Calcium Channel Currents
in Pars Intermedia Cells
of the Rat Pituitary Gland

Kinetic Properties and Washout
During Intracellular Dialysis

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ABSTRACT   Ca channel currents in primary cultured pars intermedia cells were studied using whole-cell recording with patch pipettes. Experiments were carried out at 18–21°C in cells internally dialyzed with K-free, EGTA-containing solutions and in the presence of 10 mM Ca or 10 mM Ba in the external solution. Ca and Ba currents depended on the activity of two main populations of channels, SD and FD. With Ca as the charge carrier, these two populations differed in their closing time constants at −80 mV (SD, 1.8 ms; FD, 110 μs), apparent activation levels (SD, −40 mV; FD, −5 mV), half-maximal activation levels (SD, +5 to +10 mV; FD, +20 to +25 mV), half-times of activation at +20 mV (SD, 2.5–3.5 ms; FD, 1.0–1.3 ms), and time courses of inactivation (SD, fast; FD, slow). Functional FD channels were almost completely lost within 20–25 min of breaking into a cell, whereas SD channels retained most of their functional activity. In addition, the conductance-voltage curve for FD channels shifted ~15 mV toward more negative membrane potentials within 11–14 min under whole-cell recording. At that time, 60–70% of the FD channel maximum conductance was lost. However, the conductance-voltage curve for SD channels shifted <5 mV within 25 min. The addition of 3 mM MgATP and 40 μM GTP to the internal solution slowed down the loss of FD channels and prevented the shift in their activation curve. It was also found that the amplitude of the current carried by FD channels tends to increase as a function of the age of the culture, with no obvious changes in the kinetic properties of the channels or in SD channel activity.

INTRODUCTION

Mammalian adenohypophysial cells are able to generate spontaneous or evoked spike-like depolarizations. This property has been demonstrated in pars inter-

media cells (Douglas and Taraskevich, 1978), in unidentified pars distalis cells (Taraskevich and Douglas, 1977; Ozawa and Sand, 1978), and in enriched populations of somatotrophs (Israel et al., 1983), lactotrophs (Ingram and Mason, 1985), and gonadotrophs (Mason and Waring, 1985). The use of intracellular recording techniques has allowed investigators to infer the existence of a Ca component to the spiking activity in every case examined, which suggests that the presence of Ca channels is a general characteristic of endocrine pituitary cells. Since Ca channels in a variety of excitable cells are important targets for the action of hormones and neurotransmitters (see Reuter, 1983; Tsien, 1983), modulation of Ca channel properties could be an important mechanism of action of regulatory factors in modifying electrical and secretory activity in pituitary cells. A step to address this question is the identification and characterization of the type(s) of Ca channels present in these cells.

Ca channels have been studied in clonal cells (GH3 and related subclones) derived from rat pituitary tumors (Hagiwara and Ohmori, 1982, 1983; Dubinsky and Oxford, 1984; Matteson and Armstrong, 1984b). Recently, two different types of Ca channels have been identified in these cells (Matteson and Armstrong, 1984a; C. M. Armstrong and Matteson, 1985; D. Armstrong and Eckert, 1985; Cohen and McCarthy, 1985). However, little is known about the functional properties of Ca channels in nontransformed pituitary cells. At present, there are no published records of ionic currents from pituitary cells in primary culture.

The pars intermedia of the rat pituitary gland is a naturally enriched source of opiomelanotropinergic cells that synthesize and release several biologically active peptides, including β-endorphin and α-melanocyte–stimulating hormone (Mains and Eipper, 1979; Martin et al., 1979; Goldman et al., 1983; Jackson and Lowry, 1983; for reviews, see O'Donohue and Dorsa, 1982, and Millan and Herz, 1985). Because of its cellular homogeneity, the rat pars intermedia has been a useful system for studying the origin and functional significance of the electrical activity of primary cultured pituitary cells (see Douglas and Taraskevich, 1985). The physiological mechanisms controlling hormone release from pars intermedia cells have been extensively studied. They involve a dual control by catecholamines: release is stimulated by β-adrenergic agonists and inhibited by dopamine (Bower et al., 1974; Cote et al., 1980; Tilders et al., 1979, 1981; Munemura et al., 1980; Berkenbosch et al., 1983; Jackson and Lowry, 1983). In addition, the release of pars intermedia peptides is stimulated by corticotrophin-releasing factor (Meunier et al., 1982; Sakly et al., 1982), an effect that is blocked by somatostatin (Kraicer et al., 1985). Basal and stimulated hormone release from the pars intermedia are dependent on the presence of Ca in the extracellular medium, which suggests a role for Ca channel activation in hormone release (Tomiko et al., 1984; Tsuruta et al., 1982).

In this article, it is shown that pars intermedia cells maintained in culture have two main populations of Ca channels with properties that differ in several ways. The apparent number of channels of one of these populations tends to increase with time in culture. A brief account of some of these results has been presented elsewhere (Cota and Armstrong, 1985).

METHODS

Cell Dispersion and Culturing

Pituitary pars intermedia cells were dispersed from neurointermediate (NI) lobes by using collagenase digestion followed by mechanical agitation in a Ca- and Mg-free medium. This procedure was adapted from previous work (Tomiko et al., 1984; see also Ben-Jonathan et al., 1983; Weiner et al., 1983). Pituitary glands were removed from four to six male Sprague-Dawley rats weighing 200–300 g, and placed in a plastic petri dish containing Ca- and Mg-free Hanks' balanced salt solution with 25 mM Hepes (Cell Center, University of Pennsylvania, Philadelphia, PA), supplemented with 2% minimal essential medium amino acids (Gibco Laboratories, Grand Island, NY), 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 1.25 μg/ml of Fungizone (Flow Laboratories, McLean, VA) (modified HBSS). The NI lobes were carefully separated from the anterior lobe (pars distalis), transferred to a 25-ml Erlenmeyer flask containing 5 ml of 0.3% collagenase (Gibco Laboratories) in modified HBSS, and incubated for 15 min at 37°C in a shaker bath. At the end of the incubation, the NI lobes were transferred to a conical centrifuge tube containing 5 ml of modified HBSS supplemented with 10 μg/ml of deoxyribonuclease (type I, Sigma Chemical Co., St. Louis, MO) and 0.1% bovine serum albumin. The NI lobes were then triturated with Pasteur pipettes that had progressively smaller fire-polished tips. Macroscopic fragments of undissociated tissue were allowed to settle, and the cell suspension, consisting of single cells and cell clusters of various sizes, was transferred to another centrifuge tube. Cells were collected by centrifugation at slow speed, resuspended in the medium used for culturing, and plated on fragments (2 × 10 mm) of glass coverslips placed in 35-mm plastic petri dishes (one dish per rat). The culture medium was Kennett's HY medium (Flow Laboratories) supplemented with 5% fetal bovine serum (Gibco Laboratories), 1% glutamine, and antibiotics. Cells were kept in culture for 1–9 d at 37°C in a humidified incubator gassed with air and 5 or 8% CO_2. The medium was replenished after 1 and 4 d of culture.

