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Modulation of L-Type Ca^{2+} Channels in Clonal Rat Pituitary Cells by Membrane Depolarization

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SUMMARY

The modulation of L-type Ca^{2+} channels by membrane depolarization, in terms of channel number, function, and interaction with 1,4-dihydropyridine ligands, has been characterized in clonal rat pituitary cells (GH_4C_1) and rat cerebellar granule cells. Membrane depolarization by 50 mM extracellular K^+ for 120 min caused an approximately 90% reduction in the total number of [^{3}H]PN200–110 binding sites (B_{max}) and an approximately 20-fold increase in binding affinity in a whole-cell binding assay. Similar results were obtained in a primary culture of rat cerebellar granule cells. In GH_4C_1 cells the dissociation constant (K_d) and B_{max} were changed from 2.15 nM and 214 fmol/mg at 5 mM K^+ to 110 pM and 24 fmol/mg at 50 mM K^+, respectively. The changes in affinity and B_{max} were both dependent on the extracellular K^+ concentration. The affinity change resulted from an increased association rate constant (increased from 0.17 to 3.11×10^8 M^{-1} min^{-1} after depolarization) and an unchanged dissociation rate constant (0.032 min^{-1}). Depolarization for 2 hr reduced the number of [^{3}H]PN200–110 binding sites in the membrane fraction by approximately 50%, but no significant change was detected in total cell homogenates, suggesting removal of L-type Ca^{2+} channels from the cell surface after depolarization. Blockade of the internalization process by concanavalin A and phenylarsine oxide inhibited the depolarization-induced reduction of L-type Ca^{2+} channels on the cell surface. A decrease in the number of functional channels on the cell surface, as revealed by stimulated $^{45}Ca^{2+}$ uptake, accompanied the change in [^{3}H]PN200–110 binding. Reduction of $^{45}Ca^{2+}$ uptake had two exponential components, i.e., rapid (with a time constant of about 2.5 min), with a rapid rate of recovery, and slow (with a time constant of 54 min), with a correspondingly slow rate of recovery. Depolarization of the cells with veratridine (50 μM) or treatment of the cells with the Ca^{2+} ionophore A23187 (10 μM) had effects similar to those of K^+ depolarization on [^{3}H]PN200–110 binding sites and stimulated $^{45}Ca^{2+}$ uptake. The change in [^{3}H]PN200–110 binding sites in whole-cell and membrane preparations occurred rapidly, becoming prominent within 45 min, and largely recovered when the cells were repolarized. The down-regulation of L-type Ca^{2+} channels is dependent on Ca^{2+} entry via a calmodulin-dependent process.

Ca^{2+} channels are widely distributed and serve many functions. The rapid entry of Ca^{2+} ions through VGCC generates electrical and chemical signals critical to the control of many cellular processes, including muscle contraction, neurotransmitter release, hormone secretion, and cell differentiation and growth. Four major types of VGCC have been identified (L, N, T, and P), each with characteristic pharmacological and electrophysiological properties (1, 2). Of the four types of VGCC, the L-type channel has been studied extensively because it is sensitive to the clinically available Ca^{2+} channel antagonists such as verapamil, nifedipine, and diltiazem. These antagonists are used effectively for the treatment of several cardiovascular disorders, including angina pectoris, hypertension, and certain cardiac arrhythmias (reviewed in Refs. 3 and 4).

The brain expresses a high density of L-type channels, but under normal conditions the L-type Ca^{2+} channel antagonists have little effect on neuronal function (5). However, evidence is accumulating that these antagonists can act directly on neuronal L-type Ca^{2+} channels and modulate several central neuronal functions under conditions associated with brain ischemia, brain damage, and brain dysfunction related to aging (6). These observations prompted the study of the interaction of these Ca^{2+} channels and their antagonists in neuronal and neurosecretory cells under depolarized conditions.

Ion channels, in common with other pharmacological receptors, are regulated species. L-type Ca^{2+} channels are up- and down-regulated by homologous and heterologous influences including membrane potential and activator and antagonist drug exposure (reviewed in Ref. 7). Chronic depolarization with elevated $[K^+]_o$ reduces channel number, as measured by 1,4-dihydropyridine binding and $^{45}Ca^{2+}$ uptake, in PC-12 cells (8, 9) and chick retinal neurons (10). Chronic depolarization also reduces Ca^{2+} current in cultured myenteric neurons (11).

Membrane potential also regulates the kinetic properties of

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ABBREVIATIONS: VGCC, voltage-gated Ca^{2+} channels; CGC, cerebellar granule cells; IBMX, isobutylmethylxanthine; LDH, lactate dehydrogenase; TPP^+, tetraphenylphosphonium; $[K^+]_o$, extracellular K^+ concentration.

drug binding to Ca^{2+} channels by altering the affinities or access of drug to the several states of the channels (4). The binding of Ca^{2+} channel antagonists is well established to be voltage dependent, with affinity increasing with decreasing membrane potential or increasing frequency of stimulation (12–15). Although most extensively studied by electrophysiological techniques, voltage-dependent binding can also be studied by radioligand binding in intact cells at different membrane potentials (16, 17). We have studied this phenomenon extensively in cultured cardiac cells (18, 19).

To study further the influence of membrane potential on the regulation and properties of L-type Ca^{2+} channels, we have investigated the rat anterior pituitary cell line GH_4C_1. This cell line has a high density and a relatively pure population of Ca^{2+} channels of T and L types (20, 21). Cells of pituitary gland origin have been found to be chemically and electrically excitable, and the persistent secretion induced by thyrotropin-releasing hormone is mediated by the activation of L-type Ca^{2+} channels (22). Voltage-dependent blocking of Ca^{2+} channels by nimodipine has been studied electrophysiologically in this cell line (20). The plasticity of Ca^{2+} channels is physiologically important for maintaining homeostasis of hormone secretion. Decreased activity of L-type Ca^{2+} channels induced by prolonged exposure to gonadotropin-releasing hormone contributes to the desensitization of pituitary gonadotropin secretion (23). More limited studies used rat CGC in primary culture. These cells also carry L-type current, have high affinity 1,4-dihydropyridine binding sites, and show Ca^{2+} influx upon depolarization (24, 25).

