Long-Term Regulation of Calcium Channels in Clonal Pituitary Cells by Epidermal Growth Factor, Insulin, and Glucocorticoids

ULISES MEZA, GUILLERMO AVILA, RICARDO FELIX, JUAN CARLOS GOMORA, and GABRIEL COTA
From the Department of Physiology, Biophysics, and Neurosciences, Center for Research and Advanced Studies of the National Polytechnic Institute, Mexico, D.F. 07000, Mexico

ABSTRACT In rat pituitary GH3 cells, epidermal growth factor (EGF) and insulin stimulate prolactin production, whereas glucocorticoids exert the opposite effect. In the present study, GH3 cells were subjected to whole-cell patch clamp to assess the chronic actions of such regulatory factors on voltage-dependent calcium currents. Before the electrical recording, cells were grown 5–6 d either under standard conditions or in the presence of 5 nM EGF, 100 nM insulin, 1 µM dexamethasone or 5 µM cortisol. EGF induced a twofold selective increase in high-threshold calcium current density. Insulin and glucocorticoids, on the other hand, specifically regulated low-threshold Ca channels. Current density through these channels increased by 70% in insulin-treated cells, and decreased by 50% in cells exposed to dexamethasone or cortisol. Other Ca channel properties investigated (conductance-voltage curves, deactivation rates, time course and voltage dependence of low-threshold current inactivation) were unaffected by the chemical messengers. The alterations in current density persisted for many hours after removing the regulatory factors from the culture medium. In fact, the stimulatory action of EGF on high-threshold current lasted > 3 d. The results suggest that the control of prolactin production by the factors tested involves regulation of the surface density of functional Ca channels in the plasma membrane.

INTRODUCTION

The long-term secretory behavior of GH3 cells and related clonal strains (e.g., GH3B6, GH4C1) of rat pituitary tumor cells can be regulated by a number of extracellular chemical messengers, including peptide factors and steroid hormones (Tashjian, 1979; Hinkle, 1984). Specifically, chronic treatment of GH cells with epidermal growth factor (EGF) or insulin enhances the release and synthesis of prolactin (Schonbrunn, Krasnoff, Westendorf, and Tashjian, 1980; Kiino and Daniels, 1981; White and Bancroft, 1983; Prager, Yamashita, and Melmed, 1988).
contrast, when GH cells are incubated with cortisol or its synthetic analogue dexamethasone for several days, prolactin production is drastically inhibited (Naess, Haug, and Gautvik, 1980; White, Bauerle, and Bancroft, 1981).

Prolactin synthesis and secretion are calcium-dependent processes (White et al., 1981; Ramsdell and Tashjian, 1985; Ronning and Martin, 1986; Zorec, Sikdar, and Mason, 1991) and can be strongly influenced by pharmacological manipulation of the influx of calcium ions through voltage-dependent Ca channels in the plasma membrane (Tan and Tashjian, 1984; Enyeart, Aizawa, and Hinkle, 1985; Enyeart, Sheu, and Hinkle, 1987; Enyeart, Biagi, Day, Sheu, and Maurer, 1990; Stojilkovic, Izumi, and Catt, 1988). Furthermore, the physiological control of prolactin production might include regulation of Ca channel expression, as the stimulatory actions of 1,25-dihydroxycholecalciferol \([1,25-(OH)2D3]\), a vitamin D3 metabolite, and 17β-estradiol on prolactin synthesis (Kiino and Dannies, 1981; Lieberman, Maurer, Claude, and Gorski, 1982; Wark and Tashjian, 1983) seem to be associated with changes in the number of functional Ca channels (Tornquist and Tashjian, 1989; Ritchie, 1993). In addition, subpopulations of rat lactotropes that release prolactin at distinct basal rates also differ in calcium current density and responsiveness to Ca channel blockers (Cota, Hiriart, Horta, and Torres-Escalante, 1990; Lledo, Guerineau, Mollard, Vincent, and Israel, 1991; Felix, Horta, and Cota, 1993; Horta and Cota, 1993).

Motivated by these previous studies, we have now examined the long-term effects of EGF, insulin and glucocorticoids on the voltage-dependent Ca channel activity of GH3 cells. We found that these factors all are able to induce persistent changes in calcium current density. EGF selectively enhances high-threshold Ca channel activity, whereas insulin and glucocorticoids specifically regulate low-threshold Ca channels. An additional observation is that EGF stimulates sodium current density as well. Some of the results have been presented in abstract form (Meza, Navarrete, Marin, and Cota, 1993).

