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Role of Ca^{2+} for Inactivation of N-type Calcium Current in Rat Sympathetic Neurons

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The voltage-dependence of N-type calcium current inactivation is U-shaped with the degree of inactivation roughly mirroring inward current. This voltage-dependence has been reported to result from a purely voltage-dependent mechanism. However, Ca^{2+}-dependent inactivation of N-channels has also been reported. We have investigated the role of Ca^{2+} in N-channel inactivation by comparing the effects of Ba^{2+} and Ca^{2+} on whole-cell N-current in rat superior cervical ganglion neurons. For individual cells inactivation was always larger in Ca^{2+} than in Ba^{2+} even when internal EGTA (11 mM) was replaced with BAPTA (20 mM). The inactivation vs. voltage relationship was U-shaped in both divalent cations. The enhancement of inactivation by Ca^{2+} was inversely related with the magnitude of inactivation in Ba^{2+} as if the mechanisms of inactivation were the same in both Ba^{2+} and Ca^{2+}. In support of this idea we could separate fast (τ ~150 ms) and slow (τ ~2500 ms) components of inactivation in both Ba^{2+} and Ca^{2+} using 5 sec voltage steps. Differential effects were observed on each component with Ca^{2+} enhancing the magnitude of the fast component and the speed of the slow component. The larger amplitude of fast component indicates that the more channels inactivate via this pathway with Ca^{2+} than with Ba^{2+}, but the stable time constants support the idea the fast inactivation mechanism is identical in Ba^{2+} and Ca^{2+}. The results do not support a Ca^{2+}-dependent mechanism for fast inactivation. However, the Ca^{2+}-induced acceleration of the slowly inactivating component could result from a Ca^{2+}-dependent process.

Key Words : N-current, Whole-cell patch-clamp, Voltage-clamp, Voltage-dependent inactivation

INTRODUCTION

Influx of Ca^{2+} through N-type calcium channels triggers neurotransmitter release at a variety of central and peripheral synapses.[1] Neurotransmitter-induced inhibition of N-current has been shown to reduce neurotransmitter release.[2-7] Inactivation provides another mechanism by which N-channel activity can be reduced. Indeed, it was recently been concluded that accumulated calcium channel inactivation during a train of action potentials contributes to posttetanic depression.[8]

Early studies on inactivation focused on invertebrate calcium channels and vertebrate L-type calcium channels. These studies described two mechanisms of inactivation : voltage-dependent[9-11] and Ca^{2+}-dependent.[10-15] The voltage-dependent mechanism induces a monotonic decrease in current with increasing voltage, similar to the classic type of voltage-dependent inactivation first described by Hodgkin and Huxley.[16] In Ca^{2+}-dependent inactivation, the rate and magnitude of inactivation depends on the internal Ca^{2+} concentration (near the channel). Thus, inactivation is correlated with current amplitude. This results in a U-shaped inactivation vs. voltage relation where inactivation is maximal at the voltage generating peak current and decreases as current becomes smaller at more depolarized and hyperpolarized voltages. Eckert and Chad[17] showed that Ca^{2+}-dependent inactivation was attenuated when Ca^{2+} is re-

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placed by Ba^{2+} or by monovalent cations, and can be prevented by buffering intracellular Ca^{2+}. However, the effect of buffering can be small in some preparations.[10] Voltage and Ca^{2+}-dependent mechanisms of inactivation have been shown to coexist in some calcium channels.[10, 11]

In N-type Ca^{2+} channels, the mechanisms of inactivation are more controversial. One reason for the controversy is that the inactivation vs. voltage relationship is U-shaped. Superficially this appears to argue for a Ca^{2+}-dependent mechanism. However, several properties of this inactivation deviate from predictions based on a Ca^{2+}-dependent mechanism. For example, the magnitude of inactivation is not strongly correlated with current amplitude, since substantial inactivation can be observed at voltages that generate little or no current.[18-20] In addition, the U-shaped inactivation vs. voltage relationship is observed when Ca^{2+} is replaced by Ba^{2+}.[18, 20] These differences from the Ca^{2+}-dependent hypothesis lead several investigators to propose unique voltage-dependent mechanisms for N-channel inactivation.[18, 20] Patil et al.[20] recently showed that a model where channels inactivated preferentially from intermediate closed states on the pathway to channel opening could explain the properties of N-channels inactivation. An analysis of gating currents supported predictions of the preferential closed state inactivation model.[21] A similar model has been proposed to explain the U-shaped inactivation vs. voltage relation for several potassium channels.[22, 23] Klemic et al.[23] termed this type of inactivation U-type to distinguish it from N-type and P/C-type inactivation. The existence of U-type inactivation in both calcium and potassium channels supports the idea that Ca^{2+} influx is not a requirement.

However, Ca^{2+} does have effects on N-channel inactivation. Increasing the external Ca^{2+} concentration can increase inactivation particularly when the concentration of intracellular Ca^{2+} buffering (EGTA or BAPTA) is low.[19] In addition, experiments using long depolarizations (3 sec) showed that more inactivation was observed during increased [Ca^{2+}] even with 10 mM EGTA.[24] These results were used to argue in favor of a Ca^{2+}-dependent mechanism for inactivation. Perhaps the most compelling observation in support of a Ca^{2+}-dependent mechanism was that fast inactivation was absent when N-channels passed monovalent cations instead of divalent cations.[19] Recently further supporting evidence has been obtained from measurements of gating current. Shirokov[25] demonstrated U-shaped inactivation vs. voltage relation in N-channel gating current only when Ca^{2+} was allowed to permeate the channel. Inactivation increased monotonically with voltage when calcium currents were blocked by a combination of Co^{2+} and Gd^{2+}.

We have examined N-channel inactivation in rat superior cervical ganglion neurons to determine the effect of Ca^{2+}. To ensure in this paper that gross changes in internal Ca^{2+} concentration would not interfere with our measurement of inactivation, we have recorded currents using high internal concentrations of Ca^{2+} chelators (11 mM EGTA or 20 mM BAPTA). We show that Ca^{2+} can enhance calcium current inactivation and that the major calcium channel type affected is the N-type channel. This Ca^{2+}-induced enhancement resulted from an increase in the amplitude of the fast component and an increase in the speed of the slow component of inactivation. In the next paper, we examine the effect of changes in Ca^{2+} and Ba^{2+} concentration on inactivation. We believe that our data as well as those of other investigators can be explained if divalent cations are required for fast inactivation of N-channels. Our hypothesis differs from "classic" Ca^{2+}-dependent inactivation in two ways. First, both Ca^{2+} and Ba^{2+} are effective at triggering fast U-type inactivation. Second, divalent cation permeation is not required for fast U-type inactivation.

