The neurosecretory anterior pituitary GH4C1 cells exhibit the high voltage-activated dihydropyridine-sensitive L-type and the low voltage-activated T-type calcium currents. The activity of L-type calcium channels is tightly coupled to secretion of prolactin and other hormones in these cells. Depolarization induced by elevated extracellular K+ reduces the dihydropyridine (+)-[3H]PN200-110 binding site density and 45Ca2+ uptake in these cells (22). This study presents a functional analysis by electrophysiological techniques of short term regulation of L-type Ca2+ channels in GH4C1 cells by membrane depolarization. Depolarization of GH4C1 cells by 50 mM K+ rapidly reduced the barium currents through L-type calcium channels by ~70% and shifted the voltage dependence of activation by 10 mV to more depolarized potentials. Down-regulation depended on the strength of the depolarizing stimuli and was reversible. The currents recovered to near control levels on repolarization. Down-regulation of the calcium channel currents was calcium-dependent but may not have been due to excessive accumulation of intracellular calcium. Membrane depolarization by voltage clamping and by veratridine also produced a down-regulation of calcium channel currents. The down-regulation of the currents had an autocrine component. This study reveals a calcium-dependent down-regulation of the L-type calcium channel currents by depolarization.

Voltage-gated Ca2+ channels control the flux of Ca2+ across the plasma membrane in a wide range of tissues and play a crucial role in many physiologic functions that include excitation-contraction and excitation-secretion coupling. Several major types of voltage-gated Ca2+ channels have been identified that differ in their pharmacological and biophysical properties (1–4). A revised classification scheme based on sequence, biophysical, and pharmacological properties has been proposed (5). L-type channels, which are sensitive to 1,4-dihydropyridine antagonists and activators, are distributed widely in the cardiovascular, central nervous, and neuroendocrine systems, and the Ca2+ channel antagonists active at these channels are cardiovascular drugs of clinical significance (6).

These channels represent an important category of pharmacological receptors with discrete drug binding sites. The binding sites have been characterized, and their localization on the major α1 subunit has been well established (7, 8). They are regulated by homologous and heterologous influences and are altered in their expression and function in numerous clinical and pathophysiological conditions (9–13). Activity-dependent regulation is a common regulatory mechanism in a variety of receptor systems including ion channels, and electrical activity plays an important role in the regulation of ion channels. Thus, in many receptors of the G protein category, persistent activation by agonist ligands causes down-regulation of the receptor through a process of receptor internalization that involves an initial reversible phase in which the receptor can be recycled back to the plasma membrane and a later irreversible phase in which the receptor is degraded through the lysosomal machinery (14). In a number of systems, the membrane potential as an activating signal regulates both the function and numbers of ion channels. The membrane potential exerts two distinct categories of control over voltage-gated ion channels including Ca2+ channels, both of which have the property of shutting down cellular Ca2+ influx that is in excess is a pathological signal. Inactivation is a rapid event with a time scale of milliseconds, and down-regulation is a slower event with a time frame of seconds to minutes or hours. Inactivation has both voltage- and Ca2+-dependent mechanisms, and recovery is typically both rapid and complete after the removal of the depolarizing stimulus (15, 16).

Voltage-gated Ca2+ channels are also subject to a slower and more persistent regulation by depolarizing stimuli. Thus, in PC-12 cells (17, 18) and chick retinal neurons (19) that contain L-type Ca2+ channels, chronic depolarization with elevated extracellular K+ down-regulates the channel density measured by 1,4-dihydropyridine binding and channel function measured by 45Ca2+ uptake measurement. In rat myenteric neurons, persistent depolarization causes a slowly developing long term reduction in sustained 1,4-dihydropyridine-sensitive calcium channel current (20). Membrane depolarization reduces both low and high voltage-activated Ca2+ currents in molluscan neurons (21). In the neuroendocrine GH4C1 cells, short-term depolarization (up to 2 h) with elevated K+ produces a decrease in L-type Ca2+ channel density measured by 1,4-dihydropyridine binding and a corresponding decrease in channel function as measured by 45Ca2+ uptake into these cells. This decrease in the binding site density is ac companied with an increase in affinity for the ligand as anticipated from voltage-dependent binding (22).