**MATERIALS AND METHODS**

**Cell Culture**

GH3 cells were obtained from the American Type Culture Collection (Rockville, MD), and grown as monolayers in a water-saturated atmosphere of 5% CO2 and 95% air at 37°C. The standard culture medium was RPMI 1640 medium (Sigma Chemical Co., St. Louis, MO) supplemented with 5% fetal bovine serum (Gibco Laboratories, Grand Island, NY), 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. The maintenance culture was grown in 25 cm2 polystyrene flasks (Corning Glass Works, Corning, NY). Subcultures were prepared every 7 d by incubating a nearly confluent monolayer of cells with a trypsin solution (0.05% trypsin in Hanks' balanced calcium-free salt solution, Gibco Laboratories) for 5 min. Harvested cells were then replated at 10-fold lower density in a new flask.

For patch clamp experiments, the cells were grown on glass coverslips \((10 \times 2.5 \text{ mm})\) placed in 35-mm plastic petri dishes. A constant plating density of 0.2 \(\times\) 10⁶ cells/dish was used. To assess the chronic actions of extracellular regulatory factors on ion channel activity, the cells were cultured either in standard medium alone (control cultures) or in standard medium supplemented with 5 nM EGF, 100 nM insulin, 1 µM dexamethasone or 5 µM cortisol (Sigma Chemical Co.). At the concentrations tested, and after long-term treatment (4–7 d), these
Chemical messengers exert maximal regulatory effects on prolactin production (Naess et al., 1980; Schonbrunn et al., 1980; Kiino and Dannies, 1981; Prager et al., 1988). In all cases, the culture medium was replenished every day, starting on day 3 after plating. Cells were kept in culture for 5 to 6 d until used for electrophysiological recording.

Patch Clamp Experiments

The whole-cell variant of the patch clamp technique (Hamill, Marty, Neher, Sakmann, and Sigworth, 1981) was used to compare the functional activity of voltage-gated ionic channels in the plasma membrane among control GH3 cells and cells exposed to the regulatory factors mentioned above. Such factors were not present in the recording solutions.

Electrodes. Patch pipettes were pulled from borosilicate glass capillaries (KIMAX-51; Kimble Division, Owens-Illinois, Inc., Toledo, OH) in two steps on a vertical puller (Kopf model 700D; David Kopf Instruments, Tujunga, CA). Pipette tips were then fire polished using a homemade microforge. The electrodes exhibited resistances of 1.0–2.5 MΩ when filled with internal solution.

Data recording and analysis. All experiments were carried out at room temperature (20–21°C). Cells grown on glass coverslips were transferred from the incubator to an 0.2-ml acrylic chamber mounted on the stage of an inverted microscope, and then used within 15–60 min. The bath solution was continually perfused through the chamber at a rate of 0.5 ml/min. Cells were visualized at a magnification of 600 and approached with the patch electrode. After obtaining a gigaseal, the holding potential was set at −80 mV. The membrane patch was then ruptured to obtain the whole-cell recording configuration.

Currents were recorded with an Axopatch 1B or 200A amplifier and acquired on line using a TL-1 interface with pCLAMP software (Axon Instruments, Inc., Foster City, CA). Channel activity was elicited by 6–200 ms depolarizing voltage steps. Pulses < 20 ms in duration were applied at a frequency of 0.5 Hz. When longer voltage steps were used the interval between pulses was increased to 3 s in order to permit the low-threshold Ca channels to fully recover from inactivation. Currents were filtered at 2, 5, or 10 kHz by a four-pole Bessel filter and sampled at intervals of 14–200 μs, depending on pulse duration. Capacitive transients were greatly eliminated via the transient cancellation feature on the amplifier. The remaining linear components in the current signals were subtracted out using the scaled current response to 20- or 50-mV hyperpolarizing pulses. A 386 computer was used to control pulse generation and data acquisition, as well as to store and analyze the resultant current records. Current measurements in each cell were usually completed within 2 min after establishing the whole cell mode.

Digitized data were analyzed and plotted by the combined use of pCLAMP and the Sigmaplot software set (Jandel Scientific, San Rafael, CA). The data reported in each study represent the cumulative results obtained in three different batches of cells. In each cell, measurements of current amplitude were usually made in triplicate and averaged. Calcium tail currents were fit by the sum of two exponentials following the procedure of Matteson and Armstrong (1986). To minimize possible extrapolation errors, the initial amplitudes of the fast and slow components in the tail currents were taken as the amplitudes of the fitted exponentials measured 100 μs after the onset of repolarization. Data are expressed as means ± SEM, with the number of cells in parentheses.
**Solutions.** The composition of the recording solutions is shown in Table I. Solutions A were used to record currents through Na channels and Ca channels. Solutions B were employed to isolate calcium currents. To slow down the "washout" of high-threshold Ca channels during prolonged intracellular dialysis (Cota, 1986), internal solution B was supplemented with 2 mM Na₂ATP and 50 μM GTP immediately before use.