A preliminary report of some of these results has appeared in abstract form.[26]

METHODS

1. Cell isolation procedure

Superior cervical ganglion (SCG) neurons were acutely isolated from adult Sprague Dawley rats (150-350 g) as described previously.[27] Briefly, rats were anes-

thetized with ether, decapitated and the heads placed in iced Hank's balanced salt solution. Neurons were dissociated from the isolated ganglia by enzymatic digestion 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℃ (for up to 30 hrs) until use.

2. Electrophysiological recording

The neurons were voltage-clamped using the whole-cell configuration of the patch-clamp technique. Electrodes were fabricated from Corning 7740 glass (I.D. 0.90 mm, O.D. 1.5 mm, Garner Glass Co. Claremont, CA) using a Flaming/Brown P-87 pipette puller (Sutter Instrument Co., San Rafael, CA) and had resistances of 1-2 MΩ producing series resistance (Rs) of 3.52±1.13 (mean±S.D., n=70). Series resistance was compensated by at least 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 Inc., Cambridge, MA) following analog filtering with the amplifier's 4 pole low pass Bessel filter. The digitization rate was at least 5 times the filter frequency. All experiments were conducted at room temperature.

3. Solutions

The high Cl^- internal solution contained in mM : 120 N-methyl-D-glucamine (NMG)-Cl, 10 tetraethylammonium (TEA)-Cl, 11 NMG2-EGTA, 10 NMG-HEPES, 1 $CaCl_2$, 6 $MgCl_2$, 2 Li_3GDP-β-S, 14 creatine phosphate and 5 $Tris_2ATP$. The high Cl^- external solution contained (mM) 140 TEA-Cl, 10 NMG-HEPES, 5 $BaCl_2$ or 5 $CaCl_2$, 1 $MgCl_2$ and 15 glucose. The pH of both solutions was adjusted to 7.4 using NMG base. 2 mM Li_3GDP-β-S was used to block endogenous G-protein activation.

In low Cl^- internal solution, 120 NMG-Cl and 10 TEA-Cl were substituted to 125 NMG-OH, 20 TEA-OH, respectively and titrated with methanesulfonic acid to pH 7.4.[28] The higher concentrations of NMG and TEA were required to maintain osmolarity. In low Cl- external solution, 140 TEA-Cl was substituted to 140 TEA-OH and titrated with methanesulfonic acid to pH 7.4. 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.

4. Data acquisition and analysis

Voltage steps were generated and data taken using S3 (software developed by Dr. Stephen Ikeda; Guthrie Research Institute, Sayre, PA) on a Macintosh II computer (Apple Computer, Cupertino, CA). IgorPro software (WaveMetrics, Lake Oswego, OR) was used to measure current amplitudes and to fit equations (Marquardt-Levenberg algorithm) to currents and data plots.

5. Drugs

Test solutions were applied from a gravity-fed perfusion system with five inputs and a single output. NMG, creatine phosphate, ATP, EGTA and HEPES were obtained from Sigma Chemical Co. (St. Louis, MO). GDP-β-S was obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). All other chemicals were reagent grade.

RESULTS

1. Calcium enhances inactivation

Calcium currents were recorded in single cells where the external solution was switched from 5 mM Ba^{2+} to 5 mM Ca^{2+}. This switch induced a 10 mV right shift in the I-V curve (Fig. 4) and decreased peak current by an average of 58+9% (n=43). Since we were interested in the effect of Ca^{2+} on channel inactivation, we made several adjustments to compensate for the other effects of Ca^{2+}. Test voltages were depolarized 10 mV in Ca^{2+} compared to Ba^{2+} and currents in Ca^{2+} were normalized to those in Ba^{2+}. During a 5-sec depolarization to generate peak current (0 mV in Ba^{2+}, +10 mV in Ca^{2+}) we observed more inactivation in Ca^{2+} than in Ba^{2+} (Fig. 1B). On average Ca^{2+} induced a 14 % increase in

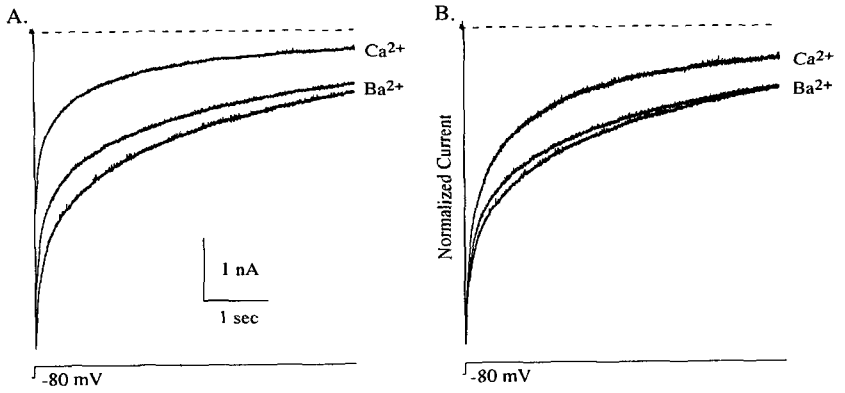

Fig. 1. Calcium enhances inactivation. A) Ca^{2+} current traces shown on the left were sequentially recorded in 5 mM Ba^{2+}, 5 mM Ca^{2+}, and 5 mM Ba^{2+} from the same cell. 5 sec depolarizing pulses were given from a holding potential of -80 mV to the potentials that generated peak current (Ba^{2+} : 0 mV, Ca^{2+} : 10 mV). Ca^{2+} reduced the size of peak current by 57.6% of that in Ba^{2+}. B) Current traces were normalized with respect to the peak current in Ba^{2+} to compare the time course of inactivation between Ba^{2+} and Ca^{2+}. All these recordings were made in high Cl^- solution ($[Cl^-]_o$=152 mM, $[Cl^-]_i$ = 144 mM).

