Calcium-dependent Inactivation of the Dihydropyridine-sensitive Calcium Channels in GH_3 Cells

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ABSTRACT The inactivation of calcium channels in mammalian pituitary tumor cells (GH_3) was studied with patch electrodes under voltage clamp in cell-free membrane patches and in dialyzed cells. The calcium current elicited by depolarization from a holding potential of −40 mV passed predominantly through one class of channels previously shown to be modulated by dihydropyridines and cAMP-dependent phosphorylation (Armstrong and Eckert, 1987). When exogenous calcium buffers were omitted from the pipette solution, the macroscopic calcium current through those channels inactivated with a half time of ~10 ms to a steady state level 40–75% smaller than the peak. Inactivation was also measured as the reduction in peak current during a test pulse that closely followed a prepulse. Inactivation was largely reduced or eliminated by (*a*) buffering free calcium in the pipette solution to $<10^{-8}$ M; (*b*) replacing extracellular calcium with barium; (*c*) increasing the prepulse voltage from +10 to +60 mV; or (*d*) increasing the intracellular concentration of cAMP, either 'directly' with dibutyryl-cAMP or indirectly by activating adenylate cyclase with forskolin or vasoactive intestinal peptide. Thus, inactivation of the dihydropyridine-sensitive calcium channels in GH_3 cells only occurs when membrane depolarization leads to calcium ion entry and intracellular accumulation.

INTRODUCTION

The inactivation of voltage-activated calcium channels contributes to the regulation of intracellular calcium, an important second messenger, by preventing unrestricted entry of this ion during depolarization. In many cell types (reviewed by Eckert and Chad, 1984), the predominant calcium current, measured with conventional microelectrodes, inactivates when channel activation leads to calcium entry and accumulation. Thus, substituting barium for calcium, reducing calcium influx by depolarizing

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close to the reversal potential for calcium, or buffering intracellular calcium concentrations below 10^{-8} M all reduce or eliminate inactivation. In other cell types, calcium currents exhibit inactivation that appears to be voltage dependent as well as calcium dependent (Brown et al., 1981; Kass and Sanguinetti, 1984; Lee et al., 1985). This raises the possibility that for a single channel, these two mechanisms may not be mutually exclusive. Alternatively, the macroscopic current may reflect the activity of more than one class of channel, each of which inactivates by one of the two mechanisms (Deitmer, 1984, 1986).

In our laboratory, we are using patch-clamp techniques to study these mechanisms in cells of the pituitary tumor line GH_3. Recent patch-clamp studies on these cells (Armstrong and Matteson, 1985; Matteson and Armstrong, 1986; Cohen and McCarthy, 1987) as well as on other vertebrate cells (Carbone and Lux, 1984; Hess et al. 1984; Bean, 1985; Fedulova et al., 1985; Nilius et al., 1985; Nowycky et al., 1985), have revealed two classes of voltage-activated calcium channels. Channels of one class have a low threshold of activation (~ -40 mV) and inactivate in a voltage-dependent manner. Channels of the other class activate at more positive voltages (~ -20 mV) and are characterized by their sensitivity to dihydropyridines. Whether dihydropyridine-sensitive channels also inactivate, and if so by what mechanisms, has not been resolved. It is clear that these channels do not inactivate significantly when barium is the charge carrier, nor in whole cells dialyzed internally with exogenous calcium buffers (e.g., Matteson and Armstrong, 1986; Cohen and McCarthy, 1987). However, these experimental conditions are also ones that eliminate calcium-dependent inactivation. In fact, the presence of such a mechanism is suggested by the similarity between these channels and the calcium-inactivating channels in molluscan neurons. Both lose activity or 'wash out' when the cytoplasmic side of the membrane is exposed to minimal saline solutions (Byerly and Hagiwara, 1982; Fenwick et al., 1982; Cavalie et al., 1983; Matteson and Armstrong, 1986), an effect reversed by cAMP-dependent phosphorylation (Doroshenko et al., 1984; Chad and Eckert, 1986; Armstrong and Eckert, 1987).

Considering this similarity and that previous studies on these channels did not optimize conditions to detect calcium-dependent inactivation, we investigated whether the dihydropyridine-sensitive channels inactivate and whether they do so in a calcium-dependent manner. We report here that with calcium as the charge carrier, and without including exogenous calcium buffers in the pipette solution, inactivation occurs when channel activation leads to calcium ion entry and accumulation. Furthermore, we find no evidence for direct effects of voltage on inactivation aside from those on calcium entry. Thus we conclude that the surface membrane of GH_3 cells has calcium channels of two classes, each of which inactivates by a separate mechanism, one dependent on calcium, the other on voltage. Some of the results have been published previously (Kalman et al., 1987*a*, *b*).

METHODS

GH_3 cells, a mammalian cell line isolated from a rat pituitary tumor (Tashjian, 1979), were obtained from American Type Culture Collection (Rockville, MD). Cells were grown in plastic tissue culture flasks (Corning Glass Works, Corning, NY) containing Ham's F10 supplemented with 15% horse serum, 2.5% fetal calf serum, penicillin (50 μg/ml), and streptomycin

(50 U/ml) (GIBCO, Grand Island, NY) and incubated in a 5% CO_2/95% air mixture at 37°C. Cells were fed every 3–4 d. For electrophysiological experiments, cells were mechanically dissociated and plated onto collagen-coated glass coverslips (No. 1, 12-mm diam; Fisher Scientific Co., Pittsburgh, PA). Each experiment was begun on a new coverslip of cells, and only cells grown on coverslips for longer than 1 d, but not more than 3 d were used in the experiments described here.

Cells (<20 μm in diameter) were voltage clamped in the whole-cell configuration and cell-free membrane patches were formed in the outside-out configuration as described by Hamill et al. (1981). Low resistance (<3 MΩ) patch pipettes were manufactured from Corning glass 7052, 7040, or 8161 (Garner Glass Co., Pomona, CA). Some of the results reported here were obtained with pipettes made from 8161 glass because they frequently made high resistance seals. Pipettes made of this glass have been reported to alter the kinetics of potassium currents in whole-cell recordings (Cota and Armstrong, 1988). We have observed that unitary barium currents are partially blocked in cell-attached recordings with 8161 glass (unpublished observations). However, in the whole-cell and outside-out patch recordings reported here, the kinetics of calcium currents appeared to be unaffected by this glass because identical results were obtained with the other glasses.

