Ca\(^{2+}\) Enhances U-Type Inactivation of N-Type (CaV2.2) Calcium Current in Rat Sympathetic Neurons

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\(^1\)Department of Physiology, Tulane University Medical School, New Orleans, Louisiana; \(^2\)Department of Physiology, Chungbuk National University Medical School, Cheongju, Korea; \(^3\)Department of Physiology, Gachon Medical School, Incheon, Korea; and \(^4\)Department of Anesthesiology, Penn State College of Medicine, Hershey, Pennsylvania

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Goo, Yong Sook, Wonil Lim, and Keith S. Elmslie. Ca\(^{2+}\) enhances U-type inactivation of N-type (CaV2.2) calcium current in rat sympathetic neurons. J Neurophysiol 96: 1075–1083, 2006. First published June 7, 2006; doi:10.1152/jn.01294.2005. Ca\(^{2+}\)-dependent inactivation (CDI) has recently been shown in heterologously expressed N-type calcium channels (CaV2.2), but CDI has been inconsistently observed in native N-current. We examined the effect of Ca\(^{2+}\) on N-channel inactivation in rat sympathetic neurons to determine the role of CDI on mammalian N-channels. N-current inactivated with fast (\(\tau \sim 150\) ms) and slow (\(\tau \sim 3\) s) components in Ba\(^{2+}\). Ca\(^{2+}\) differentially affected these components by accelerating the slow component (slow inactivation) and enhancing the amplitude of the fast component (fast inactivation). Lowering intracellular BAPTA concentration from 20 to 0.1 mM accelerated slow inactivation, but only in Ca\(^{2+}\) as expected from CDI. However, low BAPTA accelerated fast inactivation in either Ca\(^{2+}\) or Ba\(^{2+}\), which was unexpected. Fast inactivation was abolished with monovalent cations as the charge carrier, but slow inactivation was similar to that in Ba\(^{2+}\). Increased Ca\(^{2+}\), but not Ba\(^{2+}\), concentration (5–30 mM) enhanced the amplitude of fast inactivation and accelerated slow inactivation. However, the enhancement of fast inactivation was independent of Ca\(^{2+}\) influx, which indicates the relevant site is exposed to the extracellular solution and is inconsistent with CDI. Fast inactivation showed U-shaped voltage dependence in both Ba\(^{2+}\) and Ca\(^{2+}\), which appears to result from preferential inactivation from intermediate closed states (U-type inactivation). Taken together, the data support a role for extracellular divalent cations in modulating U-type inactivation. CDI appears to play a role in N-channel inactivation, but on a slower (sec) time scale.

INTRODUCTION

Influx of Ca\(^{2+}\) through N-type calcium channels triggers neurotransmitter release at a variety of central and peripheral synapses (Meir et al. 1999). Thus modulation of N-channel activity can produce profound effects on neuronal communication. One regulatory mechanism is neurotransmitter-induced inhibition of N-type channels (Elmslie et al. 1990; Koh and Hille 1997). Inactivation is another mechanism by which N-channel activity can be reduced. Several inactivation pathways have been found for calcium channels. In L-type channels (CaV1), the two primary mechanisms are voltage-dependent inactivation (VDI) and Ca\(^{2+}\)-dependent inactivation (CDI) (Giannattasio et al. 1991; Gutnick et al. 1989; Yue et al. 1990). VDI increases monotonically with increasing voltage and saturates at voltages where channels are fully activated. CDI shows U-shaped voltage dependence with a strong correlation with Ca\(^{2+}\) influx. A different form of VDI identified in N-channels has U-shaped voltage dependence (Jones and Marks 1989b; Patil et al. 1998) and has been termed U-type inactivation (Klemic et al. 2001). U-type inactivation is observed with either Ca\(^{2+}\) or Ba\(^{2+}\) as the charge carrier, which distinguishes it from CDI (Jones and Marks 1989b; Patil et al. 1998).

CDI has been observed in L-type and P/Q-type calcium channels (Giannattasio et al. 1991; Lee et al. 1999, 2000), but had been controversial for N-type channels (Cox and Dunlap 1994; Jones and Marks 1989b; Patil et al. 1998). Studies that examined N-current inactivation in bullfrog sympathetic neurons (Jones and Marks 1989a) and expressed mammalian N-channels in HEK293 cells failed to find evidence of CDI (Patil et al. 1998), whereas Cox and Dunlap (1994) provided solid evidence for CDI of chick N-current. It was recently shown that expressed N-channels showed CDI (Liang et al. 2003), but it was only obvious when N-channels were expressed with the CaV\(_{2a}\) subunit as opposed to CaV\(_{2b}\) or CaV\(_{2c}\) subunits (Patil et al. 1998). The CaV\(_{2a}\) subunit seemed to slow VDI sufficiently to reveal CDI (Liang et al. 2003). In addition, the concentration of intracellular Ca\(^{2+}\) buffer was low (0.5 mM EGTA), which was a condition also required for CDI of chick N-current (Cox and Dunlap 1994). However, N-current inactivation was not affected by intracellular Ca\(^{2+}\) chelator concentration in the other studies (Jones and Marks 1989b; Patil et al. 1998). To determine the effect of Ca\(^{2+}\) on inactivation of native mammalian N-current, we compared Ca\(^{2+}\) versus Ba\(^{2+}\) inactivation of N-current recorded from rat sympathetic neurons. We found that Ca\(^{2+}\) could affect inactivation, but some of those effects were inconsistent with CDI. We conclude that the Ca\(^{2+}\) enhancement of fast U-type inactivation (\(\tau = \sim 50\) ms) results from Ca\(^{2+}\) binding to an external site on the channel. CDI seems to play a role in N-channel inactivation, but on a slower (s) time scale.