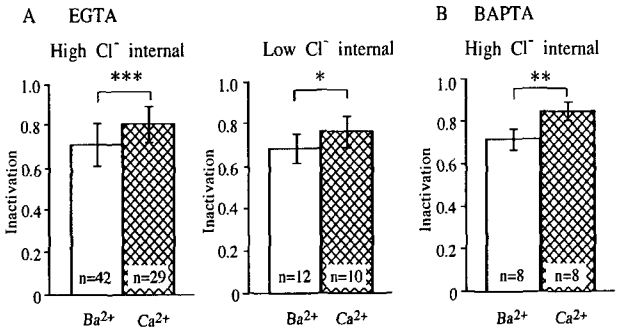

Fig. 2. Calcium effect was not due to calcium-activated chloride current. A) Inactivation between Ba^{2+} and Ca^{2+} were compared both in high Cl^- ($[Cl^-]_i$= 144 mM) and low Cl^- internal solution $[Cl^-]_i$=14 mM) in 11-mM internal EGTA. B. Inactivation between Ba^{2+} and Ca^{2+} were compared in high Cl^-, 20-mM internal BAPTA. Methanesulfonate was used to substitute for Cl^- both in external and internal solution. The magnitude of inactivation was measured as 1-(current at the end of 5 sec/peak current). Error bar represents standard deviation. ANOVA was used for statistical comparison. The inactivation was statistically different between Ca^{2+} and Ba^{2+} in both internal EGTA and in internal BAPTA. The number of cell tried is indicated. The asterisk shows significant difference (* : $p<0.05$, ** : $p<0.01$, *** : $p<0.001$).

inactivation over Ba^{2+} (Fig. 2A) and the Ca^{2+}-induced enhancement of inactivation was observed in 37 of 39 cells.

2. Calcium effect was not due to Calcium activated Chloride current

In solutions in which Cl^- is the major internal and external anion, it is possible that the 5-sec depolarizations could stimulate sufficient Ca^{2+} entry to evoke the Ca^{2+}-activated Cl^- current in rat sympathetic neurons.[29] If Ca^{2+}-activated Cl^- current activates slowly, the effect could masquerade as enhanced inactivation. To investigate the possible contamination by Ca^{2+}-activated Cl^- current, we substituted Cl^- internal in the internal solution with methanesulfonate to give a final Cl^- concentration 14 mM (reduced from 144 mM in our normal internal). With a constant external Cl^- of 144 mM, this substitution should change E_{Cl} from -0 mV to -60 mV, which would increase the amplitude of an outward chloride current at our test voltages (generally 0-20 mV). If Ca^{2+}-activated Cl^- current was present, the effect on calcium current would be observed as enhanced inactivation by the low Cl^- internal. However, Cl^- substitution had no effect on inactivation with either Ca^{2+} or Ba^{2+} as the charge carrier (Fig. 2A). Ca^{2+} still enhanced inactivation with the low Cl^- internal, but a comparison of inactivation between the high and low Cl^- showed no significant differences (Fig. 2A). Thus,

the Ca^{2+}-induced enhancement of inactivation did not result from contamination by Ca^{2+}-activated Cl^- current. Since the presence or absence of Cl^- did not affect inactivation, we grouped the data for analysis (Fig. 6).

Another potential problem is with our use of EGTA. First, H^+ released by EGTA when it binds Ca^{2+} could acidify the cell, which has been shown to decrease N-type calcium current.[30] Second, EGTA is a relatively slow Ca^{2+} buffer,[11, 31] so Ca^{2+} concentration near the channel could increase during our long depolarizations. The resulting reduction in driving force could mimic inactivation. To determine if these problems influenced our results, we repeated our experiments using an internal solution containing 20 mM BAPTA. As with EGTA, the inactivation was larger with Ca^{2+} than with Ba^{2+} as the charge carrier (n=8, $p<0.001$) (Fig. 2B). Thus, the Ca^{2+} induced enhancement of inactivation did not result from problems with EGTA.

3. Calcium enhances N-current inactivation

Though N-type calcium channel is major type in rat sympathetic neurons, non-N-type calcium current comprise 10-40% of the total current.[32-34] To determine the channel type mediating the Ca^{2+} enhancement of inactivation, we used the specific N-channel blocker ω-conotoxin GVIA (ωCGVIA). For the cell illustrated in Fig. 3, ωCGVIA blocked about 80% of calcium current and on average the block was 61±12% (n=6). This value is similar to that previously published from adult rat sympathetic neurons (62±4%).[34] In 3 cells we examined the effect of Ca^{2+} on inactivation both before and after the application of 1 μM ωCGVIA. Ca^{2+} enhanced the inactivation of the total current (before ω CGVIA) by 11±7% (n=3). In the presence of toxin, Ca^{2+} still enhanced inactivation but the effect was smaller (6±1%, n=3) (Fig. 3D). N-current was isolated from total current by subtracting the ωCGVIA-resistant current. The enhancement of inactivation by Ca^{2+} was larger for isolated N-current (13±10%) (Fig. 3C) than the total current. Although small effects of Ca^{2+} were observed in the toxin resistant current, the majority of the enhancement of inactivation was due to effects of Ca^{2+} on N-type calcium channels.

