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

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

KEY WORDS: calcium channels • ion channel selectivity • sodium • permeation

INTRODUCTION

Calcium (\( \text{Ca}^{2+} \)) channels are highly selective for \( \text{Ca}^{2+} \) over sodium (\( \text{Na}^{+} \)) and other monovalent cations. Their remarkable selectivity is accomplished by differential affinity of \( \text{Ca}^{2+} \) 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 \( \text{Ca}^{2+} \), monovalent cations conduct well through \( \text{Ca}^{2+} \) channels (Kostyuk et al., 1983; Almers et al., 1984; Fukushima and Hagiwara, 1985; Hess et al., 1986; Matsuda, 1986). Addition of \( \mu \text{M} \) \( [\text{Ca}^{2+}] \) completely blocks \( \text{Ca}^{2+} \) conductance but does not result in the generation of \( \text{Ca}^{2+} \) currents. At much higher \( [\text{Ca}^{2+}] \), \( \text{Ca}^{2+} \) currents are generated. In L-type \( \text{Ca}^{2+} \) channels, \( \text{Ca}^{2+} \) current saturates with a \( K_{d} \) for \( \text{Ca}^{2+} \) of \( \sim 14 \mu \text{M} \) (Hess et al., 1986). This concentration difference required for block of \( \text{Na}^{+} \) conductance and generation of \( \text{Ca}^{2+} \) 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 \( \text{Ca}^{2+} \) 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 \( \text{Ca}^{2+} \) for these sites, the first available site is bound by \( \text{Ca}^{2+} \) at low concentrations. At higher concentrations, a second \( \text{Ca}^{2+} \) enters simultaneously, and the electrostatic repulsion between these two tightly packed divalent ions lowers the apparent \( K_{d} \) for the sites and results in \( \text{Ca}^{2+} \) current. In such a model, the 14 mM \( K_{d} \) for \( \text{Ca}^{2+} \) current saturation can be interpreted as representing the affinity of \( \text{Ca}^{2+} \) for the second binding site once the first binding site is bound by \( \text{Ca}^{2+} \) (Tsien et al., 1987).

Alternative models that have a single high affinity binding site have been proposed that equally well explain the \( \text{Ca}^{2+} \) channel permeation data (Armstrong and Neyton, 1991; Yang et al., 1993; Dang and McGleskey, 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 \( \mu \text{M} \) \( [\text{Ca}^{2+}] \), two observations suggest that these two sites in the \( \text{Ca}^{2+} \) channel pore can be simultaneously occupied by one \( \text{Ca}^{2+} \) and one monovalent cation (Kuo and Hess, 1993a). First, in \( \text{Ca}^{2+} \) channels carrying outward \( \text{Li}^{+} \) currents, high external \( [\text{Li}^{+}] \) decreases the outward exit rate of the blocking \( \text{Ca}^{2+} \) ion from the pore, producing a “lock in” type of effect in the channel. Second, elevation of external \( [\text{Li}^{+}] \) decreases the on-rate of external \( \text{Ca}^{2+} \), which suggests that external \( \text{Li}^{+} \) interferes with access...
of Ca²⁺ to its high affinity site. When the channel is occupied by one Ca²⁺ 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 Ca²⁺ within the pore.

The interaction between Ca²⁺ and monovalents in the pore is derived from studies in low [Ca²⁺] conditions, with Li⁺ as the monovalent charge carrier. There is little information, however, about the potential interaction between Ca²⁺ 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 [Ca²⁺] suggest that there is still less than a 10-fold difference in apparent affinity between Ca²⁺ and Na⁺ for the second binding site ($\sim 14$ vs. $\sim 100$ mM). Consequently, if extrapolated to high [Ca²⁺] conditions, the model predicts that Ca²⁺ 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 Ca²⁺ less in the presence of Na⁺ than in the absence of Na⁺. Since a channel occupied by one Ca²⁺ and one monovalent cation does not conduct (Almers et al., 1984; Kuo and Hess, 1993a, b), this large decrease in double occupancy by Ca²⁺ would be expected to result in Ca²⁺ current reduction.

