Block of N-type Calcium Channels in Chick Sensory Neurons by External Sodium

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Abstract: L-type Ca\(^{2+}\) channels select for Ca\(^{2+}\) over sodium Na\(^+\) by an affinity-based mechanism. The prevailing model of Ca\(^{2+}\) channel permeation describes a multi-ion pore that requires pore occupancy by at least two Ca\(^{2+}\) ions to generate a Ca\(^{2+}\) current. At \([\text{Ca}^{2+}] < 1 \mu\text{M}, \text{Ca}^{2+} \text{ channels conduct Na}^{+}\). Due to the high affinity of the intrapore binding sites for Ca\(^{2+}\) relative to Na\(^+\), addition of \(\mu\text{M}\) concentrations of Ca\(^{2+}\) block Na\(^+\) conductance through the channel. There is little information, however, about the potential for interaction between Na\(^+\) and Ca\(^{2+}\) for the second binding site in a Ca\(^{2+}\) channel already occupied by one Ca\(^{2+}\). The two simplest possibilities, (a) that Na\(^+\) and Ca\(^{2+}\) compete for the second binding site or (b) that full time occupancy by one Ca\(^{2+}\) excludes Na\(^+\) from the pore altogether, would imply considerably different mechanisms of channel permeation. We are studying permeation mechanisms in N-type Ca\(^{2+}\) channels. Similar to L-type Ca\(^{2+}\) channels, N-type channels conduct Na\(^+\) well in the absence of external Ca\(^{2+}\). Addition of 10 \(\mu\text{M}\) Ca\(^{2+}\) inhibited Na\(^+\) conductance by 95%, and addition of 1 mM Mg\(^{2+}\) inhibited Na\(^+\) conductance by 80%. At divalent ion concentrations of 2 mM, 120 mM Na\(^+\) blocked both Ca\(^{2+}\) and Ba\(^{2+}\) currents. With 2 mM Ba\(^{2+}\), the \(I_{\text{Ca}}\) for block of Ba\(^{2+}\) currents by Na\(^+\) was 119 mM. External Li\(^+\) also blocked Ba\(^{2+}\) currents in a concentration-dependent manner, with an IC\(_{50}\) of 97 mM. Na\(^+\) block of Ba\(^{2+}\) currents was dependent on [Ba\(^{2+}\)]; increasing [Ba\(^{2+}\)] progressively reduced block with an IC\(_{50}\) of 2 mM. External Na\(^+\) had no effect on voltage-dependent activation or inactivation of the channel. These data suggest that at physiological concentrations, Na\(^+\) and Ca\(^{2+}\) compete for occupancy in a pore already occupied by a single Ca\(^{2+}\). Occupancy of the pore by Na\(^+\) reduced Ca\(^{2+}\) channel conductance, such that in physiological solutions, Ca\(^{2+}\) channel currents are between 50 and 70% of maximal.

Key words: calcium channels • ion channel selectivity • sodium • permeation

Introduction: Calcium (Ca\(^{2+}\)) channels are highly selective for Ca\(^{2+}\) over sodium Na\(^+\) and other monovalent cations. Their remarkable selectivity is accomplished by differential affinity of Ca\(^{2+}\) and monovalent cations for one or more intrapore binding sites (Almers et al., 1984; Hess and Tsien, 1984; Hess et al., 1986). In the absence of Ca\(^{2+}\), monovalent cations conduct well through Ca\(^{2+}\) channels (Kostyuk et al., 1983; Almers et al., 1984; Fukushima and Hagiwara, 1985; Hess et al., 1986; Matsuda, 1986). Addition of \(\mu\text{M}\) [Ca\(^{2+}\)] completely blocks (\(K_{d} = \sim 0.7 \mu\text{M}\)) the monovalent cation conductance but does not result in the generation of Ca\(^{2+}\) currents. At much higher [Ca\(^{2+}\)], Ca\(^{2+}\) currents are generated. In L-type Ca\(^{2+}\) channels, Ca\(^{2+}\) current saturates with a \(K_{d}\) for Ca\(^{2+}\) of \(\sim 14 \text{mM}\) (Hess et al., 1986). This concentration difference required for block of Na\(^+\) conductance and generation of Ca\(^{2+}\) currents is one of the signature characteristics of a multi-ion pore (Almers et al., 1984; Hess and Tsien, 1984; Hille, 1992).

The classical two-site model of the Ca\(^{2+}\) permeation pathway describes the pore as containing two identical high-affinity cation binding sites in the permeation pathway (Almers et al., 1984; Hess and Tsien, 1984). Due to the high affinity of Ca\(^{2+}\) for these sites, the first available site is bound by Ca\(^{2+}\) at very low concentrations. At higher concentrations, a second Ca\(^{2+}\) enters simultaneously, and the electrostatic repulsion between these two tightly packed divalent ions lowers the apparent \(K_{d}\) for the sites and results in Ca\(^{2+}\) current. In such a model, the 14 mM \(K_{d}\) for Ca\(^{2+}\) current saturation can be interpreted as representing the affinity of Ca\(^{2+}\) for the second binding site once the first binding site is bound by Ca\(^{2+}\) (Tsien et al., 1987).

