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Voltage-activated calcium channels that must be phosphorylated to respond to membrane depolarization

(cAMP-dependent protein kinase/tumor cell line/dihydropyridines)

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ABSTRACT **Two classes of calcium channels were activated by membrane depolarization in cell-free membrane patches from GH_3 cells, an electrically excitable cell line derived from a mammalian pituitary tumor. One class had a conductance of ≈10 pS in 90 mM barium, had a threshold of activation near −40 mV, and was inactivated rapidly at holding potentials more positive than −80 mV. The other class, with a conductance of ≈23 pS and a threshold nearer −20 mV, did not inactivate in barium but stopped responding to depolarization altogether when the cytoplasmic side of the patch was exposed to a standard physiological saline solution. Buffering the concentration of calcium ions to <10 nM on the cytoplasmic side did not prevent this loss of activity. However, activity was restored and maintained for the duration of the patch when the catalytic subunit of cAMP-dependent protein kinase was added with MgATP to the cytoplasmic side of the membrane. Cell-free patch formation in the presence of the dihydropyridine, BAY K 8644, also delayed the loss of activity, but unlike the catalytic subunit plus ATP, BAY K 8644 alone did not restore activity when it was added after the channels no longer responded to depolarization. Evidently the dihydropyridine-sensitive class of voltage-activated calcium channels must be phosphorylated in order to open when the membrane is depolarized. That hypothesis provides a simple framework for understanding the modulation of calcium channel gating by neurotransmitters, calcium ions, and dihydropyridines.**

Electrophysiological studies of voltage-activated calcium currents have demonstrated that the activity of calcium channels can be modulated *in vivo* by cAMP-dependent phosphorylation (1–3), but the nature of the calcium channels that are modulated by phosphorylation has not been established clearly. The question also remains whether the response to phosphorylating agents reflects a direct action of the kinase on calcium channels or an indirect action mediated by other regulatory molecules whose own activity depends on phosphorylation. Single-channel recording techniques (4) have allowed investigators to address similar questions for potassium channels in cell-free patches of membrane (5). In cell-free patches the cytoplasmic surface can be exposed to purified enzymes in the absence of much of the cell's complex regulatory machinery. At the same time functional transitions in the conformation of individual ion channels can be observed directly.

Studies of calcium channels in cell-free patches (6–8) have been impeded by the rapid, irreversible, and calcium-dependent loss of activity that occurs when the cytoplasmic side of the membrane is exposed to standard physiological saline solutions (9, 10). Chad and Eckert (11) have identified two processes responsible for the rundown of calcium current in dialyzed molluscan neurons: (*i*) an irreversible process that can be interrupted by leupeptin, an inhibitor of calcium-dependent proteases, and (*ii*) a reversible process that can be counteracted by agents that promote cAMP-dependent protein phosphorylation.

Here we describe the effects of the purified catalytic subunit of cAMP-dependent protein kinase on individual calcium channels in cell-free membrane patches under conditions that largely eliminate the irreversible loss of calcium channel activity. The results demonstrate that the dihydropyridine-sensitive class of voltage-activated calcium channels, or molecules closely associated with the channels in the membrane, must be phosphorylated for the channels to open when the membrane is depolarized. Preliminary reports of some of these data have been presented (12, 13).

METHODS

The experiments were conducted on cells of an electrically excitable cell line derived from a rat pituitary tumor (14). The biophysical properties of calcium currents in GH_3 cells have been studied extensively and closely resemble those in many other cell types (15–17). GH_3 cells were obtained from American Type Culture Collection (ATCC CCL 82.1) and were grown on collagen-coated coverslips under 5% CO_2 at 37°C in Ham's F-10 medium supplemented with 15% horse serum, 2.5% fetal calf serum, and 50 μg/units of penicillin-streptomycin per ml (GIBCO). Each experiment was begun on a fresh coverslip of cells and done at room temperature (19–22°C).

