Negative Surface Charge Density Near Heart Calcium Channels

Relevance to Block by Dihydropyridines

R. S. KASS and D. S. KRAFTE

From the Department of Physiology, University of Rochester School of Medicine and Dentistry, Rochester, New York 14642

ABSTRACT We have measured the density of negative surface charges near the voltage sensor for inactivation gating of (L-type) Ca channels in intact calf Purkinje fibers and in isolated myocytes from guinea pig and rat ventricles. Divalent cation-induced changes in the half-maximal voltage for inactivation were determined and were well described by curves predicted by surface potential theory. We measured shifts in inactivation induced by Ca, Sr, and Ba in the single cells, and by Sr in the Purkinje fibers. All of the data were consistent with an estimated negative surface charge density of 1 electronic charge per 250 Å². In addition, the data suggest that Ca, but neither Ba nor Sr, binds to the negative charges with an association constant on the order of 1 M⁻¹. We find that divalent ion-induced changes in surface potential can account for most of the antagonism between these ions and Ca channel block by 1,4-dihydropyridines.

INTRODUCTION

It is well known that an increase in the divalent ion concentration causes the relationship between voltage-dependent membrane conductances and voltage to change. Since the work of Frankenhaeuser and Hodgkin (1957), most of these effects have been explained by changes in membrane surface potential caused by the electrostatic attraction of cations to fixed negative charges on the cell membrane. The negative surface potential can be reduced by counterions in the external solution that form a diffuse double layer at the surface, or by the binding of cations to the fixed charges. Surface potential theory has been tested in nerve, muscle, and bilayers by several groups (see Chandler et al., 1965; Blaustein and Goldman, 1968; Gilbert and Ehrenstein, 1969; McLaughlin et al., 1971; Begenisich, 1975; Hille et al., 1975; Hille, 1984), but has not been systematically addressed in heart cells.

The purpose of this study was to estimate the density of negative surface charges on the membranes of isolated ventricular cells from rat and guinea pig.
hearts and of intact calf Purkinje fibers. The study was motivated by several goals. The first was to provide data to compare properties of heart Ca channels with Ca, Na, and K channels in other cells. We were also interested in determining whether differences could be observed between enzymatically dissociated and intact preparations.

A second aim of this study was to provide information that would allow estimates of the importance of different types of Ca channels in cardiac cells exposed to physiological solutions. Many experiments designed to provide evidence for different types of Ca channels in heart (Bean, 1985; Nilius et al., 1985) and other tissues (Nowycky et al., 1985) have been carried out in the presence of very high concentrations of divalent ions. In order to estimate the physiological relevance of these channel types, estimates of the changes in surface potential caused by the different divalent ion concentrations are needed. In addition, our data allow the comparison of single channel properties of L-type Ca channels that were determined under conditions of high extracellular divalent ion concentrations (for example, Hess et al., 1984) with macroscopic data on these channels obtained under more physiological conditions.

The final goal of this study was to examine the relationship between block of Ca channels by dihydropyridine Ca channel blockers and changes in the extracellular divalent cation concentration. Initial investigations of these drugs showed that elevation of extracellular Ca could reverse the drugs' negative inotropic effects in cardiac and smooth muscle (for review, see Fleckenstein, 1977). As a result of this apparent competition, the drugs were referred to as Ca antagonists. Recently, Lee and Tsien (1983) have found that Ba is more effective than Ca in antagonizing the blocking activity of nitrendipine and other organic blockers. Other experiments have shown that inhibition of Ca channel currents by 1,4-dihydropyridines is steeply voltage dependent and have suggested that these drugs bind preferentially to channels in the inactivated state (Bean, 1984; Sanguinetti and Kass, 1984; Uehara and Hume, 1985) or favor a silent mode of channel gating (Hess et al., 1984). We proposed to test the possibility that the apparent relief of Ca channel block by elevated divalent ion concentrations is due, at least in part, to surface potential–mediated changes in channel gating, and that the contrast between the actions of Ba and Ca is due to the relative effectiveness of these cations to shift gating. Our data show that changes in surface potential account for much of the apparent antagonism between divalent ions and these drugs, but, by themselves, cannot account for the observations of Lee and Tsien (1983).