METHODS

Cell isolation procedure

Superior cervical ganglion (SCG) neurons were acutely isolated from adult Sprague-Dawley rats (150–350 g) as described previously (Ehrlich and Elmslie 1995). Briefly, rats were anesthetized with ether and subjected to rapid decapitation. The cervical spinal cord was exposed, and the superior cervical ganglia were removed. The ganglia were transferred into ice-cold superfusion buffer and cut into smaller pieces using a scalpel. The tissue was then minced with forceps in a medium consisting of 125 mM NaCl, 2.5 mM KCl, 1.2 mM MgSO\(_4\), 2.0 mM CaCl\(_2\) (when Ca\(^{2+}\) was included), 10 mM glucose, and 20 mM HEPES at pH 7.4. After 30–60 min of superfusion, the ganglia were cut into smaller pieces, and ganglion cells were isolated using a dissecting microscope. The isolated cells were placed into a medium consisting of 125 mM NaCl, 2.5 mM KCl, 1.2 mM MgSO\(_4\), 2.0 mM CaCl\(_2\), 10 mM glucose, and 20 mM HEPES at pH 7.4 and superfused at 3 ml/min. The cells were loaded into a small recording chamber mounted on the stage of an inverted microscope. Axons were stimulated extracellularly using a pair of tungsten electrodes. The recordings were made using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA) and filtered at 5 kHz. Intracellular recordings were made using glass micropipettes filled with a solution containing 120 mM potassium aspartate, 5 mM KCl, 10 mM Hepes, and 0.1% biocytin adjusted to pH 7.2. The micropipettes were broken at a resistance of 4–5 M\(\Omega\) and intracellularly injected with dihydro-2,3-benzofuran (DHF) to mark the cell membrane. The recordings were then amplified, digitized, and filtered at 3 kHz using a Digidata 1322A (Axon Instruments, Foster City, CA) and were stored on computer using pClamp 9 software (Axon Instruments, Foster City, CA).
and decapitated, and the heads were placed in iced Hank’s balanced salt solution. Neurons were dissociated from the isolated ganglia by enzymatic digestion at 37°C followed by vigorous shaking. The enzymatic digestion was stopped by the addition of 10% fetal calf serum to the media. The dissociated cells were plated in 35-mm culture dishes and stored in a humidified atmosphere at 4°C (for ≤30 h) until use.

Electrophysiological recording

The neurons were voltage-clamped using the whole cell configuration of the patch-clamp technique. Electrodes were fabricated from Corning 7740 glass (ID 0.90 mm; OD 1.5 mm; Garnier Glass, Claremont, CA) using a Flaming/Brown P-97 pipette puller (Sutter Instrument, San Rafael, CA) and had resistances of 1–2 MΩ, which produced a mean series resistance (Rₛ) of 3.52 ± 1.13 (SD) MΩ (n = 70). Series resistance was compensated by ≥80%. Membrane currents were recorded using an Axopatch 200A amplifier (Axon Instruments, Foster City, CA) and digitized with a 12-bit A/D converter (GW Instruments, Cambridge, MA) after analog filtering with the amplifier’s four-pole low-pass Bessel filter. The digitization rate was ≥5 times the filter frequency. All experiments were conducted at room temperature.

Solutions

The standard internal solution contained (in mM) 120 N-methyl-D-glucamine (NMG)-Cl, 10 tetraethylammonium (TEA)-Cl, 11 MgCl₂, 10 NMG-HEPES, 1 CaCl₂, 6 MgCl₂, 2 Li₃GDP-β-S, 14 creatine phosphate, and 5 Tris₃ATP. GDP-β-S was used to block endogenous G protein activation (Ikeda 1991). The standard external solution contained (mM) 140 TEA-Cl, 10 NMG-HEPES, 5 BaCl₂ or 5 CaCl₂, 1 MgCl₂, and 15 glucose. The pH of both solutions was adjusted to 7.4 using NMG base.

For the high BAPTA internal solution, 11 mM EGTA was replaced with 20 mM BAPTA, and the concentration of NMG-Cl was reduced from 120 to 90 mM to maintain osmolarity. Experiments with low internal BAPTA (0.1 mM) and some 11 mM EGTA experiments used low Cl⁻ internal and external solutions (Cl⁻ substituted with methanesulfonate) to minimize possible artifact resulting from activation of Ca²⁺-activated chloride channels (Sanchez-Vives and Gallego 1994). We used 0.1 mM BAPTA as opposed to 0.5 mM EGTA used in other studies (Cox and Dunlap 1994; Liang et al. 2003), because it has been estimated to closely mimic the natural Ca²⁺ buffering capacity of cytoplasm (Beech et al. 1991). In addition, little or no difference in inactivation was observed between EGTA and BAPTA in this or previous studies (Jones and Marks 1989b; Patil et al. 1998).