4. Voltage dependence of inactivation

To examine the effect of Ca^{2+} on inactivation over a range of voltages we used a two pulse protocol, with 500 ms prepulses given to different voltages followed by a postpulse to a voltage producing peak inward current (usually +20 mV in Ca^{2+} and +10 mV in Ba^{2+}, Fig. 4). A plot of postpulse current amplitude vs. prepulse voltage revealed that maximal inactivation was observed at voltages near those yielding maximal inward current, with less inactivation at more positive and negative voltages (Fig. 4A, B). A similar U-shaped inactivation vs. voltage relationship has been observed for N-channels in other preparations.[18-20]

The U-shaped voltage-dependence superficially appears to mirror the amplitude of inward current. However, superimposing the inactivation-voltage and the current-voltage (I-V) relationships shows that the inactivation curve is much broader than the I-V rela-

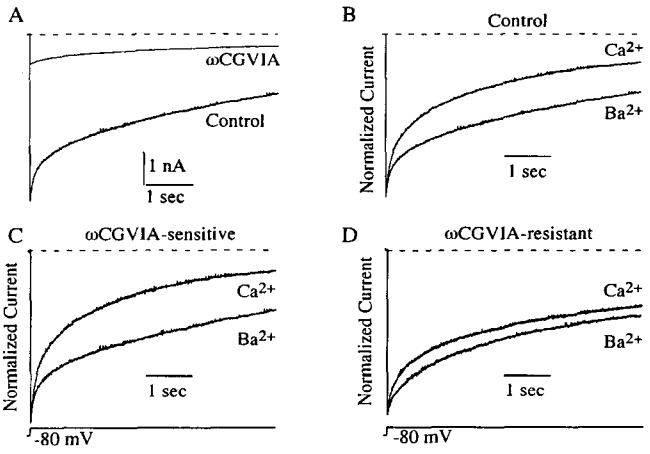

Fig. 3. Calcium enhances N-current inactivation. A) Ca^{2+} currents recorded with Ba^{2+} before and after 1 μM ω-conotoxin GVIA (ωCGVIA) application at the same cell with 5 sec depolarization from -80 mV to 0 mV were shown. B) Normalized Ca^{2+} currents recorded both in Ba^{2+} and Ca^{2+} before (control, a) and after 1 μM ωCGVIA application (ωCGVIA-resistant, b) at the same cell with 5 sec depolarization from -80 mV to 0 mV, 10 mV for Ba^{2+} and Ca^{2+}, respectively. C) ωCGVIA sensitive N-current was isolated by subtracting the current traces of ωCGVIA-resistant (D) from those of control in B.

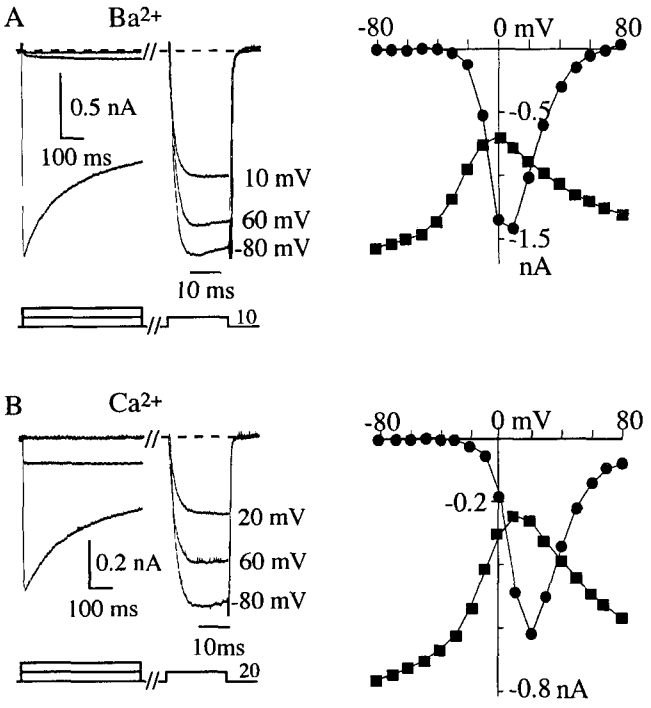

Fig. 4. Voltage dependence of inactivation. 500 ms depolarizing pulses of variable amplitude were given from a holding potential of -80 mV to induce inactivation. This prepulse step followed by a second pulse (postpulse) to the voltage giving maximal inward current (+10 mV in Ba^{2+} and +20 mV in Ca^{2+}) to assay inactivation. Data are shown for a single cell exposed to either Ba^{2+} (A) or Ca^{2+} (B). The prepulse voltage is indicated next to its corresponding current. Tail currents during the repolarization to -80 mV after prepulse were omitted from the current traces during the postpulse. The internal solution contained 20 mM BAPTA. Current-voltage curves are shown with currents measured at the peak current during prepulse (I) and postpulse (n). The data points are averages of two protocols with prepulse voltages given in ascending and descending order. This was done to compensate for slow changes in current amplitude (e.g. rundown) during these long protocols.

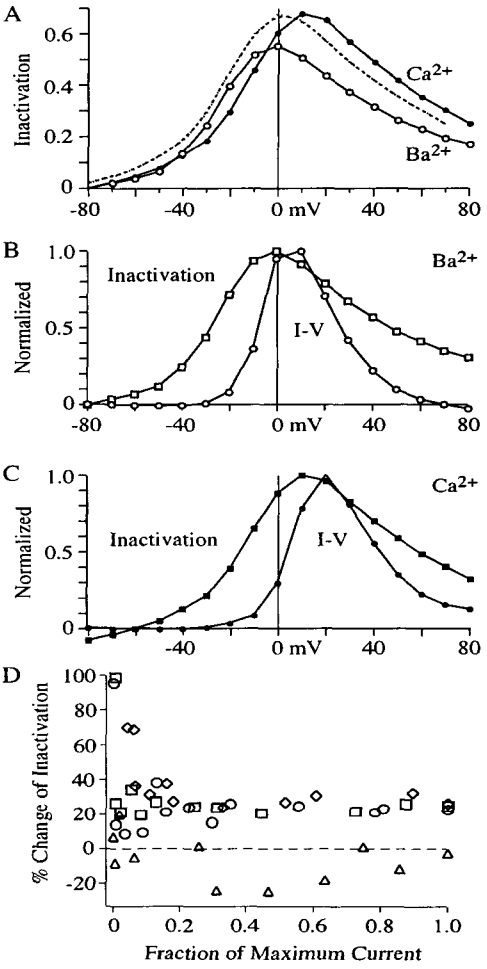

Fig. 5. Ca^{2+} influx is not required for the Ca^{2+} enhancement of inactivation. A) Data from the cell in Fig. 4 are replotted to show the effect of Ca^{2+} on inactivation over a range of voltages. The dashed line is the relationship in Ca^{2+} shifted 10 mV so that peak inactivation in Ca^{2+} matches that in Ba^{2+}. Note Ca^{2+} enhances inactivation at voltages with little or no Ca^{2+} current. B and C. Normalized current is superimposed on a plot of normalized inactivation vs. voltage (from the same cell in Fig. 4). Current (circle) was measured during the prepulse and was normalized to peak inward current (10 mV in Ba^{2+} or 20 mV in Ca^{2+}). Inactivation (square) was measured from the postpulse and was normalized with respect to the maximal inactivation (inactivation at 0 mV in Ba^{2+} and 10 mV in Ca^{2+}). D. The percent change of inactivation induced by Ca^{2+} (relative to that in Ba^{2+}) is plotted versus prepulse voltage for 4 cells where data were obtained in both Ca^{2+} and Ba^{2+}. Ca^{2+} enhanced inactivation in 3 of 4 cells tested and in each case the enhancement was observed at voltage with no measurable Ca^{2+} current. Each different symbol (○,□,△,◇) represents each cell recorded with voltage pulse of Fig. 4.

