Interactions between H^+ and Ca^{2+} Near Cardiac L-Type Calcium Channels: Evidence for Independent Channel-associated Binding Sites

Yiu Wa Kwan and Robert S. Kass
Department of Physiology, University of Rochester School of Medicine and Dentistry, Rochester, New York 14642 USA

ABSTRACT Monovalent and divalent ions are known to affect voltage-gated ion channels by the screening of, and/or binding to, negative charges located on the surface of cell membranes within the vicinity of the channel protein. In this investigation, we studied gating shifts of cardiac L-type calcium channels induced by extracellular H^+ and Ca^{2+} to determine whether these cations interact at independent or competitive binding sites. At constant pH_o (7.4), Ca_o-induced gating shifts begin to approach a maximum value ($\cong$17 mV) at concentrations of extracellular calcium of $\geq$40 mM. A fraction of the calcium-dependent gating shift could be titrated with an effective $pK_a = 6.9$ indicating common and competitive access to H^+ and Ca^{2+} ions for at least one binding site. However, if pH_o is lowered when Ca_o is $\geq$40 mM, additional shifts in gating are measured, suggesting a subpopulation of sites to which Ca^{2+} and H^+ bind independently. The interdependence of L-channel gating shifts and Ca_o and pH_o was well described by the predictions of surface potential theory in which two sets of binding sites are postulated; site 1 ($pK_a = 5.5$) is accessible only to H^+ ions and site 2 ($pK_a = 6.9$) is accessible to both Ca^{2+} and H^+ ions. Theoretical computations generated with this model are consistent with previously determined data, in which interactions between these two cations were not studied, in addition to the present experiments in which interactions were systematically probed.

INTRODUCTION

Divalent and monovalent ions can alter ion channel gating by screening of and/or binding to negative charges located on membrane surfaces in the vicinity of channel pores in a manner that is well described by Gouy-Chapman diffuse double layer theory (Begenisich, 1975; Hille, 1968; Gilbert and Ehrenstein, 1969; Hille et al., 1975; Hille, 1984; Woodhull and Hille, 1970; McLaughlin, 1977). The information gained from studying surface potential-induced changes in channel function is useful in providing a biophysical profile of the ion channel and its environment in the membrane phospholipid. Previous investigations have demonstrated that H^+ and Ca^{2+} can, by themselves, cause changes in L-type channel gating in a manner consistent with the predictions of surface potential theory (Kass and Krafte, 1987; Krafte and Kass, 1988; Wilson et al., 1983; Ohmori and Yoshii, 1977; Irisawa and Sato, 1986; Iijima et al., 1986), but these studies did not test systematically for interactions between these cations.

Possible interactions between H^+ and Ca^{2+} in the regulation of calcium entry into heart cells have been suggested by Langer (1985) and Langer et al. (1989) in studies of the effects of extracellular acidosis on the contraction of isolated rabbit ventricular cells. This possibility is particularly interesting for L-type calcium channels in the heart because of the multiple roles of H^+ and Ca^{2+} in the regulation of channel function. In addition to causing shifts in channel gating, binding of protons to an extracellular site on the L-type calcium channel protein has been postulated to promote transitions of the channel between two conducting states when ions other than calcium permeate the channel (Prod'hom et al., 1989; Pietrobon and Hess, 1990) and to cause changes in channel permeability (Konnerth et al., 1987). Binding of calcium also has important regulatory roles in the permeability (Lansman et al., 1986; Hess and Tsien, 1984; Tsien et al., 1987; Almers and McCleskey, 1984) and gating (Kass and Sanguinetti, 1984; Yue et al., 1990) properties of native L-type channels, and divalent ion binding has been postulated to induce protein conformational changes that, in turn, could affect the binding of other ions (notably H^+) (Pietrobon and Hess, 1990).

The regulation of calcium entry by dihydropyridine (DHP) calcium channel blockers is also markedly influenced by divalent ion and proton binding. Positive allosteric regulators of DHP binding increase the calcium affinity of the L-type channel and alter DHP receptor kinetics (Triggle, 1991). H^+ and Ca^{2+} modulate DHP and phenylalkalamine binding to the purified skeletal muscle α_1 subunit, and Ca^{2+} stabilizes the drug-bound DHP receptor complex (Schneider, 1991) with evidence for contributing extracellular (Ebata et al., 1990) or cytoplasmic (Babitch, 1990) regulatory calcium sites. Because recent experimental evidence based on electrophysiology, photoaffinity radioligand binding studies, and anti-peptide antibody mapping suggests an extracellularly accessible DHP binding domain on the L-channel α_1 subunit, allosteric interactions between the binding of extracellular protons and calcium and the DHP binding site are an attractive possibility (Kass et al., 1991; Nakayama et al., 1991; Catterall and Striessnig, 1992; Striessnig et al., 1991).

The purpose of this study was to test for interactions between the binding of H^+ and Ca^{2+} to sites on the extracellular membrane near L-type calcium channels of cardiac ventricular cells by investigating the interrelationship between H^+ and Ca^{2+}-induced shifts in channel gating. We sought to determine whether H^+ and Ca^{2+} act at independent or competitive sites and whether or not it is possible to estimate the

Received for publication 12 March 1993 and in final form 4 June 1993.
Address reprint requests to Dr. Robert S. Kass.

0006-3495/93/09/1188/08 $2.00

pK_a of those sites to which Ca^{2+} bind. These data will be useful in comparing the properties of native and recombinant L-type channel activity as the structural basis for function and roles of the channel environment in the cell membrane are unraveled.

MATERIALS AND METHODS

Single myocytes were isolated from either ventricle of male or female guinea pigs using an enzymatic isolation procedure as described by Mitra and Morad (1985) and modified by Kass and Arena (1989). Electrodes (resistance of 1–2 MΩ) were fabricated from Gold Seal Accu-fill micropipettes (Clay Adams, Inc., Parsippany, NJ). Calcium channel currents were measured at room temperature (20–22°C) with the whole cell arrangement of the patch clamp technique (Hamill et al., 1981) using a Yale Mark IV patch clamp amplifier and headstage. Cells were transferred to a 0.5-ml recording chamber mounted on the stage of an Olympus CK-2 inverted stage microscope (Lake Success, NY). Solutions in the chamber were changed by four electronically controlled valves (General Valve, Fairfield, NJ) or by using a multibarreled ejection pipette to change solution locally (Hamill et al., 1981). Data were sampled at 150 μs and filtered at 3–5 kHz with an in-house low-pass filter, digitized, and then stored on a PC-286 computer. Series resistance compensation was used in all experiments and was adjusted to give the fastest possible transients without producing ringing. Linear leak and capacitance currents were subtracted using a protocol consisting of hyperpolarizing pulses that produced no ionic current and/or subtraction of current measured after all L-type channels had been inactivated in inactivation protocols.

