regions of the polypeptide are available to fill the sites.

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Phosphorylation-Independent Modulation of L-Type Calcium Channels by Magnesium-Nucleotide Complexes

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Free magnesium ions and magnesium-nucleotide complexes can exert opposite effects on many fundamental cellular processes. Although increases in the intracellular concentration of magnesium ions inhibit the L-type calcium current in heart cells, magnesium–adenosine triphosphate complexes (MgATP) would be expected to increase the current by promoting channel phosphorylation. Rapid increases in the intracellular concentration of MgATP induced by flash photolysis of caged magnesium or caged ATP resulted in enhanced calcium current. The increase in calcium current was not prevented by blocking phosphorylation, revealing a previously unrecognized direct regulatory action of the magnesium-nucleotide complex.

Cellular processes as diverse as transcription, energy metabolism, and excitability are regulated by changes in the intracellular concentrations of either free magnesium ions (Mg^{2+}) or Mg-nucleotide complexes (*1*), with the two forms often exerting opposite effects on ion channels (*2*, *3*). Increases in the concentration of intracellular Mg^{2+} in the millimolar range inhibit L-type calcium current (I_{Ca}) (*4*).

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In contrast, addition of adenosine triphosphate (ATP) to the intracellular solution reduces the rate of irreversible decline (or "run-down") of I_{Ca} in dialyzed cells (*5*) and increases I_{Ca} when endogenous ATP production is inhibited (*6*). To clarify the regulatory roles of Mg^{2+} and MgATP, we used flash photolysis of caged Mg^{2+} (*7*) or caged ATP (*8*) to rapidly raise cytosolic Mg^{2+}, MgATP, or both from submicromolar concentrations to concentrations of several hundred micromolar. We found that L-type Ca^{2+} currents are increased by Mg-nucleotide complexes through a phosphorylation-independent mechanism, possibly by allosteric interaction.

Dimethoxy (DM)-nitrophen is a photolabile chelator that binds either Ca^{2+} or Mg^{2+} (*7*). We used Ca^{2+}-loaded DM-nitrophen to determine the efficiency of photolysis in our system by monitoring the concentration of free intracellular Ca^{2+} ($[Ca^{2+}]_i$) (*9*). In a whole cell–clamped myocyte loaded with caged Ca^{2+} (in the absence of Mg^{2+}), depolarizing pulses elicited small $[Ca^{2+}]_i$ transients (Fig. 1A). A flash of ultraviolet light abruptly raised the baseline Ca^{2+} signal. Two subsequent flashes evoked little further increase in basal $[Ca^{2+}]_i$, suggesting that photolysis was 80 to 90% complete after one flash. To use DM-nitrophen as caged Mg^{2+}, the cytoplasm was equilibrated with pipette solutions which included ATP, magnesium, DM-nitrophen, and 1,2-is(*o*-aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA) to maintain $[Ca^{2+}]_i$ at subnanomolar levels. Under these conditions, no change in $[Ca^{2+}]_i$ was detected in response to depolarizing pulses or to flashes (Fig. 1B).

To verify that Mg^{2+} was in fact released by photolysis of caged Mg^{2+}, we used a bioassay that exploited the capacity of cytoplasmic Mg^{2+} to block outward currents through inwardly rectifying K^+ channels (I_{K1}) (*10*, *11*). Depolarization to +20 mV elicited a large outward current before photolysis, but the subsequent release of Mg^{2+} induced inward rectification (Fig. 1C). The results verify that $[Mg^{2+}]_i$ reached concentrations sufficient to block outward current through the K^+ channels (1.7 to 30 μM) (*10*), consistent with our estimate that photolysis increased $[Mg^{2+}]_i$ from 0.06 to 58 μM in this experiment (*11*).