An alternative possibility is that Na⁺ is excluded from the pore at physiological [Ca²⁺] and [Na⁺]. This possibility is supported by data from guinea pig ventricular cells and mouse neoplastic B lymphocytes, in which Ca²⁺ currents were unaffected when external Na⁺ was replaced by Tris or choline (Matsuda and Noma, 1984; Yamashita et al., 1990). Reconciliation of these conflicting possibilities is important for understanding Ca²⁺ channel permeation, since one suggests that permeation is strictly governed by competition for binding sites and the other suggests that higher [Ca²⁺] induces an allosteric change in the channel that prevents monovalent cations from binding.

Nearly all studies of Ca²⁺ channel permeation have used L-type Ca²⁺ channels. We demonstrate here that N-type Ca²⁺ channels in chick sensory neurons display permeation properties similar to those of L-type Ca²⁺ channels. In physiological [Na⁺] and [Ca²⁺], external Na⁺ blocks N-type Ca²⁺ channels in a concentration-dependent manner, and this block appears to result from a competitive interaction between Ca²⁺ and Na⁺ in the pore.

**MATERIALS AND METHODS**

**Cells**

Dorsal root ganglion (DRG) neurons were acutely isolated from thoracic and lumbar level ganglia of 14-d-old white leghorn chick embryos (UCONN Poultry Farm, Storrs, CT). Ganglia were incubated in Tyrodes (in mM: 128 NaCl, 3 KCl, 1 MgCl₂, 27 NaHCO₃, 10 glucose, pH 7.3) containing 0.08% trypsin (#610-5050PG; Gibco Laboratories, Grand Island, NY) for 30 min at 37°C in a CO₂ incubator (both 6 and 10% CO₂ in air were used with no detectable difference). Cells were removed from the incubator and washed three times with Media 199 (#B-1202-AX plus 3.1 g/liter NaHCO₃; 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 0.8–3.0 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/pixel. Tail currents in Fig. 5 were filtered at 50 kHz and digitized at 3 μs/pixel. Unless otherwise stated, the holding potential was ~80 mV, and Ca²⁺ 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 measured 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 lowering a large bore pipet (N51A glass coated with Sigmacote) that contained the desired test solution near the cell under study. Application of test solution was terminated by removal of the large bore pipet from the bath. In general, solutions bathing the cells were exchanged within 5 s using this technique. In continuous flow experiments, a large bore quartz pipet that contained multiple solution inputs was placed near the cell before recording. 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 BaCl₂, 1 MgCl₂, 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 MgCl₂, 4 creatine phosphate, 4 ATP-Na, leupeptin, and creatine kinase, pH 7.3 (CAOH), 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 SigmaPlot 2.0 for Windows (Jandel Scientific, Corte Madera, CA). Average values

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1Abbreviation 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 signifi-
cance was tested by unpaired Student’s t test. Data on percent
block of Ca²⁺ channel currents by monovalent cations (Figs. 4 C
and 8 C) were fit by the following equation:

\[ y = 100 \cdot \frac{[\text{cation}]^3}{(IC_{50}^k + [\text{cation}]^3)}, \]

