{2+} concentrations. Recordings were made in a dissociated dorsal root ganglion neuron by conventional patch-clamp recording in voltage-clamp mode, using a voltage step command from -70mV to 0mV for 100ms . Compared to baseline conditions (Ca^{2+} concentration 2.3mM , black trace), bath solution with no added Ca^{2+} (0.35mM , red trace) decreased current, while high bath Ca^{2+} (7mM , blue trace) raised current.

Figure 3. Cell size histogram indicating distribution of diameters of Aα/β-type neurons and Aδ-type neurons included in the study. Although the average size is larger for Aα/β-type neurons (see text), there is substantial overlap between the groups.

Figure 4.

Effects of manipulations that decrease inward Ca²⁺ flux. **A.** Lowering bath Ca²⁺ concentration (*, red trace) from 2.3mM to 0.35mM in an A α / β -type neuron results in decreased action potential amplitude (arrow) and more rapid resolution of the afterhyperpolarization. **B.** Action potential traces (above) and differentiated traces (below) showing the effects of lowering bath Ca²⁺ (red traces) in an A δ -type neuron. The action potential duration is shortened and the inflection on the descending limb of the action potential (arrowheads) is lost in low bath Ca²⁺. **C.** Bath application of cadmium (200 μ M) to an A δ -type neuron decreased the amplitude and duration of the afterhyperpolarization (*, red trace). **D.** Iontophoretic injection of the Ca²⁺ chelator ethylenediamine-tetraacetic acid (EDTA; *, red trace) in an A δ -type neuron decreased amplitude and duration of the afterhyperpolarization.

Figure 5. Effects of increasing inward Ca^{2+} flux. Elevation of bath Ca^{2+} concentration from 2.3mM to 5mM increased the afterhyperpolarization amplitude (*, blue trace) of an $\text{A}\delta$ -type neuron.

Figure 6.

Influences on repetitive firing behavior of I_{Ca} modulation in three sensory neurons. In each case, the top panel shows traces recorded during baseline conditions (2.3mM bath Ca^{2+}), while the middle panel shows the response to altered bath conditions. The current commands are the same for each pair and are shown in the bottom panel. Increased noise in these current injection protocols is due to discontinuous current clamp recording mode that is employed for accurate voltage measurement. *A.* Effect of lowering bath Ca^{2+} concentration to 0.35mM on firing behavior of an $A\alpha/\beta$ -type neuron. The rheobase current at which an action potential first appears was lowered, and the number of action potentials during depolarization was increased in low bath Ca^{2+} . *B.* Effect of bath cadmium (200 μ M) application on firing behavior of an $A\delta$ -type neuron. Cadmium caused burst firing during a current injection that previously only elicited a single action potential. *C.* Effect of elevating bath Ca^{2+} concentration from 2.3mM to 5mM on firing behavior of a repetitively firing (non-accommodating) $A\alpha/\beta$ -type. The neuron fired repetitively under baseline conditions but fired only a single action potential with increased bath Ca^{2+} .

Figure 7.

Dependence of hyperpolarization-induced voltage sag on intracellular Ca^{2+} accumulation. *A.* Switching from baseline bath Ca^{2+} (2.3mM) to lowered bath Ca^{2+} (0.35mM, red trace) caused decreased voltage sag in an A δ -type neuron. *B.* Iontophoretic injection of the Ca^{2+} chelator ethylenediamine-tetraacetic acid (EDTA; red trace) in an A α/β -type neuron likewise decreased the sag towards resting membrane potential during a hyperpolarizing current injection. *C.* Current command for both neurons. Recording by discontinuous current clamp.

Figure 8.

Representative AP traces showing effects of subtype-specific blockers of I_{Ca} . Traces before and 5min after (*, red traces) blocker are superimposed at the same resting potential (dotted line). Peak amplitude is indicated by an arrow in each case. AP duration was substantially prolonged by T-type current block with mibefradil, N-type block with SNX-111, P/Q-type block with Aga-IVA, and R-type block with SNX-482. AHP area was diminished by L-type block with nitrendipine, SNX-111, Aga-IVA, and SNX-482. Approximate doses at the neuron (and resting membrane potential for the demonstration traces before/after drug application) were nickel 0.5mM ($-73.2/-72.0$ mV), mibefradil 1 μ M ($-70.2/-74.6$ mV), nitrendipine 10 μ M ($-70.3/-70.1$ mV), SNX-111 0.5 μ M ($-66.3/-62.7$ mV), Aga-IVA 0.5 μ M ($-55.0/-52.4$ mV), and SNX-482 0.5 μ M ($-75.5/-73.8$ mV).