Recording Technique

A coverslip with cultured cells was transferred from the incubator to a plastic petri dish containing 1 ml of the external recording solution (see below). After 15–20 min, the electrophysiological recordings were started. Experiments were carried out at room temperature (18–21°C) on round, highly refractile, isolated cells 11–18 μm in diameter.

Ionic channel activity in pituitary cells was recorded by using the whole-cell variant of the patch-clamp technique (Hamill et al., 1981), with a 100-MΩ feedback resistor on the current-to-voltage converter. The frequency response was improved by using low-resistance patch pipettes and series resistance compensation. The details of the experimental procedure, electronic circuitry of the patch-clamp amplifier, and data-acquisition system are described in Matteson and Armstrong (1984b). In the present experiments, patch electrodes were fabricated from low-melting-point glass capillaries (type 8161, Corning Glass Works, Corning, NY). The patch electrode resistances ranged between 0.9 and 1.5 MΩ after fire-polishing. Time- and voltage-dependent ionic currents were induced by depolarizing voltage-clamp steps applied every 2–15 s from the holding potential (−80 mV in most of the experiments). Current responses were usually sampled at 20- or 50-μs intervals, and stored and analyzed using a laboratory computer. Pulse generation, data sampling, and display were performed by an interface designed and built in this laboratory (see Matteson and Armstrong, 1984b). The linear components of the capacitive and ionic

currents were subtracted out before final storage with a P/2 or P/4 procedure (Bezanilla and Armstrong, 1977) using a subtracting holding potential of −130 or −140 mV.

Recording Solutions

The standard external//internal recording solutions contained (in mM): 135 NaCl, 5 KCl, 10 CaCl$_2$, and 10 Hepes-NaOH//105–120 K-glutamate, 20 KF, 5–20 NaCl, 2 MgCl$_2$, 10 EGTA-KOH, and 10 Hepes-KOH. To record Ca channel currents, the external//internal solutions were composed of (in millimolar units unless otherwise noted): 115–140 NaCl, 0–25 tetraethylammonium chloride (TEA-Cl), 10 CaCl$_2$ or 10 BaCl$_2$, 1–5 μM tetrodotoxin (TTX), and 10 Hepes-NaOH//80 Cs-glutamate, 30 N-methylglucamine (NMG) glutamate, 20 CsF, 20 CsCl, 2 MgCl$_2$, 10 EGTA-CsOH, and 10 Hepes-CsOH. In all cases, the pH was adjusted to 7.30 ± 0.01. The osmolarity of the external solutions was adjusted to ∼300 mosmol, 5–10 mosmol hyperosmotic with respect to the internal solutions (see Dubinsky and Oxford, 1984). The relatively elevated divalent cation concentrations in the external solutions and the presence of 20 mM internal fluoride facilitated the formation of gigaseals and allowed the recording of whole-cell currents typically over >30 min. External and internal solutions were passed through 0.2-μm Millipore filters immediately after their preparation and again before use. Internal solutions containing supplemental nucleotides (MgATP and Tris-GTP; Sigma Chemical Co.) were made up daily. All solutions were stored at 5°C before use. In the figure legends, the cationic species present in the recording solutions are indicated following the convention external//internal, with concentrations in millimolar units.

RESULTS

Identification of Ca Channel Currents

Action potentials triggered by pars intermedia cells are mediated mainly by an increase in the Na membrane permeability, but they also have a Ca component (Douglas and Taraskevich, 1978, 1980). Fig. 1 shows evidence for the presence of several voltage-dependent ionic channels, including Ca channels, in pars intermedia cells under voltage-clamp conditions. Fig. 1A illustrates the pattern of ionic currents recorded in every cell investigated using the standard external and internal recording solutions. During the depolarizing pulse, a fast inward current is followed by a delayed outward current. After repolarization, a tail current is observed that decays in two phases, fast and slow. The initial fast inward current was detected between −40 and −30 mV, reached a maximum value at about +10 mV (time to peak, 0.5–0.6 ms), and reversed direction at membrane potentials very close to the calculated Na equilibrium potential, which indicates that it was carried mainly by Na ions. Furthermore, this current was completely blocked by adding 1–5 μM TTX to the external recording solution. Fig. 1B illustrates that no inward current was observed in the presence of TTX during depolarizing steps to +20 mV.

Delayed outward currents were mainly carried by K ions since they were greatly reduced by replacing all the K by Cs and NMG in the pipette-filling solution. Under this condition, the fast Na current was followed by a smaller, relatively maintained component of inward current (Fig. 1C). The maintained component of pulse-elicited current, as well as the biphasic tail current, can be recorded even in the presence of external TTX (Fig. 1D). These TTX-insensitive

components of inward current can be ascribed to Ca channel activity since they were greatly reduced by the addition of 2 mM $CdCl_2$ to the external solution or by the complete replacement of external Ca by Mg. Furthermore, similar TTX-insensitive currents were also observed after the replacement of external Ca by Ba. These results confirm the presence of Ca channels in cultured pars intermedia cells.
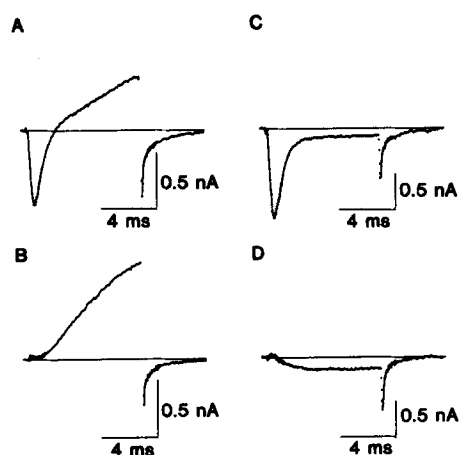

FIGURE 1.   Components of ionic current. Membrane currents were induced by 8-ms depolarizing steps to +20 mV from a holding potential of −100 mV. Each trace was recorded from a different cell within 3 min after the rupture of the membrane patch. In this and in subsequent figures, T indicates the age of the culture and t is the time elapsed under whole-cell recording. (A) Pattern of ionic currents using the standard external and internal recording solutions (see Methods). Cell 1AP185; $T = 6$ d; $t = 2.9$ min. 135 Na, 5 K, 10 Ca//160 K, 5 Na, 2 Mg. (B) Blockade of the fast inward current during the depolarizing step by external TTX. Cell 2AP185; $T = 6$ d; $t = 1.7$ min. Solutions were the same as in A, except that 1 μM TTX was added to the external solution. (C) Inward currents after the replacement of K by Cs and NMG in the pipette-filling internal solution. Cell 6AP195; $T = 7$ d; $t = 1.8$ min. 135 Na, 5 K, 10 Ca//130 Cs, 30 NMG, 2 Mg. (D) TTX-insensitive components of inward current. Cell 7AB195; $T = 7$ d; $t = 1.8$ min. Solutions were the same as in C but with 1 μM external TTX.