METHODS

Preparations, Solutions, and Recording Techniques

These experiments were carried out on isolated calf Purkinje fibers and on single myocytes obtained from adult guinea pig and rat ventricles by an enzymatic dissociation procedure modified from Powell and Twist (1976). Membrane currents were measured with a conventional two-microelectrode arrangement in the Purkinje fibers and with a patch electrode in a whole-cell configuration (Hamill et al., 1981) in the single-cell experiments.
For the Purkinje fiber experiments, the standard modified Tyrode's solution had the following composition (mM): 150 NaCl, 4 KCl, 5 glucose, and 10 Tris, pH 7.4. Experiments were carried out at 37°C and solutions were gassed with 100% O₂. Outward currents were blocked by injection of tetrabutylammonium ion (TBA) (Kass et al., 1982), and Na channel currents were blocked by tetrodotoxin (TTX) (10 µM).

Single-cell experiments were carried out at 20°C and the standard external solution consisted of (mM): 132 NaCl, 4.8 KCl, 10 HEPES, pH 7.3, and 5 glucose. Na channel currents were blocked by TTX (10⁻⁵ to 10⁻⁶ M). The patch pipette solutions consisted of (mM): 140 KCl, 2 MgCl₂, 11 EGTA, 1 CaCl₂, 5 TBA, and 10 HEPES, pH 7.3. In some experiments, CsCl replaced KCl in the patch electrode and TBA was not used. In each single-cell experiment, pipette capacitance was neutralized after forming a seal, and series resistance was compensated to provide the fastest possible capacity transient without ringing.

The divalent ion compositions of solutions for both the Purkinje fiber and single-cell experiments are specified in the figure captions.

Inactivation of (L-type) Ca channels in heart cells has been described as being both Ca and voltage dependent (Kass and Sanguinetti, 1984; Lee et al., 1985). In the Purkinje fiber experiments, we measured Ca channel currents with Sr as the charge carrier because it promotes large currents (Hess and Tsien, 1984), but not a Ca-dependent inactivation mechanism. In the single-cell experiments, we could buffer intracellular Ca by adding EGTA to the patch pipette. This minimized the contribution of a Ca-dependent mechanism (Josephson et al., 1984) and thus permitted measurement of voltage-dependent inactivation of Ca channel currents with Ca, Ba, and Sr as the charge carrier.

Theory and Curve-fitting Procedures

The density of fixed negative surface charges on cell membranes is related to surface potential via the Grahame equation (for derivation, see Grahame, 1947, or McLaughlin et al., 1971):

\[ \sigma = \frac{1}{G} \left( \sum_{i=1}^{n} C_i \exp[-z_i F \psi(0)/RT] - 1 \right)^{1/2}, \]  

(1)

where \( \sigma \) is the surface charge density in electronic charges per square angstrom, \( G \) is a constant and has a value of 270 (Å²/charge) (mole/liter)⁻¹, \( C_i \) is the concentration of the \( i \)th ion species in the bulk solution in moles per liter, \( z_i \) is its valence, \( RT/F = 25.3 \) mV at 22°C, and \( \psi(0) \) is the surface potential. If divalent ions bind to negative sites on the membrane with an association constant \( K \) (in units per molar), the apparent surface charge density is related to true surface charge density as follows:

\[ \sigma_a = \sigma /[1 + K \cdot C_i \exp[-2F \psi(0)/RT]]. \]  

(2)

We used a standard Newton-Raphson iteration procedure (Dorn and McCracken, 1972) to solve Eq. 1 for \( \psi(0) \) with appropriate ionic compositions of the solutions, binding constant, and surface charge density (\( \sigma \)).

A Boltzmann relation, \( [1 + \exp(V - V_{1/2})/k]^{-1} \), where \( V \) is membrane potential and \( V_{1/2} \) and \( k \) are constants, was used to describe Ca channel availability. Theoretical curves were fitted to experimental data using an algorithm of Marquardt (1963) as described by Bevington (1969). This procedure, which fits an arbitrary nonlinear function to the data, was thus used to determine \( V_{1/2} \) and \( k \) for each curve.
Pulse Protocols

Ca channel availability was determined by measuring current during test pulses to voltages near the peak of the Ca channel current-voltage relation after applying prepulses to several conditioning potentials. Prepulse durations were chosen for particular experiments and are so noted. Test pulses were separated from prepulses by a 10-ms return to the holding potential. Curves were normalized to currents measured after the most negative prepulses.