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

Ca²⁺ Channel Type

It was previously demonstrated that Ca²⁺ currents through
DRGs acutely isolated from 11–12-d-old chick embryos
consist entirely of N-type Ca²⁺ channels (Cox and Dun-
lap, 1992). When cells were kept in culture for several
days, however, other Ca²⁺ 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 Ca²⁺ channels in chick
DRGs at concentrations of 1–10 \( \mu \)M (Aosaki and Kasai,
1989; Cox and Dunlap, 1992). \( \omega \)-Conotoxin (10 \( \mu \)M) irre-
versibly blocked Ca²⁺ currents by 94.4 ± 2.7% \( (n = 18) \);
inhibition = 100% in 14 of 18 cells) and Ba²⁺ currents
by 93.1 ± 1.2% \( (n = 4) \). In four cells tested, 1 \( \mu \)M cono-
toxin also produced a 100% block of Ca²⁺ channel current. Also
consistent with N-type Ca²⁺ channel properties, cur-
cents were completely inactivated by depolarization to
−10 mV, with half-maximal inactivation at −64.4 ± 1.5
\( (n = 6) \). Identical pharmacological and kinetic results
were obtained from cells ranging in size from 12 to 40 µm,
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
Ca²⁺ channels.

Permeation Characteristics of the Chick N-type Ca²⁺ Channel

The theoretical framework for understanding Ca²⁺
channel permeation is derived almost exclusively from
studies on L-type Ca²⁺ channels. To determine whether
chick N-type Ca²⁺ channels used a fundamentally simi-
lar selectivity mechanism, we tested for the salient fea-
ture of Ca²⁺ channel permeation: conductance of Na⁺
in the absence of divalent cations and inhibition of Na⁺
conductance by \( \mu \)M \([\text{Ca}^{2+}]\).

In the absence of Na⁺, removal of external Ba²⁺ com-
pletely abolished inward currents (Fig. 1 A and B; \( n = 4 \)). When the Ba²⁺-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 Ca²⁺ typically contain
several \( \mu \)M Ca²⁺. Upon addition of 1 mM EGTA to che-
late this residual Ca²⁺, the magnitude of the inward current increased (Fig. 1 C3). Removal of external
Mg²⁺ in this Ca²⁺-free, Ba²⁺-free solution resulted in an
additional large increase in Na⁺ current magnitude
(Fig. 1 C4). Finally, addition of just 10 \( \mu \)M Ca²⁺ to the
Ba²⁺-free, Mg²⁺-free bath solution inhibited the inward current by 95.0 ± 0.1% \( (n = 3) \); Fig. 1 C5). Increasing the
Ca²⁺ concentration to 100 \( \mu \)M resulted in an addi-
tional 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 ab-
ence of Ca²⁺ (or Ba²⁺) Na⁺ conducts well through the
chick N-type Ca²⁺ channel, and that both Mg²⁺ and low
concentrations of Ca²⁺ inhibit Na⁺ conductance through the channel. Thus, these data suggest that the
chick N-type Ca²⁺ channel selects for Ca²⁺ over Na⁺ by

![Figure 1](https://example.com/image1.png)

**FIGURE 1.** Permeation of Na⁺ through N-type Ca²⁺ channels. (A) Two superimposed currents recorded in a Na⁺-free bath, in 2 mM
Ba²⁺, and after the external solution was switched to one contain-
ing 0 Ba²⁺. The 0 Ba²⁺ solution contained 1 mM Mg²⁺ and was
nominally free of Ca²⁺ (we assume a \([\text{Ca}^{2+}]\) between 5–10 \( \mu \)M). (B) I-V curves recorded with 2 mM Ba²⁺ (Cont., Recov.) and 0 Ba²⁺.
(C) Currents were recorded from a different cell in the conditions
described above the trace. All external solutions contained (in
mM): 30 TEA, 10 glucose, 10 HEPES, and 1 \( \mu \)M TTX. (C1) Con-
tral (external solution as described in methods). (C2) After re-
moval of Ba²⁺. (C3) After addition of 1 mM EGTA (0 Ba²⁺). (C4) After removal of 1 mM Mg²⁺ (0 Ba²⁺, 0 Mg²⁺, 1 EGTA). (C5) After removal
of EGTA and addition of 10 \( \mu \)M Ca²⁺ (0 Ba²⁺, 0 Mg²⁺). (C6) Recovery (control solution). In A and C, all currents were
evoked by 100-ms depolarizations from −80 to 0 mV.
a qualitatively similar mechanism as the L-type Ca\textsuperscript{2+} channel.