Kinetic Properties of Ca Channels

Current-voltage relations. Fig. 2A shows Ca current records obtained at various membrane potentials. It can be seen that the fast component in the tail current is clearly present only after steps to potentials more positive than 0 mV. The decay of tail currents after small depolarizations follows a simple, slow time course. These observations suggest the existence of two main types of Ca channels differing in their closing kinetics and voltage range of activation, as is the case in GH3 cells (C. M. Armstrong and Matteson, 1985). Further evidence supporting this view can be obtained from the voltage dependence of the inward current during the depolarizations (*I-V* relation; Fig. 2B). The inward current becomes

noticeable at −40 mV and reaches a maximum value around +15 mV. A hump or shoulder is detected on the descending branch of the *I-V* relationship. This characteristic has been also observed in other excitable cells (Carbone and Lux, 1984; Deitmer, 1984; Bean, 1985; Fedulova et al., 1985), and it is consistent with the presence of two main components of Ca current that differ in their activation levels. Thus, it is possible to infer that depolarizing steps to membrane potentials between −40 and −10 mV only activate a small-amplitude component of Ca current carried by slowly deactivating (SD) channels. Larger depolarizations activate an additional, high-amplitude component that flows through fast-deac-
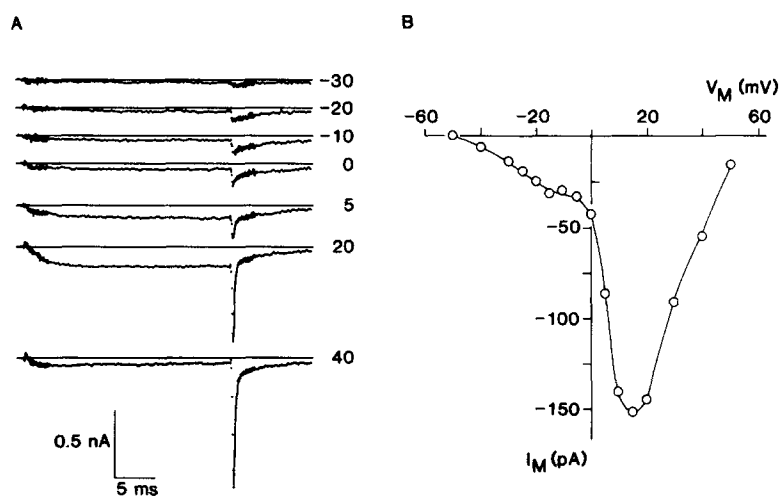

FIGURE 2.   Two components of Ca current. (*A*) Ca currents at various membrane potentials using 25-ms activating pulses from a holding potential of −80 mV. The numbers next to each trace indicate the membrane potential in millivolts during pulses. Ca currents activated during small depolarizations are followed by slow, simple tail currents. Biphasic tail currents are present only after depolarizations to 0 mV or more positive membrane potentials. Cell 4JL035; *T* = 7 d; *t* = 2–3 min. 115 Na, 25 TEA, 10 Ca//130 Cs, 30 NMG, 2 Mg. (*B*) *I-V* relation for the inward current from the same cell as in *A*. The points correspond to the average current during a 5-ms interval at the end of 25-ms pulses (up to +20 mV) or around the peak current (pulses above +20 mV). A small-amplitude component of inward current was detected at about −40 mV. An additional component of inward current was activated between −10 and 0 mV.

tivating (FD) channels. Evidence for two components of Ca current was obtained even with a holding potential of −60 mV. The results in the following sections further support the existence of two main populations of Ca channels.

Both components of Ca channel current, SD and FD, were present in 94% of the cells investigated (*n* = 51), although their relative contributions to the pulse-elicited or tail current were variable in different cells. The main source of this variability was the age of the culture (see below).

The hump in the current-voltage curve was generally less evident with Ba as the charge carrier, which can be explained by a selective shift in the activation

curve for FD channels toward more negative voltages, as described in the next section.

Conductance-voltage relations. The voltage range of activation for SD and FD channels was explored by analyzing the tail current after 8-ms depolarizing steps to different membrane potentials. With a constant repolarizing level, the amplitude of the tail current is proportional to the number of open channels at the end of the steps. In order to separate the contributions of FD and SD channels to the tail current, an exponential was fitted to the slow phase of each tail record, and its amplitude was extrapolated to the instant of repolarization. The fast component was isolated by subtracting the fitted slow component from the total tail record. Fig. 3A illustrates this separation procedure for a pulse to +20 mV. Trace *a* shows the Ca channel current recorded during the last 1-ms segment of the depolarizing step and the corresponding tail after repolarization to −80 mV. In trace *b*, the fitted slow component is shown superimposed on the data points. Trace *c* corresponds to the remaining fast component after the subtraction procedure.

The extrapolated amplitude of the slow component is plotted by the solid circles in Fig. 3B, and the amplitude of the isolated fast component is plotted by the open circles; both have been normalized relative to their maximum value. The data correspond to the average value for five cells with Ca present in the external recording solution. As expected from the *I-V* relation, the conductance-voltage curve for SD channels is significantly different from the corresponding curve for FD channels. The half-maximal activation level is between +5 and +10 mV for SD channels and between +20 and +25 mV for FD channels. In addition to this different position along the voltage axis, the curve for FD channels is steeper than that for SD channels.

The conductance-voltage curves obtained from four different cells in the presence of external Ba are shown in Fig. 3C. The shape of both curves and the position of the curve for SD channels are about the same as in the presence of Ca. The main effect of replacing Ca with Ba on the voltage dependence of Ca channel conductance is a shift of ~10 mV in the position of the curve for FD channels toward negative potentials.

Closing kinetics. The closing time constant measured at −80 mV after 8-ms steps to +20 mV was 1.75 ± 0.44 ms (mean ± standard deviation) for SD channels and 106 ± 28 μs for FD channels ($n = 19$) with Ca as the charge carrier. In the presence of Ba, the closing time constants were 1.37 ± 0.15 ms and 112 ± 27 μs, respectively ($n = 4$). Thus, the substitution of Ba for Ca does not appreciably change the closing kinetics of Ca channels. There was not a systematic relation between the closing time constant values and the amplitude or duration of the depolarizing steps.