Having confirmed that Mg^{2+} can be released from caged Mg^{2+} without concomitant changes in $[Ca^{2+}]_i$, we examined the effect of Mg^{2+} release on I_{Ca}. Currents were stable until exposure to a flash (Fig. 2A), after which the amplitude of I_{Ca} transiently decreased and then increased to a steady value approximately twice that during the control period. A second flash induced a similar biphasic effect resulting in further augmentation of I_{Ca}. Subsequent flashes had no effect. The large Mg^{2+}-induced increase in I_{Ca} was not associated with detectable alterations in the kinetics of the current, as confirmed by examination of representative records before and after the flashes (Fig. 2A). In six cells studied under identical conditions, I_{Ca} increased from 1328 ± 266 (SEM) to 2073 ± 290 pA ($P < 0.05$). Current through L-type Ca^{2+} channels was also enhanced by release of Mg^{2+} when Ba^{2+} replaced Ca^{2+} as the charge carrier (Fig. 2B). This result provides additional evidence that the response does not involve changes in $[Ca^{2+}]_i$ (*12*), nor does it require Ca^{2+} occupancy of the pore, in contrast to the phenomenon of Ca^{2+}-de-

pendent inactivation (*13*). Flashes of ultraviolet light did not alter I_{Ca} when myocytes were dialyzed with solutions containing ATP, magnesium, and either EDTA without DM-nitrophen or an equimolar mixture of EDTA and DM-nitrophen, confirming that neither the ultraviolet light itself nor the by-products of DM-nitrophen photolysis can account for the results.

The increase in I_{Ca} cannot be explained by charge-screening effects at the membrane surface resulting from changes in $[Mg^{2+}]_i$, because I_{Ca} increased at both positive and negative test potentials after Mg^{2+} release (Fig. 2C). Furthermore, a simple surface charge shift should affect Na^+ and Ca^{2+} currents in a qualitatively similar fashion. This was clearly not the case: Na^+ current elicited by a pulse to −40 mV usually decreased with time, whereas Ca^{2+} current in the subsequent test pulse increased in response to Mg^{2+} release (Fig. 2D).

Because both $[Mg^{2+}]_i$ and $[MgATP^{2-}]_i$ would be expected to increase after photolysis of caged Mg^{2+}, we sought to distinguish which of these two factors mediates the increase in I_{Ca}. To decrease cytosolic ATP to a low concentration, we incubated cells in glucose-free solution and equilibrated them with an intracellular solution containing caged Mg^{2+}, no ATP, and dinitrophenol (an inhibitor of mitochondrial ATP production). Under these conditions, I_{Ca} amplitude progressively decreased; flash photolysis of caged Mg^{2+} neither reversed the decline nor affected the kinetics of I_{Ca} (Fig. 3A). The lack of effect of Mg^{2+} release on Ca^{2+} current after ATP depletion suggested that the Mg-nucleotide complex, not Mg^{2+}, caused the increase in I_{Ca}. To test this idea, we photoreleased ATP in the presence of physiological $[Mg^{2+}]_i$. In myocytes equilibrated with an intracellular solution containing 50 μM ATP, 1 mM caged ATP, and 0.8 mM Mg^{2+}, flashes produced an increase in I_{Ca} (Fig. 3B) similar to that elicited by Mg^{2+} release (Fig. 2A). Transient decreases in I_{Ca} were not observed after ATP release, suggesting that the inhibition of I_{Ca} immediately after photolysis of caged Mg^{2+} (Fig. 2A) may be attributable to the associated increase in $[Mg^{2+}]_i$, perhaps through Mg^{2+} block of the channels at an internal divalent ion binding site (*13*, *14*).