RESULTS

Divalent Ions Shift Inactivation in Single Cells and Purkinje Fibers

Influence of Ca, on single cell inactivation. Fig. 1 illustrates the effects of variations in the extracellular Ca concentration on the 500-ms inactivation curve in the isolated ventricular cell. Normalized inactivation curves were determined in three different Ca-containing solutions. The smooth curves are the best fits to the data of Boltzmann functions for steady state inactivation (see Methods). As expected if the surface potential is affected, $V_{1/2}$ becomes progressively more positive with increasing Ca, concentrations. We obtained similar results in a total of six ventricular cells over a concentration range of 2–25 mM Ca,.

Influence of Sr, on Purkinje fiber inactivation. We carried out similar experiments in intact Purkinje fiber cell bundles, but, in this case (as explained in the Methods), we replaced extracellular Ca with Sr. We found that Sr also shifted the inactivation curve for Ca channel currents in the positive direction, but the magnitude of this effect appeared to be smaller than that caused by a comparable Ca concentration in the single-cell experiments. This can be seen in Fig. 2, which
shows normalized inactivation curves measured in three concentrations of extracellular Sr in a Purkinje fiber. These results and others obtained in different Sr concentrations are summarized in Fig. 6.

**Relative effectiveness of Ca, Sr, and Ba.** To determine whether the possible difference in the effects of Sr and Ca was related to the type of preparation used, we also carried out experiments in single cells to determine the influence of extracellular Sr on the voltage dependence of gating in this preparation. Again, we were able to measure shifts in inactivation in different Sr concentrations (Fig. 3), and the magnitude of this effect resembled that observed in the Purkinje fiber.

Fig. 4 shows that there is clearly a difference between the influence of Ca and Sr on the voltage dependence of inactivation. This figure shows inactivation curves obtained in the same cell in the presence of comparable concentrations of Ca and Sr ions. Changing the Ca concentration from 1 to 8 mM caused a 12-mV positive shift in the midpoint of the inactivation curve, whereas changing to a solution containing 8 mM Sr caused the inactivation curve to shift only 4 mV. As shown in the figure, a return to the control solution resulted in very good recovery from the high divalent ion conditions. This shows that Ca ions are more effective than Sr ions at shifting the voltage dependence of inactivation in these cells.

We also found that the effects of Ba on the relationship between inactivation and voltage in single cells are similar to those of Sr (see Fig. 6). We discuss the interpretation of these results below.
In each of the experiments illustrated so far, the change in the divalent cation concentration in the external solution caused increases in current amplitude as well as positive shifts in channel availability vs. voltage. To test for the possibility that the shifts we observed were somehow related to these changes in current...
amplitude, we also carried out experiments in which we fixed the extracellular concentration of a permeant cation (Sr) and varied the levels of extracellular Mg, a cation that blocks Ca channels (Almers and McCleskey, 1984; Hess and Tsien, 1984). As shown in Fig. 5, raising the extracellular Mg concentration also caused depolarizing shifts in inactivation, despite the fact that currents were reduced in solutions containing elevated Mg.

**Surface Potential Theory: Surface Charge Density and Binding Constants That Are Consistent with the Data**

We summarize our experiments with Ca, Ba, and Sr ions in both single cells and Purkinje fibers in Figs. 6 and 7. Here we plot divalent ion-induced shifts in inactivation (relative to control solutions) vs. the concentration of the test cation.

![Normalized current vs. pre (mV)](image)

**FIGURE 5.** Mg, shifts inactivation in the Purkinje fiber. Normalized 500-ms inactivation curves were determined in solutions containing 0.5 (○), 2.5 (●), and 5.5 (●) mM Mg. The values for $V_\text{in}$ that described the smooth curves in each solution were −32, −50, and −22 mV. Sr, was maintained at 2 mM throughout the experiment and Ca, was nominally 0 mM. Preparation 583-1.