Time course of conductance during a maintained depolarization. The time course of activation and inactivation of Ca channels was studied by analyzing the tail current after steps of various durations to +20 mV. Fig. 4A shows examples of Ca tails after pulses of 1.4 (*a*), 8.4 (*b*), and 40.4 ms (*c*). The relative contribution of the fast and slow components to the tail changed with pulse duration; the contribution of the slow component is less important in *a* and *c* than in *b*. This indicates that FD and SD channels also differ in the time course of activation

and inactivation. The plot in Fig. 4B shows the time course of conductance for SD (solid symbols) and FD channels (open symbols) with Ca as the charge carrier (different symbols indicate different cells). FD channels activate with a faster time course than SD channels. FD conductance reached a maximal value in ~4
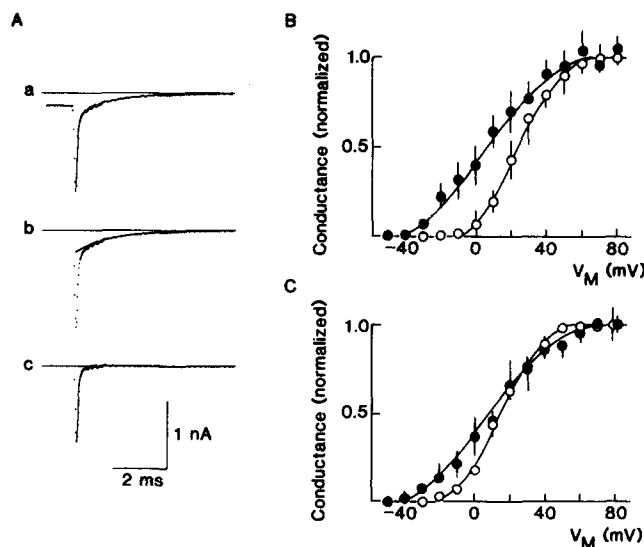

FIGURE 3. Voltage dependence of Ca channel conductance. (A) Separation procedure of the tail current in fast and slow components. Trace a corresponds to the inward current during the last 1-ms segment of an 8-ms pulse to +20 mV and the associated tail current following repolarization to the holding potential (−80 mV). A single exponential was fitted to the data points between 0.7 and 4 ms after the onset of the repolarization and extrapolated back to the end of the pulse (trace b). The fitted slow component was then subtracted from the total tail current to isolate the fast component (trace c). Cell 7JL025; $T = 6$ d; $t = 1.0$ min. 140 Na, 10 Ba//130 Cs, 30 NMG, 2 Mg. The plots are conductance-voltage curves for SD (solid circles) and FD channels (open circles) in the presence of 10 mM Ca (B) or 10 mM Ba (C) in the external recording solution. Tail Ca channel currents were recorded at the holding potential after 8-ms pulses to various voltages and the fast and slow components were separated as in A. The extrapolated amplitude of the slow component and the peak amplitude of the isolated fast component were then normalized with respect to their maximum values. Data from several cells (five in B, four in C) were averaged and plotted (means ± standard deviation) as a function of the membrane potential during the activating pulses. All data were obtained within 4 min under whole-cell recording from 3–7-d-old cells. Sigmoidal curves were drawn by eye.

ms, whereas the peak value of the SD conductance was generally reached at ~8 ms. Half-activation was reached in 1.0–1.3 and 2.5–3.5 ms for FD and SD channels, respectively. However, SD channels tend to inactivate promptly. After 100 ms, the SD conductance fell to ~30% of its peak value, whereas >80% of the maximum FD conductance remained.
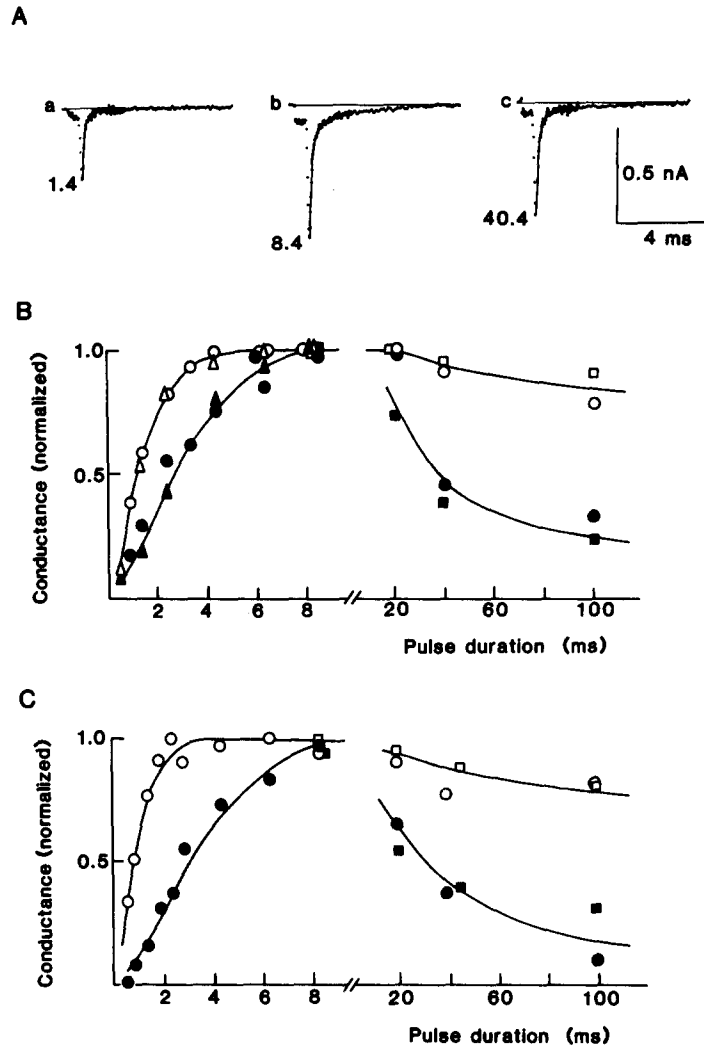
FIGURE 4.   Time dependence of Ca channel conductance. (A) Ca currents at the end of steps to +20 mV lasting 1.4, 8.4, and 40.4 ms. Each trace shows the baseline current before the depolarizing pulse, the current during the last 0.4-ms segment of the pulse, and the tail current after repolarization to the holding potential (−80 mV). Cell 2JL035; T = 7 d; t = 8–9 min. Solutions were the same as in Fig. 2A. The plots show the time course of SD (solid symbols) and FD (open symbols) channel conductance at +20 mV with Ca (B) or Ba (C) as the charge carrier. The amplitudes of the fast and slow components in the tail Ca channel current were normalized relative to their maximum values and plotted as a function of the pulse duration. It is assumed in this analysis that the driving force for Ca or Ba ions during the tail measurement remains constant with increasing duration of the activating pulse. Data were obtained from several cells (each cell is indicated by a different symbol; T = 7 d in every case) within 3–9 min after the rupture of the membrane patch. The data-gathering run required <3 min in each cell.

Fig. 4C shows the time course of FD and SD conductances with Ba replacing Ca. The curves are very similar to those in Fig. 4B, although there are some minor differences. It is possible that the FD conductance activated slightly faster, whereas the activation of SD channels was somewhat slower, with Ba as the charge carrier.

In conclusion, these results indicate that Ca and Ba currents in pars intermedia cells depend on the activity of two main populations of Ca channels with different kinetic properties.

Effect of the Age of the Culture on Ca Channels

In the course of these experiments, it was observed that the amplitude of the Ca channel currents depended on the age of the culture. In contrast, no systematic changes were observed in the Na current amplitude. Larger Ca and Ba currents

TABLE I

Ca Channel Activity Depends on the Age of the Culture

Age of the culture	Cell diameter	Tail Ca current*			
		Slow component		Fast component	
		Amplitude	Time constant	Amplitude	Time constant
d	μm	nA	ms	nA	μs
1–2	14.7±1.7‡	−0.15±0.12	1.45±0.31	−0.40±0.25	120±33
6–7	15.8±1.5	−0.18±0.05	1.79±0.32	−2.18±0.41	93±14

* Tails after 8-ms pulses to +20 mV. Holding potential and repolarization level, −80 mV. The amplitude of the fitted exponentials was extrapolated back to the onset of the repolarization. Records were taken within 3 min under whole-cell recording.

