A $[\text{Na}^+]_o$-independent, $pH_o$-dependent Mechanism for Reduction of Intracellular $[\text{Ca}^{2+}]$ after Influx through $\text{Ca}^{2+}$ Channels in Mouse Pituitary Cells

STEPHEN J. KORN and RICHARD HORN

From the Neurosciences Department, Roche Institute of Molecular Biology, Nutley, New Jersey 07110

ABSTRACT The effect of extracellular pH ($pH_o$) on the duration of calcium-dependent chloride currents ($I_{\text{Cl}_{\text{calc}}}$) was studied in voltage clamped AT-20 pituitary cells. $I_{\text{Cl}_{\text{calc}}}$ was activated by $\text{Ca}^{2+}$ influx through plasma membrane $\text{Ca}^{2+}$ channels, which were opened by step depolarization to voltages between -20 and +60 mV. Increasing $pH_o$ from 7.3 to 8.0 reversibly prolonged $I_{\text{Cl}_{\text{calc}}}$ tail currents in perforated patch recordings from cells bathed in both $\text{Na}^+$-containing and $\text{Na}^+$-free solutions. This prolongation was prevented in standard whole cell recordings when the pipette solution contained 0.5 mM EGTA. The effects of raised $pH_o$ were not due to alteration of intracellular pH, since tail current prolongation still occurred when intracellular pH was buffered at 7.3 with 80 mM HEPES. The prolongation of $I_{\text{Cl}_{\text{calc}}}$ at $pH_o$ 8 could not be accounted for by a direct action on $\text{Ca}^{2+}$ channels, since tail currents were prolonged when $pH_o$ was changed rapidly during the tail current, after all $\text{Ca}^{2+}$ channels were closed. The effects of increasing $pH_o$ on $I_{\text{Cl}_{\text{calc}}}$ also could not be explained by a direct action on $\text{Cl}^-$ channels, since changing to $pH_o$ 8 did not prolong $\text{Cl}^-$ tail currents when intracellular calcium concentration ($[\text{Ca}^{2+}]_i$) was fixed by EGTA in whole cell recordings. Raising $pH_o$ did, however, prolong depolarization-evoked $[\text{Ca}^{2+}]_i$ transients, measured directly with the $\text{Ca}^{2+}$ indicator dye, fura-2. Taken together, these data demonstrate the presence of a $\text{Na}^+$-independent, $pH_o$-sensitive mechanism for reduction of $[\text{Ca}^{2+}]_i$ after influx through $\text{Ca}^{2+}$ channels. This mechanism is associated with the plasma membrane, and is active on a time scale that is relevant to the duration of single action potentials in these cells. We suggest that this mechanism is the plasma membrane $\text{Ca}^{2+}$ ATPase.

INTRODUCTION

Many excitable cells exhibit long duration action potentials during which $\text{Ca}^{2+}$ enters the cell through voltage-gated calcium ($\text{Ca}^{2+}$) channels. The transient changes in intracellular $\text{Ca}^{2+}$ concentration ($[\text{Ca}^{2+}]_i$) that result play an important role in membrane-associated events, such as ion channel gating, neurotransmitter and
hormone secretion, and second messenger activity. The control of \([\text{Ca}^{2+}]_i\), near the plasma membrane depends not only on processes that modulate the increase in local \([\text{Ca}^{2+}]_i\), but also on the processes that reduce it. These latter processes may include:

(a) \(\text{Ca}^{2+}\) extrusion from the cell via \(\text{Na}^+/	ext{Ca}^{2+}\) exchange (Gill, Chueh, and Whitlow, 1984; Kaczorowski, Costello, Dethmers, Trumble, and Vandlen, 1984) or \(\text{Ca}^{2+}\) ATPases (Barros and Kaczorowski, 1984; Carafoli, 1991); (b) diffusion of \(\text{Ca}^{2+}\) away from the membrane into the cell interior; (c) binding of \(\text{Ca}^{2+}\) to either diffusable or membrane bound \(\text{Ca}^{2+}\) binding molecules; or (d) sequestration into intracellular organelles. While all of these processes are thought to exist in most excitable cells, little is known about the role each plays in the reduction of submembrane \([\text{Ca}^{2+}]_i\) on an electrophysiologically relevant time scale.