The pipette solution contained (in mM): 60 CsCl; 1 $CaCl_2$; 10 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES); 1 $MgCl_2$; 11 EGTA; 5 K_2ATP; 50 aspartic acid (pH 7.4 with CsOH, and the final concentration of Cs^+ is about 135–140 mM). It had been shown (Krafte and Kass, 1988) that 10 mM HEPES is adequate to buffer the intracellular pH (pH_i) over the range of extracellular pH (pH_o) studied. Extracellular solution contained (in mM): 132 *N*-methyl-D-glucamine; 4.8 CsCl; 5 dextrose; 5 HEPES; 2 $MgCl_2$; and one of the following buffers where appropriate: 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS; pK_a 10.4 for pH_o 10), TAPS (*N*-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid; pK_a 8.4 for pH_o 8.5 and 8.0), HEPES (pK_a 7.5 for pH_o 7.4 and 7.0), MES (2-(*N*-morpholino)ethanesulfonic acid; pK_a 6.1 for pH_o 6.5, 6.0, and 5.5) or Trizma succinate (pK_1 4.6 for pH_o 5.0) (all buffers were obtained from Sigma Chemical Co., St Louis, MO). For each pH_o change, the solution contained 5 mM HEPES and 5 mM of the appropriate buffer. Liquid junction potentials were measured (<3 mV) and found to be independent of pH_o and extracellular Ca^{2+}. This value was therefore not corrected for in the subsequent analysis.

Preliminary studies showed that the effect of extracellular Ca^{2+} (5–20 mM) on the gating shifts of calcium channel inactivation was unaffected by osmolarity differences. However, with higher Ca^{2+} (30–60 mM), the osmolarity of solution was compensated for by reducing by appropriate amount of *N*-methyl-D-glucamine. The extracellular free Ca^{2+} concentrations in different buffers were measured by using an ion-sensitive microelectrode (digital ionalyzer; Orion Research, Inc., Boston, MA). Preliminary studies also showed that none of buffers used in this study affected free Ca^{2+} concentrations.

The voltage protocols used in the present study have been described previously in detail (Krafte and Kass, 1988). Holding potentials were adjusted in experiments in which large shifts in surface potentials, caused by changes in pH_o and/or high Ca^{2+}, were expected. Briefly, inactivation was determined by measuring current in response to the same test pulse voltage preceded by variable conditioning pulses (500 ms). Test pulse duration was 40 ms, and test pulse voltage was adjusted for each solution to measure current near the peak of the current voltage relationship. Peak current measured during the test pulse was plotted against conditioning pulse potential and normalized to the current measured after the most negative conditioning pulse. High intracellular EGTA concentrations (11 mM) were used to minimize the contributions of Ca-dependent inactivation, and the agreement we found between divalent ion-induced gating shifts in activation and inactivation (see Results) reinforced the view that we had measured shifts in voltage-dependent parameters. For activation, current was measured in response to a series of test pulses, background current (measured at small negative potential changes and suitably scaled) was subtracted, and the calcium channel reversal potential was determined. Conductance, obtained by normalizing peak current to driving force, was plotted versus membrane potential to determine activation.

Surface potential calculations

The equations used to determine the theoretical relationship between extracellular ion concentration, surface charges, and changes in membrane surface potential, as described by the Grahame equation (McLaughlin, 1977; Grahame, 1947) and modified to include the possibility of the binding to and neutralization of surface charge by extracellular ions, are used in this paper. Its use in describing changes in channel gating has been described in detail previously (Krafte and Kass, 1988). Consequently, only the minimum information relevant to the present study is summarized here.

The Grahame equation was used as follows:

$$\sigma_a^2 = \frac{kT\epsilon}{2\pi \sum C_i(\exp[-\psi_o \cdot Z_i \cdot e/kT] - 1)} \quad (1)$$

where σ_a is the apparent surface charge density in electronic charges/Å^2, kT/e = 25.3 mV at T = 22°C, C_i is the concentration of the ith ion and Z_i is its valence, ϵ is the dielectric constant of aqueous solution, and ψ_o is the surface potential in mV. If H^+ and Ca^{2+} bind to common sites, the relationship between apparent and true negative surface charge is

$$\sigma_a = \frac{\sigma_T}{1 + K_H[H^+]^* + K_{Ca}[Ca^{2+}]^*)}. \quad (2a)$$

Binding to independent sites is given by the sum of similar, but separate, saturating relationships for surface charge:

$$\sigma_a = \frac{\sigma_{T1}}{1 + K_{H1}[H^+]^* + K_{Ca}[Ca^{2+}]^*} + \frac{\sigma_{T2}}{1 + K_{H2}[H^+]^*}. \quad (2b)$$

K_H and K_{Ca} are apparent association constants in units of M^{-1}. $[H^+]^*$ and $[Ca^{2+}]^*$ equal the surface concentrations of H^+ and Ca^{2+}, respectively, which are related to bulk concentrations $[H^+]$ and $[Ca^{2+}]$ by the following expressions:

$$[H^+]^* = [H^+]\exp(-\psi_o{}^* e/kT), \quad (3a)$$

$$[Ca^{2+}]^* = [Ca^{2+}]\exp(-2\psi_o{}^* e/kT). \quad (3b)$$

Curve fitting procedures

A Newton-Raphson iteration procedure (Dorn and McCracken, 1972) was used to solve Eq. 1 for σ_a. Boltzmann curves were determined from fits to the experimental data provided by ORIGIN (MicroCal, Northampton, MA). Results are expressed as mean ± SEM. Significance tests were performed by Student's paired or unpaired t tests where appropriate. p values of less than 0.05 were considered to be statistically significant.