**TABLE I**

**Recording Solutions**

|                         | NaCl* | CaCl | TEA-CF | Cs-Asp¹ | CsF | CaCl₂ | MgCl₂ | EGTA² | HEPES¹ |
|------------------------|-------|------|--------|---------|-----|-------|-------|-------|--------|
| External solutions     |       |      |        |         |     |       |       |       |        |
| (A)                    | 145   | —    | —      | —       | —   | 5     | —     | —     | 10     |
| (B)                    | 128   | —    | 10     | —       | —   | 10    | —     | —     | 10     |
| Internal solutions     |       |      |        |         |     |       |       |       |        |
| (A)                    | 30    | —    | 80     | 20      | 1   | —     | 10    | —     | 10     |
| (B)                    | —     | 150  | —      | —       | —   | 2     | 10    | —     | 10     |

External solution B also contained 1 μM tetrodotoxin (TTX). Exogenous nucleotides were added to internal solution B as described in the text. All solutions were supplemented with 5 mM glucose and had osmolalities ranging from 0.29 to 0.3 osmol.

*Concentrations are in millimolar.

¹Tetraethylammonium chloride.

²Cs aspartate.

¹External pH adjusted to 7.3 with NaOH. Internal EGTA and HEPES acids were neutralized to pH 7.3 with CsOH.

**RESULTS**

**EGF Increases Inward Currents**

In a first set of whole-cell patch clamp experiments, we addressed the long-term effects of EGF on inward ionic currents. To this end, the electrophysiological recording was carried out under ionic conditions that eliminate K⁺ currents. Both control cells and EGF-treated cells generated a similar pattern of inward currents in response to 6-ms depolarizations to +10 or +20 mV from a holding potential of −80 mV. Such a pattern consisted of a fast, transient current followed by a smaller, relatively maintained current, as shown in Fig. 1A.

In keeping with previous studies (Dubinsky and Oxford, 1984; Matteson and Armstrong, 1984), adding 1 μM tetrodotoxin (TTX) to the external recording solution completely blocked the transient component of inward current without affecting the amplitude of the maintained component. The replacement of 5 mM external Ca²⁺ by 2 mM Ca²⁺ and 0.2 mM Cd²⁺ had a complementary effect, that is a 70–80% reduction of the maintained current with no change in the peak amplitude of the transient current. Thus, the transient and maintained components of inward current in Fig. 1A can be ascribed to Na channel and Ca channel activity, respectively.

The apparent lack of Ca channel inactivation in the records shown in Fig. 1A is
related to the short duration of the depolarizing pulses. The calcium current does inactivate during prolonged depolarizations, as described in the next section.

Fig. 1A also illustrates our major finding in these initial experiments: EGF-treated cells exhibit larger inward currents than control cells. A summary of the results obtained in 34 control cells and 31 cells cultured in the presence of 5 nM EGF for 3–6 d is presented in Fig. 1, B and C. Chronic exposure to EGF causes a 90% increase in peak sodium current (Fig. 1B) and a 2.1-fold increase in calcium current amplitude (Fig. 1C).

Surface membrane area was also stimulated by EGF, as inferred from whole-cell capacitance (Cm) measurements (see Materials and Methods). However, this effect was much less pronounced than the changes in inward current amplitude. For example, in the same experiments of Fig. 1, Cm was 11.6 ± 0.5 pF for control cells and 13.6 ± 0.5 pF for EGF-treated cells.

The onset and reversibility of stimulation of calcium current by EGF is presented in Fig. 2. In each cell investigated, current amplitude was divided by Cm to eliminate membrane area as a variable. A small, but significant increase in calcium current density is already observed within the first 19 h after the beginning of the treatment. The increase gradually develops as a function of time and tends to reach a maximum 5 d later. Likewise, once the growth factor is removed from the culture medium, the Ca channel activity slowly returns to its control value. Little changes are detected 24 h after EGF withdrawal. Full recovery takes >3 d. The increase in sodium current density followed a similar slow time course (data not shown).