The results for single cells and Purkinje fibers are plotted separately, as are the results for Ca, Ba, and Sr ions. The curves are the solutions to the Grahame equation (Eq. 1) for a negative surface charge density of −1 e/250 Å² with no binding (solid curve in both Figs. 6 and 7) and for the same charge density, but with a binding constant of 1 M⁻¹ (dashed curve). Thus, all of our experimental results are consistent with changes in surface potential associated with divalent ion-induced screening of the same negative charge. In addition, the theory suggests that Ca ions, but neither Ba nor Sr ions, bind to and neutralize some of these negative charges, thereby causing larger changes in surface potential and, in turn, larger shifts in voltage-dependent gating.
FIGURE 6. Shift in $V_{in}$ as a function of external divalent ion concentration in single cells. Shifts in inactivation were determined relative to solutions containing 1 mM $Ca_0$ and plotted against test cation concentration. Results shown are for solutions in which $Ca_0$ (●), $Ba_0$ (△), and $Sr_0$ (□) were varied. Data are from a total of 15 cells and are shown ± SEM. The curves are the changes in surface potential computed with Eqs. 1 and 2 using a surface charge density of $-e/250$ Å$^2$ and no binding (solid curve), and with the same charge density but with a binding constant of 1 M$^{-1}$ (dashed curve). The surface potential computed for 1 mM $Ca_0$ using Eqs. 1 and 2 was $-53$ mV.

FIGURE 7. Shift in $V_{in}$ as a function of $Sr_0$ in the Purkinje fiber. Data were obtained in 12 fibers and are shown ± SEM. The smooth curve is the solution to the Grahame equation with parameters as described for the solid curve in Fig. 6.
Influence of Divalent Ions in the Presence of Nisoldipine: Apparent Relief of Block

We next tested for shifts in the voltage dependence of inactivation in the presence of the dihydropyridine nisoldipine, a well-described Ca channel blocker. We find that the presence of nisoldipine (200 nM) does not affect the shift in inactivation caused by the addition of divalent ions when the inactivation curve is determined by 500-ms prepulses.

When long (10–30 s) prepulses are used to determine the inactivation curve, divalent cations also shift the relationship in the depolarizing direction, as illustrated in Fig. 8. In this figure, 30-s prepulses were used to measure the inactivation curve, because under these conditions, nisoldipine causes inactivation to occur at voltages ~20–25 mV more negative than under drug-free conditions and thus produces an apparent block of Ca channel currents (Sanguinetti and Kass, 1984). In the present experiment, although nisoldipine was present at a fixed concentration, the elevation of Sr, caused a 6-mV depolarizing shift in inactivation and returned this relationship toward voltages measured under drug-free conditions. We obtained similar effects of divalent ions on inactivation in the presence of nisoldipine in eight other experiments. In each case, the divalent ion–induced shift in inactivation was similar to that predicted by the drug-free data.

A divalent cation–induced depolarizing shift in inactivation will result in an apparent relief of nisoldipine block of Ca channel currents. This effect will be most pronounced if currents are measured from holding potentials near −50

![Figure 8](image-url)
mV, because this region of the inactivation curve is steeply voltage dependent. Fig. 9 shows that nisoldipine block of currents measured from this holding potential is in fact very sensitive to the divalent ion concentration in the extracellular solution. In this experiment on a Purkinje fiber preparation, both extracellular Sr and Mg were elevated to keep the magnitudes of the currents in the two drug-free solutions approximately the same. It is clear that in the low

![Graph showing influence of extracellular divalent cations on inhibition of Ca channel currents by nisoldipine.](image)

**Figure 9.** Influence of extracellular divalent cations on inhibition of Ca channel currents by nisoldipine. Membrane currents were recorded from a -50-mV holding potential in the absence (○) and presence (□) of nisoldipine (200 nM). Peak inward currents were measured and plotted against test voltage. (A) External divalent ions: 3 mM Sr, 0.5 mM Mg. Inset: currents in response to voltage pulses near -5 mV. (B) External divalent cation concentrations: 10 mM Sr, 10.5 mM Mg. Inset: currents in response to voltage pulses near +10 mV. Preparation 380-1.
divalent cation solution, the addition of a moderate amount of nisoldipine (200 nM) completely inhibited Ca channel currents. The same drug concentration is much less potent in the solution containing a high concentration of divalent cations.