Experiments to examine the effect of monovalent cation permeation difference in inactivation was observed between EGTA and BAPTA capacity of cytoplasm (Beech et al. 1991). In addition, little or no inactivation was induced in neurons dialyzed with 10 mM EGTA. This enhancement did not result from a contaminating Cl⁻ current such as Ca₂⁺-activated Cl⁻ current (Sanchez-Vives and Gallego 1994), because the Ca²⁺ effect on inactivation was similar (9 ± 6%, P < 0.01, n = 36) in cells dialyzed with 10 mM EGTA. This enhancement did not result from a contaminating Cl⁻ current such as Ca₂⁺-activated Cl⁻ current (Sanchez-Vives and Gallego 1994), because the Ca²⁺ effect on inactivation was similar (9 ± 6%, P < 0.01, n = 36) when Cl⁻ in the internal and external solutions was replaced by methanesulfonate.

Because N-type channels comprises 60–90% of the total current in rat sympathetic neurons (Plummer et al. 1989; Regan et al. 1991; Zhu and Ikeda 1993), it is likely that N-channel inactivation is affected by Ca²⁺. This was confirmed using the N-channel specific blocker ω-conotoxin GVIA (ωCGVIA; Fig. 2). Inactivation was measured (5-s steps) in both Ca²⁺ and Ba²⁺ in neurons before and after exposure to 1 μM ωCGVIA. In four cells that survived the entire procedure, Ca²⁺ enhanced the inactivation of the total current (before ωCGVIA) by 12 ± 12%, but the enhancement was only 6 ± 12% after toxin application. The Ca²⁺-enhanced inactivation of ωCGVIA-sen-

1 The online version of this article contains supplemental data.
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Two components of inactivation in both Ca\(^{2+}\) and Ba\(^{2+}\)

It was clear from examining the current during 5-s voltage steps that N-current inactivated with fast and slow components. We used double exponential fitting to determine the effect of Ca\(^{2+}\) on each component. N-current was well described by double exponential equations plus an offset (noninactivating component), which provided amplitude and \(t\) for the fast and slow components (Supplemental Fig. 1). From this point, the fast and slow components of inactivation will be termed fast and slow components (Supplemental Fig. 1). From this point, the speed of inactivation will be termed fast and slow components (Supplemental Fig. 1). From this point, the speed of inactivation was derived from the \(\tau\) for each component. We derived the fractional amplitudes of fast and slow components relative to the total current (see METHODS). Ca\(^{2+}\) enhanced the amplitude of fast inactivation (\(P < 0.001\)) and the speed of slow inactivation (\(P < 0.001\); Fig. 3), which together generated the increased inactivation observed in Fig. 1.

Fast and slow inactivation resulted from the activity of N-type calcium channels because both components of inactivation were identified in \(\omega\)CGVIA-sensitive currents. The fast inactivation \(\tau\) was 167 ± 69 ms in Ba\(^{2+}\) and 191 ± 79 ms in Ca\(^{2+}\), and the slow inactivation \(\tau\) was 3253 ± 994 ms and 2278 ± 867 ms in Ba\(^{2+}\) and Ca\(^{2+}\), respectively (\(n = 4\); cf. with Fig. 3). Ca\(^{2+}\) enhanced the amplitude of the fast component by 18 ± 27%. None of the Ca\(^{2+}\)–Ba\(^{2+}\) differences were significant for the \(\omega\)CGVIA-sensitive current, which likely resulted from the small number of observations. Indeed, Ca\(^{2+}\) failed to enhance inactivation of control current (before \(\omega\)CGVIA application) in one of the four cells. However, the trend of Ca\(^{2+}\)-induced increase in fast inactivation amplitude and speeding of slow inactivation were preserved.

One obvious possibility is that the Ca\(^{2+}\)-induced enhancement results from CDI. The main characteristics of CDI are 1) the increase in the magnitude and 2) speed of inactivation on switching from Ba\(^{2+}\) to Ca\(^{2+}\), 3) a U-shaped voltage dependence where the magnitude of inactivation is correlated with Ca\(^{2+}\) influx, and 4) reduced inactivation by high concentrations of intracellular Ca\(^{2+}\) chelators (Cox and Dunlap 1994; Giannattasio et al. 1991; Haack and Rosenberg 1994; Kalman et al. 1988; Liang et al. 2003; Peterson et al. 1999). Interestingly, Ca\(^{2+}\) had no effect on the speed of fast inactivation (cf. open bars of Fig. 3, \(B_b\) and \(E_b\)), which is inconsistent with CDI. However, Ca\(^{2+}\) accelerated the slow component as expected for CDI (cf. open bars of Fig. 3, \(C_b\) and \(F_b\)). To gain a better understanding the mechanism by which Ca\(^{2+}\) affects each component, we examined each of these characteristics starting with reduced the levels of intracellular Ca\(^{2+}\) buffering, which has been particularly effective at revealing N-channel CDI (Cox and Dunlap 1994; Liang et al. 2003).