This work was designed to extend our previous study by characterizing electrophysiologically the short term regulation of L-type Ca2+ channels in GH4C1 cells by membrane depolar-
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ization. This cell line has a high density and a relatively pure population of Ca\(^{2+}\) channels of the T- and L-types (23–26), and the L-type channels are sensitive to 1,4-dihydropyridines (27). Cells of pituitary gland origin are chemically and electrically excitable, and the activation of L-type Ca\(^{2+}\) channels is involved in the regulation of persistent secretion induced by depolarization and hormones such as thyrotropin-releasing hormone (28–31). The activity and regulation of Ca\(^{2+}\) channels is physiologically important for maintaining a homeostasis of hormone secretion. Ca\(^{2+}\) influx into the cell subsequent to depolarization or hormonal response not only acts as a trigger for hormone release but also acts to inactivate the channels and prevent their sustained activation. This is analogous to the hormone-induced desensitization of receptors (32).

**EXPERIMENTAL PROCEDURES**

**Materials**

**Cell Culture Medium**

F-10 medium and fetal bovine serum were purchased from Sigma. Horsera serum was purchased from either Sigma or Life Technologies, Inc.

**Chemicals**

ATP was purchased from Sigma. BAPTA-AM\(^1\) was purchased from Molecular Probes, Inc. (Eugene, OR).

**Glass Electrodes**

Electrodes were pulled from thin-walled borosilicate glass capillaries with an outer diameter of 1.2 mm (TW-120, World Precision Instruments, Sarasota, FL). The pipettes were pulled in two stages on a vertical electrode puller (Model 750, David Kopf Instruments, Tujunga, CA) and had a resistance of 8–10 megohms when filled with internal recording solution.

**Methods**

**Cell Culture**

The rat anterior pituitary cell line, GH\(_{4}C_{1}\), was obtained from Dr. Jane Chisholm (Bayer, Inc., West Haven, CT). Cells were maintained in a monolayer culture in Ham’s F-10 medium supplemented with 15% horse serum and 2.5% fetal bovine serum at 37 °C in a humidified incubator under an atmosphere containing 5% CO\(_2\). Cells were removed from flasks once a week with 0.05% trypsin and were plated either into flasks or sterile 35-mm Petri dishes (Corning) for electrophysiology experiments. The medium was changed every 2 days. Cells that were in culture for 2–6 days were used for experiments.

**Solutions for Electrophysiology**

**Normal Tyrode’s Solution**—Normal Tyrode’s solution contained 132 mM NaCl, 5.8 mM KCl, 1.2 mM MgCl\(_2\), 2 mM CaCl\(_2\), 10 mM HEPES, and 5 mM dextrose. The pH was adjusted to 7.4 with NaOH.

**Depolarizing Tyrode’s Solution**—Depolarizing Tyrode’s solution contained 87 mM NaCl, 50 mM KCl, 1.2 mM MgCl\(_2\), 2 mM CaCl\(_2\), 10 mM HEPES, and 5 mM dextrose. The pH was adjusted to 7.4 with NaOH.

**External Recording Solution**—External recording solution contained 125 mM N-methyl-D-glucamine, 5 mM CsCl, 10 mM HEPES, 1 mM MgCl\(_2\), 5 mM dextrose, and 20 mM BaCl\(_2\). 105 ml of 1 N HCl/liter was added to the solution. The pH was adjusted to 7.4 with CsOH.

**Internal Recording Solution**—Internal recording solution contained 60 mM CsCl, 1 mM CaCl\(_2\), 30 mM HEPES, 10 mM MgCl\(_2\), 11 mM EGTA, 50 mM aspartic acid, and 5 mM Na\(_2\)ATP. The pH was adjusted to 7.4 with CsOH.

**Electrophysiology**

Whole cell voltage clamp experiments were carried out using an Axopatch 200 amplifier (Axon Instruments, Inc. Foster City, CA). Voltages were generated on a Macintosh Ici computer through a Lowpass Bessel filter.