RESULTS

Saturation of Ca_o-induced gating shifts: constant pH_o

In a previous study, Ca_o-dependent gating shifts were measured for L-type calcium channel currents in guinea pig ventricular myocytes over a limited range of extracellular calcium concentrations (Ca_o) but constant external pH (pH_o), and the results were found to be consistent with predictions of surface potential theory in which calcium ions screen and

bind to negative surface charges near the channel (Kass and Krafte, 1987). The first step of the present investigation was to determine whether or not Ca_o-dependent gating shifts approach saturation at sufficiently high Ca_o concentrations when pH_o was constant.

Fig. 1 summarizes the effects on I_{Ca} availability of varying the extracellular Ca concentration over a broad range at pH 7.4. Fig. 1 *A* illustrates results from one cell in which normalized inactivation curves were determined in five concentrations of extracellular calcium. As expected, elevation of Ca_o caused progressive depolarizing shifts in inactivation with little change in the slope of the relationship between channel availability and voltage. However, the Ca_o-dependent gating shift begins to saturate when extracellular calcium concentration is elevated above 40 mM. This experiment was verified in three other cells in which we were able to measure gating shifts in at least five different calcium-containing solutions and then a large number of cells (4–8 cells/$[Ca]_o$) in which gating shifts were measured in two to four different solutions (Fig. 1 *b*). The results confirmed that there was no significant difference in Ca_o-induced shifts in inactivation for $[Ca]_o$ that was $\geq$40 mM. Similar observations were made for Ca_o-induced shifts in activation (see Fig. 5).

H^+ and Ca^{2+}-induced gating shifts are additive: evidence for site independence?

These results suggest that the negative sites to which calcium ions bind begin to saturate when extracellular calcium is elevated higher than 40 mM. If Ca^{2+} and H^+ bind to a common set of negatively charged sites, then at these concentrations of Ca_o acidification of the extracellular solution would not be expected to cause additional shifts in channel gating. Fig. 2 shows that this is not the case. Shown in the figure are the results of experiments carried out in 40 mM (*A*) and 60 mM (*B*) Ca_o, concentrations sufficiently high that Ca_o-dependent gating shifts begin to saturate. In each case further shifts in both inactivation (*A*) and activation (*B*) were clearly apparent after changing pH_o from 7.4 to 6.0. These data show that acidification causes shifts in channel gating that add to the maximal Ca_o-induced shifts and are consistent with independent Ca^{2+} and H^+ binding sites.

Although acidification caused additional gating shifts in the presence of high extracellular calcium concentrations, the measured pH-induced gating shifts (6.3 ± 1.9 mV, inactivation, $[Ca^{2+}]_o$ = 40 mM; 4.6 ± 1.3 mV, activation, $[Ca^{2+}]_o$ = 60) were, however, much smaller than those previously reported by Krafte and Kass (1988) for similar pH changes but in much lower calcium-containing (1 mM) solutions. This raises the possibility that the pH-induced gating shifts may be affected by the high calcium concentrations of the experiments summarized in Fig. 2. Thus, despite the additivity of the proton and Ca^{2+}-induced changes in gating, some interactions between these two cations are possible. In order to test this hypothesis, we measured pH-induced gating shifts as a function of extracellular calcium over a broad calcium concentration range.

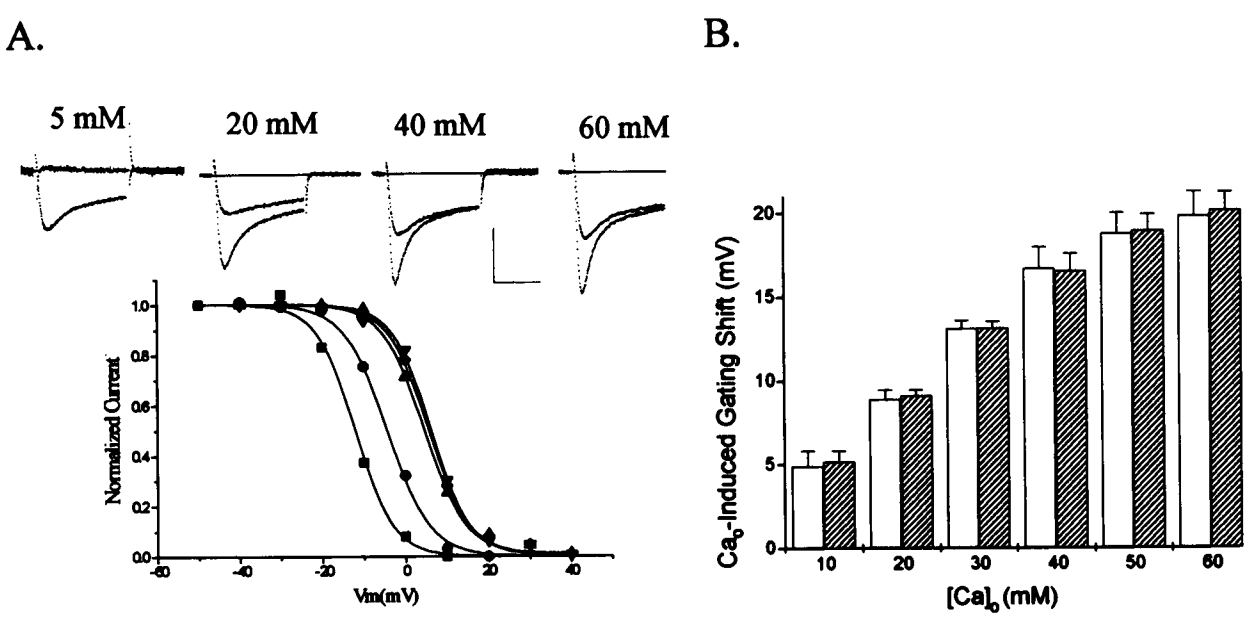

FIGURE 1 Saturation of gating shifts with high Ca_o. (*A*) Normalized inactivation (500 ms) curves were determined as described in Materials and Methods in a single cell in solutions containing (in mM) 5 (■), 20 (●), 40 (▲), 50 (◆), and 60 (▼). The dotted curves are the best-fit Boltzmann functions to the data (Materials and Methods). The parameters obtained to generate the curves shown are *k* (slope factor, all) = 4.54 mV; $V_{1/2}$ = −12.4 mV (5 Ca_o), −5 mV (20 Ca_o), +4 mV (40 Ca_o), +5 mV (50 Ca_o), and +6 mV (60 Ca_o). Current traces are recorded in the indicated calcium concentrations after conditioning pulses to −50, 0, and +30 mV. pH_o = 7.4 throughout. Calibration bars: 1 nA, 25 ms. (*B*) Summary of gating shifts induced by changes in Ca_o: activation and inactivation. $V_{1\backslash 2}$ relative to $V_{1\backslash 2}$ in 5 mM Ca_o was measured in separate cells for activation (□) and inactivation (▨) and plotted versus Ca_o. Each bar corresponds to the average relative shift in $V_{1\backslash 2}$ measured in three cells in each calcium concentration shown. There is no significant difference ($p < 0.01$) in the relative shifts measured at 40, 50, or 60 mM Ca_o for either activation or inactivation.