Calcium channels were detected as unitary inward barium currents. Patch pipettes (2–5 MΩ) were manufactured from Kovar sealing glass (Corning no. 7052 or 7040) and coated with Sylgard (Dow Corning no. 184) (18). Membrane patches were isolated by gigaohm seals (>20 GΩ), excised in either the outside-out or the inside-out configuration and voltage-clamped (4). The extracellular side of the membrane was bathed in 90 mM $BaCl_2$/15 mM tetraethylammonium chloride/2 μM tetrodotoxin/10 mM Hepes buffered to pH 7.2 with tetraethylammonium hydroxide. The minimal solution bathing the cytoplasmic side of the patch contained 120 mM CsCl and was buffered to pH 7.2 with 40 mM Hepes and CsOH and to pCa 8.0 with 5 mM EGTA or bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetate (BAPTA, Molecular Probes, Junction City, OR) (19). The minimal solution also contained 0.2 mM leupeptin (Chemicon, El Segundo, CA) to prevent the irreversible loss of activity attributed to proteolysis (11). Glucose (20 mM) was added to the solution in the chamber. The patches were depolarized for ≈100 ms every 6 s, and the records were stored on magnetic tape. The data were filtered at 2 kHz with a low-pass, 8-pole Bessel filter and digitized at 10 kHz for analysis on an Indec 11-23 system

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Abbreviation: BAPTA, bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′,-tetraacetate.

*Deceased June 16, 1986.

(INDEC Systems, Sunnyvale, CA). Events longer than 200 μs reached at least 90% of maximum amplitude.

Affinity-purified proteins were prepared by A. Nairn, Rockefeller University (catalytic subunit of cAMP-dependent protein kinase) and K. Diltz, University of Washington (protein kinase inhibitor protein). Dihydropyridines were obtained from Miles Laboratories (Naperville, IL). ATP was always added as a magnesium salt.

RESULTS

Two Types of Calcium Channels. Fig. 1 illustrates the two types of calcium channels activated by depolarization in outside-out patches voltage-clamped to −80 mV. The channels with the smaller conductance (≈10 pS) in 90 mM barium are activated by depolarization above −40 mV but inactivate significantly at holding potentials more positive than −80 mV. The other channels have a larger conductance (≈23 pS), require larger depolarizations (nearer −20 mV) for activation, and do not show voltage-dependent inactivation in barium. Because both types are blocked by cobalt (2 mM) but not by tetrodotoxin (2 μM), and both have extrapolated reversal potentials more positive than +40 mV, we believe they represent two classes of voltage-activated calcium channels that have been described in many excitable cells (8, 20–22).

The two classes of calcium channels differ not only in conductance and in the voltage-dependence of activation and inactivation, but also in the fact that the channels with the smaller conductance in barium remain active in cell-free patches exposed to the minimal solution (Fig. 1*C*). In contrast, the channels with the larger conductance stopped opening altogether within 15 min of cell-free patch formation (Fig. 2). In 61% (11/18) of the outside-out patches exposed to the minimal solution, many of which contained more than one functional channel, activity of the channels with the larger conductance ceased before 5 min had elapsed (Fig. 2), and subsequent depolarizing steps to +10 mV, where activation was initially maximal, produced no resolvable openings of the channels with the larger conductance.

The complete loss of activity of this particular class of calcium channels occurred even though the concentration of free calcium ions on the cytoplasmic side of the patch had

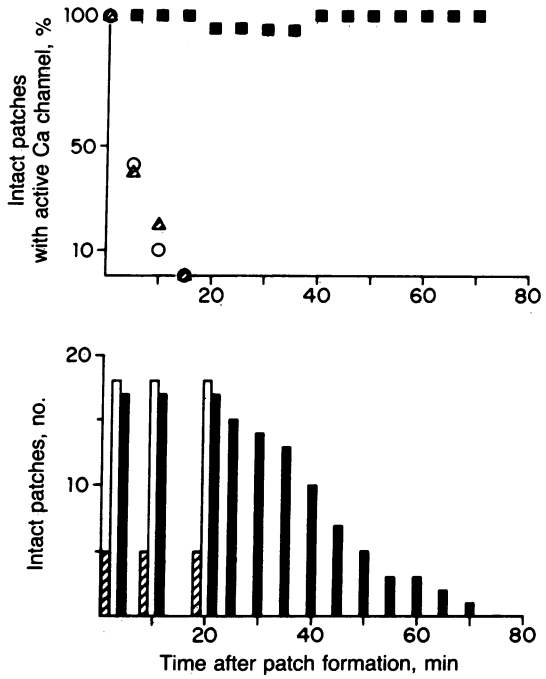

FIG. 2. Phosphorylation prevents the loss of activity in outside-out patches. Open circles and bars, pipette contained minimal solution; closed squares and bars, 1–10 μg of catalytic subunit per ml/2 mM MgATP/1 mM dibutyryl cAMP were added to the minimal solution; cross-hatched triangles and bars, only catalytic subunit and dibutyryl cAMP were added (ATP-Mg was omitted).