Figure 9.

Representative AP traces showing divergent effects of I_{Ca} amplification with the L-type current agonist BayK 8644 (approximate concentration $10\mu\text{M}$) in three different neurons. Traces before and after (# 1min, * 5min, both represented as blue traces) BayK 8644 are superimposed at the same resting potential (dotted line). Display of the AP spike is truncated in B and C. In A, the AHP was replaced by an afterdepolarization (arrowhead) during Bay K 8644. Other neurons developed a new late AHPs, either with an initial afterdepolarization (B), or without one (C). Peak AP amplitude is indicated by arrows in A.

Effects of decreased and increased bath Ca^{2+} concentration on active and passive membrane parameters of sensory neurons.

Table 1

	Condi- -tion	RMP (mV)	CV (m/S)	APamp (mV)	AP95% (mS)	AHPamp (mV)	AHP80% (mS)	AHParea (mV·mS)	dV/dt (V/S)	R _{in} (MΩ)	Sag ratio (%)	Rheobase (nA)
Auβ n=26	BL	-65.6±4.9	19.8±7.6	73.6±8.1	0.63±0.14	12.2±3.9	11.1±10.6	135±103	441±121	24.4±10.8	20.7±11.55	3.8±2.5
	Low [Ca ²⁺] _o	-64.3±5.9	19.6±6.8	70.7±13.0 [*]	0.62±0.13	12.7±3.8	8.1±7.5 ^{***}	106±88 ^{***}	430±134	23.4±9.4	14.1±11.1 ^{**}	3.0±2.0 ^{***}
Aδ n=6	BL	-70.0±5.7	7.9±2.2	82.2±14.1	1.34±0.78	9.4±4.9	40.2±42.2	318±280	358±191	37.5±23.7	15.6±13.9	1.8±1.7
	Low [Ca ²⁺] _o	-64.3±8.0 ^{**}	7.8±1.7	71.0±12.5 [*]	0.91±0.13	10.7±3.7	16.2±14.9 [*]	176±147 [*]	270±81	38.2±20.5	15.2±13.6	2.3±2.2
Auβ n=8	BL	-63.2±5.4	21.7±4.2	69.7±5.6	0.52±0.22	12.7±2.8	8.5±7.4	86.3±93.0	476±115	34.9±16.8	24.2±14.7	1.3±0.5
	High [Ca ²⁺] _o	-64.0±5.1	19.9±4.0 [*]	69.3±9.1	0.53±0.23	12.5±3.7	9.7±8.5 [*]	98.2±106.6 [*]	483±117	39.0±17.8 [*]	23.3±11.8	1.2±0.5
Aδ n=9	BL	-69.1±8.5	10.1±2.7	84.7±6.1	1.14±0.42	11.5±3.7	56.6±34.4	328.8±169.6	354±180	47.3±15.6	21.9±8.7	1.5±1.0
	High [Ca ²⁺] _o	-67.3±10.1	9.5±2.4 [*]	83.2±7.0	1.17±0.42	12.7±3.4	50.0±29.7	337.9±168.8	334±183	46.8±20.1	18.4±12.5	1.6±0.9

Values are given as mean ± SD. *BL* Baseline condition; *RMP* resting membrane potential; *CV* conduction velocity; *APamp* action potential amplitude; *AP95%* action potential duration at 95% of amplitude; *AHParea* afterhyperpolarization area under the curve; *dV/dt* maximum action potential upstroke velocity; *R_{in}* input resistance.

* *P*<0.05,

** *P*<0.01,

*** *P*<0.001 vs. baseline.

Effects of selective blockers of voltage-gated Ca²⁺ channel subtypes on active and passive membrane parameters in sensory neurons.

