Functional Characterization of Ion Permeation Pathway in the N-Type Ca$^{2+}$ Channel

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Wakamori, Minoru, Mark Strobek, Tetsuhiro Niidome, Tetsuyuki Teramoto, Keiji Imoto, and Yasuo Mori. Functional characterization of ion permeation pathway in the N-type Ca$^{2+}$ channel. J. Neurophysiol. 79: 622–634, 1998. Multiple types of high-voltage-activated Ca$^{2+}$ channels, including L-, N-, P-, Q- and R-types have been distinguished from each other mainly employing pharmacological agents that selectively block particular types of Ca$^{2+}$ channels. Except for the dihydropyridine-sensitive L-type Ca$^{2+}$ channels, electrophysiological characterization has yet to be conducted thoroughly enough to biophysically distinguish the remaining Ca$^{2+}$ channel types. In particular, the ion permeation properties of N-type Ca$^{2+}$ channels have not been clarified, although the kinetic properties of both the L- and N-type Ca$^{2+}$ channels are relatively well described. To establish ion conducting properties of the N-type Ca$^{2+}$ channel, we examined a homogeneous population of recombinant N-type Ca$^{2+}$ channels expressed in baby hamster kidney cells, using a conventional whole cell patch-clamp technique. The recombinant N-type Ca$^{2+}$ channel, composed of the α, α1, and β subunits, displayed high-voltage-activated Ba$^{2+}$ currents elicited by a test pulse more positive than −30 mV, and were strongly blocked by the N-type channel blocker ω-conotoxin-GVIA. In the presence of 110 mM Ba$^{2+}$, the unitary current showed a slope conductance of 18.2 pS, characteristic of N-type channels. Ca$^{2+}$ and Sr$^{2+}$ resulted in smaller ion fluxes than Ba$^{2+}$, with the ratio 1.0:0.72:0.75 of maximum conductance in current-voltage relationships of Ba$^{2+}$, Ca$^{2+}$, and Sr$^{2+}$, respectively. In mixtures of Ba$^{2+}$ and Ca$^{2+}$, where the Ca$^{2+}$ concentration was steadily increased in place of Ba$^{2+}$, with the total concentration of Ba$^{2+}$ and Ca$^{2+}$ held constant at 3 mM, the current amplitude went through a clear minimum when 20% of the external Ba$^{2+}$ was replaced by Ca$^{2+}$. This anomalous mole fraction effect suggests an ion-binding site where two or more permeant ions can sit simultaneously. By using an external solution containing 110 mM Na$^{+}$ without polyvalent cations, inward Na$^{+}$ currents were evoked by test potentials more positive than −50 mV. These currents were activated and inactivated in a kinetic manner similar to that of Ba$^{2+}$ currents. Application of inorganic Ca$^{2+}$ antagonists blocked Ba$^{2+}$ currents through N-type channels in a concentration-dependent manner. The rank order of inhibition was La$^{3+}$ ≥ Cd$^{2+}$ ≥ Zn$^{2+}$ > Ni$^{2+}$ ≥ Co$^{2+}$. When a short strong depolarization was applied before test pulses of moderate depolarizing potentials, relief from channel blockade by La$^{3+}$ and Cd$^{2+}$ and subsequent channel reblocking was observed. The measured rate (2 × 10$^5$ M$^{-1}$ s$^{-1}$) of reblocking approached the diffusion-controlled limit. These results suggest that N-type Ca$^{2+}$ channels share general features of a high affinity ion-binding site with the L-type Ca$^{2+}$ channel, and that this site is easily accessible from the outside of the channel pore.

INTRODUCTION

Voltage-dependent Ca$^{2+}$ channels have been commonly classified into T-, N-, L- and P-types on the basis of electrophysiological and pharmacological properties (Bean 1989; Linás et al. 1992; Tsien et al. 1991). In addition to these four types of Ca$^{2+}$ channels, two additional high-voltage-activated (HVA) Ca$^{2+}$ channels designated as R- and Q-types (Zhang et al. 1993) have been recently distinguished in cerebellar granule cells and in heterologous expression systems (Ellinor et al. 1993; Sather et al. 1993; Wakamori et al. 1994; Williams et al. 1994). It is presumed that this functional diversity has been acquired evolutionally by Ca$^{2+}$ channels to exert a pivotal role in multiple cellular processes including membrane excitability, muscle contraction, neurotransmitter release, axonal outgrowth, and synaptic plasticity at specific subcellular regions in different tissues. Because gating parameters such as time- and voltage-dependence of activation and inactivation kinetics at a macroscopic level and opening and closing time distribution at a unitary level have been major clues in distinguishing multiple Ca$^{2+}$ channel types, gating properties have been relatively well-described compared with ion permeation properties for neuronal Ca$^{2+}$ channels (Artalejo et al. 1992; Fox et al. 1987a,b; Kasai and Neher 1992; Plummer et al. 1989; Sather et al. 1993; Swandulla et al. 1991). In fact, permeation characteristics have primarily been described on L-type Ca$^{2+}$ channels in myocytes and pheochromocytoma cells, where the L-type channel can be easily distinguished from the T-type channel by altering the holding potential (Almers and McCleskey 1984; Hess and Tsien 1984; Hess et al. 1986; Kuo and Hess 1993; Rosenberg and Chen 1991; Yue and Marban 1990). Furthermore, it is possible that previous assignments of particular ion permeation characteristics to Ca$^{2+}$ channel types defined solely on the basis of gating characteristics involves some degree of confusion, because recent experimental data have revealed underestimation/overestimation of the N-/L-type component of Ca$^{2+}$ currents because of coexistence of multiple types of HVA Ca$^{2+}$ channels in individual neurons (Artalejo et al. 1992; Kasai and Neher 1992; Plummer et al. 1989; Swandulla et al. 1991). Thus there is yet little information available for the ion permeation properties of the N-type Ca$^{2+}$ channel. In the present investigation, we take advantage of using recombinant N-type channels homogeneously expressed in baby hamster kidney (BHK) cells free from contamination of other Ca$^{2+}$ channel types. Permeation properties and ion selectivity of recombinant α1B channels are similar to those of the L-type channel, suggesting that the N-type channel shares general features, including a high-affinity permeant binding site easily accessible from the extracellular surface of the channel.
**METHODS**

*Construction of expression plasmids containing the α₁β²⁺⁻ or β₁⁻ subunit cDNA*

To construct the mammalian expression plasmid carrying the entire protein-coding region of the rabbit α₁β²⁺⁻ subunit, the 7.4-kb HindIII/HindIII fragment from pKCRBIII (Fujita et al. 1993) was inserted into the HindIII site of the plasmid pK4K (Niidome et al. 1994) to yield pK4KHBBIII, pK4K contains the simian virus 40 (SV40) early gene promoter, two polyadenylation sites (derived from the plasmid pKCR; O'Hare et al. 1981), and a second transcription unit to direct the expression of the dihydrofolate reductase gene (derived from the plasmid pAdD26SV(A)(no. 3) (Kaufman and Sharp 1982). The plasmid pCAAA2, containing the entire protein-coding sequence of the α₂⁻ subunit cDNA and the plasmid pCABE, containing the entire protein-coding sequence of the β₁⁻ subunit cDNA, were described previously (Niidome et al. 1994).