Alternative models that have a single high-affinity binding site have been proposed that equally well explain the Ca\(^{2+}\) channel permeation data (Armstrong and Neyton, 1991; Yang et al., 1993; Dang and McCleskey, 1996). All of these models share the concept, however, that at least two ions must occupy the pore to generate current.

In the presence of \(\mu\text{M}\) [Ca\(^{2+}\)], two observations suggest that these two sites in the Ca\(^{2+}\) channel pore can be simultaneously occupied by one Ca\(^{2+}\) and one monovalent cation (Kuo and Hess, 1993). First, in Ca\(^{2+}\) channels carrying outward Li\(^+\) currents, high external [Li\(^+\)] decreases the outward exit rate of the blocking Ca\(^{2+}\) ion from the pore, producing a “lock in” type of effect in the channel. Second, elevation of external [Li\(^+\)] decreases the on-rate of external Ca\(^{2+}\), which suggests that external Li\(^+\) interferes with access.
of Ca\(^{2+}\) to its high affinity site. When the channel is occupied by one Ca\(^{2+}\) or less, the affinity of Li\(^{+}\) for its binding site has an apparent \(K_d\) of \(\sim 100\) mM (\(\sim 75\) mM expressed as activity). The lock-in effect suggests that Li\(^{+}\) binds externally to Ca\(^{2+}\) within the pore.

The interaction between Ca\(^{2+}\) and monovalents in the pore is derived from studies in low [Ca\(^{2+}\)] conditions, with Li\(^{+}\) as the monovalent charge carrier. There is little information, however, about the potential interaction between Ca\(^{2+}\) and Na\(^{+}\) in physiological solutions. Although Na\(^{+}\) appears to bind to the channel with somewhat lower affinity than Li\(^{+}\) (Hess et al., 1986), the studies in low [Ca\(^{2+}\)] suggest that there is still less than a 10-fold difference in apparent affinity between Ca\(^{2+}\) and Na\(^{+}\) for the second binding site (\(\sim 14\) vs. \(\sim 100\) mM). Consequently, if extrapolated to high [Ca\(^{2+}\)] conditions, the model predicts that Ca\(^{2+}\) and Na\(^{+}\) would compete for the second binding site. If the competition predicted by the model occurs at physiological concentrations, the pore will be doubly occupied by Ca\(^{2+}\) less in the presence of Na\(^{+}\) than in the absence of Na\(^{+}\). Since a channel occupied by one Ca\(^{2+}\) and one monovalent cation does not conduct (Almers et al., 1984; Kuo and Hess, 1993a, b), this large decrease in double occupancy by Ca\(^{2+}\) would be expected to result in Ca\(^{2+}\) current reduction.

An alternative possibility is that Na\(^{+}\) is excluded from the pore at physiological [Ca\(^{2+}\)] and [Na\(^{+}\)]. This possibility is supported by data from guinea pig ventricular cells and mouse neoplastic B lymphocytes, in which Ca\(^{2+}\) currents were unaffected when external Na\(^{+}\) was replaced by Tris or choline (Matsuda and Noma, 1984; Yamashita et al., 1984). Reconciliation of these conflicting possibilities is important for understanding Ca\(^{2+}\) channel permeation, since one suggests that permeation is strictly governed by competition for binding sites and the other suggests that higher [Ca\(^{2+}\)] induces an allosteric change in the channel that prevents monovalent cations from binding.

Nearly all studies of Ca\(^{2+}\) channel permeation have used L-type Ca\(^{2+}\) channels. We demonstrate here that N-type Ca\(^{2+}\) channels in chick sensory neurons display permeation properties similar to those of L-type Ca\(^{2+}\) channels. In physiological [Na\(^{+}\)] and [Ca\(^{2+}\)], external Na\(^{+}\) blocks N-type Ca\(^{2+}\) channels in a concentration-dependent manner, and this block appears to result from a competitive interaction between Ca\(^{2+}\) and Na\(^{+}\) in the pore.

**MATERIALS AND METHODS**

**Cells**

Dorsal root ganglion (DRG)\(^1\) neurons were acutely isolated from thoracic and lumbar level ganglia of 14-d-old white leghorn chick embryos (UCONN Poultry Farm, Storr’s, CT). Ganglia were incubated in Tyrodes (in mM: 128 NaCl, 3 KCl, 1 MgCl\(_2\), 27 NaHCO\(_3\), 10 glucose, pH 7.3) containing 0.08% trypsin (#610-5050PG; Gibco Laboratories, Grand Island, NY) for 30 min at 37°C in a CO\(_2\) incubator (both 6 and 10% CO\(_2\) in air were used with no detectable difference). Cells were removed from the incubator and washed three times with Media 199 (#B-1292-AX plus 3.1 g/liter NaHCO\(_3\); Hyclone Laboratories, Inc., Logan, UT) plus 10% FBS (#A-1115-L; Hyclone Laboratories, Inc.). Cells were dissociated in Media 199 by trituration with a siliconized, fire-polished pasteur pipet and plated on polyornithine-coated 35-mm culture dishes. Cells were maintained in this media in a 37°C incubator and used in experiments 1–8 h after plating.