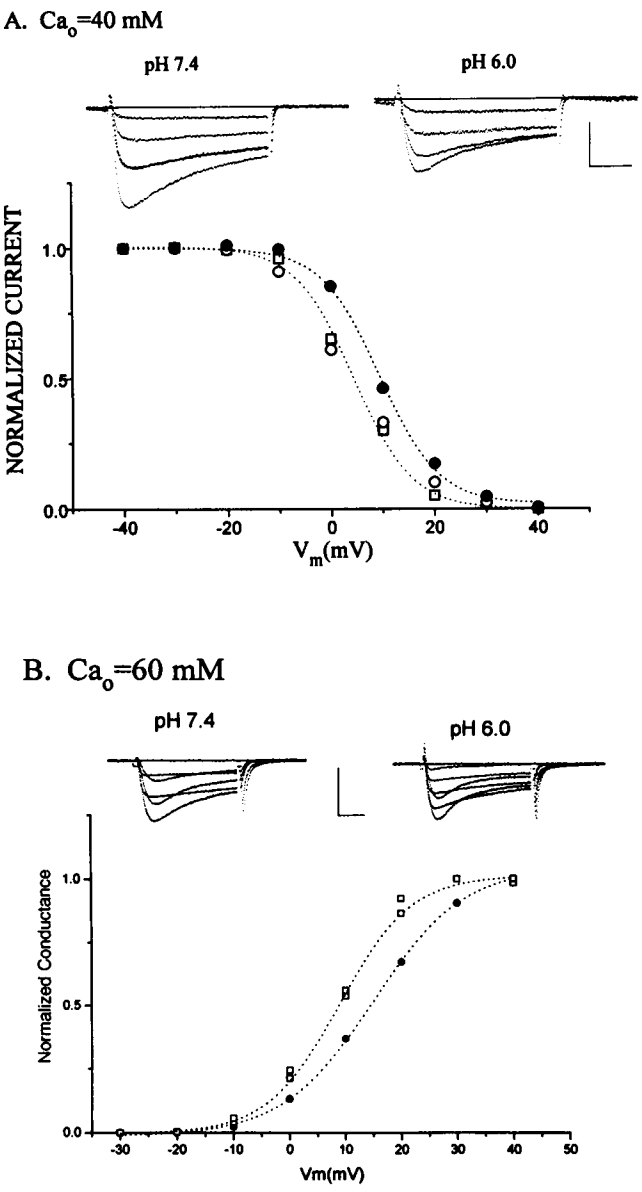

FIGURE 2 Changes in pH_o induce gating shifts in solutions containing high concentrations of calcium. (*A*) Acidification (pH 6.0) of extracellular solution shifts inactivation in the maintained presence of 40 mM Ca_o. Inactivation (500 ms) curves were measured as described in Materials and Methods in solutions buffered to pH 7.4 (○), pH 6.0 (●), and after returning to pH 7.4 (□). Ca_o was 40 mM in all solutions. Current inset calibration: 1 nA, 10 ms. The normalized inactivation curves and their best-fit Boltzmann functions are plotted. The parameters for the fitted functions are: $V_{1/2}$ = 4.3, 9.3 mV (pH 7.4, 6.0) and slope factor (k) = 5.6 mV (all). (*B*) pH_o shifts activation in 60 mM Ca_o. Activation curves determined as in Materials and Methods in the presence of 60 mM Ca_o but in pH_o = 7.4 (*left*) and 6.0 (*right*). Insets show current traces measured in both conditions, and the plot shows normalized conductance versus test voltage in control and recovery pH 7.4 (□) and in pH_o 6.0 (●). Calibration: 1.25 nA, 10 ms. Parameters are: $V_{1/2}$ = 8.9, 14.9 mV (control and recovery, pH 6.0); k = 6.4 mV (control and recovery), 8.1 mV (pH 6.0).

External calcium modifies pH-induced gating shifts

Fig. 3 confirms that the gating shift caused by a fixed change in pH_o depends on the concentration of Ca_o of the solutions in which the measurements are made. Lowering Ca_o from 40 to 5 mM roughly doubles the gating shift induced by a constant pH change (7.4 to 6.0). Voltage shifts on the order of 10 to 13 mV were also measured for the same change in pH_o in both 10 and 20 mM Ca_o for activation as well as inactivation but are not shown. The inset, which summarizes the data from 4–7 cells, clearly shows significant differences in pH-induced gating shifts measured in 5 and 60 mM Ca_o. As the concentration of Ca^{2+} in the extracellular solution is elevated, the subsequent pH_o-induced gating shifts are reduced, clearly indicating competition with or screening of H^+ by Ca^{2+}.

Titration of Ca_o-induced gating shifts

As a final test for Ca^{2+}/H^+ competition, we measured shifts in both activation and inactivation caused by a fixed change in Ca_o (5 to 40 mM) as a function of pH_o in an attempt to titrate Ca^{2+}-dependent gating shifts. We reasoned that acidification could neutralize the sites to which Ca^{2+} binds and thus reduce the magnitude of calcium-dependent gating shifts. We in fact found that Ca_o-induced gating shifts for both activation and inactivation were consistently larger in more alkaline than acidic solutions and that the relationship between Ca_o-induced gating shifts and pH was well described by a simple saturating function (Fig. 4) with an effective pK of 6.9 and a Hill coefficient of 1.8. Nevertheless, the total titratable Ca_o-dependent gating shift was small (4 mV), and sizable gating shifts could still be measured by changing Ca_o from 5 to 40 mM in very acidic solutions buffered between pH 5.5 and 5.0.