*Transfection and cell culture*

Nontransfected BHK tk⁻ ts13 (BHK⁻) (Waechter and Baserga 1982) cells, obtained from American Type Culture Collection, were grown in Dulbecco’s modified Eagle’s medium (DMEM) containing 5% fetal calf serum (FCS), streptomycin (30 μg/ml) and penicillin (30 units/ml). BHK⁻ cells (2 x 10⁵ cells) were transfected with 5 μg each of pK4KHBBIII, pCAA2, and pCABE by a modified CaPO₄ precipitation technique by using the CellPhect Transfection Kit (Pharmacia). The transfected cells were grown in DMEM containing 5 or 10% FCS, streptomycin (30 μg/ml), penicillin (30 units/ml), 600 μg/ml geneticin, and 0.25 μM methotrexate, and the BHKN101 line was selected. To have a better comparison of the pore structure formed in the N-type α₁β²⁺⁻ subunit with those in other α₁ isoforms (Niidome et al. 1994; Yatani et al. 1995), α₁β₁ was expressed in the same environment, namely, in the presence of the same accessory subunits, as those used for expressing other α₁ isoforms in BHK cells. Moreover, because β₁ is the only β subunit isoform shared by the α₁ isoforms we established a BHK line expressing the subunit combination of α₁β₁α₂β₁.

*Northern analysis*

Total RNA was isolated from stably transfected BHK (BHKN101) cells as described previously (Niidome et al. 1994). Total RNA (5 μg) was electrophoresed through 1% agarose gels and transferred to nylon membranes by standard techniques. Double-stranded cDNA probes for the Ca²⁺ channel α₁β₁, α₂β₁, and β₁ subunits were prepared from the following sources: α₁β₁, 1.4-kb EcoRI/EcoRI fragment of λCBP53 (Fujita et al. 1993); α₂β₁,
2.4-kb HindIII fragment of pCABE (Niidome et al. 1994); \( \beta_{1a} \), 0.80-kb Pst I/Sca I fragment of pCABE (Niidome et al. 1994). Each probe was then labeled by random labeling. Hybridization was carried out at 42°C overnight as described previously (Mori et al. 1991).

Electrophysiology

For electrophysiological measurements, BHK cells were seeded onto plastic cover slips, Celldesk (Sumitomo Bakelite, Tokyo, Japan) and incubated in the culture medium for 5–8 days. Cells prepared in this manner had a spherical or spindle shape with membrane capacitance of 76.3 \( \pm \) 2.9 pF (mean \( \pm \) SE, \( n = 186 \)). Currents from BHK cells were recorded at room temperature (22–25°C) with patch-clamp techniques of whole cell mode and cell-attached mode (Hamill et al. 1981) with an Axopatch 200A patch-clamp amplifier (Axon Instruments, Foster City, CA). Patch pipettes were made from borosilicate glass capillaries (1.5 mm OD, Narishige, Tokyo) by using a model P-87 Flaming-Brown micropipette puller (Sutter Instrument, San Rafael, CA). The patch electrodes were coated with Sylgard 184 (Dow Corning) and fire-polished. Resistance of patch pipettes used for whole cell recording ranged from 1 to 2 M\( \Omega \) when filled with the pipette solutions described below. The series resistance was electronically compensated to \( \geq 70\% \) and both the leakage and the remaining capacitance were subtracted by \(-P/6\) method. Currents were sampled at 10 kHz after low-pass filtering at 1 or 2 kHz (\(-3\) dB) by using an 8-pole Bessel filter (Model 900, Frequency Devices, Haverhill, MA) and analyzed with pClamp 6.02 software (Axon Instruments). Successive step depolarization for 30 ms at an interval greater than 10 s could produce a current equivalent to that of preceding one. A phenomenon known as "run-down" progressed very slowly (10% reduction of current occurred after 15–20 min of recording). Experiments in which the amplitude of inward current decreased more than 10% of the maximum inward current were discarded. Resistance of Sylgard-coated and fire-polished pipettes for single-channel recording was 4–8 M\( \Omega \) when filled with the pipette solution. Unitary Ba\( ^{2+} \) currents were sampled at 10 kHz after low-pass filtering at 1 kHz. Voltage steps were given at a 3-s interval. Single-channel records were corrected for linear leakage and capacitive currents by using subtraction with averaged blank records.

Solutions

To isolate Ba\( ^{2+} \) currents for whole cell recording, BHK cells were bathed in an external solution containing (in mM) 1 BaCl\(_2\), 145 tetraethylammonium chloride (TEA-Cl), 10 N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES), and 10 glucose (pH was adjusted to 7.4 with TEA-OH). The pipette solution contained (in mM) 100 Cs-aspartate, 40 CsCl, 2 MgCl\(_2\), 5 ethylene glycol-bis(\( \beta \)-aminoethyl ether)\( \cdot \)N\(_2\),N\(_4\),N’\(_4\)-tetraacetic acid (EGTA), 2 ATPMg, and 5 HEPES (pH 7.2 with TEA-OH). A stock solution containing 20 mM guanosine 5’-O-(2-thiodiphos-
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FIG. 4 Single-channel recording of $\alpha_{\text{in}}$ channel. Currents were recorded in cell-attached configuration using 110 mM Ba\textsuperscript{2+} as charge carrier. A: typical single-channel currents (3 consecutive traces from left to right). Unitary activities were elicited in one patch by 150 ms stepping to −5 (a), 5 (b), and 15 mV (c) from a $V_h$ of −100 mV every 3 s. ▲: beginning and end of test depolarization. −−−, unitary current levels. B: amplitude histogram at a test depolarization of 5 mV was constructed from 300 traces of (Ab). Histogram was fitted with Gaussian functions. C: unitary current-voltage relationship. Each point indicates mean of seven patches and vertical bars represent ± SE. Data were fitted by linear regression with a slope of 18.2 pS.

phate) (GDP\textsubscript{bS}) was prepared and was added in a final concentration of 0.2 mM to the pipette solution for the experiments in Fig. 10. The external solution of Ca\textsuperscript{2+} or Sr\textsuperscript{2+} was made by the replacement of equimolar Ba\textsuperscript{2+} with one of those ions. The ionic composition of the external solution containing 50 mM Ba\textsuperscript{2+} was (in mM) 50 BaCl\textsubscript{2}, 71.5 TEA-Cl, 10 HEPES, and 10 glucose (pH 7.4 with TEA-OH). Details of this technique have already appeared (Yatani et al. 1995). The external solution containing one cell recorded was completely exchanged within 10−20 ms.