tionship, which results from substantial inactivation at voltages yielding little or no current (Fig. 5B, C). In addition, the peak of inactivation is shifted by 10 mV to the left to of peak current in both Ba^{2+} and Ca^{2+} (Fig. 5A). While Ca^{2+} does not alter the voltage-dependence of inactivation, the magnitude of inactivation was increased during the 500 ms prepulse. For this example, maximum inactivation in Ca^{2+} was 70% compared to 55% in Ba^{2+} [mean 62.1±0.8% in Ca^{2+} (n=8) and 44.4

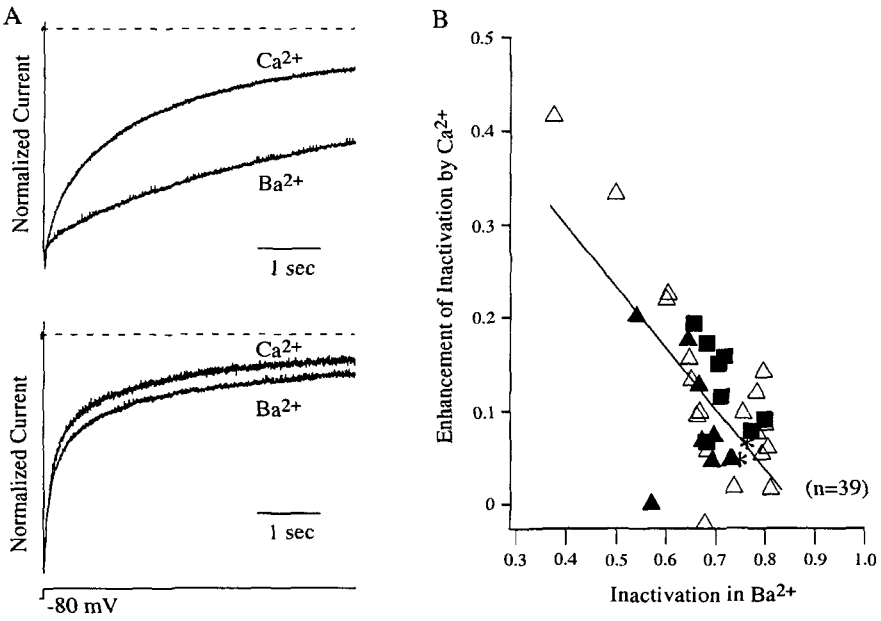

Fig. 6. Inverse relationship between the magnitude of inactivation in Ba^{2+} and the enhancement of inactivation by Ca^{2+}. A) Different cells a. and b. represent the diversity of inactivation in Ba^{2+}. Cells were depolarized for 5 sec from -80 mV to 0 mV for Ba^{2+} and 10 mV for Ca^{2+}. Both cells showed more inactivation in Ca^{2+}. B) The magnitude of inactivation in Ba^{2+} is plotted against the enhancement of inactivation by Ca^{2+}. Cells were pooled regardless of Cl^- concentration or internal Ca^{2+} buffer. Each different symbol represents solution with different Cl^- concentration. With 11 mM internal EGTA, * represents 21 cells recorded in the solution of $[Cl^-]_o$=152 mM, $[Cl^-]i$=144 mM, s represents 8 cells recorded in the solution of $[Cl^-]_o$=152 mM, $[Cl^-]i$=14 mM and * represents 2 cells recorded in the solution of $[Cl^-]_o$=10 mM, $[Cl^-]i$=14 mM. With 20 mM internal BAPTA, n represents 8 cells recorded in the solution of $[Cl^-]_o$=152 mM, $[Cl^-]i$=144 mM. Straight line is the result of linear regression on all the data represented (correlation coefficient r = -0.73, n=39).

±0.8% in Ba^{2+} (n=10)] (Fig. 5A). Thus, Ca^{2+} appears to enhance U-type voltage-dependent inactivation.

5. Two components of inactivation in both Ca^{2+} and Ba^{2+}

During these experiments we noticed the magnitude of inactivation measured during 5-sec steps in Ba^{2+} varied between neurons. Fig. 6A shows two example cells that illustrate the range of inactivation. We examined the possibility that the variability of inactivation was induced by the difference of cell size or series resistance. Plots of membrane capacitance vs inactivation (r=0.053, n=31) and series resistance vs inactivation (r=-0.008, n=31, data not shown) in Ba^{2+} showed no correlation. Interestingly, the effect of Ca^{2+} on inactivation appeared to be inversely related with the magnitude of inactivation in Ba^{2+} (Fig. 6B). This supports the idea that the same inactivation pathways are active with either Ba^{2+} or Ca^{2+} as the charge carrier, and if the inactivation pathways are maximally active in Ba^{2+}, the effect of Ca^{2+} is minimal.

The time course of inactivation during 5-sec voltage steps was nicely fitted with the sum of two exponential equations in both Ba^{2+} and Ca^{2+}. The exponential fits yielded amplitude and time constant for fast and slow components of inactivation. In addition, we derived the fractional amplitudes of fast and slow component relative to the total current (Fig. 7). We investigated the

effect of Ca^{2+} on each of these components by comparing amplitudes and time constants between Ba^{2+} and Ca^{2+}. Fractional amplitude of the fast component of inactivation in Ca^{2+} was significantly larger (0.38±0.11) than that in Ba^{2+} (0.29±0.11, n=31, $p<0.001$). However, there was no significant difference in the fractional amplitude of slow component of inactivation between Ba^{2+} and Ca^{2+} (Fig. 7A).