DISCUSSION

Our results present clear evidence for interactions between Ca^{2+} and H^+ that cause shifts in cardiac L-type channel gating and, in this manner, support the findings of Langer and colleagues (Langer et al., 1989; Langer, 1985) in studies of the effects of extracellular acidosis on the contraction of isolated rabbit ventricular cells. However, our experiments appear to reveal some contradictory properties. The additivity of pH_o- and Ca_o-induced gating shifts appears to suggest independent sites of action, but evidence for interactions between Ca^{2+} and H^+ is clear from the experiments summarized in Figs. 3 and 4. How can these apparent contradictions be resolved?

Surface potential theory: predictions for Ca^{2+} and H^+ binding sites

In order to interpret our experimental results, we returned to the predictions of surface potential theory as discussed in Materials and Methods and previously described (Krafte and Kass, 1988; Kass and Krafte, 1987). Here, however, we have assumed the existence of two titratable charge groups: one that binds only H^+ and one that can bind both Ca^{2+} and H^+ and used surface charge densities within the range previously

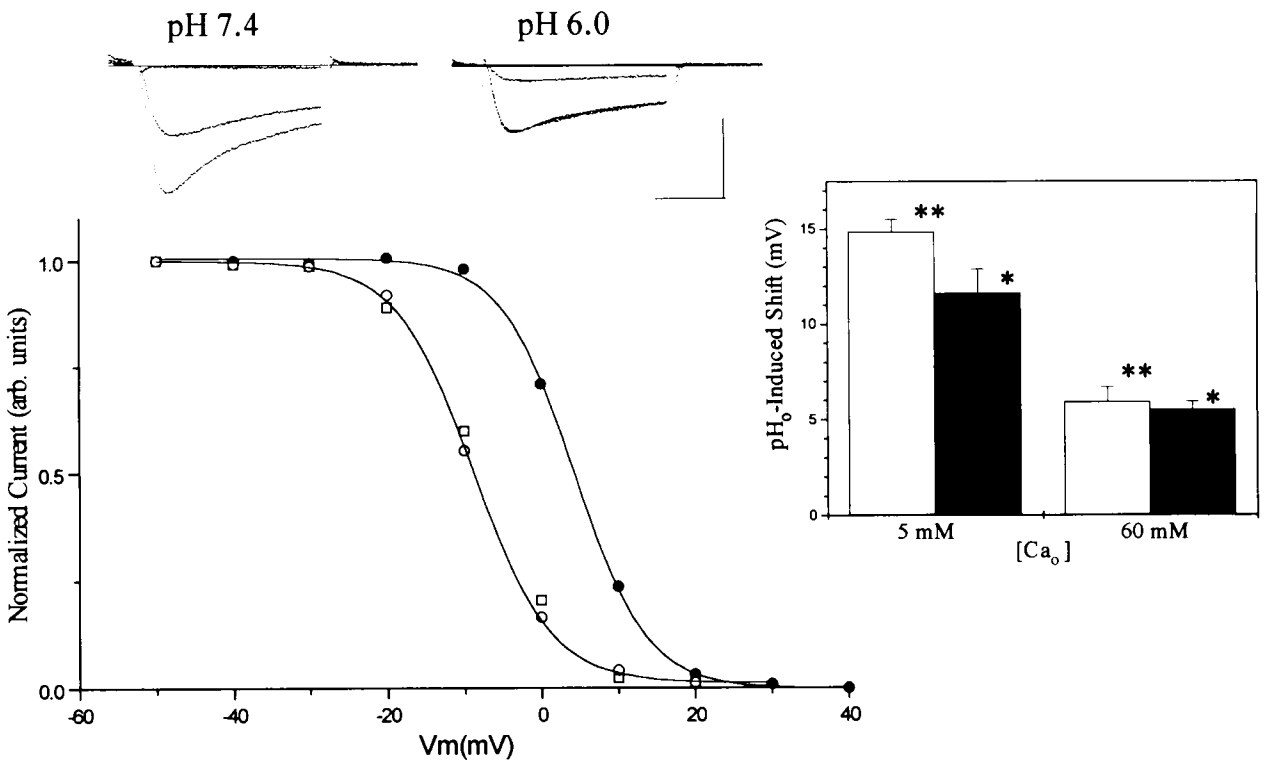

FIGURE 3 Extracellular calcium affects the amplitude of pH_o-induced gating shifts. Normalized inactivation curves were measured as described in Materials and Methods in solutions of fixed Ca_o, but in which pH_o was changed from 7.4 to 6.0 and then back to 7.4. (*A*) Representative current traces and normalized inactivation curves for pH 7.4 (both control and recovery) as open symbols and curves measured in pH_o 6.0 as filled symbols, $[Ca]_o$ = 5 mM. The best fits to the data are $V_{1/2}$ = −8.9 mV, 4.3 mV (pH 7.4, 6.0); slope factor = 4.96 mV (all). Current traces are shown after conditioning pulses to −50, −20, +10, and 20 mV. Calibration: 1 nA, 20 ms. (*Inset*) Mean and SEM gating shifts for activation (□) and inactivation (■) measured in 5 and 60 mM extracellular calcium. Gating shifts were determined as the difference between $V_{1/2}$ measured in pH 6.0 and the average $V_{1/2}$ determined in control and recovery pH 7.4 solutions in each concentration of Ca_o. Each bar is the average pH-induced gating shift measured in this manner in 4–6 cells. *, **, significantly different ($p < 0.01$).

reported (Krafte and Kass, 1988). For the Ca^{2+}-sensitive binding sites, we used pK = 6.9 as found in the data presented in Fig. 4. For the Ca^{2+}-independent binding sites, we used a pK of 5.5, a value within the range reported by Krafte and Kass (1988) for H^+-induced gating shifts. Several different computations were carried out to simulate the conditions of the experiments used in this study. First, using this model, we computed the predicted shifts in gating associated with changes in extracellular calcium when pH was constant (pH = 7.4). The results of this computation, summarized in Fig. 5 *A*, indicate that the theoretical predictions for changes in surface potential can account for the measured Ca_o-induced gating shifts, including the saturation that occurs at high Ca^{+2} concentrations.