To date, techniques have not been developed that enable direct observation of free \([\text{Ca}^{2+}]_i\), selectively in the local region where it can interact with plasma membrane ion channels. One approach to this problem is to use \(\text{Ca}^{2+}\)-dependent ion channels in the plasma membrane to monitor submembrane [\(\text{Ca}^{2+}\)] (Barish and Thompson, 1983; Pallotta, Hepler, Oglesby, and Harden, 1987; Korn and Horn, 1989). AtT-20 pituitary cells express a \(\text{Ca}^{2+}\)-dependent Cl\(^-\) current (\(I_{\text{ClCa}}\)) that is activated when \(\text{Ca}^{2+}\) enters the cell during action potentials (Korn and Weight, 1987; Korn, Bolden, and Horn, 1991a). \(I_{\text{ClCa}}\) is relatively insensitive to voltage, and does not inactivate over a period of many seconds (Evans and Marty, 1986; Korn and Weight, 1987). Consequently, once \(I_{\text{ClCa}}\) is activated and the source of \(\text{Ca}^{2+}\) turned off, the time course of \(I_{\text{ClCa}}\) at a given membrane potential can be used as a qualitative monitor of the \([\text{Ca}^{2+}]_i\) change adjacent to the cytoplasmic surface of the plasma membrane. We have previously shown, using this technique, that \(\text{Na}^+/	ext{Ca}^{2+}\) exchange removes \(\text{Ca}^{2+}\) from this submembrane region on a time scale that permits it to limit the duration of \(I_{\text{ClCa}}\) (Korn and Horn, 1989). In this paper, we present evidence that another process in the plasma membrane, which is pH dependent and \(\text{Na}^+\) independent, also plays a role in clearing \(\text{Ca}^{2+}\) from this region on a time scale that is relevant to the activation of \(\text{Ca}^{2+}\)-dependent channels during and after action potentials.

**METHODS**

**Cell Culture**

AtT-20/D16-16 pituitary cells were kindly provided by the Laboratory of Cell Biology, National Institutes of Health, and Dr. Terry Reisine, University of Pennsylvania. They were grown in Dulbecco’s modified Eagle’s medium (DMEM) plus 10% fetal bovine serum (Gibco Laboratories, Grand Island, NY), and maintained in 75-mm tissue culture flasks in a 37°C, 5% CO\(_2\) incubator. Cells were passed once weekly and used for experiments 5–8 d after plating (into 35-mm Nunc dishes for most experiments, and glass-bottom Nunc dishes for fluorescence experiments). Plated cells (passage 18–35) were fed three times weekly.

**Electrophysiology**

Perforated patch and standard whole cell patch clamp recordings were made as described previously (Korn and Horn, 1989). Perforated patch recordings were made (Figs. 1, 3, 4, 7) when it was desired to prevent washout of \(\text{Ca}^{2+}\) and \(\text{Ca}^{2+}\)-dependent currents and to maintain endogenous \(\text{Ca}^{2+}\) buffering systems, and also when the time course of intracellular \(\text{Ca}^{2+}\)
transients and Ca\(^{2+}\)-dependent currents were of interest (Korn and Horn, 1989; Korn et al., 1991a; Korn, Marty, Connor, and Horn, 1991b). Standard whole cell recordings (Hamill, Marty, Neher, Sakmann, and Sigworth, 1981) were made (Figs. 2, 5, 6) when better control of intracellular [Ca\(^{2+}\)] and/or pH was necessary. Membrane currents were recorded and filtered (2 kHz, -3 dB) with an Axopatch 1C patch clamp amplifier (Axon Instruments, Inc., Burlingame, CA). Series resistance (R\(_s\)) and membrane capacitance (C\(_m\)) were measured by cancellation of capacitive currents evoked by 20-ms, 10-mV voltage steps from -70 mV. R\(_s\) ranged from 5 to 20 M\(\Omega\) in perforated patch experiments, and was < 5 M\(\Omega\) in standard whole cell experiments (4–10 M\(\Omega\) when 80 mM HEPES was added to the pipette solution). Experiments were performed at 21–23°C. Data were acquired with pCLAMP (Axon Instruments, Inc.) and analyzed with both pCLAMP and user-written programs.

Ca\(^{2+}\)-activated Cl\(^{-}\) tail currents decay with complex kinetics and cannot be uniformly fit by the sum of a few exponentials. Therefore, the tail current duration was measured as the time for decay from peak to 20% of peak (peak to 10% of peak in Fig. 5).

**Solutions**

The volume of the bathing solution in the recording chamber (35-mm Nunc dish) was \(\sim 1\) ml, and except as described below, experiments were performed in a static bath. The standard extracellular solution in both perforated patch (except in Fig. 4, A–E) and standard whole cell experiments contained (in mM): 155–160 tetraethylammonium (TEA) Cl, 5 CaCl\(_2\), 0.8 MgCl\(_2\), 10 HEPES-NaOH, 20 glucose, pH 7.3, osmolality 335 mosM. The standard pipette solution in perforated patch experiments contained (in mM): 55 CsCl, 75 Cs\(_2\)SO\(_4\), 8 MgCl\(_2\), and 10 HEPES-CsOH, pH 7.3, 310 mosmol/kg. Substitutions for these standard solutions, and pipette solutions used for standard whole cell recordings, are listed in the figure legends.