Lowering internal BAPTA differentially affected each inactivation component. The speed of fast inactivation was increased (relative to high BAPTA) to the same degree in both Ca\(^{2+}\) and Ba\(^{2+}\) (Fig. 3, \(B_b\) and \(E_b\)). In addition, the amplitude of the fast component was reduced. CDI predicts a Ca\(^{2+}\) specific acceleration of inactivation, which was observed for slow inactivation in low BAPTA versus high BAPTA. This suggests that CDI could be affecting native N-channel availability on a time scale of seconds as opposed to 100 ms observed for N-channels expressed with CaV\(_{2.2}\) subunits in HEK293 cells (Liang et al. 2003).

Effect of altered divalent cation concentration on the components of N-channel inactivation

N-CHANNEL INACTIVATION IN THE ABSENCE OF EXTERNAL DIVALENT CATIONS. Cox and Dunlap (1994) observed a dramatic decrease of N-channel inactivation when Na\(^{+}\) was used as the charge carrier in chick sensory neurons, which was used as support for CDI. We were interested to determine if mammalian N-channels responded in a similar fashion. We used...
methylamine (MA\(^+\)) as the monovalent charge carriers, which has been shown to produce significantly better calcium current isolation than with inorganic monovalent cations (Jones and Marks 1989a; Liang and Elmslie 2002). To ensure that N-current was well isolated, we measured the block of MA\(^+\) current by oCGVIA (Fig. 4, A and B). On average, 79 ± 18% of MA\(^+\) current was blocked by 1 μM oCGVIA (n = 5), which shows that the majority of current is attributable to N-type channels. Peak MA\(^+\) current was observed at a voltage 10 mV hyperpolarized to that in 5 mM Ba\(^{2+}\), so when comparing inactivation at peak current, the stimulus voltage was hyperpolarized by 10 mV for MA\(^+\) compared with Ba\(^{2+}\). When compared in the same cell, inactivation was significantly decreased during permeation by MA\(^+\) versus Ba\(^{2+}\) (Fig. 4, C and D). It seemed that this decrease resulted from the absence of fast inactivation, because the current inactivated slowly during our 5-s voltage step. Inactivation in MA\(^+\) was best fit with a single exponential equation with \(\tau = 4.0 \pm 0.2\) s (at −20 mV, n = 3). Inactivation in the same three cells recorded in 5 mM Ba\(^{2+}\) was best fit by two exponential equations with \(\tau = 201.7 \pm 85.5\) ms and 4.1 ± 1.1 s (at −10 mV) for fast and slow inactivation, respectively. There was no significant difference in the slow inactivation \(\tau\) measured in MA\(^+\) versus Ba\(^{2+}\) (\(P = 0.34\)).

The 10-mV left shift used to compare MA\(^+\) and Ba\(^{2+}\) data were derived from the \(I-V\) curve. To further test the validity of this shift, slow inactivation \(\tau\) was compared over four voltages in cells exposed to both Ba\(^{2+}\) and MA\(^+\). Slow inactivation \(\tau\) corresponded very well, provided the MA\(^+\) data were shifted 10 mV hyperpolarized (Fig. 4E). Thus the 10-mV left shift seems to be valid for comparison of inactivation between Ba\(^{2+}\) and MA\(^+\). In addition, this analysis shows that slow inactivation \(\tau\) decreases with depolarization in both MA\(^+\) and Ba\(^{2+}\), which is consistent with a classical VDI mechanism.

Other effects of zero divalent cations were slow activation and deactivation of N-current (Fig. 4). Slow deactivation is consistent with previous observations from chick N-channels (Cox and Dunlap 1994) and may be explained by the left-shifted current-voltage relationship in zero divalent cations. However, such a shift cannot explain slowed activation. The effect on activation is similar to that observed during voltage-dependent inhibition of N-current by neurotransmitters (Elmslie et al. 1990), but we included 2 mM GDP-β-S in the internal solution to prevent the interference of tonically activated G proteins (Ikeda 1991). It is possible that divalent cations enhance gating kinetics of N-type calcium channels.