Next, we tested the model for the effects of changing Ca_o on pH_o-induced gating shifts. Here, surface potentials were computed as described in Materials and Methods for a fixed calcium concentration at pH 7.4. Then, with the concentration of calcium maintained, the computation was repeated with pH = 6.0. The difference between surface potentials predicted for pH 6.0 and pH 7.4 was determined, calcium concentration was then changed, and the computational process was restarted. This process was repeated over a wide range of calcium concentrations to generate the smooth curve

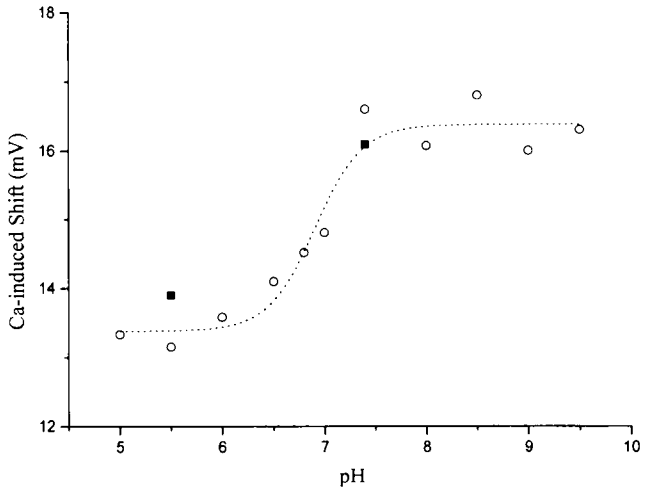

FIGURE 4 Titration of Ca_o-induced gating shifts. Experimental shifts in inactivation and activation caused by changing Ca_o from 5 to 40 mM were determined as described in Materials and Methods for solutions buffered to different values of external pH. These calcium-induced experimentally determined gating shifts for activation (■) and inactivation (○) are plotted versus pH_o. The curve is the best fit to the data of the function

$$\Delta V = V_o + V_{max}/[1 + (10^{(pK-pH)})^n]. \quad (4)$$

The experimental data are best fitted by the following parameters: V_{max} = 3.1 mV; pK = 6.9; n = 1.8; and V_o = 13.4 mV.

of Fig. 5 *B*. The result shows the predicted pH_o-induced gating shifts as a function of Ca_o. Also plotted in the figure are the experimental data for both inactivation (filled symbols) and activation (open symbols) gating shifts. This simple theoretical model predicts a calcium-dependent decrease in gating shifts caused by changes in pH_o that agrees reasonably with the experimentally determined data.

Two sets of binding sites

It has been suggested that $[Ca^{2+}]_o$ and $[H^+]_o$ can bind to distinct, negatively charged binding sites in nerve (Hille, 1968; Woodhull and Hille, 1970) and in ventricular tissue (Fry and Poole-Wilson, 1981; Langer et al., 1989). This view is consistent with the simplest interpretation of our experimental data based on the theoretical computations, which is that there exist two sets of charged groups to which H^+ bind: one with pK_a on the order of 5.5 and a second on the order of 6.9. Nevertheless, Ca^{2+} and H^+ compete at the latter site and thus cause interactive changes in channel gating in a manner that resembles the competitive effects of H^+ and Ca^{2+} on nerve sodium channels (Woodhull and Hille, 1970). The theory predicts that the effects of Ca_o on pH_o-induced gating shifts are caused, in part, by competitive binding to this subset of common sites. However, titration of all the Ca^{2+}/H^+ sites in acidic solutions reduces, but does not completely eliminate, gating shifts caused by subsequent elevation of Ca^{2+} because calcium and other divalent ions can produce significant gating shifts by screening other surface charges even in the absence of direct binding (Begenisich, 1975; Kass and Krafte, 1987). The influence of $[Ca]_o$ on pH-induced gating shifts is similarly due in part to the competition for this common set of binding sites and in part to the effects on surface potential caused by the screening of surface charges by the positive divalent cations.

Langer (1985) measured the effects of pH on calcium binding to sarcolemmal membranes and on contractile force in cultured neonatal rat cells and found that both parameters were reduced by acidification in a manner consistent with titration of a site with pK_a on the order of 6.60 to 7.15. Langer speculated that this could be due to the titration of a mildly alkaline amino group of a zwitterionic phospholipid such as phosphatidylethanolamine (PE). As pH is raised, the amino group is neutralized and no longer available to shield other negatively charged groups, which could then bind calcium (Seimiya and Ohki, 1973). This work was later extended to provide evidence for two-site (Ca^{2+} and H^+) control of ventricular contractile activity (Langer et al., 1989). The results that we have obtained are remarkably similar to those of Langer and colleagues and suggest that surface potential-mediated changes in L-type channel activity are closely related to the contractile events that they investigated.

Relationship between negative charge groups and channel distribution

The use of Guoy-Chapman theory as applied to the analysis of gating shifts in this study assumes a uniform surface charge density and thus cannot be used to accurately profile the discrete charge groups to which Ca^{2+} and H^+ bind. Nev-