**Patch Clamp Recording**

Recordings were made with the standard whole cell patch clamp configuration (Hamill et al., 1981). Patch pipets were fabricated from N51A glass (Garner Glass Co., Claremont, CA), coated with Sylgard (#184; Dow Corning, Midland, MI), and fire-polished. Series resistance ranged from 0.9–3.0 MΩ (mean = 1.9 ± 0.3 MΩ, \(n = 221\)), and membrane capacitance ranged from 9.0 to 44.6 pF (mean = 24.5 ± 0.6 pF, \(n = 221\)). Capacitive transients were electronically neutralized and series resistance compensation was used, generally at ~90% (3911A patch clamp amplifier; Dagan Corp., Minneapolis, MN). In all experiments except those described in Fig. 5, membrane currents were filtered at 2 kHz (internal patch clamp filter) and digitized at sample intervals of 100–400 μs/pt. Tail currents in Fig. 5 were filtered at 50 kHz and digitized at 3 μs/pt. Unless otherwise stated, the holding potential was ~80 mV, and Ca\(^{2+}\) currents were evoked by a 100-ms depolarizing stimulus once every 5–10 s. Experiments were performed at room temperature (20–24°C). Data were acquired and processed with pClamp 6 (Axon Instruments, Foster City, CA).

**Solutions**

Recordings were made from cells plated in 35-mm tissue culture dishes that contained 1.5 ml of bathing solution. Both static and continuous flow baths were used. When a static bath was used, the external solution bathing the cells was changed by manually switching valves on 1 of 6 input lines. Solutions flowed continuously over the cell under study and were changed by manually switching valves on 1 of 6 input lines. Except as stated otherwise, the control external solution bathing the cells contained (in mM): 150 TEA-Cl, 2–3 BaCl\(_2\), 1 MgCl\(_2\), 10 glucose, 10 HEPES, pH 7.3 (TEAOH), osmolality = 320 ± 5 mosm/kg. Tetrodotoxin (TTX; 1 μM) was added to all external solutions. Except in the experiments described in Fig. 4, Na\(^{+}\) was applied externally by equimolar replacement of 120 mM TEA. The standard pipet solution contained (in mM): 90 NMG-Cl, 30 CsCl, 30 TEA-Cl, 5 EGTA-NMG, 10 HEPES, 4 MgCl\(_2\), 4 creatine phosphate, 4 ATP-Na, leupeptin, and creatine kinase, pH 7.3 (CsOH), osmolality = 305 ± 5 mosm/kg. Addition of 0.2 mM GTP to the pipet solution did not qualitatively influence the results. Substitutions are listed in the figure legends.

**Data Analysis**

All curve fitting and statistics were done with Sigmasoft SigmaPlot 2.0 for Windows (Jandel Scientific, Corte Madera, CA). Average values

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\(^1\) Abbreviation used in this paper: DRG, dorsal root ganglion.
are expressed as mean ± standard error of the mean (SEM), with the number of samples given in parentheses. Statistical significance was tested by unpaired Student’s t test. Data on percent block of Ca$^{2+}$ channel currents by monovalent cations (Figs. 4 C and 8 C) were fit by the following equation:

$$y = 100 \cdot \frac{[\text{cation}]}{IC_{50} + [\text{cation}]}$$

where $y$ is the percentage block, $IC_{50}$ is the concentration for 50% block, and $k$ is the slope coefficient, which represents the number of molecules acting in a cooperative manner. The data in Fig. 7 were fit to the complementary function.

**RESULTS**

**Ca$^{2+}$ Channel Type**

It was previously demonstrated that Ca$^{2+}$ currents through DRGs acutely isolated from 11–12-d-old chick embryos consist entirely of N-type Ca$^{2+}$ channels (Cox and Dunlap, 1992). When cells were kept in culture for several days, however, other Ca$^{2+}$ channel types appeared (Cox and Dunlap, 1992). Since we used cells isolated from slightly older embryos, we examined the sensitivity of currents in our preparation to $\omega$-conotoxin GVIA, which selectively blocks N-type Ca$^{2+}$ channels in chick DRGs at concentrations of 1–10 $\mu$M (Aosaki and Kasai, 1989; Cox and Dunlap, 1992). Conotoxin (10 $\mu$M) irreversibly blocked Ca$^{2+}$ currents by 94.4 ± 2.7% ($n = 18$); inhibition = 100% in 14 of 18 cells) and Ba$^{2+}$ currents by 93.1 ± 1.2% ($n = 4$). In four cells tested, 1 $\mu$M conotoxin also produced a 100% block of Ca$^{2+}$ current. Also consistent with N-type Ca$^{2+}$ channel properties, currents were completely inactivated by depolarization to −10 mV, with half-maximal inactivation at −64.4 ± 1.5 mV ($n = 6$). Identical pharmacological and kinetic results were obtained from cells ranging in size from 12 to 40 $\mu$m, which indicates that the channel population in cells of all sizes studied was identical in these respects. These results indicate that the channel population in our cells was composed almost exclusively of N-type Ca$^{2+}$ channels.

**Permeation Characteristics of the Chick N-type Ca$^{2+}$ Channel**

The theoretical framework for understanding Ca$^{2+}$ channel permeation is derived almost exclusively from studies on L-type Ca$^{2+}$ channels. To determine whether chick N-type Ca$^{2+}$ channels used a fundamentally similar selectivity mechanism, we tested for the salient feature of Ca$^{2+}$ channel permeation: conductance of Na$^+$ in the absence of divalent cations and inhibition of Na$^+$ conductance by $\mu$M [Ca$^{2+}$].