In the next experiments, we focused on the changes in Ca channel activity.
EGF Stimulates High-Threshold Calcium Current Density

GH3 cells express two major types of voltage-dependent Ca channels in the plasma membrane, low-threshold channels and high-threshold channels, which differ not only in their activation voltages, but in several other properties as well, including their inactivation and deactivation rates (Armstrong and Matteson, 1985; Matteson and Armstrong, 1986; Kalman, O'Lague, Erxleben, and Armstrong, 1988; Simasko, Weiland, and Oswald, 1988). To identify the Ca channel type being regulated by EGF, we recorded isolated calcium currents after blocking the Na channels with external TTX.

Fig. 3A shows typical calcium currents recorded from a control cell by using relatively long depolarizing pulses to -30 and +20 mV. The current at -30 mV is small and tends to inactivate almost completely during the 150-ms pulse. This current flows mostly through low-threshold channels. Larger depolarizations to +20 mV induce the activation of additional low-threshold channels and also recruits the high-threshold channels. The result is a larger inward current that inactivates rapidly at first and then more slowly. The slowly inactivating phase of the current at +20 mV is carried almost exclusively by high-threshold channels.

The calcium current records in Fig. 3B were obtained from a cell treated with EGF. The growth factor enhances the amplitude of the inward current at +20 mV, but does not seem to have a major effect on the current at -30 mV. Fig. 3C confirms this impression by using pooled data from many cells. Culturing the cells during 5 to 6 days in the presence of 5 nM EGF results in a 2.2-fold stimulation of current density at the end of 150-ms pulses to +20 mV, with no significant change in peak current density at -30 mV. These observations suggest that EGF selectively stimulates the functional activity of high-threshold Ca channels.
Further supporting evidence for this view was derived from tail current measurements, as shown in Fig. 4. When the membrane is repolarized to $-80$ mV after a short activating pulse to $+20$ mV, the inward calcium current initially increases in amplitude, due to the larger driving force for the influx of calcium ions, and then declines as the channels close or deactivate (Fig. 4A, trace a). The decay of the tail current follows two phases, fast and slow, which can be easily separated by fitting exponentials to the current signal (Fig. 4A, traces b and c). As first demonstrated by Armstrong and Matteson (1985), the fast phase corresponds to the closing of high-threshold channels, whereas the slow phase is due to the closing of low-threshold channels. The initial amplitude of each tail component is a measure of the respective calcium conductance activated during the preceding depolarizing pulse.

![Figure 3](image-url)

**Figure 3.** Selective stimulation of high-threshold calcium channels by EGF. (A and B) Calcium currents induced by 150-ms pulses to $-30$ and $+20$ mV in a control cell (A) and a cell cultured in the presence of 5 nM EGF for 5 d (B). In this and all subsequent figures, the patch clamp experiments were carried out using recording solutions B. (C) Peak calcium current density at $-30$ mV (left) and calcium current density measured at the end of 150-ms depolarizations to $+20$ mV (right) in control cells and cells treated with 5 nM EGF during 5–6 d.

The separation of tail current components illustrated in Fig. 4A was carried out in every cell investigated and the resultant tail current amplitudes, normalized by $C_m$, are reported in Fig. 4B. It can be seen that current density through low-threshold Ca channels does not significantly differ between control cells and cells exposed to EGF. In contrast, current density through high-threshold Ca channels is clearly larger in EGF-treated cells.
Insulin and Glucocorticoids Regulate Low-Threshold Ca Channels

A similar approach to that described above was used to examine the long-term regulatory actions of insulin and glucocorticoids on Ca channel activity. Fig. 5A

![Diagram](image)

**Figure 4.** Tail current analysis of EGF action. (A) Calcium channels were opened by a 10-ms pulse from −80 to +20 mV and were closed by the return to the holding potential. Trace a shows the first 4-ms segment of the tail current generated by a control cell in response to membrane repolarization. The tail current was separated into fast and slow components using a two-step fitting procedure. First, a single exponential was fitted to the data points between 1.1 and 8 ms after the onset of repolarization (trace b). The slow exponential component, extrapolated back to 100 μs after the end of the activating pulse, was subtracted out and a second, fast exponential was then fitted to the remaining current (trace c). In this example, the time constants of the fitted exponentials were 0.15 and 4.7 ms. The solid line superimposed over data points in trace a is the sum of the two exponentials. (B) Initial amplitudes of the slow (left) and fast (right) tail current components, normalized by $C_m$, for same cells as in Fig. 3 C. Tail currents were recorded and analyzed using the method described in A.