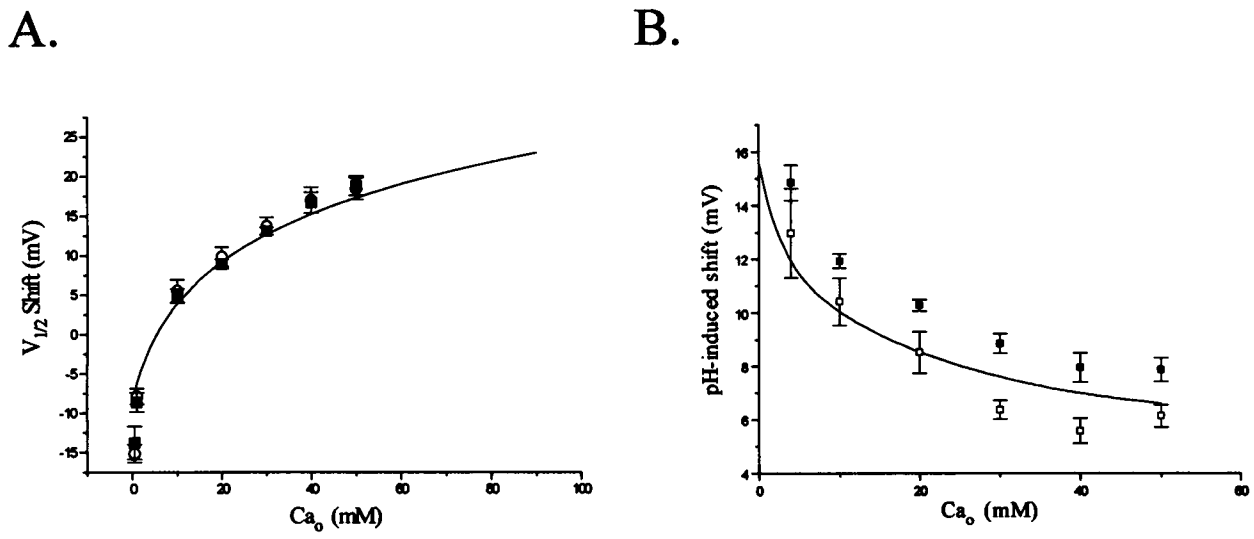

FIGURE 5 Theoretical predictions of two-site models. (*A*) Ca_o-induced gating shifts at constant pH_o (pH_o = 7.4). Surface potential changes predicted for changes in extracellular calcium concentration relative to Ca_o = 5 mM were computed as described in Materials and Methods for two independent surface charge densities, both titratable by external H^+, but only one that can also bind Ca^{+2}. The pK_a for sites that bind both H^+ and Ca^{+2} was determined from the analysis summarized in Fig. 4. The values used were: σ_T (Ca, H) = $-1e/(300\ Å^2)$; pK_a(Ca, H) = 6.9; K_{Ca} = 1.5 M^{-1}; σ_T (H) = $-1e/(250\ Å^2)$; pK_a (H) = 5.5 (pH was 7.4 in all computations). Experimental data are shown for activation (○, n = 3 cells/point) and inactivation (■, n = 3 cells/point). (*B*) Influence of Ca_o on pH_o-induced gating shifts. Using the same parameters as in *A*, shifts in surface potential caused by changing pH_o from 7.4 to 6.0 were computed for a series of extracellular calcium concentrations. The computation was carried out by fixing Ca_o and calculating the difference in surface potential between solutions buffered to pH 7.4 and 6.0. The calcium concentration was then changed and the computation repeated. The predicted pH-induced shifts (in mV) (minus a 3.0 mV baseline) as a function of extracellular calcium are plotted as the smooth curve. Experimentally determined average shifts in inactivation (open, n = 4 to 5 cells/point) and activation (filled, n = 4 to 7 cells/point) for comparable extracellular conditions are also shown.

ertheless, rough comparisons between channel densities can be made with the extracted suface charge densities to estimate whether the negative charge groups revealed by this analysis are likely reside on the channel proteins, as appears to be the case for several types of nerve K^+ channels (Spires and Begenisich, 1992; Gilly and Armstrong, 1982) or part of the membrane phospholipid. Assuming an L-channel density of 0.2 to 2 channels/μm^2 as reported by Mazzanti et al. (1991), the corresponding channel density/$Å^2$ translates to 0.2 to 2×10^{-8} channels/$Å^2$, a number several orders of magnitude lower then the negative surface charge densities described above. It thus seems likely that the negatively charged sites reside on the membrane phospholipid but within a distance defined by the Debye length (approximately 10 Å for the solutions used in this study) (Hille, 1984) of the channel pore.

It has been reported that high-affinity binding of calcium to the α_1 subunit of the L-channel protein stabilizes the binding of DHP and phenylalkalmine calcium channel blockers in addition to causing allosteric regulation of DHP binding to the α_1 subunit (Schneider, 1991; Staudinger et al., 1991), but since the sites described in the present study most likely reside in the membrane phospholipid and are probably not directly associated with the channel protein structure, it will be important to take into account the membrane environment of recombinant L-type channel expressed in other cell lines, as it is clear that channel function will be influenced by the ionization state of neighboring membrane phospholipids.

REFERENCES

Almers, W., and E. W. McCleskey. 1984. Non-selective conductance in calcium channels of frog muscle: calcium selectivity in a single-file pore. *J. Physiol. (Lond.).* 353:585–608.

Babitch, J. 1990. Channel hands. *Nature (Lond.).* 346:321–322.

Begenisich, T. 1975. Magnitude and locations of surface charges on myxicola giant axons. *J. Gen. Physiol.* 66:47–65.

Catterall, W. A., and J. Striessnig. 1992. Receptor sites for Ca channel antagonists. *Trends Pharmacol. Sci.* 13:256–262.

Dorn, W. S., and D. D. McCracken. 1972. Numerical Methods with Fortran IV Case Studies. John Wiley and Sons, New York.

Ebata, H., J. S. Mills, K. Nemcek, and J. D. Johnson. 1990. Calcium binding to extracellular sites of skeletal muscle calcium channels regulates dihydropyridine binding. *J. Biol. Chem.* 265:177–182.

Fry, C. H., and P. A. Poole-Wilson. 1981. Effects of acid-base changes on excitation-contraction coupling in guinea pig and rabbit cardiac ventricular muscle. *J. Physiol. (Lond.).* 313:141–160.

Gilbert, D. L., and G. Ehrenstein. 1969. Effects of divalent cations on potassium conductance of squid axons: determination of surface charge. *Biophys. J.* 9:447–463.

Gilly, W. F., and C. M. Armstrong. 1982. Divalent cations and the activation kinetics of potassium channels in squid giant axons. *J. Gen. Physiol.* 79:965–996.

Grahame, D. C. 1947. The electric double layer and the theory of electrocapillarity. *Chem. Rev.* 41:441–501.

Hamill, O. P., A. Marty, E. Neher, B. Sakmann, and F. J. Sigworth. 1981. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflugers Arch.* 391:85–100.

Hess, P., and R. W. Tsien. 1984. Mechanism of ion permeation through calcium channels. *Nature (Lond.).* 311:538–544.

Hille, B. 1968. Charges and potentials at the nerve surface. Divalent ions and pH. *J. Gen. Physiol.* 51:221–236.

Hille, B. 1984. Ionic Channels of Excitable Membranes. Sinauer, Sunderland, MA.