‡ Values are expressed as means ± standard deviation. Data are from seven cells in each case.

were recorded the longer the cells had been in culture, even though the diameter of the cells did not change appreciably. For example, in one set of experiments, the amplitude of the pulse-elicited Ca current measured at +20 mV within 3 min under whole-cell recording ranged from −9 to −31 pA in different cells during the first 2 d in culture, whereas 5 d later it ranged from −53 to −105 pA. However, the amplitude of the Ca current recorded at −20 or −10 mV in the same cells did not change significantly with time in culture. This selective increase in the inward current activated during large depolarizations could be due to a specific increase in the activity of FD channels. Further evidence for this view was obtained from the characteristics of the tail Ca current.

As can be seen in Table I, the main effect of the age of the culture on the tail current is the increase in the amplitude of the fast component. No obvious changes are evident in the amplitude of the slow component. The conductance-voltage curves, as well as the closing kinetics of the channels, were unaffected by the age of the culture. Thus, these data suggest that the current carried by FD channels increases as a function of time in culture, with no appreciable changes in the channel kinetic properties.

After 6–7 d in culture, more FD channels contribute to the total tail current in pars intermedia cells than in GH3 cells. With 10 mM Ca in the external recording solution, the ratio of the maximum tail amplitude of FD channels to that of SD channels in GH3 cells is usually <2, whereas it is generally >4 in pars intermedia cells, which suggests a different ratio of channel densities or single channel conductances.
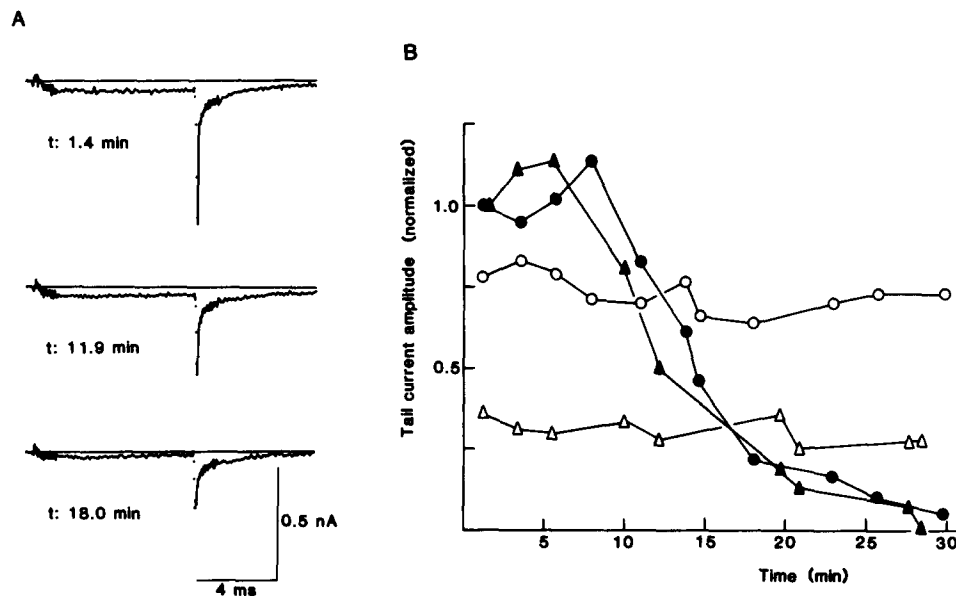
A

B

FIGURE 5.   Washout of Ca currents. (A) Ca currents induced by 8-ms pulses to +20 mV at various times under whole-cell recording (membrane patch ruptured at $t = 0$). Pulse-elicited and tail currents tended to decrease after several minutes of intracellular dialysis. Cell 3JL025; $T = 6$ d. Holding potential, −80 mV. 140 Na, 10 Ca//130 Cs, 30 NMG, 2 Mg. (B) FD (solid symbols) and SD (open symbols) channel activity during the course of intracellular dialysis. Tail Ca currents were recorded as in A from two different cells. The amplitudes of the fast and slow components were normalized relative to the value of the initial fast component and plotted as a function of time under whole-cell recording. Circles: cell 2JL025; $T = 6$ d; initial peak amplitude of the fast component, −316 pA. Triangles: cell 1JU285; $T = 2$ d; initial peak amplitude of the fast component, −143 pA.

Changes in Ca Channel Activity During Intracellular Dialysis

Loss of functional channels. It is well known that Ca currents are not stable in cells internally dialyzed with simple saline solutions because they tend to disappear after the start of cell perfusion (Fedulova et al., 1981; Byerly and Hagiwara, 1982; Fenwick et al., 1982; Doroshenko et al., 1982; Byerly and Moody, 1984). As in GH3 cells (C. M. Armstrong and Matteson, 1985), in the present experiments this "washout" process mainly affected the current carried by FD channels. For example, Fig. 5A shows Ca currents taken at various times

(*t*) after establishing continuity between the patch pipette and the cell interior. The amplitudes of both the pulse-elicited inward current and the fast component in the tail tended to decrease as a function of time, whereas the slow tail current was little affected. This decrease in Ca channel activity took place with no visual signs of cell deterioration or obvious changes in seal or series resistance. Furthermore, the time constants of decay of both the fast and slow components in the tail did not change appreciably with time. In five similar experiments, the amplitude of the FD tail current decreased by 84 ± 5% within 20–25 min,
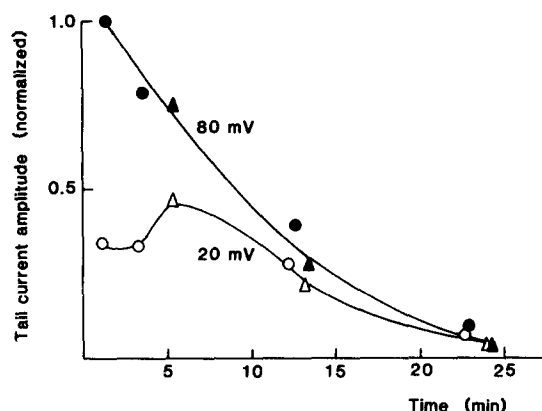

FIGURE 6. Continuous reduction in the maximum conductance of FD channels during intracellular dialysis. Tail Ca currents after steps to +20 or +80 mV were recorded at various times after establishment of the whole-cell recording. The activating pulses lasted 8 (circles) or 25 ms (triangles). The slow component was subtracted out of each tail and the fast component was normalized relative to its amplitude after the initial pulse to +80 mV (−622 pA). The FD channel maximum conductance decrease by 50% in ~8 min, as indicated by the reduction in the amplitude of the fast tail after pulses to +80 mV. In contrast, at that time, the FD channel activity induced by pulses to +20 mV remained above its initial value. Cell 1JL035; $T = 7$ d. Holding potential, −80 mV. Solutions were the same as in Fig. 2A.

whereas the amplitude of the SD component decreased by only 11 ± 8% (values compared with those obtained within 1–2 min of the rupture of the cell membrane). The time course of Ca channel washout was about the same in cells differing in the relative contribution of FD and SD components to the total tail current or in the absolute amplitude of the fast tail current.