Extracellular solutions were changed using three methods. Typically, the solution surrounding the cell being recorded was switched by manually lowering a large bore pipette that contained the desired solution into the bath adjacent to the cell. Cells were returned to control solution by removing the large bore pipette from the bath. Solution changes were accomplished within 5 s using this procedure. Faster solution changes (Fig. 4) were made with a magnetically driven stepping device (Korn and Horn, 1989). At the beginning of the experiment, a large bore pipette was placed manually 700 \(\mu\)m to the side of the cell being recorded. In these experiments, the bathing solution was flowing \(\sim 0.6\) ml/min from the direction directly opposite the large bore pipette. At the desired moment, a computer-generated TTL pulse triggered the magnet which flipped the drug-containing pipette 700 \(\mu\)m, so that it was directly apposed to the recorded cell. The new solution quickly engulfed the cell, with an on time constant that ranged from 40 to 110 ms (Korn and Horn, 1989). The experimental solution was removed by magnetically flipping the macropipette back to the starting position. Total removal of test solution from near the cell depended on the flow rate of the bath and passive diffusion away from the cell, and required 1–4 s (Korn and Horn, 1989). To insure that solution from the macropipette was applied to the cell as expected, macropipette solutions in these experiments contained 100 \(\mu\)M phenol red to allow us to visually inspect the solution flow characteristics. Phenol red had no effect on membrane currents (Fig. 4 E).

The third method of changing solutions was used in experiments that involved fluorescence measurements (see below). In these experiments (Figs. 7 and 8), solutions were changed by switching electronically (Isolatch valves from General Valve Co., Fairfield, NJ) among four teflon, gravity-fed outlet tubes nested within a 100-\(\mu\)m-diam glass pipette (Korn et al., 1991a). The glass pipette was placed in apposition to the recorded cell at the start of the experiment, with control (bath) solution flowing out of one of the four outlet tubes. The 10–90% rise time for complete solution exchange was 3–5 s.
Fluorescence Measurements of Intracellular [Ca²⁺]

Intracellular [Ca²⁺] was measured photometrically from single cells using the Ca²⁺ indicator dye, fura-2 (Molecular Probes, Inc., Eugene, OR). The method for measuring fluorescence and calculating the [Ca²⁺] has been described in detail (Korn et al., 1991a). Briefly, cells were incubated in 6.25 mg/ml of the membrane permeant, fura-2AM for 10 min at 37°C. Once deesterified in the cell (Grynkiewicz, Poenie, and Tsien, 1985), fura-2 is impermeant through both the plasma membrane and through nystatin pores. Immediately before commencing an experimental protocol, the resting [Ca²⁺] at the holding potential (usually -70 mV) was determined by measuring the fluorescence during successive 400-ms periods of excitation with 340- and 380-nm wavelengths. Stimulus-induced fluorescence transients were then measured monochromatically at 380-nm excitation wavelength to improve the time resolution of the measurements. The calculations used to determine [Ca²⁺] were exactly as described previously (Korn et al., 1991a). Bleaching was minimized by using as low an excitation light intensity as possible. The amount of bleaching was determined by comparing the fluorescence at 380 nm with a ratio measurement of [Ca²⁺] after each test run. During the brief light exposures necessary for the data presented here, significant bleaching did not occur.

RESULTS

Effect of Changing pHₒ on IₑCa₁₋₂

Voltage-activated Ca²⁺ currents (IₑCa) and Ca²⁺-dependent Cl⁻ currents (IₑCa₁₋₂) were isolated by bathing AtT-20 cells in a Na⁺- and K⁺-free extracellular solution (TEA substitution for monovalent ions) and using a K⁺-free intracellular solution (Cs⁺ substitution for K⁺). Fig. 1 illustrates currents obtained with perforated patch recording from a cell in which the membrane potential was stepped for 60 ms from -80 mV to a series of potentials between -30 and +60 mV. During the voltage step, an inward IₑCa is superimposed on an outward IₑCa₁₋₂ (Cl⁻ equilibrium potential = -22 mV; see also Fig. 3 A). Upon repolarization to -80 mV, a slowly decaying IₑCa₁₋₂ tail current is revealed (Fig. 1 A; Korn and Weight, 1987). IₑCa tail currents are not observed in these experiments, since Ca²⁺ channels close within 0.5 ms of the transition back to -80 mV (Korn and Weight, 1987), and the data were digitized at ≤1 kHz. When extracellular pH (pHₑ) was changed from 7.3 to 8.0 IₑCa₁₋₂ tail currents decayed more slowly (Fig. 1 A), regardless of size or activating voltage step (Fig. 1 B). Indeed, even tail currents that were somewhat reduced in amplitude in pHₑ 8 decayed more slowly (see activating voltages of +20 to +60 mV). For voltage steps to potentials between -20 and +10 mV, increasing pHₑ also increased the initial amplitude of the Cl⁻ tail current (Fig. 1 A). The effects of raising pHₑ reversed quickly upon return to pHₑ 7.3 (Fig. 1 A and 1, B and C, triangles).

Tail currents were similarly prolonged when pHₑ was changed to either 7.8 or 7.9 (data not shown). Experiments designed to test the effects of other pHₑ were not fruitful. Raising pHₑ to 8.1 or higher produced a steady activation of an inward current, which reversed quickly upon return to pHₑ 7.3 (data not shown). Reduction of pHₑ from 7.5 to 6.8 had no observable effect; reduction to pH 6.0 irreversibly eliminated IₑCa₁₋₂ (data not shown).