In Fig. 10, we simulated the effects of changes in surface potential on nisoldipine block of Ca channel currents by computing currents measured from a −45-mV holding potential under conditions when steady state inactivation was affected by nisoldipine and by the divalent cation concentration. We simulated steady state inactivation by using a Boltzmann relationship with $V_\infty = -30$ mV and $k = 4$ mV for drug-free conditions in 1 mM Ca (see Sanguinetti and Kass, 1984). The elevation of the divalent ion concentration was simulated by making $V_\infty$ 18 mV more positive, and the effect of nisoldipine was simulated by making it 25 mV more negative. The result, very similar to the experimental observation of Fig. 9, indicates that the change in the voltage dependence of inactivation caused by external divalent cations can account for most of the apparent antagonism of nisoldipine block of Ca channel currents.

**DISCUSSION**

**Negative Surface Charge Density Near Heart Ca Channels**

We found that the relationship between inactivation and the membrane potential of heart Ca channels was shifted by external divalent ions as it is for channels in other cells. This is the first quantitative investigation of the effects of divalent 

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**Figure 10.** Simulation of current-voltage relation for peak inward Ca channel current measured in response to test pulses applied from a −45-mV holding potential. Panels A and B illustrate simulations of low and high extracellular divalent ion concentrations, respectively. The effects of the drug were simulated by shifting inactivation by −25 mV (see Sanguinetti and Kass, 1984), and the effects of the divalent ion concentration were determined by shifting the voltage-dependent parameters for inactivation and activation as described for Fig. 7. The maximal conductance was reduced slightly in B to simulate the addition of Mg in Fig. 9. The scaling on ordinate is arbitrary, but is consistent in panels A and B.
ions on this relationship in mammalian heart cells and it is the first time data from enzymatically dispersed myocytes have been compared with data from intact multicellular preparations in the same type of experiments.

We analyzed our results in terms of changes in surface potential as predicted by screening of and/or binding to negative charges fixed on the cell membrane in the vicinity of the Ca channel. Our data were consistent with the solutions to the Grahame equation for a fixed negative charge density of $-1 \text{e/250} \text{Å}^2$, which is somewhat lower than the charge density of approximately $-1 \text{e/100} \text{Å}^2$ reported near Ca channels in other excitable cells (Ohmori and Yoshii, 1977; Kostyuk et al., 1982; Wilson et al., 1983).

Our estimate of surface charge density can also be compared with reports by other investigators for charges near Na and K channels. Begenisich (1975, Table IV) has summarized these data for nerve and muscle preparations and has suggested that there may be a difference between Na and K channel surface charge densities. The channel surface charge density near Na channels is on the order of $-1 \text{e/140} \text{Å}^2$ and that near K channels is close to $-1 \text{e/275} \text{Å}^2$. As has been described previously (see Begenisich, 1975; Hille et al., 1975; Wilson et al., 1983), it is possible to obtain reasonable agreement between theoretical and experimental data using a range of charge densities and the binding constants in the Grahame equation. We suggest, therefore, that our numbers for surface charge density and binding constants are estimates and, as such, are consistent with the data for Na and K channels.

We find that $\text{Ca}^{2+}$ is much more effective at shifting the voltage dependence of inactivation than either Ba or Sr, a result consistent with binding of Ca, but neither of these other divalent ions, to the fixed negative charges on the membrane surface. Hille et al. (1975) came to similar conclusions in their studies of the influence of these three divalent ions on Na channel activation in frog node of Ranvier, as did Wilson et al. (1983) in studies of neuronal Ca channel currents.