**INCREASING EXTRACELLULAR [CA\(^{2+}\)] ENHANCED INACTIVATION.** Because removing external divalent cation concentration prevented fast inactivation, we were interested to determine the effect of increased external Ca\(^{2+}\) and Ba\(^{2+}\) concentration on inactivation. These experiments were done using 11 mM EGTA because altering the type and concentration of internal Ca\(^{2+}\) chelator had no effect on the magnitude of inactivation measured over 5-s steps. An additional benefit of high EGTA was longer recording times, which permitted the measurement of inactivation in multiple Ca\(^{2+}\) or Ba\(^{2+}\) concentrations. To compensate for the increased surface charge screening with higher divalent cation concentrations (Zhou and Jones 1995), inactivation was always measured at the voltage generating...
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Fast N-channel inactivation is absent during monovalent cation permeation. A: calcium currents were recorded using MA⁺ as the permeant ion before and after 1 μM αCGVIA application. Voltage step duration was 150 ms. Note the slowly deactivating current at -80 mV that is lost after block by αCGVIA. B: MA⁺ current–voltage (I–V) relationship measured from peak current at each indicated voltage recorded before and after 1 μM αCGVIA. Same cell as in A. C: N-type current was normalized to the peak to compare the time-course of inactivation during permeation by 5 mM Ba²⁺ and 150 mM MA⁺. Five-second depolarizing pulses were applied using voltages that generated peak current (indicated on voltage protocol). D: inactivation was calculated as 1 – (I_{end}/I_{pk}) and was compared in the same cells when either Ba²⁺ or MA⁺ permeated the channels (***P < 0.001). I_{end} was measured at the end of the 5-s voltage step. E: voltage dependence of slow inactivation is not affected by MA⁺. Slow inactivation r from exponential fitting is shown for Ba²⁺ and MA⁺. A single exponential equation was used to fit inactivation in MA⁺, whereas a double exponential was used for inactivation in Ba²⁺. The MA⁺ data are 10 mV hyperpolarized to that in Ba²⁺ to account for surface charge effects.

Comparison of the I–V and inactivation versus voltage relationships showed that altered external divalent cation concentration induced a similar shift (data not shown). Increasing Ca²⁺ concentration from 5 to 30 mM significantly enhanced inactivation (Fig. 5). The average peak current for each Ca²⁺ concentration was 4.5 ± 4.0 (5 mM), 5.5 ± 4.2 (10 mM), and 6.2 ± 4.0 nA (30 mM; n = 5). On the other hand, there was no significant change in inactivation when Ba²⁺ concentration was increased (Fig. 5). The average peak current for each Ba²⁺ concentration was 5.3 ± 3.7 (5 mM), 9.0 ± 6.3 (10 mM), and 10.5 ± 6.6 nA (30 mM; n = 5). Perhaps the larger calcium currents resulting from higher external Ca²⁺ increased inactivation by inducing CDI. This idea was initially examined by determining the effect of changes in Ca²⁺ concentration on fast and slow inactivation, which showed that increased Ca²⁺ specifically increased the amplitude of fast inactivation (Fig. 6A) and the speed of slow inactivation (Fig. 6D). On the other hand, increasing Ba²⁺ concentration failed to significantly alter either fast or slow peak inward current.

FIG. 5. Inactivation increases with external Ca²⁺ concentration. A: currents were recorded from single cells in 5, 10, and 30 mM of either Ca²⁺ (left) or Ba²⁺ (right). Five-second voltage steps were given to potentials that generated peak current, which were +10, +20, and +30 mV for 5, 10, and 30 mM Ca²⁺, respectively, and 0, +10, and +20 mV for 5, 10, and 30 mM Ba²⁺, respectively. Currents were normalized with respect to the peak current to compare the time-course of inactivation among different concentrations of Ca²⁺ and Ba²⁺. B: a comparison of inactivation (measured as in Fig. 1) among different concentrations of Ca²⁺ (left) and Ba²⁺ (right). Number of cells tested is shown inside the bar graph (**P < 0.01; ***P < 0.001).

FIG. 6. Increased external Ca²⁺ concentration enhanced amplitude of fast inactivation. Parameters of fast and slow components of inactivation were obtained from double exponential fits to currents generated by 5-s voltage steps to voltages that generated peak current (as described in Fig. 5). Amplitude of fast (A) and slow (B) inactivation and r of fast (C) and slow (D) inactivation were compared for different Ca²⁺ concentrations. Amplitude is reported as the fraction to total amplitude, which is the sum of both inactivating and noninactivating components (**P < 0.05; ***P < 0.01; ****P < 0.001). Number of cells tested is indicated in the bar graph in A.
inactivation (data not shown). The Ca\(^{2+}\) specific acceleration of slow inactivation is consistent with CDI (Giannattasio et al. 1991).

**Voltage dependence of fast inactivation**

Up to this point, the effects of Ca\(^{2+}\) on fast inactivation are largely inconsistent with CDI. One additional test is current dependence, which tests the requirement for Ca\(^{2+}\) influx needed to activate calmodulin, the intracellular mediator of CDI. We looked for current dependence by examining inactivation over a wide range of voltages using a two-pulse protocol with 500-ms prepulses given to different voltages followed by a postpulse to the voltage producing peak inward current (Fig. 7). As a result of the shorter voltage steps (500 ms), this protocol specifically examines the voltage dependence of fast inactivation. A plot of postpulse current amplitude versus prepulse voltage revealed a U-shaped relationship in Ba\(^{2+}\), with maximal inactivation observed at voltages near those yielding maximal inward current and less inactivation at more positive and negative voltages (Fig. 7, A and B). This result from U-type inactivation, which is characterized by U-shaped voltage dependence of inactivation in Ba\(^{2+}\) and an absence of current dependence to inactivation (e.g., substantial inactivation at voltages yielding little or no current) (Jones and Marks 1989b; Patil et al. 1998). Ca\(^{2+}\) did not alter the voltage dependence of inactivation, but, as expected, the magnitude of inactivation was increased. The average peak inactivation was 62.1 ± 0.8% in Ca\(^{2+}\) (n = 8) and 44.4 ± 0.8% in Ba\(^{2+}\) (n = 10).