In order to zero the membrane potential for cell-attached recording, BHK cells were bathed in a depolarizing solution of the following composition (in mM): 140 KCl and 5 HEPES (pH 7.4 with KOH). The composition of the pipette solution was (in mM) 110 BaCl\textsubscript{2} and 10 HEPES, pH 7.4 with Ba(OH)\textsubscript{2}.

**Drugs**

Drugs used in the present experiments were DMEM, geneticin (Gibco BRL, Gaithersburg, MD), streptomycin, penicillin (Meiji Seika, Tokyo), $\omega$-CgTx-GVIA, $\omega$-Aga-IVA (Peptide Institute, Osaka, Japan), CsOH (Aldrich Japan, Tokyo), cytochrome C (Nacalai Tesque, Kyoto, Japan), S(−)-Bay K 8644, nimodipine (Research Biochemicals), FCS, melthotrexate, aspartic acid, MgCl\textsubscript{2},...
RESULTS

Stable transfection of BHK cells with \( \alpha_{1B} \), \( \alpha_{2a} \), and \( \beta_{1a} \) subunit cDNAs

Three expression plasmids pK4KHBBIII, pCAA2, and pCABE were cotransfected into baby hamster kidney tk\(^-\)ts13 (BHK\(^-\)) cells by using a modified CaPO\(_4\) precipitation technique. Total RNA was isolated from the geneticin (600 \( \mu \)g/ml) - and methotrexate (0.25 \( \mu \)M)-resistant BHK cell line, BHK101, and was subjected to Northern blot analysis using the rabbit \( \alpha_{1B} \), \( \alpha_{2a} \), or \( \beta_{1a} \) subunit cDNA probes (Fig. 1). Major positive signals ranging from ~7,700 to ~8,500 nucleotides were found in the BHK101 cells with the \( \alpha_{1B} \) probe. Multiple hybridizable RNA species may include the two \( \alpha_{1B} \) channel transcripts with/without the intronic sequence of the rabbit \( \beta \)-globin gene, if polyadenylation occurs at the site derived from the SV40 early gene. The major hybridizable RNA species of ~4,300 and ~2,200 nucleotides were detected in the BHK101 with the \( \alpha_{2a} \) and \( \beta_{1a} \) subunit probes, respectively. The sizes of these two RNA species agreed with those expected of the \( \alpha_{2a} \) and \( \beta_{1a} \) subunits in pCAA2 and pCABE, respectively. No hybridizable RNA species were detected in nontransfected BHK\(^-\) cells with an \( \alpha_{1B} \), \( \alpha_{2a} \), or \( \beta_{1a} \) subunit cDNA probe (Niidome et al. 1994), consistent with an undetectable level of endogenous \( \alpha_{1B} \) channel activity in electrophysiological measurement with an external solution containing 40 mM Ba\(^{2+}\) (data not shown).

Pharmacological sensitivity to toxins and dihydropyridines of \( \alpha_{1B} \) channel in BHK cells

High \( \omega \)-conotoxin-GVIA (\( \omega \)-CgTx-GVIA) sensitivity is the most reliable criterion in distinguishing the N-type channel from other types of Ca\(^{2+}\) channels (Mori et al. 1996). Recent combination of molecular biological and electrophysiological studies has enabled us to find the binding sites for various drugs including peptide toxins, small organic molecules, and inorganic cations in the primary sequence of channel proteins. The extracellular loop between transmembrane segment S5 and adjacent pore-lining “P” region of repeat III in the \( \alpha_{1B} \) subunit has been identified as the major interaction site of \( \omega \)-CgTx-GVIA (Ellinor et al. 1994), whereas the action sites of dihydropyridine (DHP) antagonists, that selectively inhibit L-type channel, have been located in S5, S5–S6 linker region and S6 in repeat III plus S5–S6 linker region and S6 in repeat IV of the L-type \( \alpha_{1C} \) subunit (Grabner et al. 1996; Schuster et al. 1996; Tang et al. 1993b). To pharmacologically confirm the \( \alpha_{1B} \) channel is indeed the \( \omega \)-CgTx-GVIA-sensitive N-type Ca\(^{2+}\) channel, we examined the effects of toxins and DHPs on the \( \alpha_{1B} \) channels expressed in BHK cells, in an external solution containing 1 mM Ba\(^{2+}\). A step depolarization to ~10 mV for 37.5 ms was applied every 10 s from a holding potential (\( V_h \)) of ~100 mV. Ba\(^{2+}\) currents (\( I_{Ba} \)) were time-dependently reduced by 0.1 \( \mu \)M \( \omega \)-CgTx-GVIA with a time constant of 27.9 ± 2.9 s (mean ± SE, \( n = 4 \); Fig. 2A), a value comparable to that found in rat sympathetic neurons (Boland et al. 1994). The effect of \( \omega \)-CgTx-GVIA was irreversible. On the other hand, \( I_{Ba} \) was weakly suppressed (<10%) by the DHP agonist, \( S^- \)-Bay K 8644 (3 \( \mu \)M) at potentials between ~40 and 30 mV (Fig. 2B), while \( I_{Ba} \) induced by the L-type \( \alpha_{1C} \) channel in the BHK cells was increased at least twice by the agent at the same concentration (Yatani et al. 1995). A DHP antagonist, nimodipine (3 \( \mu \)M), suppressed \( I_{Ba} \) little (3 ± 2%, \( n = 3 \)). \( \omega \)-Agatoxin (\( \omega \)-Ag-)IVA, which potently blocks P-type Ca\(^{2+}\) channels in cerebellar Purkinje neurons with an estimated \( K_0 \) of ~1.5 nM, had no effect on \( \alpha_{1B} \) channel at a concentration of 30 nM, but two out of five BHK cells showed reversible decrease in currents at 300 nM \( \omega \)-Ag-IVA (Fig. 2C). These pharmacological results indicate that the recombinant \( \alpha_{1B} \) channel coexpressed with \( \alpha_{2a} \) and \( \beta_{1a} \) subunits in BHK cells behaves similarly to native N-type Ca\(^{2+}\) channels.