In the absence of Na$^+$, removal of external Ba$^{2+}$ completely abolished inward currents (Fig. 1 A and B; $n = 4$). When the Ba$^{2+}$-free solution contained 120 mM Na$^+$, however, a residual, voltage-activated current was always present (Fig. 1 C2). Our solutions made in the absence of experimentally added Ca$^{2+}$ typically contain several $\mu$M Ca$^{2+}$. Upon addition of 1 mM EGTA to chelate this residual Ca$^{2+}$, the magnitude of the inward current increased (Fig. 1 C3). Removal of external Mg$^{2+}$ in this Ca$^{2+}$-free, Ba$^{2+}$-free solution resulted in an additional large increase in Na$^+$ current magnitude (Fig. 1 C4). Finally, addition of just 10 $\mu$M Ca$^{2+}$ to the Ba$^{2+}$-free, Mg$^{2+}$-free bath solution inhibited the inward current by 95.0 ± 0.1% ($n = 3$; Fig. 1 C5). Increasing the Ca$^{2+}$ concentration to 100 $\mu$M resulted in an additional 2–3% inhibition of inward current magnitude ($n = 3$; not shown). Upon return to the control solution, the current magnitude returned nearly to control values (Fig. 1 C6). These results demonstrate that in the absence of Ca$^{2+}$ (or Ba$^{2+}$) Na$^+$ conducts well through the chick N-type Ca$^{2+}$ channel, and that both Mg$^{2+}$ and low concentrations of Ca$^{2+}$ inhibit Na$^+$ conductance through the channel. Thus, these data suggest that the chick N-type Ca$^{2+}$ channel selects for Ca$^{2+}$ over Na$^+$ by

![Figure 1](image-url)
Ca\textsuperscript{2+} Channel Block by External Na\textsuperscript{+}

Fig. 2 shows representative currents recorded in the presence of external TEA (Cont.) and Na\textsuperscript{+}. Replacement of external TEA by Na\textsuperscript{+} (150 mM) reversibly reduced Ca\textsuperscript{2+} current magnitude with no shift in activation voltage or reversal potential (Fig. 2 B). With Ca\textsuperscript{2+} as the charge carrier, we were rarely able to record pure Ca\textsuperscript{2+} channel currents. Despite using a variety of solutions designed to eliminate other currents, depolarization usually resulted in activation of two large currents other than Ca\textsuperscript{2+} currents. One of these was a delayed rectifier K\textsuperscript{+} channel that conducts Na\textsuperscript{+} in the absence of K\textsuperscript{+} (Callahan and Korn, 1994). The tail current marked by the dotted arrow in Fig. 2 A illustrates an Na\textsuperscript{+} current through the K\textsuperscript{+} channel. Many DRG neurons also contain a Ca\textsuperscript{2+}-dependent Cl\textsuperscript{−} channel (see Callahan and Korn, 1994). To minimize the contamination of Ca\textsuperscript{2+} currents by these other currents, the remaining experiments were conducted with Ba\textsuperscript{2+} as the permeating cation. Ba\textsuperscript{2+} does not activate the Ca\textsuperscript{2+}-dependent Cl\textsuperscript{−} channel (Korn and Weight, 1987), and Ba\textsuperscript{2+} blocks Na\textsuperscript{+} currents through the Na\textsuperscript{+}-conducting K\textsuperscript{+} channel, with an IC\textsubscript{50} of ~1 mM (Callahan and Korn, 1994). To block the remaining currents through the K\textsuperscript{+} channel, we added 30 mM Cs\textsuperscript{+} to the pipet solution (to block Na\textsuperscript{+} currents) and 30 mM TEA to both the internal and external solutions (to block Cs\textsuperscript{+} current). With this combination of ions, there was little or no shift in reversal potential after application of Na\textsuperscript{+}, no detectable Na\textsuperscript{+} or Cl\textsuperscript{−} tail currents at Ba\textsuperscript{2+} concentrations of 2 mM or more (e.g., see Figs. 3 B and 4 A), and no detectable outward currents upon removal of Ba\textsuperscript{2+} and/or Na\textsuperscript{+}. This indicated that the

\textbf{Figure 2.} Inhibition of Ca\textsuperscript{2+} currents by extracellular Na\textsuperscript{+}. (A) Three superimposed Ca\textsuperscript{2+} currents recorded during control (0 external Na\textsuperscript{+}), after equimolar replacement of external TEA by 150 mM Na\textsuperscript{+}, and during recovery (0 Na\textsuperscript{+}). The stimulus was a 100-ms depolarization from −80 to −10 mV. The dashed arrow points to a tail current carried by Na\textsuperscript{+} through a delayed rectifier K\textsuperscript{+} channel. (B) Current-voltage curves in the three conditions mentioned. Pipet solution (in mM): 150 NMG, 10 EGTA, 20 HEPES, 4 MgCl\textsubscript{2}, 4 creatine phosphate, 4 ATP-Na, creatine kinase and leupeptin, pH 7.3 with NMG, osmolality 310. External solution (in mM): 150 TEA-Cl or NaCl, 2 CaCl\textsubscript{2}, 1 MgCl\textsubscript{2}, 10 HEPES, 0.001 TTX, pH 7.3 with TEAOH.