The primary physiological mechanism for increasing I_{Ca} is through phosphorylation by the cyclic AMP–dependent protein kinase (*15*, *16*). Because MgATP is a substrate for this reaction, the sudden production of Mg-nucleotide complexes might increase I_{Ca} by enhancing basal phosphorylation. We therefore examined the structural specificity of the nucleotide required to increase I_{Ca}, recognizing that phosphorylation can be effectively prevented by replacing ATP with the nonhydrolyzable analogs adenylyl-

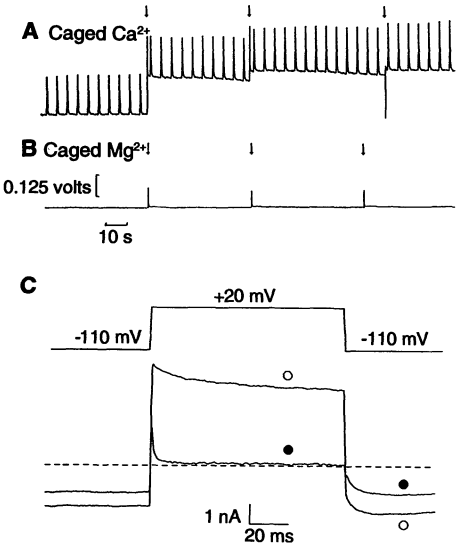
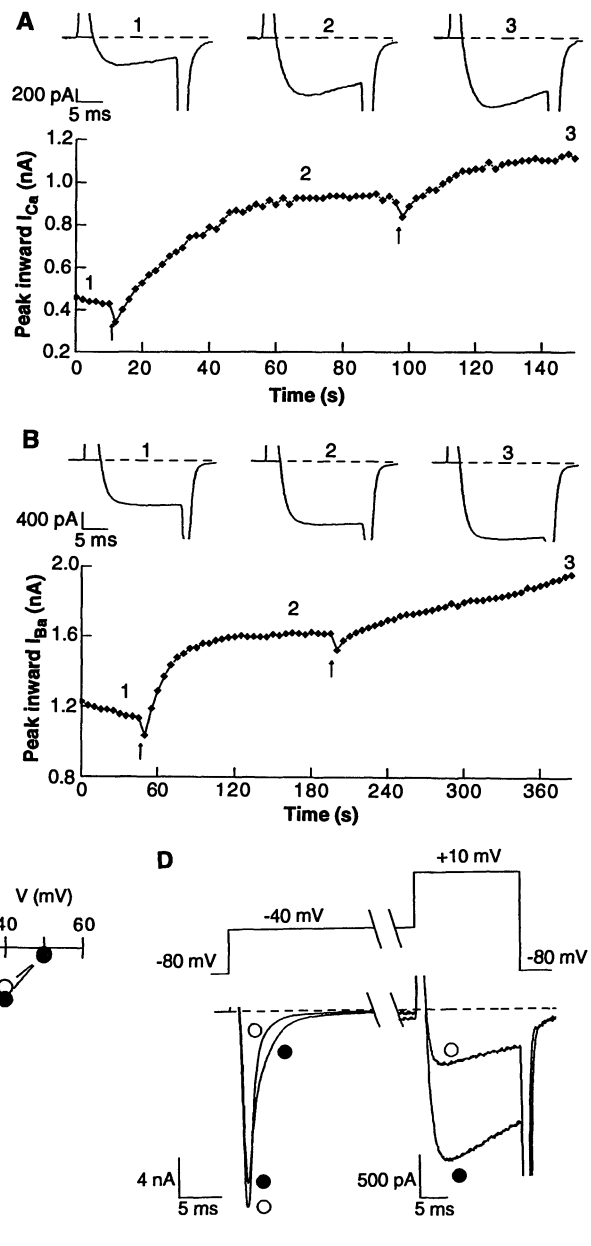