Examination of the time constant showed the speed of the slow component in Ca^{2+} (1976.0±451.3) was significantly faster than that in Ba^{2+} (2801.1±844.1, n=31, $p<0.001$). However, the speed of the fast component was not altered by Ca^{2+} (Fig. 7B). Although we measured a change in slow time constant, note that this value in Ba^{2+} (~3 sec) may have been limited by our 5-sec voltage step, which was less than two times longer than the calculated time constant. Ca^{2+} had differential effects on the two components of inactivation since it increased only the amplitude of the fast component and appeared to decrease only the time constant of slow component.

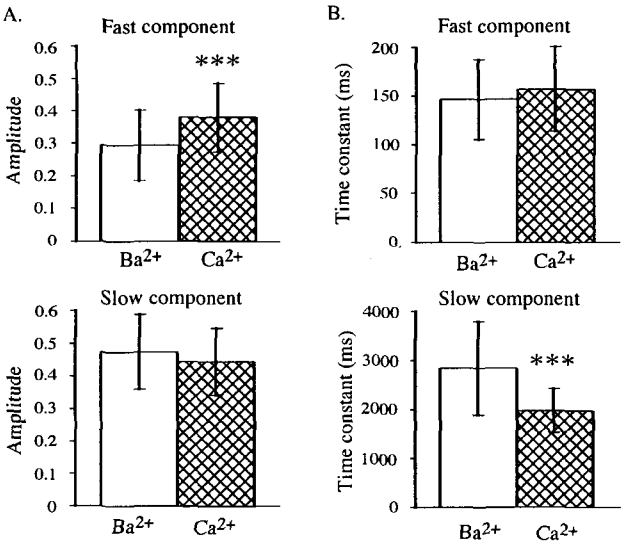

Fig. 7. Differential effects of Ca^{2+} on inactivation. A) Calcium enhances fractional amplitude of the fast component of inactivation. Amplitudes of inactivation between Ba^{2+} and Ca^{2+} were compared. The amplitudes of fast and slow components were calculated from double exponential fitting. The difference in the amplitude of fast component between Ca^{2+} and Ba^{2+} was significantly different ($p< 0.001$), while that in amplitude of slow component was not statistically different between Ca^{2+} and Ba^{2+} ($p>0.05$). B. Calcium increases inactivation rate of slow component. Time constants of inactivation between Ba^{2+} and Ca^{2+} were compared. Time constants were obtained from the two exponential fitting of currents in Ba^{2+} and Ca^{2+}. The difference in slow time constant (τ_s) between Ca^{2+} and Ba^{2+} was significantly different ($p<0.001$), while that in fast time component (τ_f) was not statistically different between Ca^{2+} and Ba^{2+} ($p>0.05$). Error bar represents standard deviation. Paired t-test was used for statistical comparison. Total 31 cells were tested.

6. Time course of the Ca^{2+} effect on inactivation

Much can be learned about a process by examining the speed by which it occurs. The enhancement of inactivation could result from a direct effect of Ca^{2+} on the channel or from Ca^{2+} influencing an enzymatic process (e.g. phosphorylation), which could be slow relative to Ca^{2+} directly effecting the channel. Using 100-ms

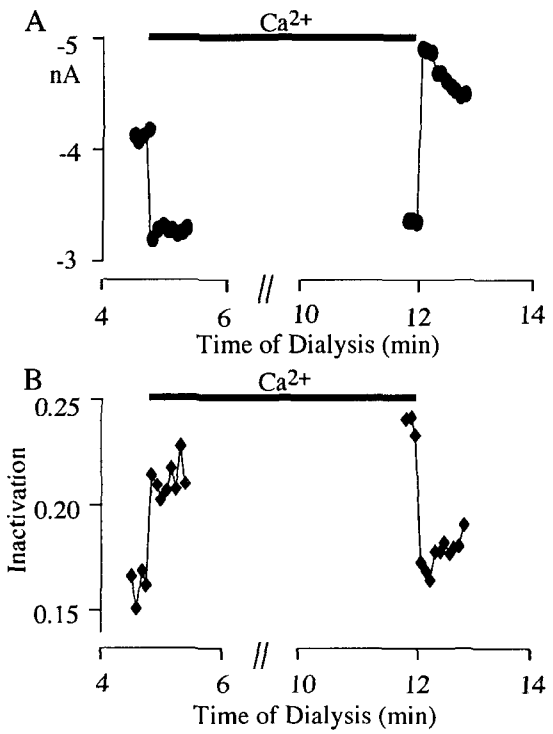

Fig. 8. Time course of changes in current amplitudes and inactivation. A) The switch effects from Ba^{2+} to Ca^{2+} and from Ca^{2+} to Ba^{2+} on peak calcium current were shown. B. The switch effects from Ba^{2+} to Ca^{2+} and from Ca^{2+} to Ba^{2+} on inactivation were shown. Stimulus voltage at 0 mV with 5-sec interval was used to monitor the switch effect. The line indicates the time period when the Ca^{2+} was used as charge carrier.

voltage steps, we compared the time course of effect of Ca^{2+} on current amplitude (a direct Ca^{2+} effect) with its effect on inactivation (an unknown). For the cell in Figure 8, switching from Ba^{2+} to Ca^{2+} reduced peak current to 79 % of that in Ba^{2+}. On average the current in Ca^{2+} was 61.6±16.4 % of that in Ba^{2+} (n=8). The mean Ca^{2+} enhancement of inactivation in these eight cells was 6±2% (n=8). The effect of Ca^{2+} on both current amplitude and inactivation were complete within the 5-sec interval between voltage steps. Although these observations are limited by the 5-sec interval between pulses, they illustrate that there were no profound differences between the temporal change in current amplitude and inactivation. Thus, rapid Ca^{2+} binding could be involved in both processes.