One possibility is that Ca\(^{2+}\) enhances U-type inactivation. Alternatively, it is possible that the enhancement results from

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**FIG. 7.** Ca\(^{2+}\) enhancement of fast inactivation is independent of current amplitude. Five hundred-millisecond prepulses ranging from −80 to +80 mV were given from a holding potential of −80 mV to induce inactivation. These prepulses were followed by a 20-ms postpulse to the voltage that generated peak current (+10 mV in Ba\(^{2+}\) and +20 mV in Ca\(^{2+}\)) to assay effect of prepulse. Interval between the prepulse and postpulse was 10 ms. Data are shown from a single cell exposed to 5 mM Ba\(^{2+}\) (A) and Ca\(^{2+}\) (B). Prepulse voltage is indicated next to its corresponding postpulse current. Current-voltage curves are shown with currents measured at peak during prepulse (△) and postpulse (○). Data points are averages of 2 protocols with prepulse voltages given in ascending and descending order, which was done to compensate for slow changes in current amplitude (e.g., rundown) during these long protocols. C: inactivation was calculated as 1 − (I\(_{peak}\)/I\(_{hold}\)), which is 1 minus the ratio of prepulse current (I\(_{prep}\)) after each prepulse step to that after a −80-mV prepulse step (I\(_{hold}\) ). Data in A and B are replotted to show the effect of Ca\(^{2+}\) on inactivation over a range of voltages. Dashed line is relationship in Ca\(^{2+}\) shifted 10 mV so that peak inactivation in Ca\(^{2+}\) matches that in Ba\(^{2+}\). Note that Ca\(^{2+}\) enhances inactivation at voltages with little or no current. D: percentage change of inactivation induced by Ca\(^{2+}\) (relative to that in Ba\(^{2+}\)) is plotted vs. normalized prepulse current in Ca\(^{2+}\) for 4 cells from which data were obtained in both Ca\(^{2+}\) and Ba\(^{2+}\). Percentage change was calculated from the inactivation vs. voltage relationship after a 10-mV left shift of the Ca\(^{2+}\) data to compensate for surface charge effects (see dashed line in C). Ca\(^{2+}\) enhanced inactivation in 3 of the 4 cells examined and each symbol represents data from a single cell. E: inactivation vs. voltage relationship from a single cell in 5, 10, and 30 mM Ca\(^{2+}\). Inactivation was measured as described above. Positive values indicate inactivation and negative values indicate facilitation. Shift in peak inactivation with increasing divalent cation concentration results from surface charge screening. This shift matches that observed for the I\(_{V}\) relationships. F: percentage change of inactivation induced by changing external Ca\(^{2+}\) from 5 to 30 mM is plotted against the resulting change in current (n = 5), which is the difference between current in 30 mM Ca\(^{2+}\) vs. that in 5 mM Ca\(^{2+}\). Data for 30 mM Ca\(^{2+}\) was left-shifted 20 mV to compensate for surface charge screening. Data from each of the 5 cells is plotted by a different symbol.
CDI that is superimposed on U-type inactivation. CDI requires Ca\textsuperscript{2+} influx, which was tested by plotting the percentage enhancement of inactivation by Ca\textsuperscript{2+} (over Ba\textsuperscript{2+}, 5 mM each) versus the normalized current amplitude in Ca\textsuperscript{2+} (Fig. 7D). Ca\textsuperscript{2+} enhanced inactivation in three of four cells examined for this analysis, and in each cell, there was no correlation between the enhancement of inactivation and current amplitude. In fact, the enhancement at voltages generating little or no current was just as large as those generating peak current (Fig. 7D). Thus the enhancement of fast inactivation does not seem to require Ca\textsuperscript{2+} influx, which is inconsistent with CDI.

Even though the above analysis showed no current dependence, it is possible that a dependence on Ca\textsuperscript{2+} influx could be revealed with higher Ca\textsuperscript{2+} concentrations (larger currents). As expected from our data using 5-s steps, inactivation observed from prepulse-postpulse protocols increased with Ca\textsuperscript{2+} concentration (Fig. 7E). Current dependence was tested by plotting the percentage increase of inactivation versus the percentage increase in current amplitude (5 vs. 30 mM Ca\textsuperscript{2+}, a within-cell comparison, n = 5). Again there was no relationship between inactivation and current amplitude (Fig. 7F). From this, we conclude that Ca\textsuperscript{2+} influx is not required for enhancement of fast inactivation.