shows representative calcium currents induced by 10-ms pulses to −30 and +20 mV in a control cell and cells treated with 100 nM insulin or 1 μM dexamethasone during 5 to 6 d. Average values for calcium current amplitudes in the different sets of cells are presented in Fig. 5, B and C. Current records and plots in Fig. 5 indicate that Ca
channel activity is increased by insulin, and decreased by dexamethasone. The inhibitory effect of dexamethasone is mimicked by the natural glucocorticoid cortisol (Fig. 5, B and C). Furthermore, unlike the effect of EGF, the changes in calcium current amplitude induced by insulin and glucocorticoids are more prominent at \(-30\) (Fig. 5 B) than at \(+20\) mV (Fig. 5 C). This suggests a preferential regulation of low-threshold Ca channels.

![Graph showing calcium current amplitude regulation by insulin and glucocorticoids](image)

**Figure 5.** Dual regulation of calcium current amplitude by insulin and glucocorticoids. (A) Calcium currents induced by 10-ms pulses from \(-80\) mV to the indicated membrane potential \((V_m)\) in three different cells. (a) Currents recorded from a control cell. Current records in b and c were obtained from cells grown 5 d in the presence of 100 nM insulin or 1 \(\mu\)M dexamethasone, respectively. (B and C) Calcium current amplitude at the end of 10-ms pulses to \(-30\) mV (B) and peak calcium current at \(+20\) mV (C) for control cells and cells treated with 100 nM insulin (I), 1 \(\mu\)M dexamethasone (D) or 5 \(\mu\)M cortisol (HC) during 5 to 6 d. Values represent means ± SEM of the results obtained in the number of cells indicated in B.

Insulin and glucocorticoids, like EGF, induced small alterations in cell capacitance, as illustrated in Fig. 6 A. These effects have been taken into account in the plots of Fig. 6 B, which present average values for tail current densities recorded on repolarization. Current density through low-threshold Ca channels increases by a
factor of 1.7 in insulin-treated cells, and decreases by 50% in glucocorticoid-treated cells. However, neither insulin nor glucocorticoids significantly affect current density through high-threshold Ca channels.

Fig. 7 displays the regulatory effects of insulin and dexamethasone on low-threshold calcium current density as a function of time. The insulin-induced increase in Ca channel activity gradually develops over the course of several days and reverses slowly after hormone removal (Fig. 7 A). This temporal pattern closely resembles the time course of EGF action observed in Fig. 2. The inhibition of low-threshold current density caused by dexamethasone also corresponds to a long-term effect. No significant change in Ca channel activity is found after a 24-h exposure to the glucocorticoid, and only a partial, small recovery is obtained 16 h after the end of a 6-d incubation period (Fig. 7 B).

In these experiments, insulin and glucocorticoids were used at concentrations that are known to induce saturating effects on hormone production in GH cells (Bancroft, Levine, and Tashjian, 1969; Naess et al., 1980; Kiino and Dannies, 1981; Prager et al., 1988). Qualitatively similar alterations in Ca channel activity to those reported above could be detected by using lower, more physiological concentrations of these hormones. For example, Fig. 8 shows that cortisol treatment decreases low-threshold calcium current density in a dose dependent manner. At 10 nM, the lowest concentration tested, cortisol reduces current density to ~80% of its control value.

**Kinetic Properties of Ca Channels**

*Conductance-voltage relationships.* Tail current analysis was also employed to estimate the relative conductances of low- and high-threshold Ca channels activated in
response to depolarizing pulses of variable amplitude. Calcium tails were recorded at 
-80 mV after 10-ms depolarizations to membrane voltages from -50 to +40 mV, as 
illustrated in Fig. 9A. The initial amplitudes of the slow and fast components in the 
tail currents were then plotted as a function of membrane voltage ($V_m$) during the 
activating pulse. To better compare the voltage dependence of Ca channel conduc-
tance among control cells and cells treated with regulatory factors, the steepness and 
midpoint of the resultant activation curves were quantified by fitting each data set to

![Figure 7](image7.png)

**FIGURE 7.** Time-dependent effects of insulin and dexamethasone on low-threshold Ca channel activity. Current density is the amplitude of the calcium current at the end of 10-ms depolarizing pulses to 
-30 mV divided by $C_m$. Before the patch clamp recording, some cells were exposed to 100 
nM insulin (A) or 1 µM dexamethasone (B) for 1, 3 or 5 to 6 
d (filled symbols). Other cells 
were grown during 6 d in the 
presence of the regulatory fac-
tors, then placed in control cul-
ture medium during 16 h or 3 
d (open triangles and squares). 
Current density was converted 
to a percentage of its average 
value in control cells (4.42 ± 
0.32 pA/pF for these experi-
ments).