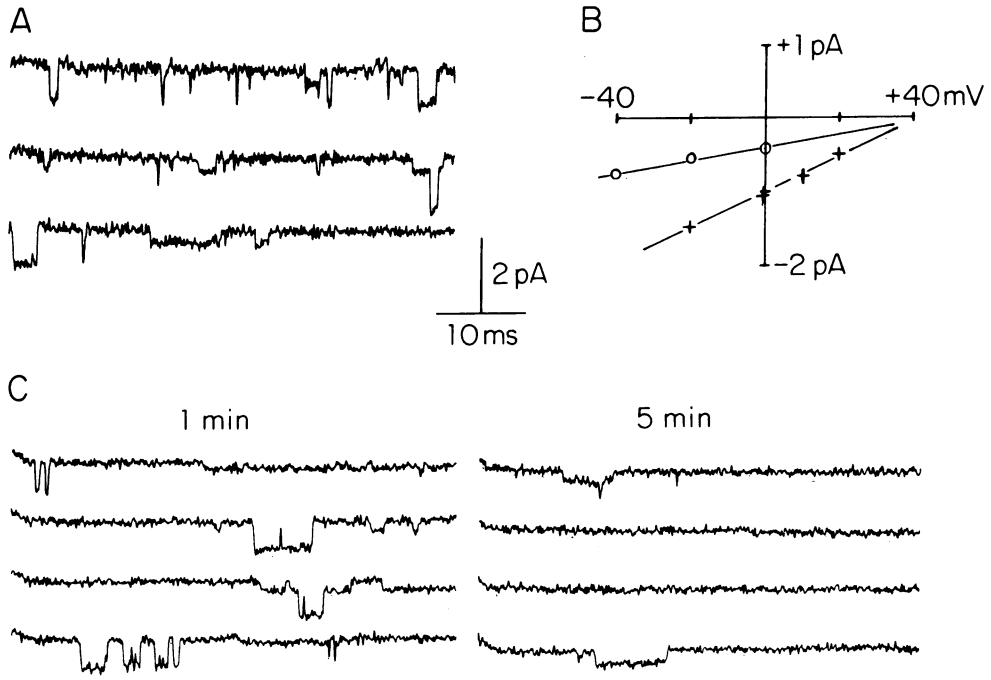

FIG. 1. Two classes of unitary barium currents in cell-free membrane patches. (*A*) Representative traces during voltage steps from −80 to 0 mV. (*B*) Current–voltage relation in 90 mM barium gives single-channel conductances of ≈10 pS and 23 pS and extrapolated reversal potentials more positive than +40 mV. (*C*) Representative traces from another outside-out patch during voltage steps from −80 to +10 mV. The patch pipette contains the minimal solution described in *Methods*. Five minutes after forming the cell-free patch, the channel with the larger conductance no longer responds to depolarization.

been buffered to <10 nM with 5 mM EGTA. Replacing EGTA with BAPTA, a more effective buffer of rapid Ca^{2+} transients (23) that might have occurred during patch formation, did not prevent the loss of activity either (Table 1). Furthermore, the activity of the channels with the larger conductance ran down even faster in inside-out patches (Fig. 3), where one might expect effective buffering to be established more quickly. Thus, it is unlikely that the observed loss of activity resulted from inadequate buffering of calcium ions at the cytoplasmic surface (10), and the continued activity of the channels with smaller conductance rules out any nonspecific damage to those patches.

Phosphorylation Sustains Activity. The catalytic subunit (1–10 μg per ml) of the cAMP-dependent protein kinase completely prevented the loss of calcium channel activity when it was included with the magnesium salt of ATP (2 mM) and dibutyryl cAMP (1 mM) in the solution bathing the cytoplasmic side of the membrane (Fig. 2). In 94% (16/17) of the outside-out patches exposed to this mixture, the activity of the channels with the larger conductance continued for as long as the gigaohm seal remained intact—more than 1 hr in some cases. Even when the membrane potential was held at 0 mV for several minutes, the channels continued to open in clusters of brief events. The unitary barium currents maintained by the presence of catalytic subunit and ATP had the same voltage-dependent properties as those recorded before the loss of activity in the minimal solution.