DISCUSSION

Our main goal in this work is to establish the effect of Ca^{2+} on N-channel inactivation. We shows that Ca^{2+} can enhance calcium current inactivation and that this enhancement does not results from artifacts such as intracellular acidification or activation of Ca^{2+}-activated Cl^- current. This effect of Ca^{2+} can be ascribed to the N-type channel, which is the dominant calcium channel type in sympathetic neurons. Ca^{2+} enhances inactivation by differentially affecting two components. The amplitude of the fast component of inactivation (τ = 150 ms) was increased by Ca^{2+}, but the τ_f was unaffected. For the slow component, the τ_s was decreased, but the amplitude was not affected by Ca^{2+}. The main question to be address is whether either of these Ca^{2+} effects can be explained by a classic Ca^{2+}-dependent mechanism of inactivation?

1. Ca^{2+}-dependent inactivation of L-type calcium channels

L-type calcium channels have been shown to inactivate via a Ca^{2+}-dependent mechanism. The manifestation of this mechanism is the increase in the magnitude and kinetics of inactivation upon switching from Ba^{2+} to Ca^{2+}.[10, 35-37] In addition, the inactivation vs. voltage relationship is U-shaped when Ca^{2+} is the charge carrier, with the magnitude of inactivation correlated with calcium current amplitude.[10, 35, 37] Thus, it was concluded that Ca^{2+}-permeating the channels could inactivate them.[38] Early work using L-type calcium channels from cardiac and smooth muscle led to the conclusion that Ca^{2+} was binding to a site on the channel that was close to the pore.[10, 38-40] However, it recently has been demonstrated that calmodulin is the Ca^{2+} sensor that triggers inactivation.[41, 42] The calmodulin appears to be bound to the channel so that it is close the mouth of the pore, as predicted from the earlier studies. P/Q-type channels have an analogous calmodulin binding motif, which binds calmodulin with an affinity similar to that of L-channels.[37, 43] The interaction between P/Q-channels and calmodulin appears to be required for a Ca^{2+}-dependent inactivation of these channels.[43, 44] Interestingly, the analogous site on N-type channels binds calmodulin very poorly.[37, 45] Thus, Ca^{2+} effects on N-channel inactivation may result from a different mechanism or from calmodulin binding to a different site on the channel.

2. Fast N-channel inactivation

The effect of Ca^{2+} on N-channel inactivation is subtler than that on L-channel inactivation. A small enhancement of inactivation has generally been observed upon substitution of Ba^{2+} by Ca^{2+}[18, 20, 46] (Fig. 1). However, inactivation kinetics are not dramatically altered by Ca^{2+} substitution[19, 46] (Fig. 7). N-current inactivation has been shown to be kinetically complex with fast (τ =100-200 ms) and slow (τ =1000-3000 ms) components.[19, 46] These two components can be identified in both Ca^{2+} and Ba^{2+} 19,46) (Fig. 7). Thus, these properties are not consistent with a Ca^{2+}-dependent hypothesis. The Ca^{2+} effect on fast inactivation is to increase the amplitude without altering the τ (Fig. 7). Our expectation was that the speed of inactivation would also be increased as in L-channels. Interestingly, the speed of slow inactivation was increased by Ca^{2+}, which may indicate a Ca^{2+}-dependent process (see below). One intriguing finding was that the size of the

Ca^{2+} enhancement of inactivation was inversely correlated with the magnitude of inactivation in Ba^{2+}. One explanation is that the same inactivation mechanisms are active in both Ca^{2+} and Ba^{2+}. The Ca^{2+} effect is small when the inactivation pathways are maximally activated in Ba^{2+}, and the effect is larger when the pathways are not fully engaged. This idea suggests that an inactivation pathway selective for Ca^{2+} has little or no impact on N-channels. We believe this statement is true for fast inactivation, but as described below there may be a Ca^{2+}-senstive component to slow inactivation.

One final attribute of N-channel inactivation is that the inactivation vs. voltage relationship is U-shaped in both Ca^{2+} and Ba^{2+}[19, 20, 46] (Fig. 4), which is against a Ca^{2+}-dependent mechanism. The voltage steps used to generate this relationship were 500 ms in duration, which primarily isolated the fast component of inactivation (τ=150 ms). The U-shaped N-current inactivation vs. voltage relationship has been modeled as a purely voltage-dependent process.[18, 20] This type of inactivation was subsequently termed U-type inactivation.[23] However, a U-shaped voltage dependence of inactivation has been observed in other calcium channel types when Ba^{2+} is the charge carrier, including L-type channels (α_{1C}) expressed in tsA201 cells[47] and E-class channels (α_{1E}) expressed in HEK293 cells.[48] These authors raised the possibility that Ba^{2+} can activate the Ca^{2+}-dependent inactivation process in these channels. Is it possible that N-channel inactivation in Ba^{2+} results from Ba^{2+} activation of a Ca^{2+}-dependent inactivation mechanism?

3. U-type inactivation

Jones and Marks[8] presented several arguments to discount a current-dependent mechanism for N-channel inactivation in Ba^{2+}. First, peak N-channel inactivation was consistently observed at a voltage -10 mV hyperpolarized to that generating peak current (see Fig. 5). Thus, peak current did not generate maximal inactivation. However, a similar observation has been made for L-channel inactivation in Ca^{2+}.[35, 37, 49, 50] This offset of maximal inactivation from peak current has been explained within the context of a Ca^{2+}-dependent mechanism of inactivation. Noceti et al.[49] were able to account for the shift by developing a model where inactivation depended on both Ca^{2+} influx and L-channel open probability. However, such a shift was predicted by a model where calmodulin was required to bind multiple Ca^{2+} ions to trigger Ca^{2+}-dependent inactivation.[50] Thus, the observed shift of maximal inactivation to voltages hyperpolarized to those generating peak current can be explained by either a voltage-dependent[18, 20] or Ca^{2+}-dependent[49, 50] mechanism. The second argument to discount a current-dependent mechanism was the observation of significant N-channel inactivation at voltages that generated little or no current.[18] This observation clearly deviates from a Ca^{2+}-dependent mechanism where inactivation requires inward calcium current.[10, 35, 37] Substantial N-channel inactivation at voltages that fail to generate inward current is a consistent observation from many preparations[19, 20, 46] (Fig. 5). In addition, we demonstrated that the Ca^{2+} enhancement of inactivation is not correlated with inward calcium current (Fig. 5C). Thus, the N-current inactivation vs. voltage relationship is inconsistent with a Ca^{2+}-dependent mechanism for inactivation. Finally, raising external Ba^{2+} concentration failed to increase inactivation,[8, 46] as expected for a current-dependent mechanism.