Fig. 1. Assessment of DM-nitrophen photolysis and Mg^{2+} release in cardiomyocytes. (**A**) Efficiency of photolysis of Ca^{2+}-loaded DM-nitrophen. Membrane depolarizations induced Ca^{2+} transients (brief upward deflections of the fluorescence signal) in a cell containing caged Ca^{2+} and fluo-3 (10 μM). Arrows indicate each exposure to a flash. (**B**) Ca^{2+} signal during depolarizing pulses [occurring at similar intervals as in (A)] or after flashes (arrows) in a cell containing caged Mg^{2+}, 10 mM BAPTA, and 10 μM fluo-3 {external $[Ca^{2+}]$ was 10 mM in (A) and (B)}. (**C**) Induction of rectification of background K^+ currents. Depolarizing pulses from −110 to +20 mV evoked a large outward K^+ current (○) with a slow time-dependent inactivation phase at 10°C. After a flash, outward current was largely eliminated (●), whereas inward current was only slightly reduced.

Fig. 2. Effects of Mg^{2+} release on I_{Ca} or I_{Ba}. (**A**) Transient reduction of I_{Ca} followed by a sustained increase in the Ca^{2+} current after flash photolysis of caged Mg^{2+} (lower panels; arrows indicate exposure to a flash). The kinetics of I_{Ca} were unaffected (upper panels; data points 1, 2, and 3 of lower time course; 2 mM external Ca^{2+}). (**B**) An experiment similar to (A) in which Ba^{2+} replaced Ca^{2+} as the charge carrier (results confirmed in six cells). (**C**) Current-voltage relation for I_{Ca} before (○) and after (●) photolysis of caged Mg^{2+}. (**D**) Specificity of the effect on I_{Ca}. Right side: current records of I_{Ca} (at +10 mV) before (○) and after (●) photolysis of caged Mg^{2+}; left side: I_{Na} in the preceding pulse to −40 mV (same symbol legends).



methylenediphosphate (AMP-PCP) or adenylylimidodiphosphate (AMP-PNP) (*15, 17*). Photolysis of caged Mg^{2+} still increased I_{Ca} when AMP-PCP or AMP-PNP substituted for ATP in the internal solutions (Fig. 4A). To verify that this was also the case when inhibition of endogenous ATP production was complete, we repeated the AMP-PNP experiment in cells exposed to 2-deoxyglucose (an inhibitor of glycolysis), P^1,P^5-di(adenosine-5′)pentaphosphate (an inhibitor of adenylate kinase) and dinitrophenol. Reduction of endogenous MgATP to concentrations of ~30 μM or less was indicated by progressive cell shortening, which results from the formation of rigor crossbridges (*18*). Nevertheless, these interventions did not attenuate the increase in I_{Ca} by Mg^{2+} release (Fig. 4A).

We obtained independent evidence against modification of Ca^{2+} channels by cAMP-dependent protein kinase by examining the responses to the β-adrenergic agonist isoproterenol and to Mg^{2+} release in the presence or absence of a specific peptide inhibitor of the kinase. Exposure of the cells to intracellular solution containing a 20–amino acid fragment of the cAMP-dependent protein kinase inhibitor protein (PKI_{5-24}) (*19*) completely abolished the β-adrenergic stimulation of I_{Ca} (1064 ± 239 pA pre-isoproterenol, 776 ± 147 pA with isoproterenol; $n = 6$; P = not significant) but did not prevent the increase in I_{Ca} elicited by Mg^{2+} release after washout of the β-adrenergic response (770 ± 69 pA preflash, 1885 ± 231 pA postflash; $n = 5$; $P < 0.001$) (Fig. 4B). Effects mediated by protein kinase C were also independently excluded by intracellular application of the pseudosubstrate peptide inhibitor PKC_{19-36}, which did not attenuate the response to photolysis of caged Mg^{2+} (*20*).