Dibutyryl cAMP was included in the initial experiments with the catalytic subunit and ATP to saturate any remaining regulatory subunit (11), but dibutyryl cAMP was not essential for preventing the loss of activity (Fig. 3, Table 1). Without ATP, however, the catalytic subunit alone was ineffective (Fig. 2) in preventing activity loss, so we conclude that the kinase sustained calcium channel activity by catalyzing phosphorylation.

Phosphorylation Restores Activity. Because outside-out patch formation involves several seconds of whole-cell recording while the solution in the pipette mixes with the cytoplasm at the tip of the pipette, it is possible that the kinase phosphorylates some other cell constituent, which, in turn, influences the behavior of calcium channels in isolated patches. That possibility was tested on inside-out patches that were excised initially into the minimal solution (Fig. 3). The experiments on inside-out patches from GH_3 cells were complicated by the membrane frequently sealing over to form a small vesicle at the tip of the pipette. Vesicle formation not only blocked access of the catalytic subunit to the cytoplasmic side of the membrane, but it also attenuated current flow across the patch so much that unitary currents were no longer detectable. Nevertheless, in the four inside-out patches that lasted long enough to complete the experiment, the catalytic subunit plus ATP restored calcium channel activity to patches lacking activity for several minutes. Because the cytoplasmic face of the membrane was perfused continuously until the catalytic subunit was added, we conclude that the phosphorylated site that regulates calcium channel gating is closely associated with the molecules forming the channel in the membrane.

Table 1. Phosphorylation prevents washout

Pipette solution*	Patches with active Ca channels 15 min after patch formation, % (*n*)
EGTA replaced by BAPTA (5 mM)	0 (0/9)
CS (1–10 μg/ml) + MgATP (2 mM)	100 (8/8)
MgATP (2 mM)	79 (11/14)
MgATP (2 mM) + PKI (~20 ng/ml)	22 (2/9)
BAY K 8644 (1 μM) before washout	67 (4/6)
BAY K 8644 (0.1–10 μM) after washout	0 (0/5)

CS, catalytic subunit of cAMP-dependent kinase; PKI, protein inhibitor of CS (26). BAY K 8644 was added to the barium solution in the bath.

*Additions to the minimal CsCl solution described in *Methods*.

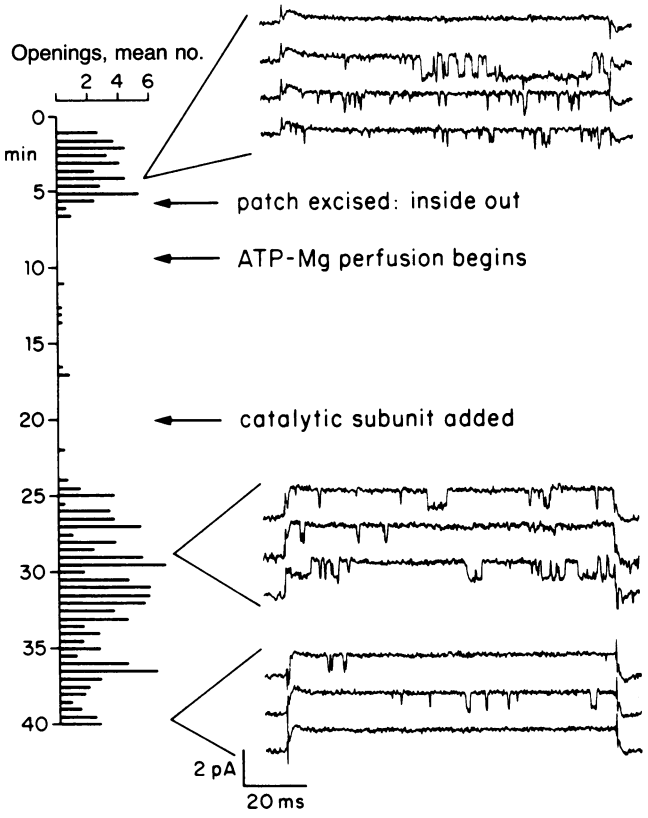

FIG. 3. Phosphorylation reverses the loss of activity in an inside-out patch. The patch was held at −40 mV and stepped to 0 mV for 120 ms every 6 s. The average number of channel openings per trace was determined over 30-s intervals and plotted versus time. The experiment ended after 40 min when the gigaohm seal was lost.