**Discussion**

We examined the effect of Ca\textsuperscript{2+} on inactivation to determine if there is a role for CDI in the inactivation of native N-type calcium channels. In accordance with previous observations of CDI, we hypothesized a Ca\textsuperscript{2+}-specific enhancement of the speed and magnitude of inactivation that was dependent on the internal Ca\textsuperscript{2+} concentration (CDI). This hypothesis was tested by lowering internal Ca\textsuperscript{2+} buffer concentration, increasing external Ca\textsuperscript{2+} concentration, and correlating inactivation with inward current amplitude. While these conditions did affect inactivation, most of the effects on fast inactivation (τ = 150 ms) were inconsistent with our expectations for CDI. Lowering the intracellular BAPTA concentration significantly accelerated fast inactivation, but that effect was not specific for Ca\textsuperscript{2+} as expected for CDI. Fast inactivation increased with external Ca\textsuperscript{2+} concentration, but that increase was not dependent on Ca\textsuperscript{2+} influx. Interestingly, fast inactivation was dependent on divalent cations (Cox and Dunlap, 1994), because it was lost in our zero divalent cation solution (MA\textsuperscript{+} permeation). We conclude that the presence of divalent cations is required for fast inactivation, but Ca\textsuperscript{2+} influx is not. CDI may play a role in N-channel inactivation, but on a time scale of 1–2 s.

**Slow N-channel inactivation**

Early work on calcium currents in rat sensory neurons showed an inactivation pathway could be blocked when external Ca\textsuperscript{2+} was replaced by Mg\textsuperscript{2+} (Schroeder et al. 1990), which led to the conclusion that CDI was involved. These experiments used 3-s voltage steps to induce inactivation, which indicates that CDI could be slow in these neurons. We have also observed effects of Ca\textsuperscript{2+} on slow inactivation that are consistent with CDI. Ca\textsuperscript{2+} accelerated slow inactivation in a concentration-dependent manner, but increased Ba\textsuperscript{2+} concentration was without effect. In addition, lowering internal BAPTA significantly accelerated slow inactivation in Ca\textsuperscript{2+}, but not Ba\textsuperscript{2+}. Thus the data support CDI as a slow regulator of N-channel availability in rat sympathetic neurons.

The speed of our putative CDI (τ ~ 1,500 ms) is slow relative to that observed in expressed N-channels (τ ~ 100 ms) (Liang et al. 2003). N-channel CDI is mediated by Ca\textsuperscript{2+} binding to the N-lobe of CaM, which makes N-channel CDI more sensitive to changes in cytoplasmic Ca\textsuperscript{2+} than that of L-type channels (Liang et al. 2003). This implies that N-channel CDI would be sensitive to both intracellular Ca\textsuperscript{2+} buffering and cell size. HEK293 cells tend to be smaller (~15 pF) than rat sympathetic neurons (~30 pF), but current amplitude (1–5 nA) can be similar in these preparations. Thus the lower current density of sympathetic neurons could delay increased intracellular Ca\textsuperscript{2+} relative to HEK293 cells, which could contribute to slower CDI. However, CDI was not observed in previous studies of N-channel inactivation using HEK293 cells (Patil et al. 1998) that co-expressed CaV\textsubscript{β3} with the N-channel as opposed to CaV\textsubscript{β2} used by Liang et al. (2003). CaV\textsubscript{β3} has been shown to be the dominant isoform in rat sympathetic neurons (Lin et al. 1996), which could explain the apparent slow CDI in our recordings (Patil et al. 1998). Perhaps differences in CaV\textsubscript{β} subunit expression can explain the disparate N-current inactivation observations from different neuronal preparations (Cox and Dunlap 1994; Jones and Marks 1989b; Schroeder et al. 1990).

In Ba\textsuperscript{2+}, slow inactivation seems to involve a classic VDI mechanism, where inactivation increases monotonically with voltage (Fig. 3E). In Ca\textsuperscript{2+}, this component should also be present as has been shown for L-type channels (Giannattasio et al. 1991). We believe that CDI is an additional inactivation component with a 1- to 2-s τ. Theoretically, this should appear as a third component to inactivation. However, this τ is not sufficiently different from that of VDI (2–4 s) to permit separation by exponential fitting. Thus CDI is superimposed on VDI, and we observe the effect of CDI as a Ca\textsuperscript{2+}-induced increase in the speed of slow inactivation.

**Physiological effect of slow CDI**

The slow time-course of CDI suggests that its most prominent impact would be on long periods of action potential activity (>500 ms). Such activity would supply the Ca\textsuperscript{2+} needed over a sufficiently long period to activate CDI in a manner that is mimicked by our long (5 s) voltage steps. In addition, slow VDI would also be increasing during the long activity period. The resulting N-channel inactivation would reduce Ca\textsuperscript{2+} influx/action potential, which could lower excitability by reducing neurotransmitter release from presynaptic terminals. On the other hand, decreased Ca\textsuperscript{2+} influx in the soma could increase excitability as a result of reduced activation of Ca\textsuperscript{2+}-activated potassium channels. These predictions are difficult to test because conditions that reduce CDI will also impair the other physiological effects of increased intracellular Ca\textsuperscript{2+}.