Additional evidence against current dependence comes from experiments showing U-type inactivation in potassium channels.[22, 23] In these experiment, the outward potassium current increases with voltage, but the inactivation shows a U-shaped voltage dependence. Together, the N-channel and potassium channel observations demonstrate that U-type inactivation is independent of current amplitude. These data are best fit by a voltage-dependent model where channels inactivate preferentially from intermediate closed states on the pathway to channel opening.[20, 22, 23]

One riddle with the proposed current-dependent mechanism for inactivation in Ba^{2+}[47, 48] is the mechanism by which such an effect would occur. Haack and Rosenberg[36] demonstrated that fast Ca^{2+}-dependent in-

activation could not be activated in L-channels with intracellular Ba^{2+} concentrations up to 1 mM. However, fast inactivation was induced with low concentrations of internal Ca^{2+} (10 μM). These results are consistent with the affinity of calmodulin and other E-F hand proteins for Ca^{2+} (~2.5 μM) and Ba^{2+} (>1000 μM).[51] Thus, it appears unlikely that Ba^{2+} could activate the calmodulin-mediated Ca^{2+}-dependent mechanism of inactivation.

4. Slow N-channel inactivation

During 5-sec voltage steps we were able to observe a slow component to inactivation. Exponential fitting estimated the τ to be ~3 sec in Ba^{2+} and ~2 sec in Ca^{2+}. Given that the voltage step was less than 2X the slow inactivation τ in Ba^{2+}, the derived value may be limited by the step duration. The estimate of slow inactivation ? in Ca^{2+} should be more accurate. Since the τ in Ba^{2+} could be larger than we estimate, the 1/3 reduction by Ca^{2+} may be an underestimate. Thus, Ca^{2+} increases the speed of slow inactivation, which is consistent with a Ca^{2+}-dependent inactivation mechanism. Previous work has generally focused on fast inactivation, since slow inactivation of N-channels is more difficult to study. However, one noteworthy study examined slow inactivation in rat sensory neurons using a 3-sec prepulse to inactivate the channels and a postpulse to assess channel availability.[24] Current amplitude during the postpulse was compared when either Ca^{2+} or Mg^{2+} was present during the prepulse. Mg^{2+} was rapidly removed prior to generating the postpulse. These experiments showed significantly more inactivation when Ca^{2+} was present during the prepulse than when Mg^{2+} was present, which supported the idea that inactivation measured over seconds had a Ca^{2+}-dependent component.[24]

5. Hypothesized mechanism for Ca^{2+} enhancement of fast inactivation

It is clear that Ca^{2+} has effects on fast inactivation, but much of the available evidence does not support a classic Ca^{2+}-dependent mechanism. To explain the N-channel inactivation data, Patil et al.[20] proposed that divalent cations are required for N-channels to undergo fast U-type inactivation. This hypothesis differs from the classic Ca^{2+}-dependent inactivation in it is the occupancy of the channels by divalent cations permits the channel to inactivate. Thus, Ca^{2+} and Ba^{2+} do not need to permeate the channel to trigger the inactivation mechanism. The enhancement of inactivation by Ca^{2+} may correspond to stronger binding of Ca^{2+} than Ba^{2+} to the channel site that permits N-channels to undergo U-type inactivation.

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흰쥐 교감신경 뉴론 N형 칼슘전류의 비활성화에 미치는 칼슘효과

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N형 칼슘전류의 비활성화 vs 전압곡선은 U형을 보인다 - 즉 칼슘 내향전류의 크기와 비활성화 정도가 어느 정도 일치한다. 이러한 U형 비활성화는 순수한 전압의존성 기전으로 설명되어져 왔으나 칼슘의존성 비활성화 기전 또한 보고되었다. 이 연구에서는 흰쥐 상행 경동맥 결절뉴론을 단일 세포로 얻은 후, whole cell patch clamp technique를 사용하여 N형 칼슘전류를 기록하고, 세포외액의 charge carrier 로서 바륨과 칼슘을 사용하면서, 칼슘이 N형 칼슘통로의 비활성화에 미치는 역할을 알아보았다. charge carrier 로 칼슘을 사용하였을 경우에 바륨을 사용하였을 때에 비하여 비활성화 정도가 증가하였으며 이러한 증가는 세포속 Ca^{2+} Chelator 가 11 mM EGTA 로부터 20 mM BAPTA 로 치환되어도 계속 관찰되었다. 비활성화 vs 전압 곡선은 바륨과 칼슘 모두에서 U형이었다. charge carrier 를 칼슘으로 치환시 추가로 유도되는 비활성화 정도는 바륨사용시의 비활성화 정도와 역비례관계를 보여 두 이온에서 같은 기전으로 비활성화가 일어날 가능성을 시사하였다. 이러한 가능성을 지원해 주는 결과로 5초의 긴 저분극 자극시 바륨과 칼슘을 써서 얻은 전류기록은 2중 지수함수로 잘 그려낼 수 있었고, 그 결과 빠른 성분(시정수 : -150 ms) 과 느린 성분(시정수 : -2500 ms) 를 얻었다. 칼슘이 각각의 성분에 미치는 효과는 각기 달라서 빠른 성분의 amplitude는 증가하였고 느린 성분의 시정수는 빨라졌다. 칼슘에 의해 빠른 성분의 amplitude는 증가하였으므로 이는 더 많은 채널이 빠른 경로로 비활성화되었음을 시사한다. 빠른 성분의 시정수는 변화하지 않았으므로, 이는 비활성화의 빠른 경로는 칼슘과 바륨에서 같음을 시사하며 즉 비활성화 기전이 칼슘의존성이 아님을 보여주는 증거이다. 그러나 비활성화의 느린 성분은 칼슘에 의해 그 시정수가 빨라졌으므로 칼슘의존성일 가능성이 있다.

중심단어 : N형 칼슘전류, 비활성화, 패치클람프 테크닉, 전압의존성, 칼슘의존성, U형 비활성화