Voltage-dependent characteristics of \( \alpha_{1B} \) channel in BHK cells

The recombinantly expressed \( \alpha_{1B} \) channel in BHK cells was activated by step depolarization above ~40 mV, from a \( V_h \) of ~100 mV, in 1 mM Ba\(^{2+}\) external solution. The

![Fig. 6. Na\(^+\) currents through \( \alpha_{1B} \) channel. A: families of currents in external solution containing 3 mM Ba\(^{2+}\) (a) and 100 mM Na\(^+\) (b) as charge carriers. N-hydroxymethyleneiminetricetric acid (HEDTA; 2 mM) was added to 100 mM Na\(^+\) solution and 1 \( \mu \)M tetrodotoxin (TTX) was added in both solutions. Inward currents were evoked by 30-ms step depolarization from ~50 to 40 mV for (a) and from ~50 to 20 mV for (b) with 10 mV increments, from a \( V_h \) of ~100 mV. B: corresponding peak I-V relationships for Ba\(^{2+}\) currents (○) and Na\(^+\) currents (●). Smooth curves were fitted as in Fig. 3.](http://jn.physiology.org/)

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CaCl\(_2\), BaCl\(_2\), SrCl\(_2\), LaCl\(_3\), CdCl\(_2\), NiCl\(_2\), CoCl\(_2\), ZnCl\(_2\), HEDTA, EGTA, ATP Mg\(_2\), HEPES, TEA-OH, tetrodotoxin, and GDP/BS (Sigma, St Louis, MO, USA).
current amplitude increased with increments of depolarization, reaching a peak in the current-voltage (I-V) relationship around −10 mV (Fig. 3A). Smooth curve was fitted with the equation:

\[ I(V_m) = G_{Ba}(V_m - E_{rev})/[1 + \exp ((V_m - V_{0.5})/k)] \]  

where \( I(V_m) \) is the peak Ba\(^{2+} \) current at the membrane potential of \( V_m \), \( G_{Ba} \) is the maximum Ba\(^{2+} \) conductance, \( E_{rev} \) is the apparent zero-current potential in the I-V relationship, \( V_{0.5} \) is the potential to give a half-value of conductance, and \( k \) is the slope factor that determines the steepness of the curve. The values of \( E_{rev}, V_{0.5}, \) and \( k \) are 39.5, −18.7, and 5.7 mV, respectively. To determine the voltage-dependence of activation more accurately, we measured the tail currents evoked by clamp-back to a fixed potential of −60 mV after 10-ms step depolarizations from −40 to 35 mV with increments of 5 mV. The tail current amplitude was normalized to the value after the step depolarization to 35 mV. The mean values were plotted as a function of voltage of the step depolarization (n = 5) and fitted to the Boltzmann’s equation:

\[ n_\alpha = 1/[1 + \exp ((V_m - V_{0.5})/k)] \]  

The values of \( V_{0.5} \) and \( k \) were −20.3 and 6.3 mV, respectively (Fig. 3Bb). According to this equation, about 4% of the \( \alpha_{1B} \) channels are activated at −40 mV and more than 96% of the channels are activated at 0 mV. Voltage-dependence of inactivation of the \( \alpha_{1B} \) channel was measured by the use of a conventional double-pulse protocol. Peak current amplitude induced by the test pulse to −10 mV from various \( V_h \) was normalized to the amplitude induced by the test pulse from a \( V_h \) of −120 mV and was plotted against the \( V_h \) from −120 to −30 mV. The continuous curve was also fitted with the Boltzmann’s equation. The estimated half-inactivation potential and the slope factor were −74.3 and −9.2 mV for 2 s \( V_h \)-displacement (n = 11), and −79.1 and −9.4 mV for 10-s \( V_h \)-displacement (n = 5) (Fig. 3Bb). The activation and inactivation curves crossed around −45 mV and had a very small overlap, or “window current.” The voltage dependence of activation and inactivation of the recombinant \( \alpha_{1B} \) channel is thus comparable to the native N-type Ca\(^{2+} \) channel seen in bullfrog sympathetic neurons (Jones and Marks 1989a,b).

**Single-channel recordings of \( \alpha_{1B} \) channel**

In addition to whole cell current measurements of the \( \alpha_{1B} \) channel in BHK cells, we determined the single-channel conductance of the \( \alpha_{1B} \) channel with a 110 mM Ba\(^{2+} \) solution, because unitary conductance is considered a hallmark for identification of N-type channels in native tissues. Figure 4A illustrates three sets of consecutive single-channel current traces from \( \alpha_{1B} \) channels obtained from the same cell-attached patch in response to test pulses of different potentials, −5, 5, and 15 mV. Channel openings were relatively rare at −5 mV and became increasingly frequent at more positive potentials. As many as two channels were observed to open simultaneously in this patch. Figure 4B shows an amplitude histogram constructed from the same data used in Figure 4Ab. A plot of unitary current-voltage relationship yields a single-channel conductance of 18.2 pS (Fig. 4C). This value is comparable with previously published values for native N-type channels (for review see Bean 1989).

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**FIG. 7.** Dependence of current amplitude and kinetics of \( \alpha_{1B} \) channel on extracellular Ba\(^{2+} \) concentration ([Ba\(^{2+} \)]\(_e\)). A: families of \( I_{Ba} \) recorded from same cell at 2 (a), 5 (b), and 10 mM [Ba\(^{2+} \)]\(_e\). \( I_{Ba} \) were evoked by step depolarization from −40 to 30 mV (a) or −30 to 40 mV (b and c) from a \( V_h \) of −100 mV. B: I-V relationships of \( I_{Ba} \) obtained from same cell in external solutions containing 1 (○), 2 (△), 5 (□), 10 (●), 20 (△), and 50 mM (■) [Ba\(^{2+} \)]\(_e\). Smooth curves were fitted as in Fig. 3. C: dependence of activation parameters of \( \alpha_{1B} \) channel on [Ba\(^{2+} \)]\(_e\). \( E_{rev} \) (○), \( V_{0.5} \) (△), \( k \) (□) were calculated from I-V relationships in 6 different external solutions and plotted as a function of [Ba\(^{2+} \)]\(_e\). D: relationship between maximum of peak current amplitude and [Ba\(^{2+} \)]\(_e\). Peak current amplitude in various [Ba\(^{2+} \)]\(_e\), was normalized to that obtained in [Ba\(^{2+} \)]\(_e\) of 5 mM (†). A continuous curve was fitted with Eq. 3 in text, where dissociation constant and Hill coefficient are 6.0 mM and 1.1, respectively. Each point in C and D represents average value of 5 cells and vertical bars show mean ± SE if they are larger than symbols.
Ion permeability of N-type α_{1B} channel in BHK cells