\textbf{Figure 3.} Inhibition of Ba\textsuperscript{2+} currents by extracellular Na\textsuperscript{+}. The external solution contained 3 mM Ba\textsuperscript{2+}. (A) Control family of currents, evoked by depolarizations between −40 and +40 mV. (B) Currents following substitution of 120 mM Na\textsuperscript{+} for TEA. (C) I-V curves before, during and following removal of 120 mM Na\textsuperscript{+}. (D) Activation curves, normalized to the peak control current at −5 mV. Symbols represent mean ± SEM for five cells in each condition. Solid lines represent the best fit to the data, using a modified Boltzmann equation, \[ g = g_{\text{max}} \cdot \frac{1}{1 + \exp\left(\frac{V - V_{1/2}}{K}\right)}^{-1}. \] Values for \( V_{1/2} \) (in mV) were −18.3 ± 0.16 (Cont.) and −18.6 ± 0.17 (120 mM Na\textsuperscript{+}). Values for \( K \) (in mV) were 4.18 (Cont.) and 4.33 (120 mM Na\textsuperscript{+}).
currents observed were essentially pure Ca\textsuperscript{2+} channel currents.

**Ba\textsuperscript{2+} Current Block by Na\textsuperscript{+}**

Replacement of external TEA with Na\textsuperscript{+} (120 mM) reversibly inhibited Ba\textsuperscript{2+} currents (Fig. 5) with no change in reversal potential (Fig. 3 C), activation voltage (Fig. 3 D), or voltage-dependence of inactivation (not shown). The concentration dependence of Ba\textsuperscript{2+} current block by Na\textsuperscript{+} was examined in two ways. In one set of experiments, Na\textsuperscript{+} concentration was increased to 10, 30, or 120 mM by equimolar replacement of external TEA (as in Fig. 3). In the second set of experiments, Na\textsuperscript{+} concentration was increased to 30, 120, or 300 mM in the presence of 150 mM TEA (Fig. 4). Concentration dependence of Ba\textsuperscript{2+} current block by Na\textsuperscript{+} was nearly identical at all potentials. Between -70 and 0 mV, block was essentially voltage independent. Block between -30 and +30 mV, measured during the depolarizing voltage step, was similarly voltage independent (not shown).

**Competition between Ba\textsuperscript{2+} and Na\textsuperscript{+}**

If Na\textsuperscript{+} blocked Ba\textsuperscript{2+} currents by binding to a Na\textsuperscript{+}-selective regulatory binding site, Na\textsuperscript{+} block would be predicted to persist regardless of Ba\textsuperscript{2+} concentration. In contrast, if Na\textsuperscript{+} and Ba\textsuperscript{2+} bound to a common site, Na\textsuperscript{+}-induced inhibition should be dependent on Ba\textsuperscript{2+} concentration. We tested these alternative hypotheses...

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**Figure 4.** Concentration dependence of Na\textsuperscript{+} block of Ba\textsuperscript{2+} currents. The external solution contained 2 mM Ba\textsuperscript{2+}. (A) Representative currents evoked by 100-ms depolarization to 0 mV, in the presence of the external Na\textsuperscript{+} concentration shown above the current. External Na\textsuperscript{+} was added to the control bath solution, so there was an increase in osmolality with increasing [Na\textsuperscript{+}]. Currents were recorded 10 s after addition of Na\textsuperscript{+}. At Na\textsuperscript{+} concentrations of 30 and 120 mM, results were statistically identical when cells were exposed to added Na\textsuperscript{+} (n = 4 [30 mM], 7 [120 mM]) or substitution of Na\textsuperscript{+} for TEA (n = 9 [30 mM], 8 [120 mM]). (B) IV curves recorded before, during and after addition of 120 mM Na\textsuperscript{+} in the presence of TEA. (C) Concentration dependence of block by Na\textsuperscript{+}. Circles represent mean and SEM of pooled data for substitution and addition of Na\textsuperscript{+}. The number of cells tested is shown in parentheses. The solid line represents the best fit of the data by Eq. 1.
by examining block by 120 mM Na⁺ in the presence of different Ba²⁺ concentrations. In the presence of 1 mM Ba²⁺, 120 mM Na⁺ inhibited Ba²⁺ currents by 72.5 ± 8.0% (n = 4; Fig. 6 A and B). In the presence of 10 mM Ba²⁺, 120 mM Na⁺ had little effect on Ba²⁺ currents, with an average inhibition of 13.6 ± 2.2% (n = 4; Fig. 6 C and D). Channel block by 120 mM Na⁺ was half maximal at a Ba²⁺ concentration of 2 mM (Fig. 7). These data, in combination with those of Fig. 4 C, suggest that Na⁺ blocks Ba²⁺ currents via a competitive interaction with Ba²⁺ for a binding site.