Macroscopic currents through calcium channels were isolated from currents through voltage-activated sodium and potassium channels as described by others (Hagiwara and Ohmori, 1982; Dubinsky and Oxford, 1984; Matteson and Armstrong, 1984, 1986). In most experiments, including those comparing barium and calcium currents, the bath contained either 25 mM $CaCl_2$ or 25 mM $BaCl_2$, 106 mM tetraethylammonium chloride (TEA-Cl), 10 mM HEPES (pH 7.2), 1 mM $MgCl_2$, 5 mM KCl, 19 mM glucose, and 2 μM tetrodotoxin (TTX) (Sigma Chemical Co., St. Louis, MO), and the pipette was filled with a minimal saline solution consisting of 140 mM CsCl, 10 mM HEPES (pH 7.2), and 2 mM $MgCl_2$. In earlier experiments, the bath solution contained 130 mM NaCl, 10 mM $CaCl_2$, 10 mM HEPES (pH 7.2), 2 mM $MgCl_2$, 5 mM KCl, and 2 μM TTX. Where indicated, the free calcium concentration in the pipette solution was buffered to 10^{-8} M with 5 mM ethyleneglycol-bis-(β-aminoethylether) *N,N,N',N'*-tetraacetic acid (EGTA; Sigma Chemical Co.) or 5 mM bis-(*o*-aminophenoxy)-ethane-*N,N,N'N'* -tetraacetic acid (BAPTA, tetrasodium salt; Molecular Probes, Junction City, OR). For single-channel recordings from outside-out patches, the bath solution contained 90 mM $BaCl_2$, 20 mM TEA-Cl, 10 mM HEPES (pH 7.2), and 2 μM TTX, and the pipette was filled with 125 mM CsCl, 2 mM $MgCl_2$, 5 mM EGTA (pCa = 8), and 40 mM HEPES (pH 7.2). In some experiments, the pipette solution was supplemented with 2 mM ATP-Mg and 2 μg/ml purified catalytic subunit of cAMP-dependent protein kinase (cf., Armstrong and Eckert, 1987). Experiments were carrried out at room temperature (20–23°C).

Currents elicited every 6 s by depolarizing voltage steps were low-pass filtered at 2 kHz with an 8-pole Bessel filter, and digitized at 10 kHz for storage and analysis on an Indec 11-23 computer system (Indec Systems, Sunnyvale, CA). Linear leakage and uncancelled capacitive currents were subtracted digitally from the traces by a $P/-4$ procedure. In this procedure the scaled average of currents elicited by four hyperpolarizing pulses of amplitude $P/4$, where P is the difference between the test and holding potential, was added to the test-pulse current. The subtraction procedure did not affect the kinetics of the calcium current during the step because leakage currents during the hyperpolarizing step were flat. Moreover, leakage currents were always <5% of the current elicited by an equivalent depolarizing step.

Nimodipine (Miles Laboratories, New Haven, CT), a dihydropyridine antagonist of calcium channels in pituitary tumor cells (Armstrong and Eckert, 1987; Cohen and McCarthy, 1987), and forskolin (Calbiochem, San Diego, CA), an activator of adenylate cyclase (Seamon et al., 1981), were dissolved in dimethylsulfoxide (DMSO) before their addition to the bath solu-

tion. The final concentration of DMSO in the bath was always <0.1%, a concentration which produced no detectable effects on the calcium current. Vasoactive intestinal peptide (Boehringer Mannheim Biochemicals, Indianapolis, IN) and dibutyryl-cAMP (Sigma Chemical Co.) were dissolved in the bath solution.

RESULTS

Separation of Two Classes of Calcium Channels

To study inactivation of dihydropyridine-sensitive channels in whole cell experiments, the current through those channels was isolated from that which went through dihydropyridine-insensitive channels. To do this, we took advantage of the finding that insensitive channels undergo voltage-dependent inactivation at holding potentials more positive than −80 mV (Matteson and Armstrong, 1986; Cohen and McCarthy, 1987). We chose −40 mV because it was the most positive holding potential below the activation threshold of either class of calcium channel, and we measured the extent to which dihydropyridine-insensitive channels inactivated at that holding potential.

With barium as the charge carrier, we determined the relative contribution of the two types of calcium channels to the currents elicited from different holding potentials. This is illustrated in Fig. 1. In the whole-cell records in Fig. 1 *A*, the current elicited from a holding potential of −40 mV (trace a_1) decreased by ~90% (i.e., washed out; trace b_1) within 10 min of establishing the whole-cell recording with the minimal saline solution in the pipette. Under these conditions, wash-out was irreversible (but see Chad and Eckert, 1986; and Armstrong and Eckert, 1987), that is, no further current could be elicited from −40 mV despite holding at −80 mV for several minutes. In addition, the current elicited from −40 mV was almost completely blocked by 1 μM nimodipine, a dihydropyridine antagonist (e.g., Fig. 2 *B*). In contrast, depolarization from a holding potential of −80 mV elicited an additional transient current (Fig. 1, trace a_2) that did not wash out (trace a_2 ~ trace a_1 + trace b_2). The wash-out resistant component (trace b_2) inactivated almost completely at a holding potential of −40 mV (trace b_1). Similar separation was obtained whether barium or calcium was the charge carrier. On average (n = 18), only 8% of the current elicited from ~40 mV was resistant to wash-out or nimodipine block.