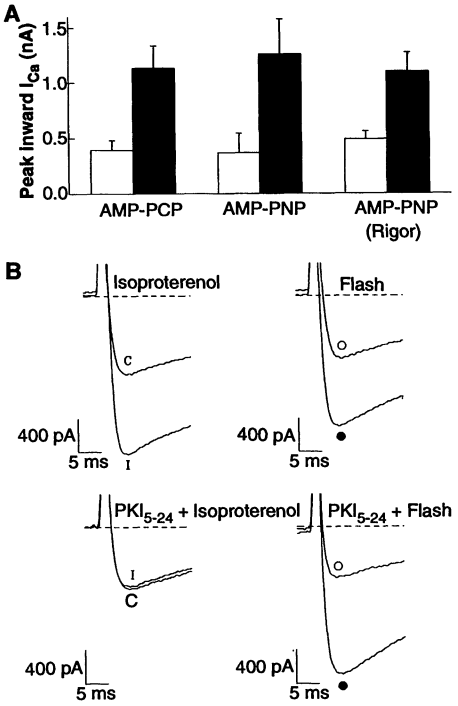


Fig. 4. Phosphorylation-independence of Mg-nucleotide effect on I_{Ca}. (**A**) Peak inward I_{Ca} before (open bars) and after (filled bars) Mg^{2+} release for cells equilibrated with intracellular solutions containing either AMP-PCP (left) or AMP-PNP (middle) as a substitute for ATP. Right: results of a similar experiment for cells in the rigor state induced by combined blockade of oxidative phosphorylation, glycolysis, and adenylate kinase. Bar heights and error limits represent the mean and standard error for at least four cells in each group. The increases in peak I_{Ca} were statistically significant with $P < 0.02$ for AMP-PCP, and $P < 0.05$ for the AMP-PNP and AMP-PNP (rigor) groups (paired t test). Pipette contained the following: 0.5 mM MgCl, 4 mM DM-nitrophen, 5 mM AMP-PCP (left) or 5 mM AMP-PNP (middle and right), and 200 μM dinitrophenol; 20 μM P^1,P^5-di(adenosine-5′)pentaphosphate was added in the rigor experiment (right). Bath was glucose-free and for rigor conditions contained 10 mM 2-deoxyglucose and 200 μM dinitrophenol. (**B**) Blockade of the β-adrenergic effect on I_{Ca} by 1 μM PKI_{5-24} (left panels: C, control; I, 100 nM isoproterenol). Lack of effect of PKI_{5-24} on the response to Mg^{2+} release (right panels; ○, before flash; ●, after flash). Current records in the upper panels were from a cell not exposed to PKI_{5-24}; records in the lower panels were from a different cell.

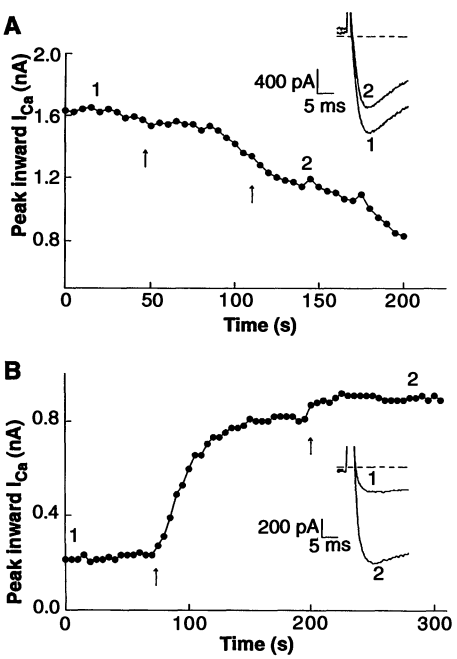


Fig. 3. Nucleotide-dependence of the effect of Mg^{2+} release on I_{Ca}. (**A**) Effects of photolysis of caged Mg^{2+} (arrows) on the amplitude of I_{Ca} when ATP was omitted from the internal solution and the cells were incubated in dinitrophenol (200 μM in internal and external solutions) (results confirmed in four cells). The continual decline in I_{Ca} resulted in a smaller calcium current after photolysis (inset; data point 2 of time course) compared to control (inset; data point 1 of time course). (**B**) Time course of the response to photolysis of caged ATP (arrows) (results confirmed in five experiments). ATP increased I_{Ca} without changing the kinetics of the current (inset: 1, control; 2, after flashes).