Hille, B., A. M. Woodhull, and B. Shapiro. 1975. Negative surface charge near sodium channels of nerve: divalent ions, monovalent ions, and pH. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* 270:301–318.

Iijima, T., S. Ciani, and S. Hagiwara. 1986. Effects of the external pH on Ca channels: experimental studies and theoretical considerations using two-site, two-ion model. *Proc. Natl. Acad. Sci. USA* 83:654–658.

Irisawa, H., and R. Sato. 1986. Intra- and extracellular actions of proton on the calcium current of isolated guinea pig ventricular cells. *Circ. Res.* 59:348–355.

Kass, R. S., and J. P. Arena. 1989. Influence of pH_o on calcium channel block by amlodipine, a charged dihydropyridine compound: implications for location of the dihydropyridine receptor. *J. Gen. Physiol.* 93:1109–1127.

Kass, R. S., and D. S. Krafte. 1987. Negative surface charge density near heart calcium channels. Relevance to block by dihydropyridines. *J. Gen. Physiol.* 89:629–644.

Kass, R. S., and M. C. Sanguinetti. 1984. Calcium channel inactivation in the cardiac Purkinje fiber. Evidence for voltage- and calcium-mediated mechanisms. *J. Gen. Physiol.* 84:705–726.

Kass, R. S., J. P. Arena, and S. Chin. 1991. Block of L-type calcium channels by charged dihydroyridines. Sensitivity to side of application and calcium. *J. Gen. Physiol.* 98:63–75.

Konnerth, A. H., H. D. Lux, and M. Morad. 1987. Proton-induced transformation of calcium channel in chick dorsal root ganglion cells. *J. Physiol. (Lond.).* 386:603–633.

Krafte, D. S., and R. S. Kass. 1988. Hydrogen ion modulation of Ca channel current in cardiac ventricular cells: evidence for multiple mechanisms. *J. Gen. Physiol.* 91:641–657.

Langer, G. A. 1985. The effect of pH on cellular and membrane calcium binding and contraction of myocardium: a possible role of sacrolemmal phospholipid in EC coupling. *Circ. Res.* 57:374–382.

Langer, G. A., T. L. Rich, and S. Ji. 1989. Contractile shortening response of ventricular cells to extracellular acidosis. Evidence for two-site control of H^+-Ca^{2+} interaction. *Circ. Res.* 57:374–382.

Lansman, J. B., P. Hess, and R. W. Tsien. 1986. Blockade of current through single calcium channels by Cd, Mg, and Ca. Voltage and concentration dependence of calcium entry into the pore. *J. Gen. Physiol.* 88:321–347.

Mazzanti, M., L. J. DeFelice, and Y. M. Liu. 1991. Gating of L-type Ca channels in embryonic chick ventricle cells: dependence on voltage, current and channel density. *J. Physiol. (Lond.).* 443:307–334.

McLaughlin, S. 1977. Electrostatic potentials at membrane solution interfaces. *Curr. Top. Membr. Transp.* 9:71–144.

Mitra, R., and M. Morad. 1985. A uniform enzymatic method for dissociation of myocytes from hearts and stomachs of vertebrates. *Am. J. Physiol.* 249:H1056–H1060.

Nakayama, H., M. Taki, J. Striessnig, H. Glossmann, W. A. Catterall, and Y. Kanaoka. 1991. Identification of 1,4-dihydropyridine binding regions within the α_1 subunit of skeletal muscle Ca^{2+} channels by photoaffinity labeling with diazepine. *Proc. Natl. Acad. Sci. USA.* 88:9203–9207.

Ohmori, H., and M. Yoshii. 1977. Surface potential reflected in both gating and permeation mechanisms of sodium and calcium channels of the tunicate egg cell membrane. *J. Physiol. (Lond.).* 267:429–463.

Pietrobon, D., and P. Hess. 1990. Novel mechanism of voltage-dependent gating in L-type calcium channels. *Nature (Lond.).* 346:651.

Prod'hom, B., D. Pietrobon, and P. Hess. 1989. Interactions of protons with single-open L-type calcium channels. Location of protonation site and dependence of proton-induced current fluctuations on concentration and species of permeant ion. *J. Gen. Physiol.* 94:23–42.

Schneider, T. 1991. The devapamil-binding site of the purified skeletal muscle receptor for organic-calcium channel blockers is modulated by micromolar and millimolar concentrations of Ca^{2+}. *Eur. J. Biochem.* 200: 245–253.

Seimiya, T., and S. Ohki. 1973. Ionic structure of phospholipid membranes and binding of calcium ions. *Biochim. Biophys. Acta.* 298:546–561.

Spires, S., and T. Begenisich. 1992. Chemical properties of the divalent cation binding site on potassium channels. *J. Gen. Physiol.* 100:181–193.

Staudinger, R., H. G. Knaus, and H. Glossmann. 1991. Positive heterotropic allosteric regulators of dihydrophyridine binding increase the Ca^{2+} affinity of the L-type Ca^{2+} channel. *J. Biol. Chem.* 266:10787–10795.

Striessnig, J., B. J. Murphy, and W. A. Catterall. 1991. Dihydropyridine

receptor of L-type Ca channels: identification of binding domains for H-PN200–110 and H-azidopine within the α_1 subunit. *Proc. Natl. Acad. Sci. USA.* 88:10769–10773.

Triggle, D. J. 1991. Calcium -channel drugs: structure-function relationships and selectivity of action. *J. Cardiovasc. Pharmacol.* 18:S1–S6.

Tsien, R. W., P. Hess, E. W. McCleskey, and R. L. Rosenberg. 1987. Calcium channels: mechanisms of selectivity, permeation, and block. *Annu. Rev. Biophys. Biophys. Chem.* 16:265–290.

Wilson, D. L., K. Morimoto, Y. Tsuda, and A. M. Brown. 1983. Interaction between calcium ions and surface charge as it relates to calcium currents. *J. Membr. Biol.* 72:117–130.

Woodhull, A. M., and B. Hille. 1970. Competition between Ca and H ions at the nerve surface. *Biophys. Soc. Annu. Meet. Abstr.* 111a.

Yue, D. T., P. H. Backx, and J. P. Imredy. 1990. Calcium-sensitive inactivation in the gating of single calcium channels. *Science (Washington DC).* 21:1735–1738.