At the single-channel level, we observed two classes of unitary barium currents during depolarizations of outside-out patches exposed to a similar minimal pipette solution. This is illustrated in Fig. 1 *B*. Channels of one class (three traces, a_2 and b_2) have a small conductance in 90 mM barium (~10 pS; measured previously by Armstrong and Eckert, 1987) and a threshold of activation near −40 mV. Channels of the other class (traces a_1) have a larger conductance in barium (~25 pS; measured previously) and a higher threshold of activation, near ~20 mV. Channels of both classes are blocked by 2 mM cobalt (not shown), but not by tetrodotoxin, and have extrapolated reversal potentials more positive than +40 mV. 6 min after forming the outside-out patch with a minimal saline solution lacking reagents to support cAMP-dependent phosphorylation, the larger conductance channel stopped opening in response to depolarization (traces b_1). In contrast, the smaller conductance channel continued to respond to depolarization (traces b_2) as long as the seal

remained intact (~20 min). In the absence of the larger conductance channel openings, the smaller conductance channels (traces b_2) undergo nearly complete steady state inactivation at the holding potential of −40 mV (traces b_1). Together, these results demonstrate that the macroscopic current elicited from −40 mV is primarily

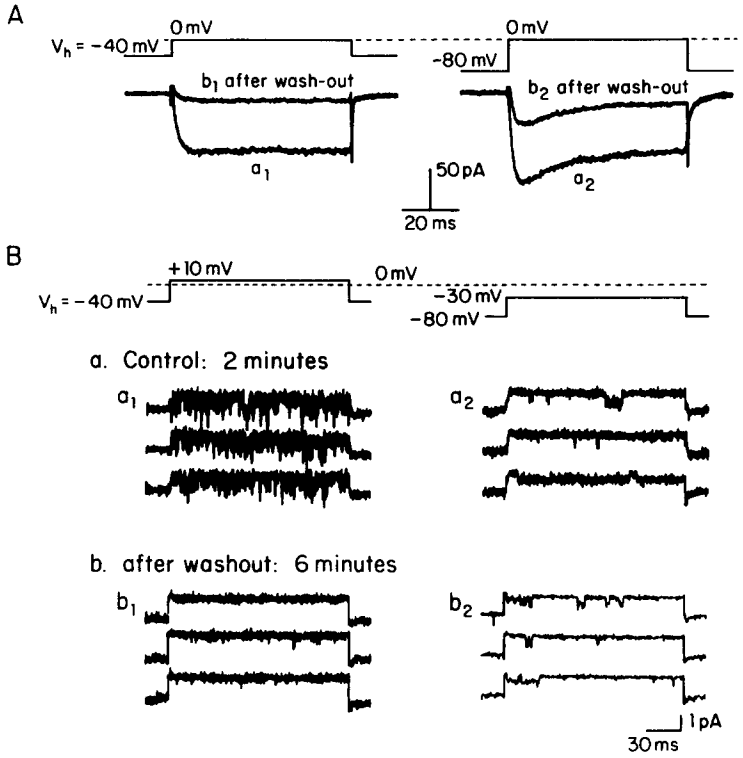

FIGURE 1. Whole-cell and single-channel barium currents before and after wash-out. Voltage steps are shown above current records. (*A*) Whole-cell barium currents elicited by a depolarizing step to 0 mV from a holding potential of −40 mV (trace a_1) washed out almost completely (~90%) after 10 min (trace b_1). In contrast, the current elicited from −80 mV (trace a_2) only partially washed out (~67%; trace b_2). Note that the wash-out–resistant current elicited from −80 mV (trace b_2) was almost completely inactivated at a holding potential of −40 mV (trace b_1) because little current could be elicited by the step to 0 mV. (*B*) Unitary barium currents in an outside-out patch. (*B*, a_1 and a_2) Current records taken at the indicated voltages (top traces) 2 min after excising the patch. (*B*, b_1 and b_2) The response to the same voltage steps as *a*, 4 min later. Records were filtered at 2 kHz except those of b_2, which were filtered at 1 kHz.

due to the high-threshold, large-conductance calcium channels that wash out entirely in minimal saline solutions.

Dihydropyridine-sensitive Channels Inactivate when Calcium Carries the Current

With calcium as charge carrier and with exogenous calcium buffers omitted from the pipette solution, currents elicited by steps from −40 mV relaxed during sus-