**Ca\textsuperscript{2+} Channel Block by External Na\textsuperscript{+}**

Fig. 2 A 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 \(\sim 1\) 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

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**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.

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**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 \pm SEM for five cells in each condition. Solid lines represent the best fit to the data, using a modified Boltzmann equation, \([1 + \exp((V_1/2 - V)/K)]^{-1}\). Values for \(V_1/2\) (in mV) were \(-18.3 \pm 0.16\) (Cont.) and \(-18.6 \pm 0.17\) (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. 3) 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 A and B). This latter approach resulted in an increase in osmolality with increasing [Na\textsuperscript{+}] but eliminated the possibility that removal of TEA (rather than addition of Na\textsuperscript{+}) caused the change in Ba\textsuperscript{2+} current magnitude. There was no shift in the current-voltage relationship with increasing osmolality with either 120 mM Na\textsuperscript{+} (Fig. 4 B) or 300 mM Na\textsuperscript{+} (not shown). The percent inhibition by 30 and 120 mM Na\textsuperscript{+} in each of these types of experiments was statistically identical, and the data were pooled for the analysis in Fig. 4 C. With 2 mM Ba\textsuperscript{2+} as the charge carrier, Na\textsuperscript{+} block of the channel was well fit by Eq. 1, with an IC\textsubscript{50} of 119 mM and a slope coefficient near 1.

Block of Ca\textsuperscript{2+} channel currents by Ca\textsuperscript{2+}, Ba\textsuperscript{2+}, Cd\textsuperscript{2+}, and Mg\textsuperscript{2+} is voltage dependent (Fukushima and Hagiwara, 1985; Lansman et al., 1986; Rosenberg and Chen, 1991; Kuo and Hess, 1993a), which places the location of the high affinity Ca\textsuperscript{2+} binding site inside of the membrane field. Although the site of Li\textsuperscript{+} interaction in the Ca\textsuperscript{2+} channel may be external to the membrane field (Kuo and Hess, 1993a), evidence strongly suggests that the interaction between divalents and monovalents in the Ca\textsuperscript{2+} channel occurs inside the pore (Fukushima and Hagiwara, 1985; Lansman et al., 1986; Yamashita et al., 1990; Kuo and Hess, 1993a, b). To examine the voltage dependence of Na\textsuperscript{+} block of Ba\textsuperscript{2+} currents, we examined block of tail currents at different repolarization potentials (Fig. 5). Panels A and B illustrate currents from one cell evoked in TEA bath (Fig. 5 A) and Na\textsuperscript{+} bath (Fig. 5 B). The tail I-V curve was reasonably linear between −70 and 0 mV (Fig. 5 C), and block by Na\textsuperscript{+} was almost identical at all potentials. Fig. 5 D plots the percent block by 120 mM Na\textsuperscript{+} as a function of voltage, averaged over six cells. 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.

![Figure 4](https://example.com/figure4.png)

**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) I-V 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 Ba\(^{2+}\) concentrations. In the presence of 1 mM Ba\(^{2+}\), 120 mM Na\(^+\) inhibited Ba\(^{2+}\) currents by 72.5 ± 8.0% (n = 4; Fig. 6 A and B). In the presence of 10 mM Ba\(^{2+}\), 120 mM Na\(^+\) had little effect on Ba\(^{2+}\) 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 Ba\(^{2+}\) concentration of 2 mM (Fig. 7). These data, in combination with those of Fig. 4 C, suggest that Na\(^+\) blocks Ba\(^{2+}\) currents via a competitive interaction with Ba\(^{2+}\) for a binding site.