Table 2

	Condi-tion	RMP (mV)	CV (m/S)	APamp (mV)	AP95% (mS)	AHPamp (mV)	AHP80% (mS)	AHParea (mV·mS)	dV/dT (V/S)	R _{in} (MΩ)	Sag ratio (%)	Rheobase (nA)
Vehicle	BL	-69.6±4.9	28.4±11.7	74.2±8.3	0.96±0.37	9.2±5.2	17.9±17.6	130±158	383±139	44.3±22.9	18.9±13.0	2.8±2.4
	n=10 drug	-69.5±6.8	27.1±12.0	73.5±9.3	1.07±0.27	6.0±9.3	14.0±10.8	110±111	329±127*	41.9±20.1	21.2±17.0	2.9±1.8
Nickel	BL	-67.9±5.9	26.9±4.3	71.3±11.5	0.88±0.17	11.1±3.6	11.5±8.5	107±88	346±101	45.7±19.2	12.5±11.2	3.4±1.1
	n=10 drug	-67.9±6.3	25.3±4.7	74.1±11.5*	1.02±0.13**	11.1±4.4	6.5±6.6*	76±86*	286±78**	61.6±41.2	5.9±6.6	3.5±1.0
Mibefradil	BL	-68.3±6.5	26.8±6.2	71.4±10.8	0.75±0.15	12.5±4.9	12.9±6.8	179±140	396±96	51.1±33.1	18.1±11.7	4.3±1.3
	n=10 drug	-71.4±7.4***	23.2±5.5***	73.6±12.0	1.05±0.21***	13.2±5.5	9.1±5.2*	163±141	307±93***	43.4±58.9	13.5±11.7*	3.2±1.3***
Nitrendipine	BL	-63.9±9.5	34.1±9.7	65.8±11.5	1.23±1.18	11.5±3.1	14.3±9.0	129±77	346±179	63.4±35.8	15.2±3.8	2.7±0.5
	n=8 drug	-65.1±10.6	34.1±9.1	60.6±12.0*	1.44±1.43*	11.0±2.8	12.3±9.1	93±52*	270±169*	46.3±24.8	16.8±5.5	2.0±0.5
SNX-111	BL	-67.5±7.4	28.6±6.8	67.6±11.7	0.92±0.15	11.8±4.9	22.3±14.5	175±130	302±46	52.7±27.9	12.2±5.8	3.8±1.9
	n=11 drug	-68.1±6.8	24.6±6.2***	69.6±14.2	1.36±0.27***	10.9±4.9**	20.0±23.2	122±86*	225±77**	70.5±48.6*	9.7±7.4	2.8±1.5**
Aga-IVA	BL	-65.7±6.8	25.8±3.7	78.4±10.8	0.73±0.12	11.5±4.6	10.8±6.8	131±108	459±74	37.5±17.9	25.4±16.7	3.6±1.5
	n=10 drug	-67.7±8.9	23.0±3.4***	76.4±8.6	1.03±0.24***	10.1±5.8	7.1±7.1*	89±77**	354±71***	30.0±36.8	11.3±8.9**	2.6±1.4***
SNX-482	BL	-68.4±4.3	23.0±4.9	72.0±11.4	0.80±0.15	9.7±4.3	16.4±16.4	120±142	382±80	40.5±16.4	15.5±10.8	3.3±1.7
	n=10 drug	-69.9±5.2	19.5±4.9***	76.7±12.7**	1.17±0.27***	9.0±5.2	17.0±24.1	104±151*	309±86***	58.3±17.6**	13.3±14.8*	2.1±1.2**
BayK 8644	BL	-67.4±5.2	29.6±9.6	78.7±8.0	0.78±0.34	10.1±2.5	20.2±21.7	191±204	461±65	32.1±15.8	27.0±12.4	3.2±1.4
	n=11 drug	-68.1±5.8	26.4±8.6***	84.2±8.3**	2.57±1.98**	3.9±2.7***	50.8±48.6*	100±58	337±96**	43.6±16.1**	26.3±12.4	1.8±0.9***

Approximate concentration of agent: nickel 0.4mM, mibefradil 1μM, nitrendipine 10μM, BayK 8644 10μM, SNX-111 0.5μM, Aga-IVA 0.5μM, SNX-482 0.5μM. Values are given as mean ± SD. BL Baseline condition; RMP resting membrane potential; CV conduction velocity; APamp action potential amplitude; AP95% action potential duration at 95% of amplitude; AHPamp afterhyperpolarization amplitude; AHP80% afterhyperpolarization duration at 80% of amplitude; AHParea afterhyperpolarization area under the curve; dV/dt maximum action potential upstroke velocity; R_{in} input resistance.

* P<0.05,

** P<0.01,

*** P<0.001 vs. baseline.