To examine whether the change in pHₑ influenced the duration of IₑCa₁₋₂ via a change in intracellular pH, the same experiment was performed with the standard whole cell recording technique (Hamill et al., 1981) using 80 mM HEPES in the...
recording pipette to heavily buffer intracellular pH at 7.3 (Deutsch and Lee, 1989). Changing pHo from 7.3 to 8.0 under these conditions produced identical results (in nine of nine cells) as under perforated patch conditions. Alkalinizing pHo increased the magnitude and duration of Ictus after activation steps to 0 mV (Fig. 2A), and increased the duration but not the magnitude of Ic	extsubscript{c} after more positive activation steps (Fig. 2B). These data demonstrate that the effects of changing pHo did not require changes in intracellular pH.

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Effect of raising pHo on Ic	extsubscript{Cl} in perforated patch recording. Currents were activated by 60-ms depolarizing voltage steps from a holding potential of −80 mV to potentials between −30 and +60 mV. (A) Currents evoked by steps to 0, 20, and 40 mV, in control (pH 7.3), pH 8, and after return to pH 7.3 external solutions. Time of voltage step is shown below lowest current traces. (B) Tail current duration, measured from peak to 20% of peak, as a function of activation potential, in control (open circles), pH 8 (filled circles), and after return to pH 7.3 (triangles). (C) Tail current peak amplitude, measured 2 ms after repolarization, as a function of activation potential. R, 20 MΩ, C, 8.4 pF.

Three general mechanisms could account for the effects of raising pHo on Ic	extsubscript{Cl}. First, decreasing the concentration of extracellular protons (H\(^+\)) could increase Ca\(^{2+}\) influx during the depolarizing voltage step, either from a shift in Ca\(^{2+}\) channel activation due to a change in surface potential (Hille, 1984), or by a direct action on Ca\(^{2+}\) channels (cf. Prod’hom, Pietrobon, and Hess, 1987). Second, H\(^+\) or OH\(^-\) might interact directly with Cl\(^-\) channels to influence their closing kinetics. Finally, increasing pHo might reduce the rate of removal of intracellular free Ca\(^{2+}\) from near
the plasma membrane. The remaining experiments in this study tested these three possibilities.

**Did Increasing pHo Prolong I_{Ca} by Increasing Ca^{2+} Influx?**

In AtT-20 cells, I_{Ca} activates relatively quickly, reaching its peak in 4–5 ms. In contrast, I_{Cl_{Ca}} activates more slowly, and is insignificant within 4–5 ms of the start of the step depolarization (Korn and Weight, 1987). Consequently, an estimate of the magnitude of I_{Ca} was obtained by measuring the inward current within 4–5 ms after the start of the depolarizing step (Fig. 3A, open arrow). The current–voltage relationship for I_{Ca} in these experiments was qualitatively similar to that observed when activation of I_{Cl_{Ca}} is prevented by high intracellular [EGTA] in whole cell recordings or when Ba^{2+} is used as the charge carrier (Korn and Weight, 1987), which supports the assumption that the inward current 4–5 ms after the start of the voltage step is not significantly contaminated by I_{Cl_{Ca}}.

![Figure 2. Prolongation of I_{Cl_{Ca}} in standard whole cell recording with 80 mM intracellular HEPES. (A) Currents were evoked by 100-ms voltage pulses to 0 mV from a holding potential of −70 mV. The repolarization potential was −100 mV. Three current traces are shown: control (pH_{o} 7.3), after cells were exposed to pH_{o} 8.0 for 10 s, and recovery (pH_{o} 7.3). (B) Currents were evoked in a different cell by 150-ms voltage pulses to +30 mV from a holding potential of −70 mV (repolarization potential = −70 mV). The pipette solution contained (in mM): 100 CsCl, 80 HEPES, and 2 MgCl_{2}, titrated to pH 7.3 with CsOH.](image)

If the prolongation of I_{Cl_{Ca}} in pH_{o} 8 were due simply to an increase in the magnitude of I_{Ca}, the relationship between I_{Ca} magnitude and tail current duration would be expected to remain relatively constant. Fig. 3C illustrates that this was not the case; tail currents were greatly prolonged at pH_{o} 8 even for significantly smaller Ca^{2+} currents. As described earlier, changing to pH_{o} 8 also resulted in larger peak I_{Cl_{Ca}} only at activation voltages of ≤ +10 mV. This is consistent with a shift in Ca^{2+} channel activation due to a change in surface potential (Hille, 1984). At most voltage steps, however, increases in I_{Ca} amplitude were not observed with pH_{o} changes. In addition, for a given I_{Ca} amplitude, there was a slight increase in I_{Cl_{Ca}} tail current amplitude (Fig. 3B). These data suggest that at least some of the increase in I_{Cl_{Ca}} amplitude following changes in pH_{o} was not due to an increase in I_{Ca}, but was due to an increased coupling between I_{Ca} and I_{Cl_{Ca}}.
Due to the mixture of currents during the 100-ms voltage step, it could not be determined in these experiments whether the integrated $I_{\text{Ca}}$ was influenced by pH$_o$. For example, increasing pH$_o$ could have decreased $I_{\text{Ca}}$ inactivation, and thus increased Ca$^{2+}$ influx during the voltage step. Such an effect could account for the increased $I_{\text{Cl}_{\text{Ca}}}$ amplitude and duration for a given $I_{\text{Ca}}$.