ATP Acts Through an Endogenous Kinase. Earlier experiments on calcium channels that did not run down in cell-free patches or dialyzed neurons routinely included ATP in the solution bathing the cytoplasmic side of the membrane (10, 20, 24). We have also observed that activity runs down much less frequently in the presence of 1 mM ATP (Table 1); however, unlike the effects observed with the catalytic subunit plus ATP, the effect of ATP alone varied widely, preventing the loss of activity in some patches but not in others. Because the cAMP-dependent kinase in brain tissue is often membrane bound (25), it is possible that ATP prevents loss of activity by serving as a substrate for an endogenous cAMP-dependent kinase isolated with the patch. We have tested that possibility in GH_3 cells by asking whether the specific protein inhibitor of the cAMP-dependent kinase (26) eliminated calcium channel activity in the presence of ATP. When the inhibitor (≈20 ng per ml) was included in the solution with ATP, calcium channel activity in most patches (7/9) ran down as though no ATP had been added (Table 1).

Dihydropyridines. Dihydropyridines are potent modulators of the voltage-activated calcium channels that have a larger conductance in barium, require larger depolarizations for activation, and do not inactivate in barium (8, 21, 27). These dihydropyridine-sensitive channels have the same voltage-dependent properties as the calcium channels that require phosphorylation to remain active in our experiments. However, dihydropyridines have been used to reconstitute calci-

um channel activity in the absence of exogenous ATP (28–30), which raises the possibility that phosphorylation may not be required for the activity of calcium channels modulated by dihydropyridines.

Dihydropyridines also modulate the gating of calcium channels maintained by exogenous phosphorylation in cell-free patches from GH_3 cells (Fig. 4). In particular, BAY K 8644 (31, 32) produced a dramatic increase in the mean open time of the channels. If phosphorylation were not required for the activity of those channels, one might expect BAY K 8644 to be as effective as the catalytic subunit and ATP at reversing the loss of activity, but that was not the case. BAY K 8644 (0.1–10 μM) had no effect on calcium channel activity when it was added to patches in the minimal solution after rundown was complete. However, when intact cells were exposed to BAY K 8644 before the patches were excised, BAY K 8644 not only modified calcium channel gating, it also delayed the loss of activity in the minimal solution (Table 1). Thus, BAY K 8644 appears to modulate calcium channel activity only when it is applied in the presence of phosphorylating enzymes. Because the channels modulated by BAY K 8644 are also less susceptible to rundown, we propose that BAY K 8644 inhibits dephosphorylation of the channels. That inhibition could explain why reconstitution of these phosphorylation-dependent channels from membrane fragments without exogenous ATP has been achieved more frequently in the presence of BAY K 8644 (28–30).

DISCUSSION

The experiments reported here demonstrate that cAMP-dependent phosphorylation is both necessary and sufficient for the activity of one class of voltage-activated calcium channels in the plasma membrane of GH_3 cells. This confirms the hypothesis developed from earlier studies on intact cardiac muscle cells (2, 33) and dialyzed molluscan neurons (3, 11) that dephosphorylation of a prominent class of calcium channels leaves the channels in a state from which they do not open in response to membrane depolarization. We have identified those channels as the dihydropyridine-sensitive calcium channels that do not inactivate in barium at depolarized holding potentials. In addition, biochemical studies have established that the putative calcium channel protein, purified from skeletal muscle by its affinity for dihydropyridines, contains a protease- and phosphatase-sensitive, cAMP-dependent phosphorylation site on one of its subunits (34, 35). We propose that dephosphorylation of that site, not voltage, inactivates the channel and produces an irreversible loss of activity in the absence of rephosphorylation.