**Fast inactivation**

**REDUCED INTRACELLULAR CA\textsuperscript{2+} CHELATION.** Previous publications showed no effect on the speed of fast inactivation in Ba\textsuperscript{2+} when lowering the concentration of intracellular Ca\textsuperscript{2+} chelator (Liang et al. 2003). Thus we were surprised to find that fast...
inactivation in both Ca\textsuperscript{2+} and Ba\textsuperscript{2+} was significantly accelerated by lowering internal BAPTA concentration from 20 to 0.1 mM. The increased fast inactivation \(\tau\) in low BAPTA seems to be inconsistent with CDI. However, two studies have concluded that Ba\textsuperscript{2+} influx could trigger L-channel CDI (Ferreira et al. 1997; Jouveneau et al. 2000). This seems highly unlikely because calmodulin has been shown to be the Ca\textsuperscript{2+} detector for CDI in both CaV1 and CaV2 channels (Lee et al. 1999; Liang et al. 2003; Peterson et al. 1999; Zuhlke et al. 1999), and calmodulin and other E-F hand proteins have a very low affinity for Ba\textsuperscript{2+} (>1,000 \(\mu\)M) versus Ca\textsuperscript{2+} (≈2.5 \(\mu\)M) (Gu and Cooper 2000). In addition, Haack and Rosenberg (1994) showed that CDI could not be induced in L-channels with intracellular Ba\textsuperscript{2+} concentrations \(\approx\)1 mM, but CDI was induced with low concentrations of internal Ca\textsuperscript{2+} (10 \(\mu\)M). Thus it is unlikely that Ba\textsuperscript{2+} could activate the calmodulin-mediated CDI observed for N-type calcium channels (Liang et al. 2003).

It is also unlikely that accelerated fast inactivation results from the activity of contaminating currents, such as Ca\textsuperscript{2+}-activated Cl\textsuperscript{−} current, because Cl\textsuperscript{−} in the internal and external solution was replaced by methanesulfonate and the only permeant cations were Ba\textsuperscript{2+} or Ca\textsuperscript{2+}. Alternatively low intracellular BAPTA could invite a build-up in the intracellular concentration of Ca\textsuperscript{2+} or Ba\textsuperscript{2+} that would decrease driving force during the 5-s voltage steps. This should affect both inactivation components, but slow inactivation was not affected when Ba\textsuperscript{2+} was the charge carrier. In addition, Goldman-Hodgkin-Katz rectification predicts a substantial increase of intracellular divalent cation concentration is needed to significantly affect calcium current amplitude. For example, raising internal Ca\textsuperscript{2+} from 1 nM to 100 \(\mu\)M decreases calcium current at 0 mV by 2% (5 mM external Ca\textsuperscript{2+}), which is much too small an effect to explain the changes in inactivation we observe using low internal BAPTA. It seems more likely that fast inactivation is a U-type inactivation model where the channels require this divalent cation-induced N-channel conformation. Perhaps fast inactivation can be observed in either ion, but not in their absence. One candidate for site 1 is the selectivity filter because it binds both Ca\textsuperscript{2+} and Ba\textsuperscript{2+} (Almers and McCleskey 1984; Carbone et al. 1997; Hess and Tsien 1984). The second site (site 2) seems to be selective for Ca\textsuperscript{2+}, but its affinity for Ba\textsuperscript{2+} is low (mM). Inactivation selectively increases with Ca\textsuperscript{2+} concentrations >5 mM. EF-hand containing proteins are highly Ca\textsuperscript{2+} selective (Gu and Cooper 2000), but these protein are intracellularly located and Ba\textsuperscript{2+} influx does not seem to be required for fast inactivation. A putative EF-hand motif has recently been identified on the extracellular face of the N-channel (domain III, S5-H5) (Feng et al. 2001), which could provide the specific Ca\textsuperscript{2+} binding to site 2. However, it should be noted that site 2 seems to have very low Ca\textsuperscript{2+} affinity (millimolar) compared with the micromolar affinity of typical EF-hand binding proteins. On the other hand, ion sensitivity may be a general feature of U-type inactivation, because increased extracellular K\textsuperscript{+} concentration enhances U-type inactivation of potassium channels (Klemic et al. 1998, 2001). The mechanism by which increased ion concentration enhances U-type inactivation remains to be determined.

Extracellular ions seem to affect the function of many voltage-dependent ion channels. Ca\textsuperscript{2+} has been shown to be an important co-factor in sodium channel closing (Armstrong 1999; Armstrong and Cota 1991, 1999). K\textsuperscript{+} occupancy of the Shaker potassium channel pore seems to be required for the channel to maintain a functional permeation pathway and gating charge movement (Lobeda et al. 2001; Melishchuk et al. 1998). In addition, K\textsuperscript{+} occupancy of the pore seems to retard C-type inactivation (Baukrowitz and Yellen 1995; Kiss and Korn 1998). Ca\textsuperscript{2+} has recently been shown to modulate dihydropyridine binding to L-type calcium channels (Peterson and Catterall 2006). Thus regulation by physiological ions seems to be a common theme among voltage-dependent ion channels. Extracellular divalent cations have recently been shown to regulate the binding of \(\alpha\)CGVIA to N-channels (Liang and Elmslie 2002). \(\alpha\)CGVIA is a very slowly reversible N-channel blocker in the presence of Ca\textsuperscript{2+} or Ba\textsuperscript{2+}, but rapidly dissociates from the channel in divalent cation-free solutions. This effect was interpreted to result from a divalent cation induced conformational change in N-channel structure. Perhaps fast inactivation also requires this divalent cation-induced N-channel conformation.
GRANTS

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