Shift of the activation curve along the voltage axis. Fig. 5 B shows the amplitude of the fast (solid symbols) and slow (open symbols) components of the Ca tail after 8-ms pulses to +20 mV, measured as a function of time under whole-cell recording from two different cells. Before the rundown of the FD component, there was an increase in its amplitude, which reached a peak value within 3–8 min. This characteristic could be due to an initial transient increase in the FD channel maximum conductance after the start of cell dialysis. The experiment shown in Fig. 6 examined this possibility by measuring the FD tail after depolar-

izations to +20 mV (open symbols) and comparing it with that induced after steps to +80 mV (solid symbols). These values are proportional to the maximum channel conductance (see Fig. 3B). In contrast to the complex behavior after pulses to +20 mV, the FD tail after steps to +80 mV continuously decreased as a function of time, which indicates that there was a monotonic fall in the maximum conductance during intracellular dialysis.
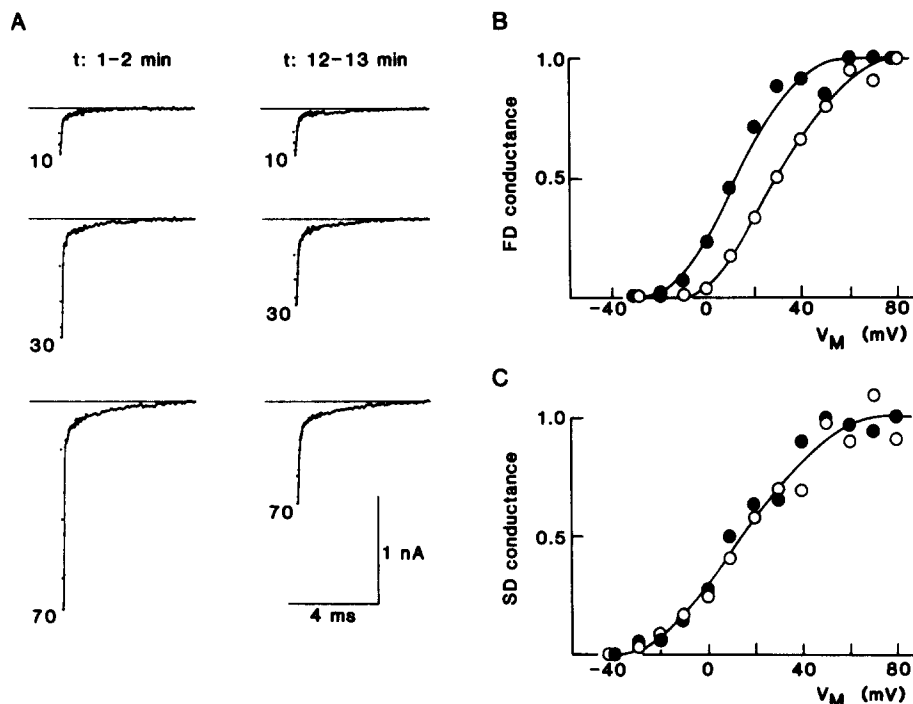

FIGURE 7.   Time-dependent shift along the voltage axis of the activation curve for FD channels. (A) Tail Ca currents recorded at two different intervals after the rupture of the membrane patch. 8-ms activating pulses were used, followed by repolarization to the holding potential (−80 mV). The numbers next to each trace indicate the membrane potential in millivolts during the pulses. The degree of reduction of the tail current amplitude with the time of whole-cell recording depends on the voltage during the activating pulse. Same cell as in Fig. 6. (B) Conductance-voltage relationships for FD channels at $t = 1–2$ min (open circles) and $t = 12–13$ min (solid symbols). At each interval, the amplitude of the fast component in the tail was normalized relative to its value after the largest pulse. (C) Conductance-voltage relationships for SD channels from the same tail records as in B. A time-dependent voltage shift was detected for FD channels, but not for SD channels. The curves in B and C were drawn by eye.

Previous studies using whole-cell recording have shown a shift with time toward more negative potentials in the voltage dependence of kinetic parameters for Na channels (Marty and Neher, 1983; Fernandez et al., 1984) and K channels (Cahalan et al., 1985). A similarly time-dependent, negative shift in the activation

curve for FD channels could explain the initial increase in the FD tail (and pulse-elicited) current after small depolarizations, despite the continuous reduction in FD channel maximum conductance. Fig. 7 presents evidence supporting this hypothesis. Fig. 7A shows tail Ca currents after 8-ms pulses to various potentials, recorded at two different intervals after rupture of the membrane patch. The
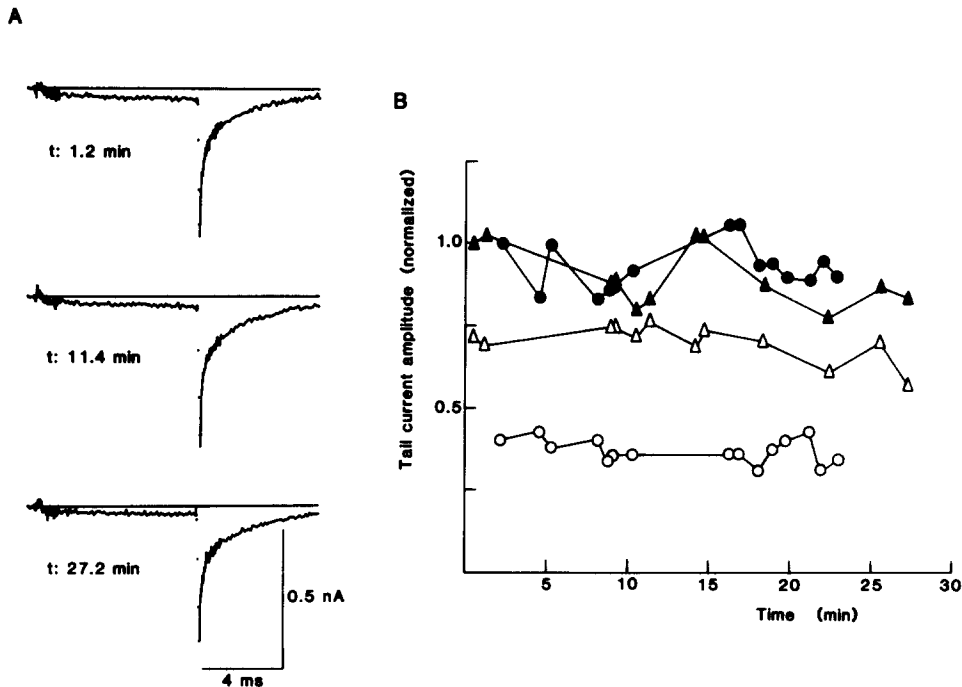