**Ba\(^{2+}\) Current Block by Li\(^+\)**

L-type Ca\(^{2+}\) channels are somewhat selective for Li\(^+\) over Na\(^+\), both in the presence and absence of external...
Ba$^{2+}$ (Hess et al., 1986). In addition, the single channel conductance is lower for Li$^+$ than Na$^+$ (Hess et al., 1986). These observations are consistent with a model in which Li$^+$ has a somewhat higher affinity for the channel than Na$^+$. If N-type Ca$^{2+}$ channel permeation is indeed similar to that of L-type channels, Li$^+$ would be expected to inhibit Ba$^{2+}$ currents at similar or slightly lower concentrations than Na$^+$. Fig. 8 illustrates that this was the case. Application of increasing concentrations of Li$^+$ in the presence of a constant concentration of TEA produced a concentration-dependent, reversible inhibition of the Ba$^{2+}$ current (Fig. 8, A and B). The IC$_{50}$ for Ba$^{2+}$ current block by Li$^+$, 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$^{2+}$ and Na$^+$, external Na$^+$ blocks Ca$^{2+}$ (and Ba$^{2+}$) currents through N-type Ca$^{2+}$ channels. This observation is of interest from a physiological perspective, since it suggests that Ca$^{2+}$ channels are normally conducting at <100% efficiency. Our results also indicate that the permeation selectivity mechanism in chick N-type Ca$^{2+}$ channels is similar to that of L-type Ca$^{2+}$ channels. Consequently, these experiments provide additional insight into the Ca$^{2+}$ channel permeation mechanism.

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

Ca$^{2+}$ channel permeation has been studied almost exclusively in L-type Ca$^{2+}$ channels. These channels select for Ca$^{2+}$ over Na$^+$ via differential affinity of intrapore binding sites for these ions. At high [Ca$^{2+}$], Ca$^{2+}$ channels are almost perfectly selective for Ca$^{2+}$ over monovalent cations. Reduction of external Ca$^{2+}$ to submicromolar levels permits Na$^+$ and other monovalent cations to pass through the channel. This permeation selectivity is thought to be mediated by the intrapore Ca$^{2+}$ selectivity filter, which is highly selective for Ca$^{2+}$ over Na$^+$.

**Figure 7.** Inhibition of Na$^+$ block by Ba$^{2+}$. Currents were recorded from cells bathed in one of four Ba$^{2+}$ concentrations. I-V curves were generated as in Fig. 6, and the percent inhibition by 120 mM Na$^+$ was measured at the peak of the I-V. The number of cells tested is shown in parentheses. The solid line represents the best fit of the data to the complement of Eq. 1. The calculated IC$_{50}$ was 1.98 ± 0.14 mM with a slope coefficient of 1.34 ± 0.17.

**Figure 8.** Ba$^{2+}$ current block by Li$^+$. (A) Currents recorded in the presence of the Li$^+$ concentrations shown. Li$^+$ was added to the control external solution. Currents were evoked by 100-ms depolarizations from −80 to 0 mV. (B) Ba$^{2+}$ currents recorded over time in the presence of 0, 10, 30, 100, and 300 mM [Li$^+$]. After recovery from 300 mM Li$^+$, cells were exposed to 100 mM Na$^+$ for comparison. (C) Concentration dependence of Li$^+$ block of Na$^+$ currents. Circles represent the mean ± SEM for six cells in each condition (error bars are smaller than the symbols). The solid line represents the best fit of the data to Eq. 1. The calculated IC$_{50}$ was 96.8 ± 17.2 mM and the slope coefficient was 1.16 ± 0.24.
to conduct. Indeed, Na⁺ conducts almost 10 times better than Ca²⁺ (Hess et al., 1986). Addition of μM external [Ca²⁺] (Kₐ = 0.7 μM) 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⁺ from this site to 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. (1985), 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 Kₐ, addition of high external Li⁺ reduced the on-rate of Ca²⁺ for the high affinity site, with an apparent Kₐ 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 Kₐ 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 Ca²⁺ channel was voltage insensitive, and therefore outside the membrane field.