**RESULTS**

**Current Characteristics**—GH\(_{4}C_{1}\), cells express L-type calcium channels. The currents through these channels have been previously characterized both by whole cell and single channel experiments. To establish a base line for studying the calcium channel regulation, the cells were bathed in resting Tyrode’s solution (5.8 mM K\(^+\)) for up to 2 h at 37 °C after removing the culture medium. Barium currents were measured from the cells at the end of the incubation. The total barium current density measured after preincubation in Tyrode’s solution at 37 °C remained within 85–90% of the amplitude of the current observed without any preincubation. The current traces at +10 mV after different incubation times are shown in Fig. 1A, and the current-voltage relationship is shown in Fig. 1B. A plot of normalized currents elicited by step depolarization to +10 mV from a holding potential of −40 mV at different incubation times is shown in Fig. 1C. This shows that the preincubation of GH\(_{4}C_{1}\), cells in serum-free buffer at 37 °C over 2-h time periods does not alter the stability of L-type calcium channel currents. However, once the whole cell configuration was established, the current rapidly ran down to smaller levels within 10 min. All recordings were therefore completed within 3–5 min after establishing the whole cell configuration at the end of any preincubation. The average L-type calcium channel current density obtained using 20 mM barium as an external charge carrier was 17–19 pA/pF. Cell capacitance measured during the experiment using a hyperpolarizing pulse to −60 mV from a holding potential of −40 mV was approximately 23 pF.

**Effects of Cellular Depolarization on Currents**—Cellular depolarization by preincubating the cells in depolarizing Tyrode’s solution (50 mM K\(^+\)) rapidly down-regulated the currents. The current density decreased from 17.39 ± 0.82 pA/pF to 5.36 ± 0.35 pA/pF within 5 min of depolarization, indicating a 69% decrease in the current amplitude (Fig. 2A). The time course of

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\(^1\) The abbreviations used are: BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-N,N',N''-tetraacetic acid tetra(acetoxymethyl)ester; pA/pF, picamperes/picofarad; GH, growth hormone.
down-regulation was established by preincubating cells in depolarizing Tyrode’s solution (50 mM K⁺) for different time periods. Down-regulation reached a maximal level within 5 min, and longer durations of preincubations up to 1 h did not further increase the extent of down-regulation (Fig. 2A and B). Depolarization-induced (50 mM K⁺) down-regulation was reversible. After 30 min of down-regulation by preincubating the cells in depolarizing 50 mM K⁺-Tyrode’s solution, the cells were allowed to recover in resting Tyrode’s solution for different time periods (Fig. 2C). Currents elicited by pulses to +10 mV are shown to depict the recovery from down-regulation. The currents recovered to 80–85% of control levels within 30–45 min of repolarization (Fig. 2D). This also suggests that the phenomenon observed is down-regulation and not the calcium and voltage-dependent inactivation of calcium channels as the recovery from inactivation is relatively fast and complete within seconds.

Down-regulation of L-type calcium channel currents depended on the extent of depolarization. Increasing the concentration of K⁺ ions in the external buffer increased the depolarization stimulus and the extent of down-regulation. The resting cell potential measured in 5.8 mM K⁺-Tyrode was
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**FIG. 3. Down-regulation depends on the strength of the depolarizing stimulus.** Cells were incubated in buffers containing different concentrations of K⁺ for 30 min at 37°C. At the end of incubation, the whole cell currents were measured. Data represent averages of 6–8 cells. 

A. Current traces at −60 mV and +10 mV after incubation in different concentrations of K⁺. B. Current voltage profiles (symbols representing in mV the concentration of potassium ions in extracellular buffer are shown in A). C. Percent down-regulation of current at +10 mV as a function of extracellular [K⁺]. Voltage clamping the cell at −10 mV also down-regulated L-type calcium channel currents. Cells were bathed in 5 mM K⁺ buffer, and the whole cell barium currents recorded from a holding potential of −40 mV. The same cell was then held at −10 mV for 5 min, and then the barium current was recorded from a holding potential of −40 mV. Data represent averages of 6–8 cells. D. Current traces at −60 mV and +10 mV. E. Current voltage profiles (filled circles represent control, and open circles represent traces after holding the cell at −10 mV for 5 min).