Single Cell and Purkinje Fiber Results Are in Agreement

We were particularly interested in comparing the results obtained from Purkinje fibers using the two-microelectrode technique with those obtained in ventricular myocytes with a whole-cell patch recording procedure. As shown in Figs. 6 and 7, the data from both preparations are well described by the same theoretical curve. It should be noted that we were able to obtain results only in Sr-containing solutions for the Purkinje fiber experiments because these preparations do not tolerate high levels of extracellular Ba (see Siegelbaum and Tsien, 1980) or Ca (see Kass and Tsien, 1982). On the other hand, Purkinje fibers are very stable preparations and, as such, are very useful in studies that might require long-duration pulse protocols. Thus, this preparation was very useful in studies of the dihydropyridine derivatives, because very long prepulses are needed to measure the steady state effects of these drugs (see Bean, 1984; Sanguinetti and Kass, 1984).

Relevance to Different Ca Channel Types in the Heart

Recent reports have provided evidence for the existence of more than one kind of Ca channel in heart cells (Nilius et al., 1985; Bean, 1985). One of the criteria
for distinguishing channel types is the voltage dependence of inactivation. One channel (T-type) inactivates rapidly and completely at relatively negative potentials. A second channel (L-type) inactivates more slowly and at more positive voltages. In both of these important studies, voltage ranges for inactivation were reported for Ba currents in experiments in which the external Ba concentration was very high (115 mM). Thus, the voltages reported for inactivation were considerably more positive than would be the case for more physiological solutions.

Using the values for surface charge density obtained in the present study in Eq. 1, we find that 115 mM Ba in the external solution will depolarize the surface potential, and thus shift gating, by 26 mV. Bean (1985) found that the average midpoint of the inactivation curve for T-type channels in canine atrial cells was near −45 mV in 115 mM Ba. According to our results, in more physiological solutions that contain external divalent ion concentrations on the order of 1–5 mM, the midpoint of the inactivation curves will be closer to −70 mV, a value similar to the midpoint of Na channel inactivation in ventricular cells (Brown et al., 1981). Thus, under these conditions, at voltages more positive than −60 mV, almost all of these channels are unavailable for conduction.

Are Dihydropyridines Ca Antagonists?

After determining the negative surface charge density, we were in a position to investigate the nature of the apparent antagonism between divalent ions and Ca channel block by 1,4-dihydropyridines. Our results (Fig. 10) show that surface potential–induced shifts in channel gating can account for most of the apparent antagonism between divalent cations and 1,4-dihydropyridines. Divalent ions restore the voltage dependence of the drug-modified inactivation curve toward drug-free conditions by causing depolarizing shifts of this relationship. A similar mechanism has been proposed to explain the effects of Ca, on Na channel block by local anesthetics (Hille, 1978).

On the other hand, our results cannot explain the observation of Lee and Tsien (1983) that Ca currents were more sensitive to dihydropyridine block than Ba currents. If all of the antagonism between these drugs and divalent ions were due to surface potential–mediated changes in inactivation, then for equal concentrations, Ca, which binds to negative charges, should be more potent at reversing the effects of the 1,4-dihydropyridines than either Ba or Sr, which do not appear to bind to the surface charges. The experimental observation was just the opposite: nitrendipine was more effective at blocking Ca currents than Ba currents. Thus, we cannot rule out some form of competition between divalent ions and the 1,4-dihydropyridines in addition to the effects caused by changes in surface potentials.

Summary

We have obtained estimates of negative surface charge density in both enzymatically dispersed ventricular cells and calf Purkinje fibers. We found the same charge density in the two preparations, even though there is a species difference. In fact, the values we obtained are quite similar to those found for channels in non-mammalian preparations. Very little previous work has been done on mammalian preparations, and, to our knowledge, there has been none in heart,
although the effects of $Ca_o$ on voltage-dependent processes in cardiac tissue have
been known for some time (Weidmann, 1955).

Our results are important to the understanding of the apparent competitive
actions of divalent ions and 1,4-dihydropyridines (Janis and Triggle, 1984) as
well as to the physiological roles of different types of cardiac Ca channels.
Furthermore, the similarity in surface charge densities for all of these channel
types in such a wide variety of preparations suggests a certain homogeneity in
the architecture of these structures that might be revealed as we learn more
about their molecular constituents.

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