To conclusively rule out the possibility that the effect of changing pH$_o$ could be accounted for by an action on Ca$^{2+}$ channel function, a fast solution changer (see Methods) was used to change the external solution after the termination of the voltage step. Fig. 4, A–E, shows currents evoked by voltage steps to +10 mV in a cell that was bathed in a Na$^+$-containing solution. Each panel in Fig. 4, A–E, illustrates two superimposed currents, one in the Na$^+/\text{pH}_7.3$ bath solution and one evoked 30 s later with the extracellular solution changed as described. In Fig. 4A, the solution surrounding the cell was changed to pH 8 five seconds before the depolarizing stimulus. The typical prolongation was observed. The solution was then returned to pH 7.3. In Fig. 4B, the solution was switched to pH 8 twenty milliseconds after the termination of the depolarizing stimulus (arrow), after all Ca$^{2+}$ channels were presumably closed. Following a short delay, the tail current was prolonged. Similar effects on the Cl$^-$ tail current were observed (Fig. 4, C and D)

**Figure 3.** $I_{\text{Cl}_{\text{Ca}}}$ amplitude and duration as a function of $I_{\text{Ca}}$. (A) $I_{\text{Ca}}$ magnitude was measured 4 ms after the start of the voltage step (open arrow). $I_{\text{Cl}_{\text{Ca}}}$ amplitude was measured 2 ms after the termination of the voltage step (closed arrow). (B) Tail current amplitude as a function of $I_{\text{Ca}}$ amplitude. (C) Tail current duration (measured as peak to 20% of peak) as a function of $I_{\text{Ca}}$ amplitude. Currents were evoked by 100-ms voltage steps to between −15 and +5 mV (pH 7.3) and between −20 and 0 mV (pH 8.0). $R_s$ 19 MΩ, $C_m$ 7.4 pF.
when extracellular Na\(^+\) (Na\(\text{\textit{o}}\); pH\(\text{o}\) 7.3) was replaced by TEA (pH\(\text{o}\) 7.3). Na\(\text{\textit{o}}\) removal prolongs \(I_{\text{\textit{lc}o}}\) by inhibiting Na\(^+\)/Ca\(^{2+}\) exchange (Korn and Horn, 1989). Fig. 4 E shows that the effects of switching solutions during the tail were due neither to a mechanical artifact of the solution change nor to application of 100 \(\mu\)M phenol red, which was used to visualize the flowing test solution. Rapidly changing to pH\(\text{o}\) 8 also prolonged \(I_{\text{\textit{lc}o}}\) in cells bathed in Na\(^-\)-free solution (Fig. 4 F), showing that the rapid effects of pH\(\text{o}\) on \(I_{\text{\textit{lc}o}}\) were not due to its action on the Na\(^-\)-Ca\(^{2+}\) exchanger. In conclusion, raising pH\(\text{o}\) prolonged \(I_{\text{\textit{lc}o}}\) tail currents by a mechanism that did not involve a direct action on Ca\(^{2+}\) channels.

**Dependence of the pH\(\text{o}\) Effect on [Ca\(^{2+}\)]\(\text{i}\)**

Although the effects of changing to pH\(\text{o}\) 8 were not due to effects on Ca\(^{2+}\) channel function, the increase in peak and duration of \(I_{\text{\textit{lc}o}}\) in pH\(\text{o}\) 8 were consistent with the effect being dependent on intracellular [Ca\(^{2+}\)] (\([\text{Ca}\(^{2+}\)]\)). Two possible mechanisms could account for this dependence: (a) a decrease in intracellular Ca\(^{2+}\) buffering or removal at pH\(\text{o}\) 8, or (b) a change in binding kinetics (e.g., a slower off rate) between
Ca^{2+} and the Cl⁻ channel. To distinguish between these two possibilities, we used standard whole cell recordings in which cells were loaded with 200–500 μM EGTA. Ca^{2+} influx was varied incrementally by stepping the voltage to between −30 mV and +40 mV. In EGTA-loaded cells, the peak amplitude of I_{c_{ino}} still increased with increasing Ca^{2+} influx, but the dependence of I_{c_{ino}} duration on the peak I_{c_{ino}} (which reflects Ca^{2+} influx) was greatly reduced (Fig. 5 B). This presumably occurs because chelation by EGTA becomes the dominant Ca^{2+} buffering mechanism in standard whole cell recordings (Korn and Weight, 1987; Korn and Horn, 1989). The prediction was that if increasing pHo prolonged I_{c_{ino}}, due to a decrease in Ca^{2+} buffering, changing pHo in the presence of intracellular EGTA would have little or no effect on I_{c_{ino}}. In contrast, if changing pHo changed the binding kinetics of Ca^{2+} to the Cl⁻ channel, or if it prolonged I_{c_{ino}} by a mechanism unrelated to intracellular Ca^{2+}, then adding EGTA would not prevent the effects of changing pHo. Addition of EGTA eliminated the effect of changing pHo on tail current duration (Fig. 5 A), consistent with the hypothesis that prolongation of I_{c_{ino}} involved alterations in the buffering of intracellular Ca^{2+}.