Historically two distinct mechanisms have been described for the inactivation of voltage-activated calcium currents in whole cells (36): some calcium channels inactivate directly in response to membrane depolarization, whereas others only become inactivated when depolarization results in the accumulation of calcium ions inside the cell. Calcium channels showing voltage-dependent inactivation do not require exogenous phosphorylation to remain active in cell-free membrane patches (8, 12). In contrast, dihydropyridine-sensitive calcium channels showing calcium-dependent inactivation in snail neurons and GH_3 cells do require phosphorylation to respond fully to depolarization (11, 37), and Chad and Eckert have suggested that both calcium-dependent inactivation and rundown result from dephosphorylation of the channels by an endogenous calcium-dependent phosphatase (11).

Calcineurin, a calcium and calmodulin-dependent phosphatase originally purified from mammalian brain (38, 39), accelerates calcium-dependent inactivation of calcium current when it is perfused intracellularly with calmodulin in dialyzed molluscan neurons (11). A large fraction of that enzyme in brain tissue is membrane-bound, often in association with the cAMP-dependent kinase (40). GH_3 cells contain calcineurin (41), and its basal, calcium-independent activity may be responsible for the loss of activity we observe in barium solutions in the absence of phosphorylating agents

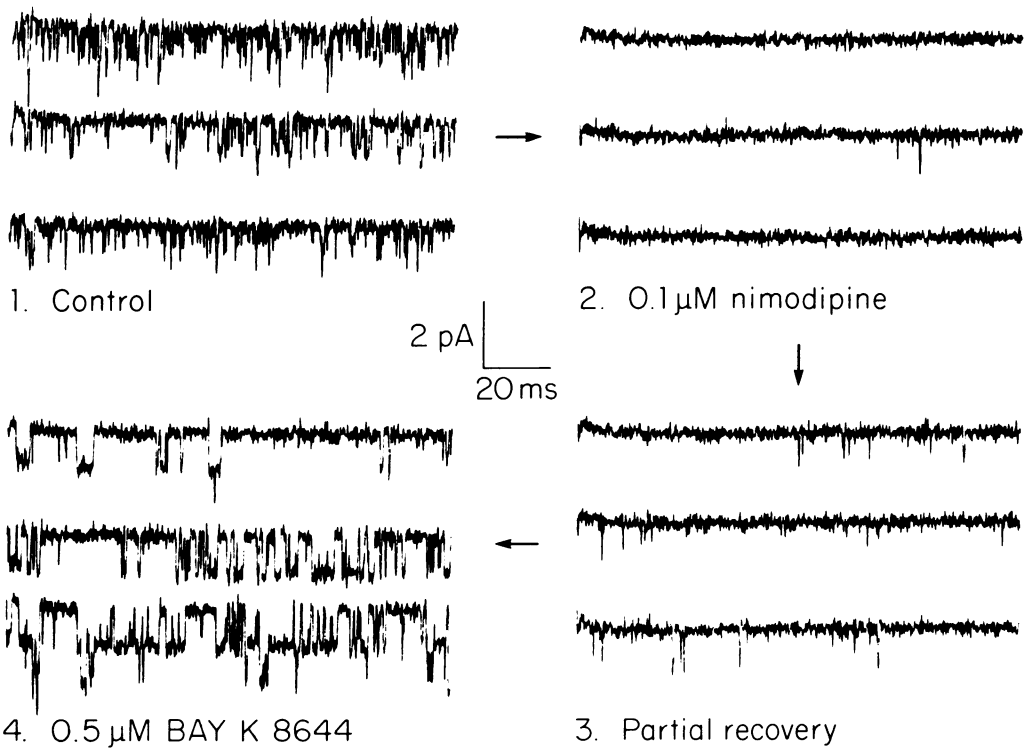

FIG. 4. Dihydropyridines modulate gating of calcium channels maintained by phosphorylation. Representative traces from an outside-out patch during voltage steps from −40 mV to 0 mV. The pipette contained 2 μg of catalytic subunit per ml/2 mM ATP-Mg in addition to the minimal solution.

and for the brief periods of inactivity that occur even in the presence of exogenous kinase.

Calcium ions trigger vital processes in a wide variety of cells. Our experiments have revealed a new property of the membrane protein best adapted to inject calcium rapidly into the cell (22). The phosphorylation dependence of voltage-activated calcium channels demonstrated here provides the cell with a dynamic mechanism for modulating calcium fluxes in response to external signals, fluctuating levels of internal calcium, and the metabolic state of the cell. Thus, calcium channels are a likely focus for the regulatory pathways that underlie the plasticity of nerve cell behavior.

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