**Ba²⁺ Current Block by Li⁺**

L-type Ca²⁺ channels are somewhat selective for Li⁺ over Na⁺, both in the presence and absence of external

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**Figure 5.** Voltage dependence of Na⁺ block. Currents in TEA bath (A) and Na⁺ bath (B) were evoked by the protocol illustrated at the bottom of B. Repolarization potentials ranged from −70 to 0 mV. Currents were carried by 3 mM Ba²⁺, and 120 mM Na⁺ was substituted for TEA. 150 µs of data were blanked from each trace during the capacitive transient before and after the depolarizing voltage step. (C) I-V relationship, measured at the peak of the tail currents illustrated in A and B. (D) Percent block by Na⁺, plotted semilogarithmically. Data represent mean ± SEM of six cells.

**Figure 6.** Na⁺ block of Ba²⁺ currents in low and high [Ba²⁺]. (A) Currents recorded in 1 mM Ba²⁺, with 0 Na⁺ (0 Na and Recov.), and with 120 mM Na⁺. (B) I-V curves from cell in A. (C) Currents recorded in 10 mM Ba²⁺, before, during (Na) and after substitution of 120 mM Na⁺ for TEA. (D) I-V curves for the cell in C. The stimulus in A and C was a 100-ms depolarization from −80 to −15 mV (A) or 0 mV (C).
Ba\textsuperscript{2+} (Hess et al., 1986). In addition, the single channel conductance is lower for Li\textsuperscript{+} than Na\textsuperscript{+} (Hess et al., 1986). These observations are consistent with a model in which Li\textsuperscript{+} has a somewhat higher affinity for the channel than Na\textsuperscript{+}. If N-type Ca\textsuperscript{2+} channel permeation is indeed similar to that of L-type channels, Li\textsuperscript{+} would be expected to inhibit Ba\textsuperscript{2+} currents at similar or slightly lower concentrations than Na\textsuperscript{+}. Fig. 8 illustrates that this was the case. Application of increasing concentrations of Li\textsuperscript{+} in the presence of a constant concentration of TEA produced a concentration-dependent, reversible inhibition of the Ba\textsuperscript{2+} current (Fig. 8, A and B). The IC\textsubscript{50} for Ba\textsuperscript{2+} current block by Li\textsuperscript{+}, derived from the best fit of the data to Eq. 1, was 97 mM, with a slope coefficient near 1 (Fig. 8 C).

**Discussion**

The primary result described in this paper is that at physiological concentrations of Ca\textsuperscript{2+} and Na\textsuperscript{+}, external Na\textsuperscript{+} blocks Ca\textsuperscript{2+} (and Ba\textsuperscript{2+}) currents through N-type Ca\textsuperscript{2+} channels. This observation is of interest from a physiological perspective, since it suggests that Ca\textsuperscript{2+} channels are normally conducting at <100\% efficiency. Our results also indicate that the permeation selectivity mechanism in chick N-type Ca\textsuperscript{2+} channels is similar to that of L-type Ca\textsuperscript{2+} channels. Consequently, these experiments provide additional insight into the Ca\textsuperscript{2+} channel permeation mechanism.

**Comparison of Permeation Mechanism in N-type and L-type Ca\textsuperscript{2+} Channels**

Ca\textsuperscript{2+} channel permeation has been studied almost exclusively in L-type Ca\textsuperscript{2+} channels. These channels select for Ca\textsuperscript{2+} over Na\textsuperscript{+} via differential affinity of intrapore binding sites for these ions. At high [Ca\textsuperscript{2+}], Ca\textsuperscript{2+} channels are almost perfectly selective for Ca\textsuperscript{2+} over monovalent cations. Reduction of external Ca\textsuperscript{2+} to submicromolar levels permits Na\textsuperscript{+} and other monovalent cations...
to conduct. Indeed, Na⁺ conducts almost 10 times better than Ca²⁺ (Hess et al., 1986). Addition of μM external [Ca²⁺] \( (K_a = 0.7 \mu M) \) prevents monovalent cations from carrying inward current (Kostyuk et al., 1983; Almers et al., 1984; Fukushima and Hagiwara, 1985; Hess et al., 1986; Matsuda, 1986), and external Mg²⁺ blocks Na⁺ currents with an IC₅₀ of about 60 μM (Matsuda, 1986). A similar mechanism appears to operate in a T-like Ca²⁺ channel in a B lymphocyte cell line (Fukushima and Hagiwara, 1985).

N-type channels from bullfrog sympathetic neurons and chick sensory neurons also conduct Na⁺ in the absence of Ca²⁺ (Kuo and Bean, 1993; Cox and Dunlap, 1994), which suggests that the permeation mechanism in these channels is similar to that in L-type channels. Our experiments extend these observations. Removal of divalent cations resulted in large Na⁺ currents through the Ca²⁺ channel that were inhibited 95% by 10 μM Ca²⁺ (consistent with an IC₅₀ near 0.7 μM). Addition of 1 mM external Mg²⁺ inhibited Na⁺ currents through N-type channels by 80%, consistent with an IC₅₀ near 250 μM. Although not examined in great detail, these results suggest that the selectivity mechanism in N-type Ca²⁺ channels is quite similar to that of L-type Ca²⁺ channels.