To investigate whether the down-regulation was because of cellular depolarization and not non-specifically because of excess potassium in the external depolarizing buffer, we explored alternate depolarization mechanisms. 50 mM K⁺ depolarizes the cell to approximately −15 mV. A similar change in membrane potential can be introduced in cells in resting Tyrode’s buffer by voltage clamping them. If depolarization were to produce a down-regulation, this should produce a similar decrease in the current amplitude. We examined this hypothesis in an experiment where we measured the whole cell currents from a holding potential of −40 mV before and after voltage clamping the cell at −10 mV for 5 min in 5.8 mM K⁺-Tyrode’s solution. Voltage clamping at −10 mV for 5 min decreased the current amplitude in a manner similar to the down-regulation observed with 50 mM K⁺ treatment (Fig. 3C).

Another mode of depolarization also produced a down-regulation of L-type currents. Cells were exposed to the alkaloid veratridine, a sodium channel activator, for 30 min (34, 35). Veratridine decreased the calcium channel currents in a concentration-dependent manner (concentration response data not shown). Treatment with 50 μM veratridine for 30 min decreased the current density by 22%. The current density decreased from 15.87 ± 0.61 pA/pF to 12.34 ± 1.06 pA/pF (Fig. 4, A and B). The decrease in the current density was also accompanied with an enhanced inactivation of the current. Thus, the different modes of depolarization had different effects on down-regulation. Normalized current-voltage curves for cells depolarized with 50 mM K⁺ or with veratridine are shown in Fig. 4, C and D. Cells depolarized with elevated extracellular [K⁺] shifted the peak current to +20 mV from +10 mV, but cells depolarized with veratridine did not show any voltage shift in peak current. Over the 200-ms pulse duration, cells depolarized with elevated extracellular [K⁺] did not show any kinetic changes, but cells depolarized with veratridine showed a faster inactivation of currents (Fig. 4A).

**Ca²⁺ Dependence of K⁺ Depolarization-induced Down-regulation**—The presence of [Ca²⁺] in the extracellular depolarizing buffer was essential for the down-regulation of currents. The removal of [Ca²⁺] from depolarizing buffer to the contaminating levels in double distilled water inhibited the down-regulation, and the current density remained the same as in the cells incubated in normal Tyrode’s buffer. The current traces at +10 mV and the current-voltage relationships in depolarizing buffers having different Ca²⁺ concentrations are shown in Fig. 5, A and B. The down-regulation was enhanced with an increasing concentration of Ca²⁺ in the depolarizing 50 mM K⁺ buffer (Fig. 5C).

BAPTA-AM, a cell permeant analog of BAPTA, chelates intracellular calcium after the hydrolysis of the acetoxyfunctional group (AM) inside the cell. Preloading the cells with 25 μM BAPTA-AM before depolarization did not inhibit the down-regulation caused by depolarization (Fig. 5, D and E). This suggests that the down-regulation may not be attributed to the accumulation of excess Ca²⁺ inside the cell. It might also be because of the fact that the molecular determinants of the Ca²⁺ channel responsible for the down-regulation may be located in microdomains inaccessible to BAPTA-AM.

**Autocrine Component of Down-regulation**—Depolarization-induced down-regulation appeared to have an autocrine component. When the cells were bathed in a stream of depolarizing 50 mM K⁺ buffer as opposed to stagnant buffer, a part of the down-regulation was relieved (Fig. 6). This suggests that hormones or other factors that are released by these anterior pituitary cells into the surrounding medium on depolarization may act on the channels and inhibit their activity. This could be prevented in the stream of depolarizing buffer if the factors are washed out from the vicinity of the cells. However, a substantial component of down-regulation still persisted that was independent of the factors released from the cell, suggesting alternate mechanisms for the down-regulation of the remaining component of the L-type current.

**DISCUSSION**

Membrane potential is an important regulator of ion channel activity. Whereas cellular depolarization on one hand is an important physiologic signal, it also serves as a pathophysiologic trigger. Membrane depolarization can bring about both...
up- and down-regulation of the channel activity. In PC-12 cells, prolonged depolarization by elevated extracellular K⁺ causes concomitant time- and concentration-dependent decreases in both [³H]nitrendipine binding and depolarization-dependent uptake of ⁴⁵Ca²⁺, indicating a decrease in the channel number and function (18). In chick neural retinal cells, chronic depolarization reduces (+)-[³H]PN200-110 binding and ⁴⁵Ca²⁺ uptake (19). Chronic depolarization decreases the density of the macroscopic Ca²⁺ currents in the rat myenteric neurons (36). In pituitary GH4C1 cells, short term depolarization for periods