Recognition of this mechanism helps to explain a number of previous observations regarding the responsiveness of L-type calcium channels to changes in cellular energetics. The ability of calcium channels to sense MgATP may underlie the direct effects of glucose metabolism on I_{Ca} in pancreatic β cells (*21*) and the reduction of I_{Ca} during metabolic inhibition or ischemia in heart cells (*22*) (Fig. 3A). Direct modulation of L-type calcium channels must also be considered in relation to previous observations that ATP can retard "run-down" (*5*) or enhance I_{Ca} during intracellular perfusion (*23*), two phenomena previously assumed to be mediated by phosphorylation.

The effects on I_{Ca} that we observed were elicited only by an increase in Mg-nucleotide complexes and thus differ from the Mg^{2+}-independent effects of ATP on ATP-sensitive K^+ channels ($I_{K,ATP}$) (*2*) or on sarcoplasmic reticular Ca^{2+}-release channels (*3*). The increase in I_{Ca} did not require transfer of the γ phosphate of the nucleotide, excluding a mechanism involving phosphorylation. Phosphorylation-independent activation of ion channels by Mg-nucleotide complexes does have precedents: Both $I_{K,ATP}$ and the cystic fibrosis transmembrane conductance regulator (CFTR) are subject to this type of regulation. Mg-nucleoside diphosphates activate $I_{K,ATP}$ channels after loss of channel activity in cell-free patches (*2, 24*). Mg-nucleotide complexes activate the CFTR channel without inducing phosphorylation, but the effect requires prior phosphorylation of the protein and is not supported by nonhydrolyzable ATP analogs (*25*). Direct ion channel modulation by Mg-nucleotide complexes may therefore represent a widespread form of biological regulation. Elucidation of this mechanism, coupled with findings indicating that high concentrations of Mg^{2+} have the opposite effect on I_{Ca} (*4*), implies that L-type calcium channel activity may be altered in vivo by deviations away from optimal concentrations of either Mg^{2+} or Mg-nucleotide complexes. Calcium channel function, like metabolism and growth, can thus be counted among the many cellular processes sensitive to the balance between magnesium in the free state and in a complex with nucleotides.