![Figure 8](image8.png)

**FIGURE 8.** Dose-dependent inhibition of 
low-threshold Ca channel activity by cortisol. 
Cells were cultured 5 to 6 d in the presence 
of the indicated concentration of the gluco-
corticoid, then subjected to whole-cell patch 
clamp. Calcium current density was evalu-
ated by using 10-ms test pulses to -30 mV.
a Boltzmann function:

\[ I = \frac{I_{\max}}{1 + \exp \left[ -(V_m - V_{1/2})/s \right]} \]  

where \( I \) is tail current amplitude, \( I_{\max} \) the saturating value of \( I \) after activating pulses to positive membrane potentials, \( V_{1/2} \) the voltage for half-maximal activation, and \( s \) the slope factor expressed in mV's. Subsequently, each curve was normalized by the respective \( I_{\max} \) to yield the conductance-voltage (\( G-V \)) relationships shown in Fig. 9, B–D. The smooth lines in these plots are calculated \( G-V \) curves using the mean values for \( V_{1/2} \), \( s \) and relative \( I_{\max} \) given in Table II.

In control cells (Fig. 9 B), the activation of high-threshold Ca channels is first detected at membrane voltages close to \(-30 \) mV, increases with depolarization following a sigmoidal function that is well fit by Eq. 1, and tends to saturate above
+40 mV. Half of the maximum high-threshold conductance is activated at \( \sim +10 \) mV. The \( G-V \) curve for low-threshold Ca channels is also a sigmoidal function, but it is steeper and its midpoint is located \( \sim -40 \) mV to the left relative to that for high-threshold channels. Neither the shape of the \( G-V \) curves nor their position along the voltages axis are significantly modified after chronic treatment with EGF, insulin or dexamethasone (Fig. 9, C and D; Table II). Thus, the alterations in calcium conductance produced by these regulatory factors do not involve any significant changes in the voltage dependence of Ca channel activation.

**Deactivation kinetics.** The chemical messengers investigated had no effect either on the deactivation rates of the Ca channels measured at \(-80 \) mV after 10-ms pulses to \(+20 \) mV. For example, in the same experiments as in Fig. 4 B, the deactivation time constant for high-threshold channels \( (\tau_{d,ht}) \) was \( 0.12 \pm 0.01 \) ms in control cells and \( 0.12 \pm 0.01 \) ms in cells exposed to EGF, whereas the deactivation time constant for low-threshold channels \( (\tau_{d,lt}) \) was \( 3.2 \pm 0.1 \) and \( 3.2 \pm 0.2 \) ms, respectively.

**Table II**

| Experimental condition | Relative \( I_{\text{max}}^{*} \) | \( V_{1/2} \) mV | \( s \) mV |
|------------------------|-------------------------------|-----------|---------|
| Low-threshold Ca channels | Control | 1.00 ± 0.11 | -27.6 ± 2.1 | 8.0 ± 0.6 |
| | EGF | 0.95 ± 0.14 | -29.7 ± 1.0 | 8.0 ± 1.0 |
| | Insulin | 1.72 ± 0.18 | -28.6 ± 1.9 | 7.9 ± 0.5 |
| | Dexamethasone | 0.33 ± 0.04 | -28.5 ± 1.3 | 8.9 ± 1.3 |
| High-threshold Ca channels | Control | 1.00 ± 0.10 | 9.8 ± 1.5 | 11.8 ± 0.9 |
| | EGF | 2.15 ± 0.17 | 11.2 ± 1.0 | 13.6 ± 0.5 |
| | Insulin | 1.03 ± 0.13 | 8.9 ± 1.3 | 12.1 ± 0.6 |
| | Dexamethasone | 1.00 ± 0.09 | 8.9 ± 1.5 | 13.5 ± 1.0 |

*\( I_{\text{max}}^{*} \) was determined in each cell and then normalized relative to its average value in control cells.

**Time course of low-threshold current.** Fig. 10 A illustrates typical calcium currents recorded during maintained depolarizations to \(-30 \) mV from a control cell (trace a) and cells cultured in the presence of insulin or cortisol (traces b and c, respectively). These three current records superimpose almost perfectly after appropriate scaling (trace d), suggesting that there are no major changes in the activation and inactivation kinetics of low-threshold Ca channels associated with the dual regulation of calcium current amplitude by insulin and glucocorticoids. In addition, the decaying phase of the transient current at \(-30 \) mV was well fitted by a single exponential in all cases investigated. The corresponding time constant \( (\tau_{\text{ina}}) \) is an index of the rate of low-threshold Ca channel inactivation. Fig. 10 B shows that the average value of \( \tau_{\text{ina}} \)
at −30 mV is ~30 ms in control cells as well as in cells treated with insulin or glucocorticoids.