![Diagram of regulation of L-type calcium channels by depolarization](image)

**Fig. 4.** Comparison of down-regulation induced by 50 mM K⁺ and 50 μM veratridine. Comparison of the effect of depolarization produced by veratridine and by 50 mM K⁺. Cells were incubated either in 5.8 mM K⁺ buffer in the presence and absence of 50 μM veratridine or in 50 mM K⁺ buffer at 37 °C for 30 min. At the end of incubation, the whole cell currents were measured. Depolarization by 50 mM K⁺ did not affect the channel inactivation over 200 ms, but depolarization with veratridine enhanced the inactivation. Data represent averages of 6–8 cells. A, current traces at −60 mV and +10 mV. B, current voltage profiles (symbols as shown in A). C, comparison of normalized I-V plots from cells without 50 μM veratridine (filled circles) and with 50 μM veratridine (open circles) treatment. D, comparison of normalized I-V plots from 5.8 mM K⁺-treated (filled circles) and 50 mM K⁺-treated (open circles) cells. Depolarization by 50 mM K⁺ shifted the voltage dependence of activation by +10 mV, but depolarization by veratridine did not induce a shift in the voltage dependence of activation. Data represent averages of 6–8 cells.

**Fig. 5.** Down-regulation of L-type calcium channels requires the presence of Ca²⁺ in extracellular buffer. The effect of extracellular Ca²⁺ on depolarization-induced down-regulation of currents is shown. Cells were incubated in 50 mM K⁺ buffers containing different concentrations of Ca²⁺ for 30 min at 37 °C. At the end of incubation, the whole cell currents were measured. Data represent averages of 6–9 cells. A, current traces at −60 mV and +10 mV. B, current voltage profiles (symbols as shown in A). C, Percent inhibition of current at +10 mV as a function of extracellular [Ca²⁺]. Preloading the cells with BAPTA-AM did not prevent the down-regulation produced with 50 mM K⁺. Cells were incubated in 5.8 mM K⁺-Tyrode containing 25 μM BAPTA-AM for 30 min at 37 °C and then switched to either 5.8 mM K⁺ or 50 mM K⁺-Tyrode containing 25 μM BAPTA-AM for another 30 min at 37 °C. At the end of incubation, the whole cell currents were measured. Data represent averages of 8–10 cells. D, current traces at −60 mV and +10 mV. E, current voltage profiles (filled circles, 5.8 mM K⁺ and open circles, 50 mM K⁺ in the presence of 25 μM BAPTA-AM).
of up to 2 h decreases the (+)-[^3]H]PN200-110 binding site density by 10-fold accompanied by a 20-fold increase in drug affinity (22). In all of these studies the regulation depended on the presence of extracellular calcium. Cellular depolarization can also cause an up-regulation of the surface channels as well. Depolarization of chick myotubes has been shown to trigger the appearance of (+)-[^3]H]PN200-110 binding sites (37). Binding studies with the radioligand ^125^I-ω-CgTx on subcellular fractions show that the intracellular pool of neuronal N-type Ca^{2+} channels in PC-12-251 cells and IMR-32 cells constituted 60–80% of the total calcium channels (38, 39). This intracellular pool of channels is recruited to the cell membrane on depolarization with KCl. The cell surface ^125^I-ω-CgTx binding sites increased by >200% within 10 min of depolarization with 55 mM KCl. This transiently decreased over a period of 1 h and then stabilized at a level that was higher than the control level.

In this study, we show an electrophysiologic correlation of the calcium channel regulation in pituitary GH_{3}C_{1} cells by short term membrane depolarization. GH_{3}C_{1} and GH_{4} pituitary clonal cells express L-type calcium channels. Currents through these channels have been characterized both by whole cell and single channel experiments (23–25, 40, 41). Short term regulation studies using radioligand binding were also done on these cells (22). Hence, we chose these cells as a model system to study the functional aspects of calcium channel regulation electrophysiologically.