**Direct Effects of Increasing pHo on I_{c_{ino}}**

To examine whether the effects of changing pHo could be due to a direct effect of OH⁻ (or H⁺ removal) on Cl⁻ channels, we used standard whole cell recordings to activate I_{c_{ino}} by voltage pulses in the presence of a fixed [Ca^{2+}] of 0.5 μM (Korn and Weight, 1987; Korn and Horn, 1989). In these experiments, Ca^{2+} entry was prevented by removal of extracellular Ca^{2+}. At pHo 8 (arrows in Fig. 6), I_{c_{ino}} was reduced in magnitude (Fig. 6 B) but not prolonged (Fig. 6 C; n = 4 cells). The effects

---

**FIGURE 5.** Prevention of tail current prolongation in pH 8 by intracellular EGTA in standard whole cell recording. (A) Currents evoked by 200-ms voltage steps to +10 mV from a holding potential of −80 mV, in control (pHo 7.3) and pHo 8 bathing solutions. The intracellular solution contained 0.5 mM EGTA, in addition to 160 mM CsCl, 10 mM HEPES, and 2 mM MgCl₂. (B) Tail duration (peak to 10% of peak) as a function of I_{c_{ino}} magnitude in two different cells. Data are plotted from one cell (R, 3.0, Cm 11.1) that was recorded with a pipette solution that contained 0.5 mM EGTA (open circles), and one cell (R, 3.5, Cm 11.4) recorded without the addition of intracellular EGTA (closed circles).
of changing to pHo 8 rapidly reversed upon returning to pHo 7.3 (Fig. 6 A ), which eliminated the possibility that the changes observed were due to a washout phenomenon. These data indicate that a direct effect of extracellular OH⁻ addition or H⁺ removal on Cl⁻ channels did not contribute to the prolongation of I_{cl,fast} at pHo 8.

**Effect of Changing pHo on \([Ca^{2+}]_i\) Transients**

The above experiments show that the effects of pHo on Cl⁻ channel currents cannot be explained by direct actions on the function of either Ca²⁺ or Cl⁻ channels, but that they are prevented by adding the Ca²⁺ chelator, EGTA, to the cytoplasm. These observations are consistent with the hypothesis that the prolongation of I_{cl,fast} in pHo 8 was due to changes in the cell's handling of Ca²⁺ after influx. To further explore this hypothesis, we made perforated patch recordings on cells previously loaded with the Ca²⁺ indicator dye, fura-2. With this procedure, we could directly observe [Ca²⁺]ᵢ transients in voltage clamped cells. Fig. 7 illustrates I_{cl,fast} (middle traces) and [Ca²⁺]ᵢ transients (top traces) evoked by a 200-ms voltage step to +10 mV (bottom trace). At pHo 7.3, depolarization caused a rapid increase in [Ca²⁺]ᵢ together with activation of I_{cl,fast}. As shown previously (Korn et al., 1991a, b), the cell averaged [Ca²⁺]ᵢ decayed more slowly than I_{cl,fast}; 10 s after switching to pHo 8, the same stimulus was repeated. Both I_{cl,fast} and the [Ca²⁺]ᵢ transient were potentiated and prolonged. This effect reversed within 30 s of returning to pHo 7.3 (Fig. 7). Note also in Fig. 7 that the
[Ca\(^{2+}\)] measured with fura-2 does not correspond with the [Ca\(^{2+}\)] required to activate \(I_{Ca_{off}}\). At pH\(_8\), [Ca\(^{2+}\)], 1 s from the end of the illustrated trace is higher than the peak [Ca\(^{2+}\)] at pH\(_7.3\), yet \(I_{Ca_{off}}\) is completely turned off. This indicates that the [Ca\(^{2+}\)] near the cell membrane is different than the average internal [Ca\(^{2+}\)] measured by fura-2.