FIGURE 8. Effect of the intracellular introduction of exogenous nucleotides on the washout of Ca currents. (A) Experiment similar to that in Fig. 5A but with 3 mM MgATP and 40 μM GTP added to the internal pipette solution. The presence of the exogenous nucleotides stabilizes the Ca channel activity during >25 min of intracellular dialysis. Cell 2JL075; T = 3 d. Holding potential, −80 mV. 115 Na, 25 TEA, 10 Ca//130 Cs, 30 NMG, 2 Mg, supplemental MgATP and GTP. (B) FD (solid symbols) and SD (open symbols) channel activity as a function of time after the rupture of the membrane patch in two cells internally dialyzed with the nucleotide-containing solution. Tail Ca currents were recorded after 8-ms pulses to +20 (triangles) or +30 mV (circles). Data are plotted as in Fig. 5B. Circles: cell 4JL055; T = 9 d; initial peak amplitude of the fast tail, −354 pA. Triangles: same cell as in A.

decrease in the amplitude of the FD tail with time under whole-cell recording was faster after large depolarizations. Fig. 7B shows the corresponding conductance-voltage relations for FD channels obtained within 1–2 (open circles) and 12–13 min (solid circles). The most obvious difference between these two curves is their distinct positions along the voltage axis. There is a shift with time of ~17 mV toward negative potentials at the half-maximal activation level. A consistent
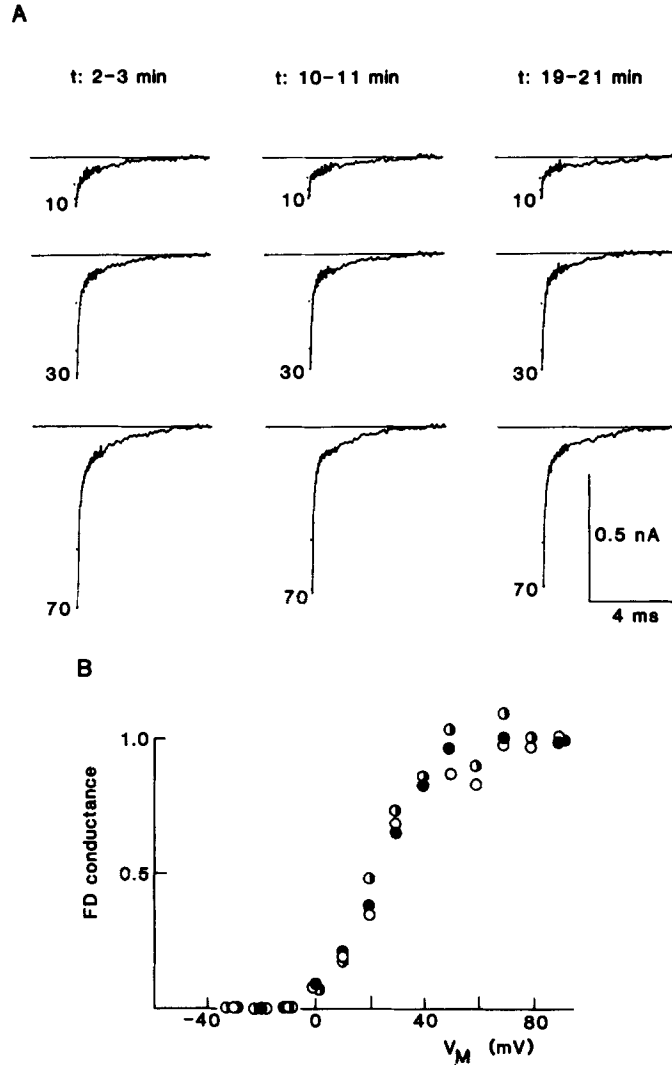
A

B

FIGURE 9.   The addition of exogenous nucleotides to the internal solution prevents the voltage shift of the activation curve for FD channels during intracellular dialysis. (A) Tail Ca currents recorded at three intervals after establishing the whole-cell recording mode, with 3 mM MgATP and 40 μM GTP added to the internal solution. The tails followed 8-ms depolarizing pulses to the voltages shown next to each trace. The amplitude of the tail current slowly declined as a function of the time of whole-cell recording. The degree of current reduction was about the same at each depolarization, which indicates the absence of important time-dependent shifts in the activation curve for Ca channels. Cell 4JL055; T = 9 d. Holding potential, −70 mV. Solutions were the same as in Fig. 8A. (B) Conductance-voltage relationships for FD channels obtained from the data in A at t = 2-3 min (open circles), t = 10-11 min (half-filled circles), and t = 19-21 min (solid circles). The data were normalized as in Fig. 7B.

shift of 12–20 mV within 11–14 min was observed in every cell investigated. At that time, the maximum FD channel conductance had decreased to 30–40% of its initial value. The severe decrease in FD conductance made it difficult to obtain reliable values for the shift after longer periods.

On the other hand, the activation curve for SD channels practically did not shift with time (Fig. 7C). An upper-limit value for this shift was 5 mV within ~25 min. Thus, the main effects of intracellular dialysis on Ca channel activity in pars intermedia cells are a loss of functional FD channels and a shift in the position of their activation curve toward more negative potentials.

Stabilizing effect of exogenous nucleotides. The loss of functional Ca channels in perfused neurons seems to be a consequence of dephosphorylation of a regulatory site associated with the channel caused by the decrease in the activity of cAMP-dependent protein kinase after the washout of cAMP and ATP during intracellular dialysis (for recent reviews, see Kostyuk, 1984, and Levitan, 1985). The intracellular addition of phosphorylating agents (several combinations of ATP, Mg, cAMP, and the catalytic subunit of the cAMP-dependent protein kinase) slows down the loss of Ca channels and can even restore Ca channel activity to its initial level (Fedulova et al., 1981, 1985; Doroshenko et al., 1982, 1984; Forscher and Oxford, 1985).

Fig. 8A shows that the addition of 3 mM MgATP and 40 μM GTP to the internal pipette solution had an obvious stabilizing effect on FD channel activity in pars intermedia cells, but did not seem to affect the SD channel current. Under this condition, the amplitude of the FD tail current after 8-ms pulses to +20 or +30 mV decreased by <15% within 25 min (Fig. 8B). In these experiments, GTP was added to the internal solution, taking into account that the activity of cAMP-dependent protein kinase is affected by regulatory membrane proteins that cannot be active unless they bind GTP (see Gilman, 1984). This seems to be the case for the protein kinase C as well (see Berridge and Irvine, 1984). Thus, the washout of GTP may be an additional factor contributing to the loss of functional Ca channels.

The addition of MgATP and GTP to the internal solution not only slowed down the loss of FD channels but also prevented the shift in their activation curve, as can be seen from Fig. 9, A and B. No systematic shifts were observed in the presence of the exogenous nucleotides even 20–27 min after rupture of the membrane patch. The data in Fig. 9B can be fitted relatively well by the average conductance-voltage curve for FD channels obtained in the absence of supplemental MgATP and GTP within 4 min under whole-cell recording (Fig. 3B, open circles).