We show that cellular depolarization with extracellular potassium produced an ~70% decrease in the barium current density in GH_{3}C_{1} cells. This process is very rapid, and the decrease in currents is evident with depolarizations as short as 5 min. Even though the earlier studies involving (+)-[^3]H]PN200-110 binding have evaluated the regulation of the channel activity for periods shorter than half an hour, these studies may not be accurate because of the limitation posed by equilibration of the ligand with receptor. In addition and unlike the (+)-[^3]H]PN200-110 binding experiments, the electrophysiologic experiments evaluate the channel activity in the absence of any exogenous 1,4-dihydropyridines, thus precluding any possible additional regulation of the channel by the antagonist itself. Our study also shows that the down-regulation of the L-type calcium channels by depolarization is a reversible phenomenon, and that the currents recover to near control levels when the cells are placed in normal 5.8 mM K^{+} Tyrode. The extent of down-regulation depended on the depolarizing stimuli and increased as the membrane potential was raised by increasing the extracellular potassium. Qualitatively, no appreciable changes in the channel kinetics were observed between the control cells and the cells depolarized with 50 mM K^{+} using barium as charge carrier. However, the down-regulation was accompanied with a +10 mV shift in the voltage dependence of activation to a more depolarized potential. Depolarization of cells by voltage clamping at −10 mV also produced a down-regulation similar to depolarization produced by 50 mM K^{+}. The depolarization of cells with veratridine, a sodium channel activator, also produced a decrease in the channel currents. However, this decrease is not as dramatic as seen with 50 mM K^{+} and also does not shift the voltage dependence of activation.

In our experiments, we observed that the down-regulation of the channel activity could be partially relieved by depolarizing the cells in a stream of depolarizing 50 mM K^{+} buffer rather than in the stagnant buffer. This finding suggests that a factor or factors may be released by these cells on depolarization with 50 mM K^{+} to act on the cell in a feedback manner under the conditions of sustained depolarization and to inhibit the channel activity. In a stream of the buffer, these factors are removed, which prevents their feedback activity on the cells. Hormone-induced modulation of channels is inherent to neurosecretory cells. The activation of calcium channels is essential for the release of hormones like prolactin, GH, and adrenocorticotropic hormone from the anterior pituitary cells (42, 43). Physiologically, this can be achieved by the action of hormones like thyrotropin-releasing hormone. Depolarization with elevated potassium also leads to hormonal release in vitro assays. On the other hand, certain hormones like somatostatin inhibit calcium channel activity and suppress the release of the pituitary hormones. This autocrine regulation of calcium channels is thought to be mediated through G proteins-G_{i} in pituitary cells (29, 42, 44).

The presence of extracellular calcium ions was essential for the down-regulation, indicating that it is a calcium-dependent process. Elimination of Ca^{2+} from depolarizing buffer abolished the down-regulation. Here we show that as the calcium concentration is increased to physiologic levels in the depolarizing buffer, the down-regulation is gradually enhanced. However, the preincubation of cells with 25 μM BAPTA-AM, a cell permeant calcium chelator before depolarization, failed to relieve the down-regulation. This suggests two possibilities: (a) even though BAPTA-AM was hydrolyzed to BAPTA inside the cell, there was a constant flux of Ca^{2+} from the extracellular medium, saturating BAPTA and thereby preventing its action, and (b) the site of action of Ca^{2+} on the channel to produce regulation is very close to the channel pore and is inaccessible to intracellular chelators.

Calcium- and voltage-dependent inactivation of L-type calcium channels are well studied phenomena (16, 45–48). In our study, the depolarizing buffer (50 mM K^{+}) contained 2 mM Ca^{2+}
channels in GH4C1 cells. This is a reversible phenomenon. The down-regulation of currents through L-type calcium channels is consistent with the decrease in the binding site density observed in our studies, suggesting that it is a down-regulation and not inactivation. This finding is also consistent with the decrease in the binding site density observed in response to depolarization in the earlier experiments (22).

In conclusion, we present evidence for depolarization-induced down-regulation of currents through L-type calcium channels in GH4C1 cells. This is a reversible phenomenon. The down-regulation of L-type calcium channels is calcium-dependent and requires the presence of external calcium. The data suggest but do not prove the direct action of calcium on calcium channel to induce the down-regulation. The possible mechanisms for down-regulation may involve an autocrine component.

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