Since changing to pH\(_8\) not only slowed the decline of [Ca\(^{2+}\)], but also increased Ca\(^{2+}\) influx, it was possible that the reduced rate of decay was due to the increased Ca\(^{2+}\) load. However, the decline of intracellular Ca\(^{2+}\) was slowed at pH\(_8\) even when Ca\(^{2+}\) influx was less (Fig. 8). The top traces (Fig. 8) show the typical effect of raising pH\(_8\) on the decay of [Ca\(^{2+}\)], following a constant stimulus; at pH\(_8\), [Ca\(^{2+}\)], reached a higher peak and decayed more slowly. The second set of traces compares the [Ca\(^{2+}\)] transient after the stimulus to +10 mV at pH\(_7.3\) (same as top trace) and a subsequent stimulus to −5 mV at pH\(_8\). The [Ca\(^{2+}\)] peaked at a significantly lower concentration at pH\(_8\) than at pH\(_7.3\), yet decayed more slowly. These data demonstrate that the slowed reduction of [Ca\(^{2+}\)] at pH\(_8\) was not due solely to an increase in the intracellular Ca\(^{2+}\) load. Furthermore, they show that pH\(_8\) has a direct effect on the rate of intracellular [Ca\(^{2+}\)] decrease after Ca\(^{2+}\) entry through Ca\(^{2+}\) channels.

**Figure 7.** Effect of increasing pH\(_8\) on intracellular [Ca\(^{2+}\)] transient. Simultaneous recording of intracellular [Ca\(^{2+}\)] (top) and membrane currents (middle) using the perforated patch technique on a fura-2–loaded cell. Ca\(^{2+}\) influx was activated by a 500-ms voltage pulse from −70 to 0 mV (protocol shown in lower trace). Three superimposed traces are shown: responses in control (pH\(_7.3\)), pH\(_8\) and after return to pH\(_7.3\). \(R_i\) 19 MΩ, \(C_m\) 11.6 pF.

**Figure 8.** [Ca\(^{2+}\)] transients were prolonged at pH\(_8\), even after less Ca\(^{2+}\) influx. (Top) [Ca\(^{2+}\)] transients were produced by 500-ms voltage steps from −70 mV to +10 mV in pH\(_7.3\) and, 30 s later, in pH\(_8\). (Middle) [Ca\(^{2+}\)] transient produced by voltage step to −5 mV in pH\(_8\) is superimposed on larger [Ca\(^{2+}\)] transient produced by stimulus to +10 mV in pH\(_7.3\) (same trace as in top panel). \(R_i\) 16 MΩ, \(C_m\) 11.6 pF.
This study began following the observation that, in voltage clamp experiments, raising pH₀ increased the magnitude and duration of I_{Cl<->}. In AtT-20 pituitary cells, I_{Cl<->} is activated by Ca²⁺ that enters the cell through plasma membrane Ca²⁺ channels. Since I_{Cl<->} does not inactivate (Evans and Marty, 1986; Korn and Weight, 1987), the primary determinants of I_{Cl<->} magnitude and duration are the amount and time course of Ca²⁺ influx, the rate of [Ca²⁺] reduction near the intracellular surface of the plasma membrane, the binding kinetics of Ca²⁺ to the Cl⁻ channel, and the intrinsic Cl⁻ channel kinetics. The data presented in this study demonstrate that the prolongation of I_{Cl<->} produced by raising pH₀ cannot be accounted for by a direct action of H⁺ removal (or OH⁻ addition) on Ca²⁺ or Cl⁻ channels, nor on Ca²⁺ binding to the Cl⁻ channel. The remaining hypothesis is that raising pH₀ prolonged I_{Cl<->} by slowing the removal of Ca²⁺ from near the plasma membrane after Ca²⁺ influx. This hypothesis was supported by experiments with the [Ca²⁺] indicator dye, fura-2, which directly demonstrated that raising pH₀ prolonged intracellular [Ca²⁺] transients that resulted from Ca²⁺ channel activation.

Which Ca²⁺ Buffering Mechanism Was Affected by Raising pH₀?

Three observations suggest that the site of pH₀ influence was the plasma membrane. First, the effect occurred very quickly, in ~100 ms or less (Fig. 4), and reversed within seconds. Second, effects remained when intracellular pH was heavily buffered (Fig. 2). This ruled out the possibility that the effects of raising pH₀ were due to changes in intracellular pH, which are known to influence Ca²⁺ buffering in the cytoplasm (Alvarez-Leefmans, Rink, and Tsien, 1981; Zucker, 1981). Third, the magnitude of I_{Cl<->} was not closely related to the [Ca²⁺] measured with fura-2 in the cell interior. In the experiment illustrated in Fig. 7, for example, the whole cell [Ca²⁺] measured after a fully decayed I_{Cl<->} in pH₀ 8 was higher than the whole cell [Ca²⁺] that corresponded with the peak I_{Cl<->} at pH 7.3. This would not be predicted if I_{Cl<->} magnitude were closely coupled to Ca²⁺ buffering by a deep intracellular buffering mechanism, such as uptake into a Ca²⁺-sequestering organelle.