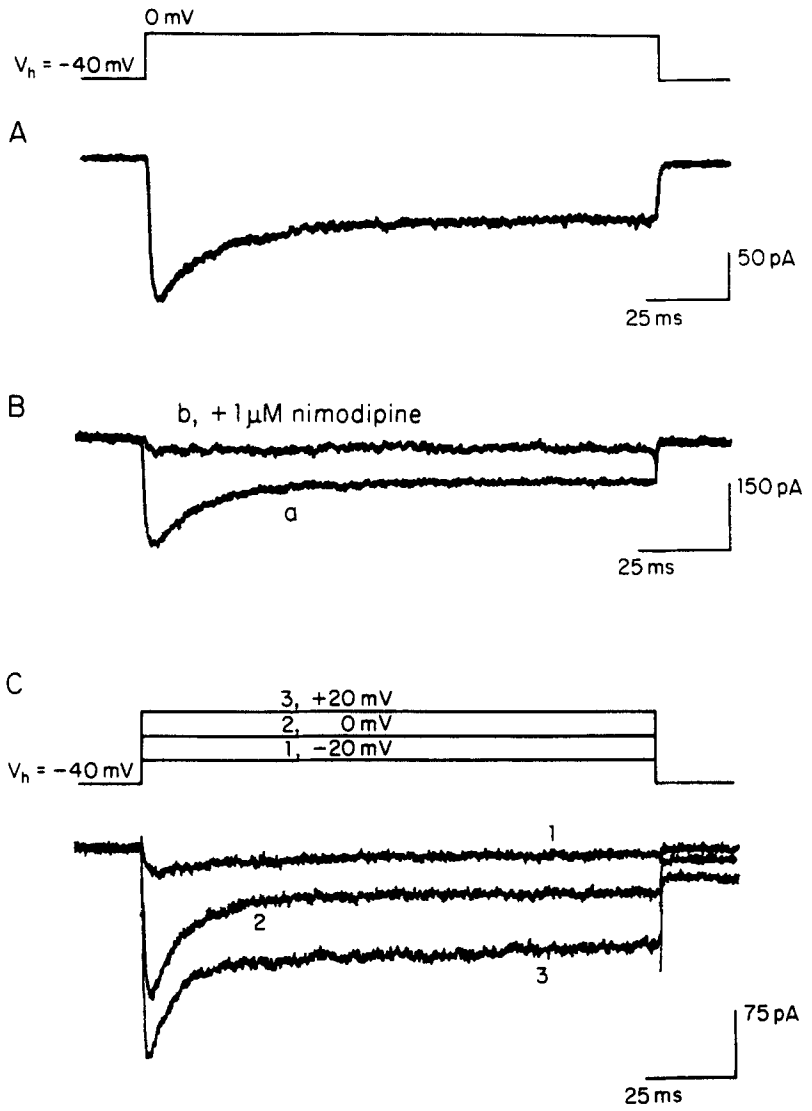

FIGURE 2. Wash-out–sensitive calcium currents inactivate when no calcium buffers are included in the pipette solution. (*A*) Calcium current elicited by a step from −40 to 0 mV. (*B*) Calcium current elicited by a step from −40 to 0 mV (trace *a*) is blocked by 1 μM nimodipine (trace *b*). (*C*) Calcium currents elicited by voltage steps (top superimposed traces) to −20 mV (trace *1*), 0 mV (trace *2*), and +20 mV (trace *3*) from a holding potential of −40 mV. Note the long-lasting inward tail currents seen after the steps to 0 mV and +20 mV, which may result from nonselective cation channels or chloride channels activated by calcium entry during the step. Also note the marked inactivation in *B* despite the absence of the long-lasting inward tail current. The pipette solution contained cesium chloride but no calcium buffers.

tained depolarizations. This is shown in Fig. 2 *A*. During the step to 0 mV, the current rose to a peak in ~5 ms, then decreased with a half time of ~10 ms to a steady state level ~57% smaller than the peak. In general, calcium currents elicited by depolarizations from −40 to 0 mV relaxed by 40–75% (60% on average, $n = 35$).

Several experiments were done to show that this relaxation results from inactivation of calcium channels rather than activation of voltage- or calcium-dependent outward currents. As illustrated in Fig. 2 *B*, the inward current was almost completely blocked by 1 μM nimodipine (92% on average; $n = 12$) or 2 mM cobalt (98% on average; $n = 3$; not shown), and revealed no underlying voltage-activated outward currents. Also, replacing potassium with cesium in the pipette and adding TEA to the bath eliminated contamination by calcium-activated potassium channels (Ritchie, 1987). Finally, Fig. 2 *C* illustrates that outward current through calcium-activated channels that are insensitive to block by TEA or cesium, such as the nonselective cation channel (Yellen, 1982) or the calcium-activated chloride channel (Korn and Weight, 1987), cannot account for the relaxation. Here, the calcium current relaxes at 0 mV (trace *2*), which is near the reversal potential in our solutions for both the nonselective cation channels and the chloride channels. In addition, the calcium current does not continue to increase during smaller steps to negative potentials (trace *1*) where current through these other channels would be inward. Although current through those channels may contribute to the slowly decreasing inward tail current observed after repolarization to −40 mV, significant inactivation of the calcium current is also observed in cases where tail current contamination is negligible (e.g., Fig. 2 *B*). However, in cases where either the nonselective cation channels or the chloride channels are present, they may contribute to the relaxation of the inward current at positive potentials (e.g., trace *3*). Therefore, inactivation was studied in steps to 0 mV whenever possible.

Inactivation Depends on Calcium Ion Entry and Accumulation

To determine whether inactivation of dihydropyridine-sensitive channels is calcium dependent, we measured the amount of inactivation under three experimental conditions: (*a*) in cells with or without exogenous calcium buffers in the pipette solution, (*b*) when barium was substituted for calcium as the charge carrier, and (*c*) when calcium influx was reduced by depolarizing close to the reversal potential for calcium.

The effects of exogenous calcium buffers in the pipette solution are illustrated in Fig. 3. In all cells tested ($n = 15$), the current elicited from −40 mV in the absence of exogenous calcium buffers inactivated to a steady state level ranging between 40 and 75% of the peak current (e.g., 75%, Fig. 3 *A*). In contrast, when EGTA (5 mM; pCa = 8) was included in the pipette solution, the currents never inactivated by >30% in 62% of the cells tested ($n = 24$; e.g., Fig. 3 B_1), and did not inactivate at all in the remaining 38% (e.g., Fig. 3 B_2). The residual inactivation in the presence of EGTA probably resulted from inefficient buffering of rapid calcium transients by EGTA (Neher and Marty, 1985). When BAPTA (5 mM; pCa = 8; Tsien, 1980) was used to buffer calcium, inactivation was abolished in steps to 0 mV in the majority of cells ($n = 9/11$; e.g., Fig. 3 *C*).

Inactivation of the macroscopic current through dihydropyridine-sensitive chan-

nels did not occur when barium carried the current, even in the absence of exogenous buffers. Replacing extracellular calcium with equimolar barium caused the peak current to increase by up to threefold but did not produce any inactivation in voltage pulses lasting 160 ms (Fig. 4 *A*). A small relaxation was observed during longer steps (1,600 ms) to very positive potentials (e.g., +40 mV; Fig. 4 *B*, trace *2*), even when BAPTA (5 mM) was included in the pipette solution to reduce intracellular accumulation of barium. However, there was no inactivation of barium currents