DISCUSSION

The present results indicate that Ca and Ba currents in pars intermedia cells depend on the activity of two main populations of channels. The kinetic properties and degree of lability under whole-cell recording of these two populations seem to be similar to those previously described for SD and FD channels in pituitary tumor GH3 cells (C. M. Armstrong and Matteson, 1985). In both cases, slowly deactivating (SD) channels have a lower activation threshold and a faster time course of inactivation than fast-deactivating (FD) channels.

Two main populations of Ca channels, differing in the voltage range of activation as well as in other voltage-dependent properties, have also been discovered in a variety of excitable cells, including egg cells (Hagiwara et al., 1975; Fox and Krasne, 1984), neurons (Llinas and Yarom, 1981a, b; Carbone and Lux, 1984; Fedulova et al., 1985; Tsunoo et al., 1985; Yoshii et al., 1985), and muscle cells (Beam and Knudson, 1985; Bean, 1985; Bean et al., 1985; Cota and Stefani, 1985; Mitra and Morad, 1985; Sturek and Hermsmeyer, 1985). In addition to the differences in kinetics, several of these studies report differences in selectivity and pharmacological properties (see, for example, Bean, 1985). It is possible that future investigations, studying in more detail the functional properties of Ca channels, may demonstrate the presence of subtypes in these two main populations. In fact, a recent study in sensory neurons gives evidence for two distinct components of high-voltage–activated Ca current (Nowycky et al., 1985).

The functional relevance of SD and FD channels to the electrical activity of pars intermedia cells remains unknown at present. It has been suggested that Ca channels, in addition to contributing to spikes, also participate in the "pacemaker" mechanism that makes possible the triggering of spontaneous action potentials (Douglas and Taraskevich, 1982). If so, pacemaking activity may involve SD channels because of their low activation level, as has been proposed for GH3 cells (C. M. Armstrong and Matteson, 1985). In pars intermedia cells, there are small, TTX-resistant fluctuations in the membrane potential (Douglas and Taraskevich, 1982, 1985) that may be correlated with basal hormone secretion (Tomiko et al., 1984). It will be of considerable interest to see whether such electrical activity depends on SD channels.

The current carried by FD channels tends to increase the longer the cells have been in culture, with no appreciable changes in the kinetic properties of the channels. This characteristic is not obvious for SD or Na channels, which suggests that there is a selective effect of the age of the culture on FD channels. An increase in the unitary current carried by each channel or an increase in the number of FD channels with time in culture could explain these results. Further investigation is required to distinguish between these two possibilities and to explore the mechanism involved. A possibly related finding in normal pituitary cells is that rat somatotrophs are able to generate evoked Ca-dependent responses only after 7 d in culture (Israel et al., 1983).

Washout of Ca Channels and Shift in Their Activation Curve

Functional FD channels are almost completely lost within 25 min after the patch pipette breaks into a cell. This washout of FD channels is slowed by adding 3 mM MgATP and 40 μM GTP to the internal solution. There is evidence that a shift toward dephosphorylation caused by the decrease in the activity of cAMP-dependent protein kinase is a contributing factor in the loss of functional Ca channels in perfused neurons (Fedulova et al., 1981, 1985; Doroshenko et al., 1982, 1984; see also Levitan, 1985). Furthermore, prevention of the washout of unitary Ca channel currents in isolated membrane patches from GH3 cells by phosphorylating agents has been recently reported (D. Armstrong and Eckert, 1985). Thus, the stabilizing effect of MgATP and GTP can be explained by the

maintenance of a certain balance between the phosphorylation and dephosphorylation reactions affecting FD channel activity. It is expected that an investigation of the sensitivity of FD channels to regulatory factors and drugs that modify the activity of protein kinases or endogenous phosphatases will help to evaluate this hypothesis in pars intermedia cells. The presence of the exogenous nucleotides, in addition to supporting phosphorylation, may also help to maintain low intracellular Ca levels by increasing the ability of the cell to sequester this ion, as has been suggested in avian sensory neurons (Forscher and Oxford, 1985) and *Lymnaea* neurons (Byerly and Yazejian, 1986). This action would slow Ca channel washout by preventing several possible Ca-stimulated processes (Byerly and Yazejian, 1986), including the Ca-mediated inactivation of the channels (see also Eckert and Chad, 1984; Chad and Eckert, 1985b) and the proteolysis of the channels or some associated protein by a Ca-activated protease (see also Chad and Eckert, 1985a).

In contrast to the lability of FD channels, functional activity of SD channels is observed during the entire course of experiments lasting as long as 70 min. A substantial difference in the washout of distinct types of Ca channels has been also observed in dialyzed GH3 cells (C. M. Armstrong and Matteson, 1985) and rat sensory neurons (Fedulova et al., 1985), as well as in isolated membrane patches from heart ventricular cells (Nilius et al., 1985) and GH3 cells (D. Armstrong and Eckert, 1985). These observations suggest that the functioning of distinct Ca channels depends on different metabolic inputs and, perhaps, on different modulatory pathways. In fact, in mammalian cardiac cells, low-voltage–activated channels, unlike high-voltage–activated channels, are little affected by stimulation of β-adrenergic receptors (Bean, 1985).

Another main effect of intracellular dialysis on Ca channel activity in pars intermedia cells is a shift in the position of the activation curve for FD channels along the voltage axis. This curve shifts ~15 mV toward negative membrane potentials within 11–14 min under whole-cell recording. At this time, 60–70% of the FD channel maximum conductance is lost. However, the activation curve for SD channels shifts <5 mV within 25 min. The presence of time-dependent shifts in the voltage dependence of ionic channels has been noted in several previous studies using whole-cell recording (Marty and Neher, 1983; Fernandez et al., 1984; Cahalan et al., 1985). It has been postulated that such shifts arise from the gradual dissipation of a Donnan-type potential between the cytoplasm and the pipette solution (Marty and Neher, 1983; Fernandez et al., 1984), in addition to a time-dependent change in the electrical field within the membrane caused by desorption of anions or adsorption of cations bound to the inner surface of the plasma membrane (Fernandez et al., 1984). However, a generalized phenomenon such as the dissipation of a liquid junction potential or a uniform change in surface charge density cannot explain the fact that the shift in the activation curve for SD channels is smaller than that for FD channels. Instead, a more specific mechanism seems necessary. Interestingly, the addition of MgATP and GTP to the internal solution not only slows the washout of FD channels but also prevents the shift in their activation curve. This suggests a similarity between the mechanism underlying the loss of functional channels and that involved in

the shift in their voltage dependence. If it is assumed that FD channel activity is controlled by the phosphorylation state of the channel protein or a regulatory membrane protein associated to the channel, then the loss of phosphate groups may modify the ability of the channel to respond to membrane voltage changes. We can speculate that the result may be either a change in the voltage dependence of the channel or a nonfunctional channel, depending on the site that is dephosphorylated. Finally, it will be interesting to investigate whether agents that support phosphorylation can also prevent shifts in the voltage dependence of Na and K channels.

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