It has been previously shown that three highly permeant divalent cations, Ba^{2+}, Ca^{2+}, and Sr^{2+} display different permeation characteristics through multiple types of Ca^{2+} channels (for review, see Tsien et al. 1987). However, these measurements were done mostly with the L-type Ca^{2+} channel, before other Ca^{2+} channel types were resolved in single cells (Varadi et al. 1995; Zhang et al. 1993). To investigate ion permeability, we measured currents through α_{1B} channels expressed in BHK cells with three different divalent cations. Substitution of Ca^{2+} for Ba^{2+} not only reduced the current amplitude but also shifted the I-V relationship in the depolarizing direction. The changes in parameters are 10.8 ± 1.3 mV for \( E_{rev} \), 8.1 ± 0.4 mV for \( V_{0.5} \), and 0.9 ± 0.2 mV (n = 11) for \( k \). Surface potential changes as a result of the divalent ion solution changes contributed to the shift in gating (Hille et al. 1975). On the other hand, replacement of Ba^{2+} with Sr^{2+} reduced the current amplitude without significantly changing \( E_{rev} \) (1.5 ± 1.3 mV), \( V_{0.5} \) (2.0 ± 0.5 mV), or \( k \) (0.1 ± 0.6 mV). The ratio of the maximum conductance for Ba^{2+}, Ca^{2+}, and Sr^{2+} currents was 1:0.72 ± 0.05:0.75 ± 0.06 (n = 11); (Fig. 5). These results suggest that Ca^{2+} has the highest affinity and Ba^{2+} has the highest mobility in the N-type channel pore.

Under physiologic conditions, L-type Ca^{2+} channels demonstrate an extraordinary selectivity to divalent cations and exclusion of monovalent cations, even though Na^{+} and K^{+} are present at comparatively much higher concentrations (Almers and McCleskey 1984; Hess and Tsien 1984; Kostyuk et al. 1983). When extracellular Ca^{2+} is absent, however, Ca^{2+} channels become highly permeable to monovalent cations (Almers and McCleskey 1984). We tested whether this characterization is also applicable to the N-type channel in Ca^{2+}/Ba^{2+}-free external solution containing 100 mM Na^{+} and 2 mM HEDTA (100 mM Na^{+} solution). Figure 6 shows inward currents recorded from the same BHK cell in an external solution containing 3 mM Ba^{2+} or 100 mM Na^{+}, as the charge carrier. When the external solution was changed from the 3 mM Ba^{2+} solution to the 100 mM Na^{+} solution, the I-V relationship shifted in the hyperpolarizing direction by ~20 mV, and further, the peak current amplitude increased about 1.5 ± 0.1-fold (n = 4). The inward currents found using the 100 mM Na^{+} solution, were induced by expressed Ca^{2+} channels but not by voltage-gated Na^{+} channels. This is evident because 1 μM tetrodotoxin (TTX) did not block currents in the Ba^{2+}-containing or the Ba^{2+}-free 100 mM Na^{+} solution. Secondly, the current decay in the Ba^{2+}-free 100 mM Na^{+} solution was extremely slow, compared with that of the typical TTX-sensitive Na^{+} channel currents. Finally, inward currents were completely inhibited by Cd^{2+} at concentrations that would marginally effect Na^{+} channel currents (3 μM) (data not shown). Interestingly, decay of tail currents in the 100 mM Na^{+} solution was slower than that in the 3 mM Ba^{2+} solution (Fig. 6 A). This phenomenon may be comparable to the finding that a decrease in the pipette Ba^{2+} concentration dramatically increased the frequency of repolarization openings of L-type channels in cell-attached recordings from hippocampal neurons (Thibault et al. 1993). We did not carry out further detailed analysis of the tail currents in the 100 mM Na^{+} solution.

Dependence of N-type α_{1B} current on extracellular Ba^{2+} concentration

Measurements of ionic current as a function of the permeant ion concentration is a useful approach to understanding the binding of ions to a saturable site within the pore. To examine the dependence of α_{1B} currents on the extracellular Ba^{2+} concentration ([Ba^{2+}]_o), we varied the [Ba^{2+}]_o from 1 to 50 mM. Figure 7A shows families of \( I_{Na} \) recorded in a cell, where the test pulses were changed from ~40 to 30 mV for 2 mM [Ba^{2+}]_o, and ~30 to 40 mV for both 5 and 10 mM [Ba^{2+}]_o. Figure 7B shows peak I-V relationships corresponding to Fig. 7A and those for 1, 20, and 50 mM Ba^{2+}.
[Ba\(^{2+}\)]\(_0\). An increase in the [Ba\(^{2+}\)]\(_0\) augmented the amplitude of peak \(I_{\text{rev}}\) and shifted the I-V relationship in the depolarizing direction. Each curve was fitted with Eq. 1, which provided \(E_{\text{rev}}\), \(V_{0.5}\), and \(k\). In Fig. 7C, [Ba\(^{2+}\)]\(_0\)-dependency of these parameters was summarized. \(E_{\text{rev}}\) and \(V_{0.5}\) shifted to more positive potentials with increasing [Ba\(^{2+}\)]\(_0\), while the slope factor \(k\) was independent of [Ba\(^{2+}\)]\(_0\). The peak current amplitude at each [Ba\(^{2+}\)]\(_0\) was normalized to the peak current amplitude at [Ba\(^{2+}\)]\(_0\) of 5 mM and their mean values from eight cells were plotted as a function of [Ba\(^{2+}\)]\(_0\). The solid curve was fitted with the equation:

\[
\frac{I}{I_{\text{rev}}} = A [([\text{Ba}^{2+}]_0)^n + [\text{Ca}^{2+}]_o + K_D].
\]

where \(I\) is the peak current amplitude at [Ba\(^{2+}\)]\(_0\), \(I_{\text{rev}}\) is the peak current amplitude at [Ba\(^{2+}\)]\(_0\) of 5 mM, \(A\) is the maximum ratio, \(K_D\) is the dissociation constant, and \(n\) is the Hill coefficient. The \(K_D\) and the \(n\) are 6.0 mM and 1.1, respectively.