**Simultaneous Binding of Ca²⁺ and Na⁺ in the Pore**

There is a wealth of evidence to suggest that Ca²⁺ channels are multi-ion pores (Almers et al., 1984; Hess and Tsien, 1984; Fukushima and Hagiwara, 1985; Friel and Tsien, 1989; Yue and Marban, 1990; Kuo and Hess, 1993a, b). Although the total number and location of binding sites in the pore is still somewhat controversial, molecular and biophysical studies suggest that a single molecular location near the outer mouth of the pore binds Ca²⁺ with high affinity, and constitutes the selectivity filter (Heinemann et al., 1992; Tang et al., 1993; Yang et al., 1993; Ellinor et al., 1995; Parent and Gopalakrishnan, 1995). This single locus is postulated to form a structure that is capable of binding two Ca²⁺ ions (Yang et al., 1993; Ellinor et al., 1995). In low [Ca²⁺], the on-rate of a single Ca²⁺ to this site from the exterior of the pore is similar when the channel is conducting monovalent cation currents in either inward or outward directions, and the on-rate of Ca²⁺ to the pore is nearly diffusion-limited (Kuo and Hess, 1993b). This suggests that the Ca²⁺ blocking site is easily accessible from the external solution. Ca²⁺ channel block by divalent cations is voltage dependent (Fukushima and Hagiwara, 1985; Lansman et al., 1986; Rosenberg and Chen, 1991; Kuo and Hess, 1993a), which places the high affinity binding site inside the membrane field.

Although many studies argue against the single-site allosteric model of Kostyuk et al. (1983), other models of Ca²⁺ permeation have been proposed that include only a single high affinity Ca²⁺ binding site (Armstrong and Neyton, 1991; Yang et al., 1993; Dang and McCleskey, 1996). The Armstrong and Neyton model is conceptually quite similar to the Kuo and Hess model; in the former, two ions can bind to one site, in the latter, two cation binding sites are separated by little or no energy barrier. The Yang et al. (1993) model similarly postulates that a single location can bind either one or two ions. In all three models, the first Ca²⁺ in does not leave the high affinity site until it is “knocked off” by an incoming cation. The Dang and McCleskey model suggests that a single high affinity site flanked by low affinity sites could account for much of the Ca²⁺ channel permeation data. Although the energies that propel the ions through the pore are derived from different sources, each of these models postulates that two ions bind in very close proximity at a single location. Molecular studies have identified the EEEE locus as the likely location of this binding (Heinemann et al., 1992; Tang et al., 1993; Yang et al., 1993; Ellinor et al., 1995; Parent and Gopalakrishnan, 1995).

At very low [Ca²⁺], high external Li⁺ can impede the outward exit of Ca²⁺ from the channel (Kuo and Hess, 1993b), which suggests that Li⁺ can bind in the pore externally to Ca²⁺. Whether this occurs at the EEEE locus while it is singly bound by Ca²⁺, or binds to an independent site external to the EEEE locus, is unknown. At [Ca²⁺] near its \( K_d \), addition of high external Li⁺ reduced the on-rate of Ca²⁺ for the high affinity site, with an apparent \( K_d \) for Li⁺ of ~100 mM. This reduction of on-rate presumably results from binding of Li⁺ to a pore unoccupied by Ca²⁺. Although the conditions of these experiments do not reflect a true equilibrium situation, this suggests that the \( K_d \) of Li⁺ for the binding site in the absence of Ca²⁺ is on the order of 100 mM.

Our data extend these observations to the interaction of Ca²⁺ and Na⁺ in Ca²⁺ channels at physiological [Ca²⁺] and [Na⁺]. Although our data do not directly address the issue of whether the interaction occurs inside the pore, our data are consistent with the framework laid by many others that monovalent and divalent cations do indeed interact inside the pore. Consequently, we will restrict our discussion to this assumption.

At 2 mM Ca²⁺, one Ca²⁺ is always bound to the high affinity binding site. The observation that Na⁺ inhibits Ca²⁺ currents indicates that occupancy of the channel by Ca²⁺ does not prevent Na⁺ from entering the pore. The observation that Na⁺ block of Ca²⁺ channel currents is reduced by divalent cations in a concentration-dependent manner suggests that Na⁺ is binding to a Ca²⁺ binding site. We observed little or no voltage dependence of block by Na⁺. Taken together, these observations suggest that Na⁺ is binding externally to a Ca²⁺ ion bound to a high affinity Ca²⁺ binding site.
Largely on indirect evidence, Kuo and Hess (1993a) also concluded that the site of Li\(^+\) occupancy in a Li\(^+\)-conducting Ca\(^{2+}\) channel was voltage insensitive, and therefore outside the membrane field.

At 2 mM Ba\(^{2+}\), we observed an IC\(_{50}\) of Na\(^+\) and Li\(^+\) for the pore that was similar to that observed by Kuo and Hess (1993b) for reduction of Ca\(^{2+}\) on-rate to the high affinity binding site with \([\text{Ca}^{2+}]\) near 1 \(\mu\text{M}\). These measurements were made differently, in that the apparent \(K_d\) in our studies were derived from near equilibrium measurements whereas those of Kuo and Hess were derived from experiments in which [Li\(^+\)] was in steady state. Nonetheless, the similarity between these two measurements is intriguing. The measurement of apparent \(K_d\) made by Kuo and Hess reflected the binding of Li\(^+\) to a pore occupied by Li\(^+\) but unoccupied by Ca\(^{2+}\). Kuo and Hess argued, however, that the binding affinity of Li\(^+\) to the second site may be similar whether the first site is bound by Ca\(^{2+}\) or Li\(^+\). Our measurement of apparent \(K_d\) reflected binding of Li\(^+\) (or Na\(^+\)) in a pore always occupied by at least one Ca\(^{2+}\). The similar apparent \(K_d\) suggest that the affinity of Na\(^+\) (or Li\(^+\)) for the pore is not dramatically influenced by the occupancy of the first site by Ca\(^{2+}\). Consequently, these data suggest that the binding site for monovalent cations is unchanged by occupancy of the first Ca\(^{2+}\). Whereas these results do not preclude the hypothesis that binding of the second cation (either Ca\(^{2+}\) or Na\(^+\)) has a lower affinity than the first due to electrostatic repulsion considerations, they do suggest that the binding affinity does not change due to an allosteric effect on the cation binding site produced by binding of the first Ca\(^{2+}\).