**Voltage dependence of low-threshold current inactivation.** The voltage-dependent steady state inactivation of low-threshold Ca channels was studied by applying test pulses to −30 mV from different holding potentials. In Fig. 11, the peak amplitude of the calcium current during the test pulse is plotted against holding potential for a control cell (circles), an insulin-treated cell (triangles) and a cell exposed to cortisol (squares). Data were fitted with smooth lines according to the following function: $I = I_{\text{max}} / (1 + \exp([V_m - V_{1/2}]/s))$. The values for $V_{1/2}$ and $s$ were, respectively, −62.9 and 6.9 mV in the control cell, −63.3 and 6.9 mV in the cell treated with insulin, and −61.1 and 7.0 mV in the cell exposed to cortisol. (Inset) Calcium currents recorded from the control cell showing inactivation at the indicated holding potentials.
In all cases, changing the holding potential from $-80$ to $-90$ or $-100$ mV has little or no effect on current amplitude, which indicates that the fraction of noninactivated Ca channels at $-80$ mV is close to 1.0. At less negative holding potentials, the inward current decreases as a larger fraction of channels inactivate. Inactivation is practically complete at voltages positive to $-40$ mV. The smooth lines in Fig. 11 represent the best fits of the data to a Boltzmann function. As reported in the figure legend, the parameters of the fits are about the same for all curves.

**DISCUSSION**

The results show that current density through voltage-dependent Ca channels in GH$_3$ cells can undergo marked changes in response to the chronic influence of EGF, insulin or glucocorticoids. The type of calcium current being regulated depends on the chemical messenger. EGF increases high-threshold current density, but has no significant effect on current density through low-threshold channels. By contrast, insulin and glucocorticoids selectively modify the low-threshold current.

**Possible Mechanism of Ca Channel Regulation**

The molecular basis of these regulatory actions have not been yet determined. Nevertheless, it is important to mention that the changes in Ca channel activity we have observed take several days to develop. In addition, they are long lasting and can be recorded for many hours after removing the chemical messengers from the extracellular medium. Furthermore, the kinetic properties of the Ca channels (voltage dependence of channel activation, deactivation kinetics at $-80$ mV, time course and voltage dependence of low-threshold current inactivation) are unaffected by the regulatory factors investigated.

These characteristics suggest that the alterations in calcium current density might depend on changes in the number of functional Ca channels in the plasma membrane. Along this regard, it is known that EGF and insulin are recognized by specific cell surface receptors with an intrinsic protein tyrosine kinase activity (Ullrich and Schlessinger, 1990). A signaling cascade that is still poorly understood links receptor activation to nuclear responses. Glucocorticoids on the other hand bind to intracellular receptors that are themselves transcription factors (Evans, 1988). Therefore, we speculate that the reported changes in Ca channel activity possibly involve regulation of gene expression.

**Significance of Ca Channel Regulation**

The altered calcium current density in cells treated with EGF, insulin or glucocorticoids could mediate, at least in part, the regulatory effects of these factors on prolactin production (Naess et al., 1980; Schonbrunn et al., 1980; Kiino and Dannies, 1981; White et al., 1981; White and Bancroft, 1983; Prager et al., 1988). GH cells exhibit spontaneous electrical activity, which consists in action potentials and small fluctuations in membrane voltage around the resting level (Kidokoro, 1975; Taraskevich and Douglas, 1980). Each action potential appears to engage a brief, concerted opening of high-threshold Ca channels, and leads to a transient rise in cytosolic free calcium concentration that is sufficient to trigger prolactin release (Schlegel, Winiger, Mollard, Vacher, Wuarin, Zahnd, Wollheim, and Dufy, 1987).
Over a more extended time scale, calcium entry through high-threshold Ca channels also sustains prolactin synthesis (Enyeart et al., 1987) by enhancing prolactin gene expression (Day and Maurer, 1990). Thus, the expected result of the stimulatory action of EGF on high-threshold calcium current density would be an increased release and synthesis of prolactin due to a larger influx of calcium during action potentials. Since action potential generation in GH cells also depends on Na channel activity (Biales, Dichter, and Tischler, 1977), the enhanced sodium current density in EGF-treated cells may further promote the opening of high-threshold Ca channels.