Effects of inorganic Ca\(^{2+}\) channel blockers on N-type \(\alpha_B\) channel

It has been recognized that divalent and trivalent cations, known as inorganic Ca\(^{2+}\) channel blockers, compete with permeant ions such as Ba\(^{2+}\) and Ca\(^{2+}\) at a common binding site in the channel pore (Chow 1991; Hagiwara and Takahashi 1967; Kim et al. 1993; Tang et al. 1993a; Yang et al. 1993). Therefore, inorganic blockers are useful probes to distinguish fine structural differences at the high affinity Ca\(^{2+}\)-binding site among Ca\(^{2+}\) channel types (Mori et al. 1996). To test divalent and trivalent inorganic blockers, currents were evoked every 10 s by step depolarization to −10 mV for 37.5 ms from a \(V_o\) of −100 mV in 1 mM [Ba\(^{2+}\)]\(_0\). The inhibitory potency at steady-state (maximum inhibition) was plotted as a function of blocker concentration. The actions of inorganic Ca\(^{2+}\) channel blockers were concentration-dependent with the half-maximum inhibition concentration (IC\(_{50}\)) of 0.05 \(\mu\)M for La\(^{3+}\), 0.08 \(\mu\)M for Cd\(^{2+}\), 8.5 \(\mu\)M for Zn\(^{2+}\), 44.8 \(\mu\)M for Ni\(^{2+}\), and 71.8 \(\mu\)M for Co\(^{2+}\). The blocking actions of Cd\(^{2+}\), Zn\(^{2+}\), Ni\(^{2+}\), and Co\(^{2+}\) were re-
versible, however, blockade by La$^{3+}$ was only partially reversible (data not shown).

We have observed that the $\alpha_{1B}$ Ba$^{2+}$ currents induced by test pulses to more positive than 10 mV were facilitated by a preceding strong depolarization (conditioning pulse to 90 mV for 50 ms) in the presence of Cd$^{2+}$ at relatively high concentrations, by using a pulse protocol shown in Fig. 10 where two identical 25 ms test pulses were applied from −30 to 40 mV with 10 mV increments. The ratios of the peak current amplitude induced by the second test pulse to that by the first pulse in the absence of Cd$^{2+}$ (control) and in the presence of 0.6 and 1 μM Cd$^{2+}$ were 0.54, 0.76, and 0.76 at 0 mV and 0.69, 1.0, and 1.1 at 20 mV, respectively. In the presence of 0.6 or 1 μM Cd$^{2+}$, the peak current amplitude induced by the second pulse, at potentials more positive than 0 mV, was larger than the amplitude at the end of the first pulse, although in the absence of Cd$^{2+}$ the peak amplitude induced by the second pulse was smaller than the amplitude at the end of the first pulse at any potentials. This facilitation of the $\alpha_{1B}$ channel was presumably the result of removal of Cd$^{2+}$ from the binding site in the pore during strong depolarizing pulses. Current decay of the facilitated currents was much faster than that of currents induced by the first pulse (Fig. 10). The current decay phase was well fitted by a single exponential function (Fig. 10Ba), suggesting that the faster decay phase may represent reblocking of $\alpha_{1B}$ channel by Cd$^{2+}$. The mean values of the exponential time constants are 54.9 ± 25.6 ms for control, 11.3 ± 1.3 ms for 0.3 μM Cd$^{2+}$, and 4.1 ± 0.1 ms ($n = 4$) for 1 μM Cd$^{2+}$ at a test potential of 30 mV. The current kinetics can be simply represented by the following scheme, which was applied to the analysis of blockade in L-type Ca$^{2+}$ channel (Lansman et al. 1986).

\[
\begin{align*}
\text{Closed channel} & : \frac{k_x}{k_{c-x}} \\
\text{Open channel} & : \frac{k_b}{k_{c-b}} \\
\text{Blocked channel} & : \frac{k_x}{k_{c-x}} \\
\end{align*}
\]

where $k_{c-x}$ and $k_x$ are the voltage-dependent rate constants for channel opening and closing, $k_b$ is a second-order rate constant for association of the blocker and $k_{c-b}$ is the first order rate constant for dissociation of the blocker. According to the model the inverse of the decay time constant ($\tau$) should be equal to $k_c + k_b$ ($\tau^{-1} = k_c + k_b$), and further $k_b$ is a linear function of the Cd$^{2+}$ concentration ($k_b = l \times [\text{Cd}^{2+}]$, where $l$ is a blocking rate coefficient and $[\text{Cd}^{2+}]$ is the Cd$^{2+}$ concentration). The inverse of the decay time constant (‘‘on-rate’’) is plotted as a function of Cd$^{2+}$ concentration at various test potentials (Fig. 10Bb). The on-rate is linearly correlated with Cd$^{2+}$ concentration, where the slope of the fitted lines corresponds to blocking rate coefficient $l$ of $1.5 \times 10^8, 2.0 \times 10^8, 2.2 \times 10^8$, and $2.2 \times 10^8 \text{M}^{-1}\text{s}^{-1}$, at 10, 20, 30, and 40 mV, respectively. However, we must take it into account that for whole cell currents, the mathematical formulas for individual time constants describing kinetic components such as block, activation, or inactivation will include all of the rate constants for

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**Fig. 10.** Facilitation of $\alpha_{1B}$ channel by strong depolarization in presence of Cd$^{2+}$. $I_{\text{Ba}}$ were recorded after completing internal perfusion of BHK cells with 0.2 mM guanosine 5'-O-(2-thiodiphosphate) to avoid contamination of direct G-protein inhibition. A: left, families of $I_{\text{Ba}}$ recorded in one cell in external solution containing 3 mM Ba$^{2+}$ without Cd$^{2+}$ (a), with 0.3 μM Cd$^{2+}$ (b), and with 1 μM Cd$^{2+}$ (c). Pulse protocol was designed to evoke currents by 25 ms test pulses from −30 to 40 mV before and after a 50 ms conditioning pulse to 90 mV. Pulse interval between conditioning pulse and 2nd test pulse was set for 10 ms. This protocol was applied to $\alpha_{1B}$ channel every 15 s from a $V_s$ of −100 mV. Right: corresponding I-V relationships for $I_{\text{Ba}}$ before (Ο) and after (●) conditioning pulses. Smooth curves were fitted as in Fig. 3. B: analysis of ‘‘on-rate’’ for Cd$^{2+}$-block. a: Ba$^{2+}$ currents evoked by 2nd test pulse to +30 mV without Cd$^{2+}$ (left) and with 1 μM Cd$^{2+}$ (right). Current decay was fitted by a single exponential function (thick curve) with time constant of 27.3 ms (left) or 4.1 ms (right). b: inverse of decay time constant of inward current induced by 2nd test pulse is plotted as a function of Cd$^{2+}$ concentration. Slopes of fitted lines are $1.5 \times 10^8$ (Ο), $2.0 \times 10^8$ (●), $2.2 \times 10^8$ (●), and $2.2 \times 10^8$ M$^{-1}\text{s}^{-1}$ (Ο), at test potentials of 10, 20, 30, and 40 mV, respectively. Each point represents average value of four cells and vertical bars show mean ± SE if they are larger than symbols.
each one of the state transitions. Because Cd$^{2+}$ does not clearly change time to peak (activation) nor voltage-dependent inactivation curve (inactivation) (data not shown), suggesting that the concentration-dependent acceleration may represent blocking rate by Cd$^{2+}$, we applied the above kinetic model to the present analysis. A relief from blockade by strong depolarizing pulses has been reported for N-type channels when the N-type channel is suppressed by neurotransmitters via G-protein(s) (Grassi and Lux 1989). However the possibility that G-protein(s) are involved in the blockade by Cd$^{2+}$ can be excluded, because we observed the facilitation and subsequent fast reblocking of $\alpha_{1B}$ channel by Cd$^{2+}$ in the presence of 0.2 mM GDP/βS.