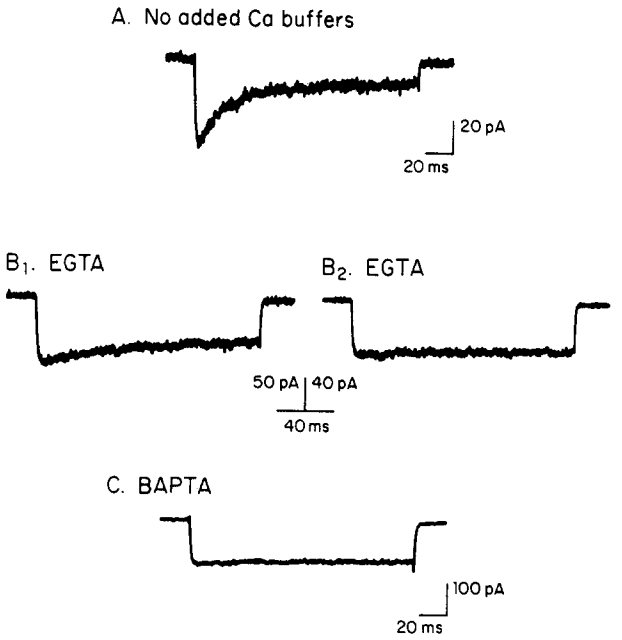

FIGURE 3. Exogenous calcium buffers significantly slow or prevent inactivation. (*A*) Calcium current elicited by a step from a holding potential of −40 to 0 mV in the absence of exogenous calcium buffers. Current inactivated by ~75%. (*B*) Effects of 5 mM EGTA in the pipette solution. Currents recorded in other cells from the same culture dish as in *A* showed substantially less inactivation (~30%; B_1) or no inactivation at all (B_2). (*C*) Effects of 5 mM BAPTA, a more efficient buffer of rapid calcium transients than EGTA.

during long pulses to less positive voltages (e.g., 0 mV; Fig. 4 *B*, trace *1*), during which outward current contamination was less likely.

Further support for this lack of inactivation in barium can be seen in the single-channel records from cell-free patches in the outside-out configuration (Fig. 4, *C* and *D*). In these experiments, the dihydropyridine-sensitive channels were prevented from washing out by including Mg-ATP and the purified catalytic subunit of the cAMP-dependent protein kinase in the pipette solution (Armstrong and Eckert, 1987). Fig. 4 *C* shows five representative traces of the unitary barium currents recorded during 160 ms depolarizations from −40 to 0 mV, and an ensemble average of all the records from that patch (bottom trace); with barium as the charge

carrier the probability of channel opening did not diminish during depolarization. Fig. 4 *D* shows the same result on a longer time scale and at a more positive voltage. In this figure, five successive traces were taken at 3-s intervals during 80-ms steps from a holding potential of +40 down to 0 mV (see top trace for voltage protocol).

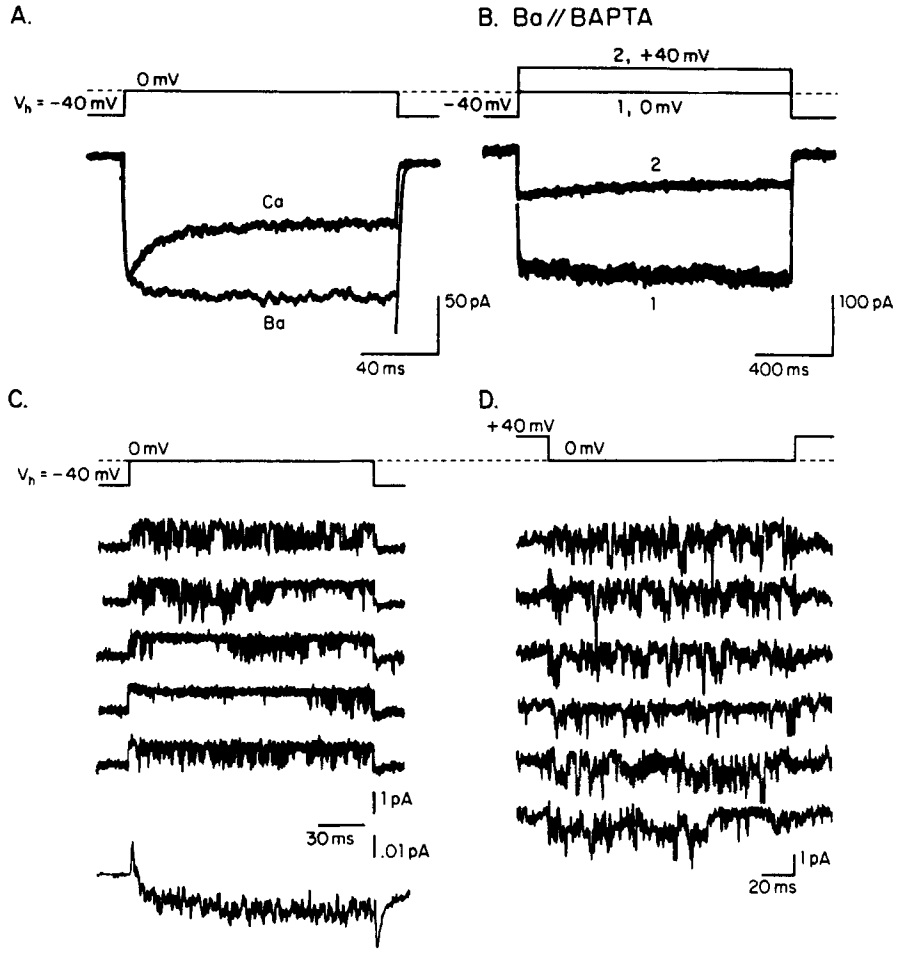

FIGURE 4. Effects of barium on inactivation. (*A*) Whole-cell calcium current inactivates, but barium current does not (voltage steps shown in top trace). (*B*) Barium current elicited by long step (1,600 ms) to 0 mV does not inactivate (trace *1*). However some relaxation is evident in the current elicited by a step to +40 mV (trace *2*). BAPTA (5 mM, pCa = 8) was present in the pipette solution in *B*, but not in *A*. (*C* and *D*). Unitary barium currents through calcium channels maintained by 2 mM ATP-Mg and 1 μg/ml catalytic subunit of cAMP-dependent protein kinase in an outside-out patch do not inactivate. (*C*) Above: selected records of unitary barium currents during voltage steps from −40 to 0 mV. Below: ensemble average of 110 records taken at 3 intervals from the same patch. (*D*) Consecutive records from a different outside-out patch than *C*, in which the membrane was stepped to 0 mV for 80 ms from a holding potential of +40 mV. Records were taken consecutively at 3-s intervals.