At 2 mM Ba²⁺, we observed an IC₅₀ of Na⁺ and Li⁺ for the pore that was similar to that observed by Kuo and Hess (1993b) for reduction of Ca²⁺ on-rate to the high affinity binding site with [Ca²⁺] near 1 μM. These measurements were made differently, in that the apparent $K_8$ 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 $K_8$ made by Kuo and Hess reflected the binding of Li⁺ to a pore occupied by Li⁺ but unoccupied by Ca²⁺. 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 Ca²⁺ or Li⁺. Our measurement of apparent $K_8$ reflected binding of Li⁺ (or Na⁺) in a pore always occupied by at least one Ca²⁺. The similar apparent $K_8$s suggest that the affinity of Na⁺ (or Li⁺) for the pore is not dramatically influenced by the occupancy of the first site by Ca²⁺. Consequently, these data suggest that the binding site for monovalent cations is unchanged by occupancy of the first Ca²⁺. Whereas these results do not preclude the hypothesis that binding of the second cation (either Ca²⁺ 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 Ca²⁺.

Comparison with Other Published Results

Almers et al. (1984) observed a slight reduction of Ca²⁺ 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 (Matsuda and Noma, 1984) or choline (Yamashita et al., 1990) did not influence Ca²⁺ currents in guinea pig ventricular cells or neoplastic lymphocytes. While these differences may, of course, be due to different Ca²⁺ channel preparations, they may also be related to the choice of ion substitute. As observed by Kuo and Hess (1993b) in L-type Ca²⁺ channels, NMG produced some block of the Ca²⁺ current in our experiments (data not shown). Thus, as with NMG, Tris and choline may also inhibit Ca²⁺ channel conductance, and thus mask inhibitory effects of Na⁺. Our results are also consistent with the possibility that cations did not block the Ca²⁺ channel but that TEA potentiated currents through the channel. We tested this in two ways. First, Na⁺ reduced the Ca²⁺ 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⁺ isosmotically 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 Ca²⁺ channels, even in the presence of 2.5 mM external Ca²⁺. The differential ability of Na⁺ to conduct in the outward vs. the inward direction in the presence of normal external [Ca²⁺] may be partially due to the different competitive situations at the internal and external face of the channel. Thus, the very low internal [Ca²⁺], combined with positive voltages, may create a competitive advantage for Na⁺ over Ca²⁺ not possible at the external face of the channel.

Physiological Significance

Our data suggest that in physiological solutions, open Ca²⁺ channels are conducting submaximally when compared with the conductance expected for 2 mM Ca²⁺. Since it is not clear that external [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 Ca²⁺ currents similarly to Na⁺ (data not shown). It is possible that under conditions of extreme increase in extracellular [K⁺], perhaps coupled with a small decrease in local [Ca²⁺] (for example, during high frequency neuronal activity or ischemia; cf. Hansen and Zeuthen, 1981), extracellular K⁺ could inhibit Ca²⁺ influx. Second, both Cs⁺ and Na⁺ will pass outward current through Ca²⁺ channels (Fenwick et al., 1982; Yamashita et al., 1990). It is intriguing to consider that intracellular K⁺, or local changes in intracellular [Na⁺] during high frequency activity, could influence Ca²⁺ channel permeation properties in a physiologically meaningful way. Finally, the sensitivity of the Ca²⁺ channel to Na⁺ suggests that compounds that bind to Na⁺ binding sites may also inhibit Ca²⁺ channels. For example, amiloride analogs, which inhibit many Na⁺-dependent processes, inhibit N-type, T-type and L-type Ca²⁺ channels (Tang et al., 1988; García 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 Ca²⁺ influx through either pre- or postsynaptic Ca²⁺ channels. An understanding of the Na⁺ binding site in Ca²⁺ channels in physiological [Ca²⁺] and [Na⁺] may lead to a novel approach to the modulation of Ca²⁺ channel function.
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