**DISCUSSION**

This is the first systematic analysis of ion permeation/selectivity properties of N-type Ca$^{2+}$ channel currents free from contamination of currents generated by other high-voltage-activated Ca$^{2+}$ channels such as L-, P-, Q-, and R-types. This homogeneous expression system, which lacks any detectable endogenous Ca$^{2+}$ channel activity served as suitable tool for our study. The $\alpha_{1B}$ subunit together with the $\alpha_{2A}$ and $\beta_{1A}$ subunits in BHK cells directed the formation of voltage-dependent Ca$^{2+}$ channels, activated at membrane potentials more positive than 40 mV in an external solution containing 1 mM Ba$^{2+}$. The channel was blocked by the N-type Ca$^{2+}$ channel blocker ω-CgTx-GVIA but was not affected by ω-Aga-IVA and DHPs, selective inhibitors of P/Q- and L-type Ca$^{2+}$ channels, respectively. The single-channel conductance of the $\alpha_{1B}$ channel in the 110 mM Ba$^{2+}$ solution was 18 pS. These electrophysiological and pharmacological characteristics of the $\alpha_{1B}$ channel indicate a functional correlation between the recombinant $\alpha_{1B}$ channel in BHK cells and the N-type Ca$^{2+}$ channels found in neuronal cells (Bean 1989; Hess 1990; Tsien et al. 1991).

**Inorganic Ca$^{2+}$ channel antagonists**

The potency of inorganic Ca$^{2+}$ antagonists in inhibiting the $\alpha_{1B}$ channel was (IC$_{50}$ in μM) La$^{3+}$ (0.05) ≫ Cd$^{2+}$ (0.08) ≫ Zn$^{2+}$ (8.5) ≫ Ni$^{2+}$ (44.8) ≫ Co$^{2+}$ (71.8). This rank order is the same as that reported for the N-type Ca$^{2+}$ channel (La$^{3+}$ > Cd$^{2+}$ ≫ Ni$^{2+}$) in NG-108-15 cells (Kasai and Neher 1992). The IC$_{50}$ value of Cd$^{2+}$ in our experiments is lower than those reported for native N-type channels (0.4 μM, Jones and Marks 1989a; 1 μM, Kasai and Neher 1992), which is probably derived from a difference in the charge carrier and its concentrations used in the recordings. Native L-type Ca$^{2+}$ channels have also shown the same order (La$^{3+}$ > Cd$^{2+}$ ≫ Ni$^{2+}$ > Co$^{2+}$, Narahashi et al. 1987; La$^{3+}$ > Cd$^{2+}$ ≫ Ni$^{2+}$, Kasai and Neher 1992). However, the low-voltage-activated T-type Ca$^{2+}$ channels have shown different orders (La$^{3+}$ ≫ Ni$^{2+}$ > Cd$^{2+}$ = Co$^{2+}$, Narahashi et al. 1987; La$^{3+}$ > Zn$^{2+}$ > Cd$^{2+}$ > Ni$^{2+}$ > Co$^{2+}$, Akaike et al. 1989a). It has been recognized that inorganic Ca$^{2+}$ channel blockers compete with permeant ions at a common high affinity binding site in the channel pore (Chow 1991; Hagiwara and Takahashi 1967; Kim et al. 1993; Tang et al. 1993a; Yang et al. 1993). In other words, inorganic Ca$^{2+}$ antagonists can be used to probe properties of the ion binding site in the channel pore (Mori et al. 1996). Blockade of the $\alpha_{1B}$ channel by La$^{3+}$ or Cd$^{2+}$ was partially removed by a 50-ms step depolarization to 90 mV (Fig. 9). This experiment provided us with information as to the reblocking process of Ca$^{2+}$ to the high affinity site. The calculated blocking rate coefficient of Cd$^{2+}$ was about $2 \times 10^7$ M$^{-1}$ s$^{-1}$ at membrane potentials above 0 mV (Fig. 10). The value is similar to the blocking rate coefficient of Cd$^{2+}$ (4 × 10$^7$ M$^{-1}$ s$^{-1}$) and Ca$^{2+}$ (4 × 10$^7$ M$^{-1}$ s$^{-1}$) estimated from the external Ca$^{2+}$ block of inward Ba$^{2+}$ current (Lansman et al. 1986) and the external Ca$^{2+}$ block of inward Li$^+$ current (Kuo and Hess 1993), respectively, at the single-channel level. Both the blocking rate coefficients of Cd$^{2+}$ and Ca$^{2+}$ are close to the diffusion-controlled limit of the association rates between Cd$^{2+}$ and channel or Ca$^{2+}$ and channel. Fast reblocking of the N-type Ca$^{2+}$ channel by Cd$^{2+}$ suggests that the permeant and blocking ions can easily access the high-affinity site from outside of the membrane. In other words, the high-affinity site is located at the external mouth of the channel pore.

Unlike other inorganic Ca$^{2+}$ channel antagonists, Zn$^{2+}$ is ubiquitously present in CNS and serves as a cofactor or a structure component for enzymes. Zn$^{2+}$ is released from presynaptic terminals in large quantities during synaptic activity (Assaf and Chung 1984). Concentration of Zn$^{2+}$ at the synaptic cleft in hippocampal CA3 region has been estimated to be as high as 100–300 μM (Frederickson et al. 1983; Xie and Smart 1991), although free Zn$^{2+}$ in other regions of the brain is lower, in the range of 1–20 μM. In the present experiments, $I_{ba}$ was concentration-dependently inhibited by Zn$^{2+}$ at the concentration between 0.3 and 300 μM with the IC$_{50}$ of 8.5 μM. It is possible that Zn$^{2+}$ presynaptically influences neurotransmission, in addition to post-synaptic action of Zn$^{2+}$ on N-methyl-D-aspartate (NMDA), γ-aminobutyric acid (GABA), glycine, and ATP responses (Bloomenthal et al. 1994; Cloues et al. 1993; Li et al. 1993; Westbrook and Mayer 1987).