Two active mechanisms for reduction of free intracellular [Ca²⁺], Na⁺/Ca²⁺ exchange and a Ca²⁺ ATPase, are known to reside in the plasma membrane of pituitary and other excitable cells (Dipolo and Beauge, 1983; Barros and Kaczorowski, 1984; Gill et al., 1984; Kaczorowski et al., 1984; Carafoli, 1991). The effects of raised pH₀ occurred in the absence of Na⁺, which rules out the possibility that Na⁺/Ca²⁺ exchange is involved in the effect. Ca²⁺ ATPases, however, display two forms of pH dependence, both of which are consistent with the hypothesis that raising pH₀ may have slowed the rate of Ca²⁺ reduction via inhibition of a Ca²⁺ ATPase. Activity of the sarcoplasmic reticulum Ca²⁺ ATPase, measured both as ATPase-mediated Ca²⁺ transport and ATPase-mediated ADP production, is reversibly inhibited at alkaline pH (Tate, Chu, McMillin-Wood, Van Winkle, and Entman, 1981; Bishop and Al-Shawi, 1988). Of perhaps greater relevance to this study, the plasma membrane Ca³⁺ ATPase appears to couple the extrusion of Ca³⁺ to countertransport of one or more H⁺ (Carafoli, 1991). As predicted for this type of mechanism, Ca³⁺ transport by the Ca²⁺ ATPase in human red blood cell membranes is inhibited by raising pH₀ in...
the presence of extracellular Ca\(^{2+}\) (Milanick, 1990). Similarly, removal of extracellular H\(^+\) by alkalinizing extracellular pH in our experiments would be expected to inhibit Ca\(^{2+}\) extrusion from the cell interior.

An alternative possibility is that raising pH\(_o\) prolonged I\(_{\text{Ca,at}}\) by indirectly delaying the diffusion of Ca\(^{2+}\) away from the plasma membrane into the cell interior. In theory, alkalinizing pH\(_o\) could change the capacity or affinity of plasma membrane molecules for Ca\(^{2+}\), which would then slow the exodus of Ca\(^{2+}\) from the local compartment (cf. Barish and Thompson, 1983; Sala and Hernandez-Cruz, 1990). This hypothesis, however, requires that the molecules involved are either integral or closely associated with the membrane, and that deprotonating an extracellular domain would alter Ca\(^{2+}\) binding to an intracellular binding site. At least one integral Ca\(^{2+}\) binding protein has been described (Kowarski, Cowen, Takahashi, and Shachter, 1987), and many others associate with membranes in the presence of Ca\(^{2+}\) (Smith and Dedman, 1990). For the time being, however, this argument is purely speculative. Given the demonstrated existence of a plasma membrane Ca\(^{2+}\) ATPase, and its inhibition by extracellular alkalinization, we favor the hypothesis that raising pH\(_o\) prolonged I\(_{\text{Ca,at}}\) via inhibition of a Ca\(^{2+}\) ATPase. An unequivocal test of this hypothesis must await the availability of a specific inhibitor (Note: Including vanadate in the pipette solution in whole cell recordings led to immediate and permanent activation of I\(_{\text{Ca,at}}\) and within seconds, cell death [Korn, S. J., and R. Horn, unpublished observations]).

**Conclusion**

Our data indicate that a pH-dependent, Na\(^+\)-independent Ca\(^{2+}\) buffering mechanism in the plasma membrane influences [Ca\(^{2+}\)] near ion channels after Ca\(^{2+}\) influx, on a time scale that is relevant to Ca\(^{2+}\)-dependent membrane currents and action potentials. While not unequivocal, present evidence suggests that this buffering mechanism is the plasma membrane Ca\(^{2+}\) ATPase. Based on kinetic data, the Ca\(^{2+}\) ATPase has been characterized as a high affinity, low capacity transporter, whereas the Na\(^+\)/Ca\(^{2+}\) exchanger is a low affinity, high capacity transporter (Dipolo and Beauge, 1983). Based on theoretical arguments, it has been proposed that the ATPase is relatively more important for the maintenance of resting [Ca\(^{2+}\)], whereas Na\(^+\)/Ca\(^{2+}\) exchange is more important for removing Ca\(^{2+}\) following large transients (Dipolo and Beauge, 1983; Gill et al., 1984). Together with a previous study (Korn and Horn, 1989), the data presented here suggest that in pituitary cells, both transporters can act to influence the duration of Ca\(^{2+}\)-dependent membrane currents produced by Ca\(^{2+}\) influx. Consequently, the activity of these transporters may influence not only Ca\(^{2+}\) removal following influx, but also Ca\(^{2+}\) influx via modulation of Ca\(^{2+}\)-dependent ion channel activity (Hume and Thomas, 1989; Korn et al., 1991a).

We thank Dr. Terry Reisine for quickly sending us AtT-20 cells, Mr. Arthur Bolden for unwavering dedication to quality cell culture maintenance, and Dr. William Chapple for his comments on the manuscript.

This work was supported in part by National Institutes of Health postdoctoral fellowship NS-08117 to Dr. Korn.

*Original version received 2 May 1991 and accepted version received 21 June 1991.*
REFERENCES