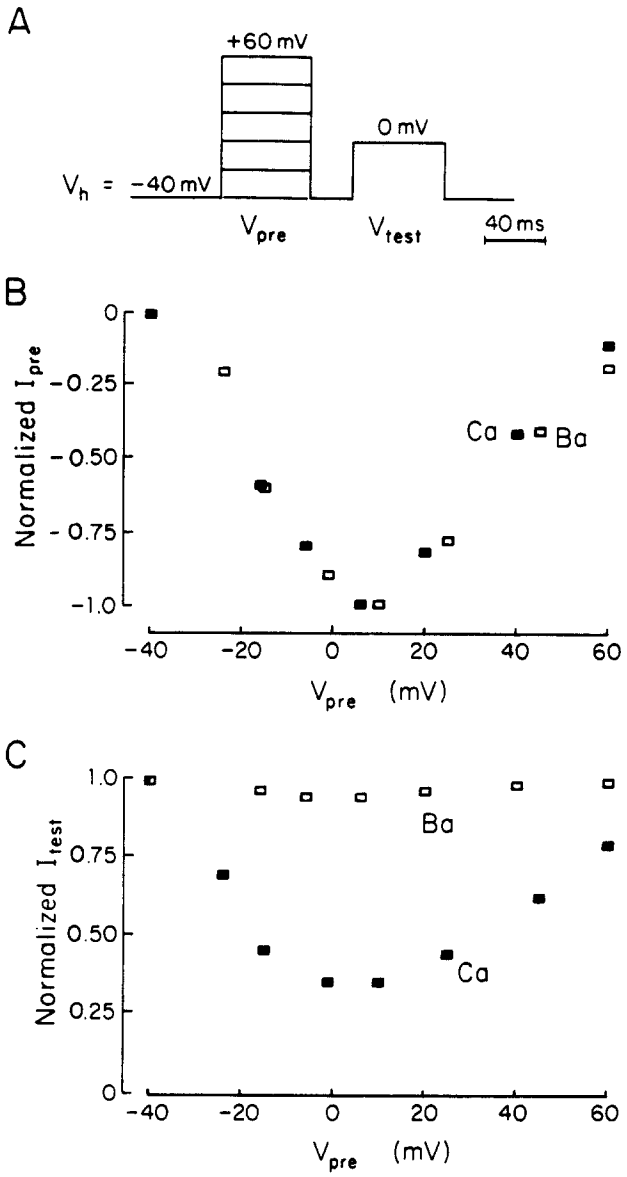

FIGURE 5. Inactivation of dihydropyridine-sensitive current (whole-cell recording) depends on voltage only to the extent that calcium entry depends on voltage. (*A*) Paired-pulse protocol: calcium or barium currents were elicited from a holding potential of −40 mV (two different cells). A 60-ms prepulse of various amplitudes between −40 and +60 mV was followed after 20 ms by a 60-ms test pulse to 0 mV. (*B*) Peak current recorded during the prepulse is plotted as a function of the prepulse voltage. The currents are normalized to the maximum current for each charge carrier. Both calcium (■) and barium (□) currents peaked near +10 mV. As the prepulse voltage is increased from +10 to +60 mV, the prepulse current declines because of the decrease in driving force. (*C*) The peak current during the test pulse is plotted as a function of the prepulse voltage. The test-pulse current is normalized to the current recorded in the absence of a prepulse. The calcium current (■) during the test pulse is maximally suppressed at potentials where calcium entry during the prepulse is maximal. Barium current (□) is not suppressed by prepulses to any voltage.

Although the membrane was held continuously at +40 mV between steps, activity at 0 mV was robust. Together, these results provide no evidence for a voltage-dependent component of inactivation.

In addition, no voltage-dependence is indicated by the double-pulse experiments shown in Fig. 5. Here, inactivation was measured as the reduction in peak current during a test pulse that followed a prepulse. We used the paired-pulse protocol (Eckert and Tillotson, 1981) illustrated in Fig. 5 *A* to investigate whether inactivation occurred as a result of calcium entry during the prepulse or as a result of the prepulse depolarization itself. The peak amplitudes of calcium (■, Fig. 5 *B*) and barium currents (□, Fig. 5 *B*) elicited by the prepulse in two different cells are plotted in Fig. 5 *B* as a function of the prepulse voltage. They show the typical current-voltage relation for the dihydropyridine-sensitive calcium channels: a threshold near −20 mV, a maximum near +10 mV, and an extrapolated reversal potential near +70 mV. As the prepulse voltage was increased from −40 to +10 mV, calcium entry during the prepulse increased (Fig. 5 *B*), and the calcium current during the unchanging test pulse to 0 mV was correspondingly suppressed (■, Fig. 5 *C*). When the prepulse and the test pulse were equal (0 mV in this case), the peak current observed during the test pulse was approximately equal to the inactivated current amplitude at the end of prepulse. Thus, the inactivation produced by the prepulse showed little or no recovery during the 20-ms delay between the two pulses, and both the single- and paired-pulse protocols yielded equivalent measures of inactivation.

As the prepulse voltage was increased further from +10 to +60 mV (Fig. 5 *B*), less suppression occurred and the amplitude of the calcium current during the test pulse increased. This reduction in the amount of inactivation produced by the prepulse paralleled the decrease in calcium entry during the prepulse in the same voltage range (compare Fig. 5 *B* with *C*). In contrast, when barium replaced calcium, currents during the prepulse did not inactivate, and little or no suppression during the test pulse was observed at any prepulse voltage (□, Fig. 5 *C*). If inactivation were voltage dependent, higher prepulse voltages would have enhanced, not diminished, inactivation of the test-pulse current carried by either calcium or barium. Such behavior is observed for isolated dihydropyridine-insensitive currents in steps from −80 mV (not shown). Thus, inactivation of dihydropyridine-sensitive channels appears voltage dependent only to the extent that calcium entry is voltage dependent.