**Permeation and selectivity of N-type $\alpha_{1B}$ channel**

The dependency of the peak $\alpha_{1B}$ current amplitude on extracellular Ba$^{2+}$ concentration showed a definite saturation ($K_D = 6.0$ mM, $n = 1.1$; Fig. 4), which was comparable to that of native Ca$^{2+}$ channels, whose $K_D$ value for Ca$^{2+}$ is 1–10 mM in T-type channels (Akaie et al. 1989a,b; Bossu et al. 1985; Carbone and Lux 1987a; Hagiwara et al. 1988), 11.6 mM in the N-type channel (Zhou and Jones 1995), and 3–15 mM in L-type channels (Aibara et al. 1992; Hagiwara et al. 1988). This saturation of divalent cation influx as the ionic concentration is increased, indicates the existence of a binding site for permeants in the channel pore.

In the $\alpha_{1B}$ channel, replacement of Ba$^{2+}$ by Ca$^{2+}$ or Sr$^{2+}$ resulted in smaller current amplitude ($I_{ba} > I_{ba} = I_{ba}$). The L-type Ca$^{2+}$ channel produced currents that showed a similar sequence in amplitude by using three different charge carriers (Ba$^{2+}$ > Sr$^{2+}$ > Ca$^{2+}$) (Fox et al. 1987a; Kasai and Neher 1992). Unitary amplitude of L-type Ca$^{2+}$ current was almost a half of that of Ba$^{2+}$ current (Friel and Tsien 1989). Another neuronal Ca$^{2+}$ channel, Ni$^{2+}$-sensitive $\alpha_{1B}$ channel transiently expressed in HEK293 cells, exhibited whole cell currents ~80% larger in Ba$^{2+}$ solution than Ca$^{2+}$ solution (Williams et al. 1994, but see Bourinet et al. 1996). By contrast, T-type Ca$^{2+}$ channels are equally permeable to
Ba²⁺ and Ca²⁺ at the whole cell level (Akaike et al. 1989a) and in single-channel recordings as well (Carbone and Lux 1987b). The time and amplitude distributions of elementary events for T-type currents were indistinguishable in Ba²⁺, Ca²⁺, or Sr²⁺-containing solutions. Thus high voltage-activated Ca²⁺ channels share similar energy profiles at the saturable binding site for permeants, different from that of low voltage-activated channels.

The α₁₈ channel showed an anomalous mole fraction behavior: in solutions with [Ba²⁺] + [Ca²⁺] held constant, the Ca²⁺ channel current in a mixture can be smaller than that measured in the presence of either Ba²⁺ or Ca²⁺ alone. This contradicts the simple hypothesis, Ca²⁺ selectivity through binding to a single site within the channel, but is rather consistent with the idea that the α₁₈ channel has a single-file pore with a binding site occupied simultaneously by at least two divalent permeants (Yang et al. 1993). High-affinity binding of Ca²⁺ secures high selectivity to Ca²⁺ over other ions and the electrostatic repulsion of two Ca²⁺ ions at the site in the single-file pore enables the Ca²⁺ channel to conduct ions rapidly. The mole fraction dependence of the N-type Ca²⁺ channel current in this work was qualitatively similar to that of the L-type channel (Hess and Tsien 1984), which suggests similarity between the two types of Ca²⁺ channels in the permeant-translocating pathways along the pore. Single-channel analysis would provide more information of the anomalous mole fraction effect. Yue and Marban (1990) reported that the L-type Ca²⁺ channel in ventricular myocytes did not show the paradoxical decrease in single-channel conductance nor absolute unitary current amplitude, although Friel and Tsien (1989) reported that the anomalous mole fraction effect on the L-type Ca²⁺ channel was found in PC-12 cells under restrictive conditions of permeation ion concentration (10 mM but not 110 mM) and membrane potential (more depolarized potentials than 0 mV). In the present study, however, we could not examine this controversial but interesting issue by using single-channel recording, mainly because of lack of N-type Ca²⁺ channel agonists that prolong openings of unitary activity so that we can evaluate reduction in single-channel amplitude more precisely in these restrictive conditions.

A recent combined approach using electrophysiological and molecular biological techniques has provided several lines of evidence that cation permeability, selectivity, and sensitivity to inorganic Ca²⁺ channel blockers are altered by substitution of residues in the conserved linker region of Ca²⁺ channels (Varadi et al. 1995). The glutamic acid residues in the pore-lining region between S5 and S6 of each repeat are involved in the high affinity binding of divalent cations in P/Q- and L-type Ca²⁺ channels, although sensitivity to Cd²⁺ blockade of Ba²⁺ currents differed among mutants of the glutamic acid residues in four repeats (Ellinor et al. 1995; Kim et al. 1993; Tang et al. 1993a; Yang et al. 1993). The four negatively charged residues and surrounding residues are also conserved in the pore region of the α₁₈ subunit, supporting the above-mentioned concept that the nature of ionic pores of the L- and N-type channels are similar. However, the net charge of the extracellular linkers, S1–S2, S3–S4, and S5–S6 linker of four repeats, in α₁₈ channel (−27) is less negative than that in α₁₉ channel (−29) (Mikami et al. 1989). Moreover in repeat III, where the glutamic acid residue has the biggest contribution to high affinity binding of divalent cations (Ellinor et al. 1995; Kim et al. 1993; Tang et al. 1993a; Yang et al. 1993), the net charge of the extracellular linker in α₁₈ channel (−5) is less negative than that in α₁₉ channel (−7). These differences in the pore-lining region may cause the difference of single-channel conductance between N- and L-type Ca²⁺ channels (Bean 1989).

Taken together, our experiments in whole cell mode of patch-clamp recording have revealed that the N-type Ca²⁺ channel (α₁₉ + α₂ + β₁₉) shares general features of a high affinity-binding site easily accessible from the extracellular side with the L-type Ca²⁺ channels. It would be interesting to perform single-channel recording for more detailed analyses of permeation of the N-type Ca²⁺ channel and to examine possible effects of various subunit combinations on permeation and gating of the N-type Ca²⁺ channel.

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