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9. We monitored $[Ca^{2+}]_i$ with the long-wavelength fluorescent Ca^{2+} indicator fluo-3 [A. Minta, J. P. Y. Kao, R. Y. Tsien, *J. Biol. Chem.* **264**, 8171 (1989)] to minimize photobleaching of the indicator during flash photolysis and to prevent continual degradation of the caged compounds by ultraviolet fluorescence excitation. Enzymatically dissociated guinea pig ventricular myocytes were equilibrated with intracellular solutions through patch-clamp pipettes (1- to 4-megohm tip resistance; 2.5- to 5-megohm series resistance) in the whole-cell configuration. Current recording, flash photolysis, and fluorescence measurement were done as described [P. H. Backx, B. O'Rourke, E. Marban, *Am. J. Hypertension* (*suppl.*) **4**, 416 (1991)]. Caged Ca^{2+} internal solution contained the following: 150 mM *N*-methyl glucamine (NMG), 10 mM Hepes, 1 mM glutathione, 1 mM ATP (sodium salt), 0.1 mM $CaCl_2$, 0.2 mM DM-nitrophen (sodium salt), 10 μM fluo-3 (ammonium salt), and 10 mM butanedione monoxime (pH 7.2 with CH_4O_3S). Caged Mg^{2+} pipette solutions contained 100 mM NMG, 50 mM Hepes, 1 mM glutathione, 10 mM BAPTA (cesium salt), 0.2 to 1 mM MgATP, and 4 to 6 mM DM-nitrophen (sodium salt) (pH 7.2 with CH_4O_3S); fluo-3 was included when $[Ca^{2+}]_i$ was measured. Photolysis-induced changes in $[Mg^{2+}]_i$ and $[MgATP^{2-}]_i$ were estimated with an iterative computer program with the approach of A. Fabiato and F. Fabiato [*J. Physiol.* (*Paris*) **75**, 463 (1979)], assuming 100% lysis after several flashes. For a 0.5 mM MgATP, 4 mM DM-nitrophen internal solution, the flash-induced increases in $[Mg^{2+}]_i$ and $[MgATP^{2-}]_i$ were 0.12 to 47 μM and 0.63 to 111 μM, respectively. External solutions contained 140 mM NaCl, 5 mM CsCl, 10 mM Hepes, 10 mM glucose, 1 mM $MgCl_2$ with Ca^{2+} or Ba^{2+} as the charge carrier where indicated. Na^+ current was inactivated by a 60-ms pulse from the holding potential (−80 to −40 mV) before a test pulse to 0 or +10 mV (test-pulse duration was 20 ms to limit Ca^{2+} entry into the cell). Frequency of stimulation was 0.2 Hz. In some experiments, Na^+ and Cl^- were replaced by NMG and CH_4O_3S, respectively. Unlike cAMP-dependent regulation of I_{Ca} [R. D. Harvey, J. A. Jurevičius, J. R. Hume, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 6946 (1991)], the effects of Mg^{2+} release on I_{Ca} were not influenced by Na^+ removal.
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Molecular Localization of an Ion-Binding Site Within the Pore of Mammalian Sodium Channels

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Sodium channels are the major proteins that underlie excitability in nerve, heart, and skeletal muscle. Chemical reaction rate theory was used to analyze the blockage of single wild-type and mutant sodium channels by cadmium ions. The affinity of cadmium for the native tetrodotoxin (TTX)-resistant cardiac channel was much higher than its affinity for the TTX-sensitive skeletal muscle isoform of the channel (μI). Mutation of Tyr^{401} to Cys, the corresponding residue in the cardiac sequence, rendered μI highly susceptible to cadmium blockage but resistant to TTX. The binding site was localized approximately 20% of the distance down the electrical field, thus defining the position of a critical residue within the sodium channel pore.

Sodium channels support the flux of millions of Na^+ ions per second while selecting against the many other ions that are present inside and outside cells (*1*). To understand how the pores of Na^+ channels function at the molecular level, one must define which residues line the permeation pathway and how those residues interact with the ions that cross the membrane. Analysis of the protein sequences deduced from cDNAs led to the prediction that Na^+ channels consist of four major symmetrical domains that assemble to form a central pore (*2*, *3*). In K^+ channels, which are encoded as monomers homologous to each of the four Na^+ channel domains (*4*), the loop linking the fifth and sixth transmembrane segments determines ionic selectivity (*5*) and contains the sites that bind K^+ channel–specific drugs and toxins that block the ionic current (*6*). Neutralization of negatively charged amino acids in the analogous segment of the mammalian brain Na^+ channel, known as the SS1-SS2 region, results in decreased sensitivity to TTX and saxitoxin (STX), altered cation selectivity, and decreased single-channel conductance (*7*). Other experiments suggest the presence of overlapping TTX and STX and divalent ion binding sites that are sensitive to modification by sulfhydryl alkylating agents (*8*). To investigate whether the SS1-SS2 region forms the Na^+ channel pore, we first characterized ion permeation for the cardiac and skeletal muscle isoforms of the Na^+ channel. We then mutated a residue in this region that is different in the two isoforms and determined whether it conferred phenotypic idiosyncracies in ion transport and TTX sensitivity.

Both Cd^{2+} and Zn^{2+} block cardiac Na^+ channels much more potently than they block Na^+ channels from nerve or skeletal muscle (*8*, *9*). Cd^{2+} blockage of the μI

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