Raising Internal cAMP Slows Calcium-dependent Inactivation

The mechanism by which calcium produces inactivation is unknown. However, several previous observations have indicated a role for phosphorylation/dephosphorylation reactions in the control of calcium channel activity in GH_3 cells (Armstrong and Eckert, 1987; Chad et al., 1987; Armstrong and Kalman, 1988). Therefore, we tested the effect of increasing intracellular cAMP on the dihydropyridine-sensitive calcium current (Fig. 6). As seen in Fig. 6 *A*, even though the current was washing out, both the peak (■) and the steady state (□) current increased when a membrane-permeable analogue of cAMP (dibutyryl-cAMP, 2 mM) was added to the bath. These effects cannot have resulted simply from a shift in the current-voltage rela-

tion because similar effects were observed at all voltages (not shown). In all cells tested, the percentage increase in steady state current (e.g., 100% in Fig. 6 *A*) was substantially larger than the increase in peak current (e.g., 35% in Fig. 6 *A*). Thus, the net effect of dibutyryl-cAMP was to decrease the effectiveness by which calcium produced inactivation. The continued wash-out of peak current in the presence of

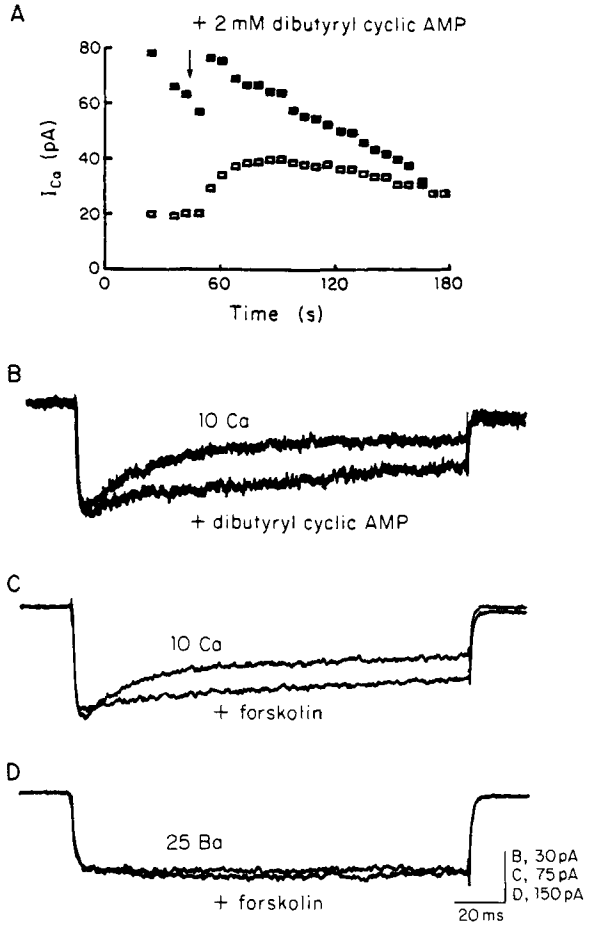

FIGURE 6. Increasing intracellular cAMP increases peak and steady state currents and slows calcium-dependent inactivation. (*A*) The peak (■) and steady state (□) amplitudes of calcium currents elicited by depolarizing steps from −40 to 0 mV are plotted as a function of time after establishing the whole-cell recording. Although the current was washing out, the addition of dibutyryl-cAMP increased the peak current by 35% and the steady state current by 100%. The larger percentage increase of the steady state current indicates that inactivation slowed. (*B*) Inactivation of equal amplitude calcium currents elicited by steps from −40 to 0 mV is slower after the addition of 2 mM dibutyryl-cAMP to the bath. The increase in the peak current is not evident because the current had washed out further by the time the effect on inactivation had developed fully. (*C*) Addition of 50 μM forskolin to the bath slows the inactivation of calcium currents elicited by steps to 0 mV from a holding potential of −40 mV. The step to 0 mV minimized contamination of the current during the pulse by chloride or nonselective cation channels. (*D*) In contrast, the addition of 50 μM forskolin has little effect on currents carried by barium, which do not inactivate.

dibutyryl-cAMP allowed the effect of this substance on inactivation to be seen more clearly. In Fig. 6 *B*, current traces with roughly equal peak amplitudes in steps to 0 mV, recorded before and after dibutyryl-cAMP application, are superimposed. Similar effects on inactivation were observed at other voltages (−20 and +20 mV). The decrease in inactivation produced by dibutyryl-cAMP contrasts sharply with the

effect observed when the peak current is increased by other means, such as voltage or higher extracellular calcium concentrations. In those cases the larger calcium current inactivates more rapidly, presumably because a larger calcium influx results in a higher intracellular concentration (cf., Chad et al., 1984).

Forskolin (Seamon et al., 1981) and vasoactive intestinal peptide both stimulate adenylate cyclase and increase cAMP levels in GH_3 cells (Gourdji et al., 1979; Drust et al., 1982; Sobel and Tashjian, 1983), and their effects were very similar to those of dibutyryl cAMP. Both compounds, forskolin at 50 μM (Fig. 6 *C*) and vasoactive intestinal peptide at 100 nM (not shown) increased the peak calcium current by up to 30% and markedly slowed the rate of inactivation. In contrast to its effect on calcium currents, forskolin had no significant effect on currents carried by barium ($n = 9$; Fig. 6 *D*). It is unlikely that an increase in the barium current was masked by wash-out because in our experiments barium currents washed out more slowly than calcium currents (not shown). Instead, the lack of an effect on barium currents is probably due to the absence of calcium-dependent inactivation in barium.