On the other hand, the dual regulation of low-threshold calcium current density by insulin and glucocorticoids could be significant for prolactin production in two respects. First, a small fraction of low-threshold channels can be open under steady state conditions at negative voltages, near the resting membrane potential (Ritchie, 1993), and may thereby allow a steady influx of calcium at rest that could contribute to support the basal rate of prolactin secretion (Zorec et al., 1991; Horta and Cota, 1993). Second, because of their ability to be open at relatively negative membrane potentials, the low-threshold channels are presumably involved in the pace-making mechanism that controls the triggering of spontaneous spikes (Matteson and Armstrong, 1986; Cota, 1986). If so, the spiking frequency, and consequently the pattern of high-threshold Ca channel activation, should depend on the functional activity of low-threshold Ca channels.

Comparison with Previous Studies

Chronic treatment of GH4C1 cells with 1,25-(OH)2D3 stimulates the production of prolactin (Wark and Tashjian, 1983) and amplifies the influx of extracellular calcium triggered by membrane depolarization with high external K+ (Tornquist and Tashjian, 1989). The latter action depends on new protein synthesis and is totally blocked by nimodipine (Tornquist and Tashjian, 1989). Thus, 1,25-(OH)2D3, like EGF, seems to promote the functional expression of high-threshold L-type Ca channels in the plasma membrane of the GH cells.

The insulin effect on low-threshold Ca channel activity reported here is similar to that previously demonstrated in GH3 cells chronically exposed to 17β-estradiol (Ritchie, 1993). Interestingly, the estrogen-induced increase in low-threshold calcium current density is prevented by cycloheximide and actinomycin-D, which suggests that it could be due to synthesis and insertion of new Ca channels into the plasma membrane (Ritchie, 1993). Insulin and 17β-estradiol together appear to act synergistically to increase the intracellular amount of prolactin in GH4C1 cells (Kiino and Dannies, 1981). It will therefore be of interest to determine the effects of the combined treatment of GH cells with insulin and 17β-estradiol on calcium current density.

Rat melanotropes have provided another useful model system for examining the long-term effects of intercellular chemical signals on pituitary hormone production and voltage-dependent Ca channel activity. For instance, experimental evidence obtained in cultured melanotropes from adult animals suggests that the tonic inhibitory influence of dopaminergic presynaptic neurons on the synthesis and release of proopiomelanocortin-derived peptides (Beaulieu, Goldman, Miyazaki,
Frey, Eskay, Kebabian, and Cote, 1984; Cote, Felder, Kebabian, Sekura, Reisine, and Affolter, 1986) involves a decreased expression of high-threshold Ca channels (Cota and Hiriart, 1989). Consistent with this idea, preliminary data indicate that the high-threshold Ca channel activity of neonatal melanotropes is drastically inhibited after the onset of dopaminergic innervation (Gomora, Navarrete, Marin, and Cota, 1993).

These previous observations, taken together with our present findings, suggest that the long-term regulation of voltage-dependent Ca channels in the plasma membrane may be a general mechanism by which growth factors, hormones, and neurotransmitters give rise to enduring changes in the secretory behavior of pituitary cells. It remains to be seen whether similar regulatory mechanisms can be detected in other types of electrically excitable endocrine cells.

It is important to keep in mind, however, that the chronic effects of extracellular chemical messengers on pituitary cell function may include regulation of other ionic transport systems in the plasma membrane, in addition to voltage-dependent Ca channels. In fact, dexamethasone has been shown to influence potassium channel gene transcription and expression in GH3 cells (Levitan, Hemmick, Birnberg, and Kaczmarek, 1991; Takimoto, Fomina, Gealy, Trimmer, and Levitan, 1993) and our current data indicate that EGF also stimulates sodium current density. Furthermore, in GH4C1 cells, 1,25-(OH)2D3 not only enhances calcium influx through voltage-dependent Ca channels, but also greatly increases the extrusion of calcium from the cell by activating Na+/Ca2+ exchange (Tornquist and Tashjian, 1989). Chronic treatment of GH3 cells with EGF may exert similar dual actions on cellular calcium homeostasis, since it enhances by 50% the average 340/380 fluorescence ratio reported at rest by the calcium indicator fura-2, but reduces the transient increase in cytosolic free calcium induced by the addition of 30 mM KCl to the external medium (Hinkle, Nelson, and Haymes, 1993). Further experiments will be necessary to test this possibility.

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