**Comparison with Other Published Results**

Almers et al. (1984) observed a slight reduction of Ca\(^{2+}\) channel currents in frog skeletal muscle upon partial replacement (32 mM) of TEA with Na\(^+\). This reduction is similar in magnitude to that which we observed. In contrast, replacement of external Na\(^+\) with Tris (Matsumi and Noma, 1984) or choline (Yamashita et al., 1990) did not influence Ca\(^{2+}\) currents in guinea pig ventricular cells or neoplastic lymphocytes. While these differences may, of course, be due to different Ca\(^{2+}\) channel preparations, they may also be related to the choice of ion substitute. As observed by Kuo and Hess (1993b) in L-type Ca\(^{2+}\) channels, NMG produced some block of the Ca\(^{2+}\) current in our experiments (data not shown). Thus, as with NMG, Tris and choline may also inhibit Ca\(^{2+}\) channel conductance, and thus mask inhibitory effects of Na\(^+\). Our results are also consistent with the possibility that cations did not block the Ca\(^{2+}\) channel but that TEA potentiated currents through the channel. We tested this in two ways. First, Na\(^+\) reduced the Ca\(^{2+}\) current when substituted for NMG (not shown), which indicates that the effect did not depend on the initial presence of TEA. More importantly, we observed an identical concentration-dependence of block whether Na\(^+\) isomotically replaced TEA or was applied in addition to already present TEA.

Finally, our results may be considered in light of those obtained by Yamashita et al. (1990), which demonstrated that, at positive potentials, internal Na\(^+\) could pass outward current through Ca\(^{2+}\) channels, even in the presence of 2.5 mM external Ca\(^{2+}\). The differential ability of Na\(^+\) to conduct in the outward vs. the inward direction in the presence of normal external \([\text{Ca}^{2+}]\) may be partially due to the different competitive situations at the internal and external face of the channel. Thus, the very low internal \([\text{Ca}^{2+}]\), combined with positive voltages, may create a competitive advantage for Na\(^+\) over Ca\(^{2+}\) not possible at the external face of the channel.

**Physiological Significance**

Our data suggest that in physiological solutions, open Ca\(^{2+}\) channels are conducting submaximally when compared with the conductance expected for 2 mM Ca\(^{2+}\). Since it is not clear that external \([\text{Na}^{+}]\) would ever vary dramatically, the significance of these findings must be speculative. There are, however, situations where the monovalent cation-sensitivity of the channel could become meaningful. First, K\(^+\) blocks Ca\(^{2+}\) currents similarly to Na\(^+\) (data not shown). It is possible that under conditions of extreme increase in extracellular \([\text{K}^{+}]\), perhaps coupled with a small decrease in local \([\text{Ca}^{2+}]\) (for example, during high frequency neuronal activity or ischemia; cf. Hansen and Zeuthen, 1981), extracellular K\(^{+}\) could inhibit Ca\(^{2+}\) influx. Second, both Cs\(^+\) and Na\(^+\) will pass outward current through Ca\(^{2+}\) channels (Fenwick et al., 1982; Yamashita et al., 1990). It is intriguing to consider that intracellular K\(^+\), or local changes in intracellular \([\text{Na}^{+}]\) during high frequency activity, could influence Ca\(^{2+}\) channel permeation properties in a physiologically meaningful way. Finally, the sensitivity of the Ca\(^{2+}\) channel to Na\(^+\) suggests that compounds that bind to Na\(^+\) binding sites may also inhibit Ca\(^{2+}\) channels. For example, amiloride analogs, which inhibit many Na\(^+\)-dependent processes, inhibit N-type, T-type and L-type Ca\(^{2+}\) channels (Tang et al., 1988; Garcia et al., 1990; Polo-Parada et al., 1996). Indeed, amiloride analogs are considered of potential use in the treatment of cardiac ischemia, especially during reperfusion (cf. Scholz et al., 1992). These drugs have both cardioprotective and antiarrhythmic properties, which may be due, in part, to inhibition of Ca\(^{2+}\) influx through either pre- or postsynaptic Ca\(^{2+}\) channels. An understanding of the Na\(^+\) binding site in Ca\(^{2+}\) channels in physiological \([\text{Ca}^{2+}]\) and \([\text{Na}^{+}]\) may lead to a novel approach to the modulation of Ca\(^{2+}\) channel function.
Ca\textsuperscript{2+} Channel Block by External Na\textsuperscript{+}

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