DISCUSSION

Isolation of the Dihydropyridine-sensitive Calcium Channels

These experiments demonstrate that the calcium current through one class of channels, which are sensitive to dihydropyridines and cAMP-dependent protein phosphorylation, can be studied in virtual isolation by holding the membrane at −40 mV. These channels resemble those called HVA, L, or FD, on the basis of their high-activation threshold, large conductance, and fast-deactivation kinetics, respectively, in many vertebrate cell types including GH_3 (Carbone and Lux, 1984; Bean, 1985; Fedulova et al., 1985; Nilius et al., 1985; Nowycky et al., 1985; Matteson and Armstrong, 1986). At −40 mV, other voltage-activated calcium channels, which resemble those designated LVA, T, or SD, inactivate almost completely (Fig. 1). In previous studies on GH_3 cells, tail-current kinetics recorded with patch pipettes filled with EGTA-containing solutions were used to estimate the relative contribution of the two types of calcium channels to the current elicited from different holding potentials (Matteson and Armstrong, 1986; Cohen and McCarthy, 1987). However, when we omitted calcium buffers from the pipette solution in order to investigate the calcium-dependence of inactivation, the tail currents were often contaminated by calcium-activated channels that were insensitive to block by cesium and TEA (Fig. 2 *C*). Therefore, we estimated the degree of separation at −40 mV by measuring how much current washed out. By those criteria, 92% of the current, on average, elicited by steps from −40 mV, passed through dihydropyridine-sensitive channels (Fig. 1 *A*; see also single-channel recordings in Fig. 1 *B*).

Inactivation Is Calcium-dependent

Several observations show that inactivation of dihydropyridine-sensitive channels is calcium dependent and only depends on voltage insofar as calcium entry does. First, inactivation was eliminated by buffering intracellular calcium to $\sim 10^{-8}$ M with 5 mM BAPTA (Fig. 3 *C*). Like others (Brown et al., 1981; Matteson and Armstrong, 1984; Bean, 1985), we found that EGTA was less effective than BAPTA at suppress-

ing inactivation (Fig. 3 *B*). Yet this cannot be taken as evidence for an additional voltage-dependent component of inactivation because EGTA, unlike BAPTA, is not an effective buffer of fast calcium transients (Tsien, 1980; Byerly and Moody, 1984; Neher and Marty, 1985). Thus, it is likely that any residual inactivation reflects inadequate buffering of such transients by EGTA. Second, inactivation was eliminated in double-pulse experiments by setting the prepulse to potentials where little or no calcium entered (Fig. 5). Finally, when barium carried the current, neither macroscopic nor unitary currents inactivated during steps to 0 mV (Figs. 4 *A* and *C*), even when the membrane was stepped to 0 mV for prolonged periods (1,200 ms; Fig. 4 *B*, trace *1*). A small relaxation of macroscopic barium currents was observed during prolonged steps to more positive potentials (e.g., +40 mV; Fig. 4 *B*, trace *2*). Similar relaxations in heart muscle cells have been interpreted as evidence for either a joint dependence of inactivation on voltage and calcium (Kass and Sanguinetti, 1984, Lee et al., 1985) or for contamination by outward current (Mentrard et al., 1984; Becham and Pott, 1985). There is no indication in our single-channel experiments of steady state inactivation over tens of seconds at a holding potential of +40 mV (Fig. 4 *D*). It is likely, therefore, that the slow relaxations in our experiments are due to contamination by an outward current, which is insensitive to block by cesium and TEA.

In contrast, our results with dihydropyridine-insensitive channels indicate that they inactivate in a strictly voltage-dependent manner, as has been reported previously by others (Matteson and Armstrong, 1986), and do not indicate any role for intracellular calcium. After wash-out, the barium currents elicited by steps from −80 mV inactivate just as effectively as calcium currents (e.g., Fig. 1 *A*, trace b_2), and undergo steady state inactivation at −40 mV, a voltage below the activation threshold for either class of calcium channels.

Thus, calcium channels of both classes inactivate during sustained depolarization, one in response to voltage and the other in response to the intracellular accumulation of calcium ions that accompanies channel activation. These two independent mechanisms of inactivation seem well suited to the different roles in cellular physiology postulated for the two types of calcium channels (Armstrong and Matteson, 1985); sustained depolarization produces inactivation of the channels that appear better adapted to generate spontaneous electrical activity, and calcium accumulation produces inactivation of the channels better adapted to inject calcium into the cell.

Molecular Mechanisms for Calcium-dependent Inactivation

Although the results presented above indicate that dihydropyridine-sensitive channels undergo calcium-dependent inactivation, they do not suggest a molecular mechanism for that process. A number of connections have been noted between calcium channel activity and cAMP-dependent phosphorylation (Reuter, 1983; Kostyuk, 1984; Byerly and Hagiwara, 1988). Neither the dihydropyridine-sensitive channels in GH_3 cells nor the predominant calcium channels in molluscan neurons activate in response to membrane depolarization under conditions that preclude cAMP-dependent phosphorylation (Chad and Eckert, 1986; Eckert et al., 1986; Armstrong and Eckert, 1987; Chad et al., 1987). Moreover, increasing the intracel-

lular concentration of cAMP has been reported to slow calcium channel inactivation in several cell types (Doroshenko et al., 1982; Bean et al., 1984; Bean, 1985; Chad and Eckert, 1986; Eckert et al., 1986; Chad et al., 1987). Here, we also found that raising cAMP levels reduced the effectiveness of calcium in producing inactivation (Fig. 6). Together, these observations suggest that calcium-dependent inactivation may be a general property of calcium channels regulated by cAMP-dependent phosphorylation.

Eckert and Chad (1984) proposed that inactivation results from dephosphorylation of the channel or a closely associated molecule in the membrane by an endogenous calcium-dependent phosphatase. They found that perfusing molluscan neurons internally with calcineurin, a calcium- and calmodulin-dependent phosphatase purified from mammalian brain (Klee et al., 1979; Stewart et al., 1982), accelerates inactivation in a calcium-dependent manner (Chad and Eckert, 1986). While GH_3 cells contain calcineurin (Farber et al., 1987), it remains to be determined whether it or another phosphatase participates in the inactivation process. Alternatively, inactivation might result from calcium binding directly to the channel. In this view, dephosphorylation at some other site might alter allosterically the efficacy of calcium action. Elucidating the precise role of calcium and calcineurin, or other endogenous phosphatases, in calcium channel gating will require the measurement of channel activitity under conditions in which phosphatase activity is blocked directly.

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