Materials and Methods

Cell culture. The rat anterior pituitary cell line GH_4C_1 was obtained from the American Type Culture Collection. Cells were maintained in monolayer culture at 37°, in a humidified incubator under an atmosphere containing 5% CO_2, in Ham's F-10 medium supplemented with 15% horse serum and 2.5% fetal bovine serum. Cells were removed from flasks with 0.04% trypsin and plated at about 10^4 cells/cm^2 on plastic tissue culture dishes for experiments (Falcon). Usually the medium was changed on day 4 and the cells were used in experiments in the next 2 days, when monolayers were essentially confluent but the cells were still dividing.

Primary cultures of rat CGC were prepared as described previously (24). Briefly, cerebella were dissected from 6–8-day-old newborn rats. The cells were dissociated with trypsin and DNase I and cultured in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum and 25 mM KCl, on polylysine-coated Petri dishes. Fibroblast cells were removed by treatment of the culture with 1 μM cytosine arabinoside.

Intact cell binding assays. (+)-[^{3}H]PN200–110 was used as a 1,4-dihydropyridine ligand for Ca^{2+} channel binding assays. It has been used extensively in previous studies, shows high specificity and high affinity for L-type Ca^{2+} channels, and is a single enantiomer (10, 18, 19). Saturation and kinetic analyses of (+)-[^{3}H]PN200–110 binding were performed with intact cells attached to the culture dishes (35 mm or 60 mm). The medium was replaced with Hanks' buffer containing varied concentrations of K^+, in which the Na^+ concentration was adjusted to maintain osmolarity, and the cells were incubated with various concentrations of (+)-[^{3}H]PN200–110, with or without unlabeled ligand, at 37° for 90 min (for saturation assays) or for varying periods of time (for kinetic studies). At the end of the incubation period, the radioligand-containing buffer was aspirated under vacuum and the culture was washed twice in 15 sec with ice-cold buffer with the same concentration of K^+. Cells were solubilized overnight with 1 or 1.5 ml of 0.5 M NaOH and radioactivity was determined by liquid scintillation counting. Nonspecific binding was routinely determined in the presence of 1–6 μM unlabeled PN200–110. Protein was determined by the method of Bradford (26), using bovine serum albumin as standard.

The kinetics of [^{3}H]PN200–110 binding were studied in depolarized and polarized cells as described by Wei *et al.* (18). The time course of association was determined by incubating cells with various concentrations of [^{3}H]PN200–110 for various periods of time. To determine the time course of dissociation, [^{3}H]PN200–110 was equilibrated with cells for 90 min. Dissociation was initiated by addition of excess unlabeled PN200–110 (10^{-6} M) and the bound radioligand was determined at various times. The dissociation of radioligand was described by the equation

$$[RB]_t = [R]_0 e^{-k_{-1}t}$$

where $[RB]_t$ is the amount of radioligand bound at time t, $[R]_0$ is the amount of radioligand bound at time 0, and k_{-1} is the dissociation rate constant. For association a pseudo-first-order process was assumed, holding the ligand concentration relatively constant by ensuring that <10% was bound. Thus, association was described by the equation

$$[RB]_t = [R]_e(1 - e^{-k_{obs}t})$$

where $[R]_e$ is the amount of radioligand bound at equilibrium and k_{obs} is the apparent association rat constant. The actual association rate constant k_1 was calculated by the equation

$$k_1 = (k_{obs} - k_{-1})/[L]$$

where [L] is the ligand concentration.

This intact cell binding assay appears to detect binding sites on the cell surface, because PN200–110 was found to be distributed mainly in the plasma membrane (27). Additionally, use of a permanently charged 1,4-dihydropyridine [SDZ 207–180, an analog of PN200–110 bearing a $(CH_2)_{10}$-$N^+(CH_3)_3$ group at the C_3 ester position] defined a level of specific binding similar to that found with PN200–110.

[^{3}H]PN200–110 binding to homogenate and membrane preparations. Binding of [^{3}H]PN200–110 was studied using cell homogenate or membrane preparations. Briefly, cells were scraped from the plates in 5 mM Tris buffer, pH 7.2, and homogenized by 10 strokes of a glass-Teflon pestle homogenizer. The homogenate was used directly in the binding assay or for the membrane preparation. The latter was made by centrifugation of the homogenate at 30,000 × *g* for 10 min and resuspension of the pellet in 50 mM Tris buffer, pH 7.2. This procedure generates a "heavy" microsomal membrane preparation, and the "light" membrane fraction remains in the supernatant (28). The cell homogenate or membrane protein was incubated with various concentrations of [^{3}H]PN200–110 in 5 ml of Tris buffer (50 mM, pH 7.2) for 90 min at 25°. Incubation was terminated by rapid filtration under vacuum through Whatman GF/B filters, followed by two washes (total, 15 ml) with ice-cold Tris buffer, using a cell harvester (Brandel Instruments, Gaithersburg, MD). Radioactivity was determined by liquid scintillation counting at an efficiency of 45–50%.

$^{45}Ca^{2+}$ uptake. Ca^{2+} uptake studies were carried out at 37° as described (18, 29). Growth medium was removed, monolayers were washed with Hanks' solution, and 2 ml of resting (5 mM $[K^+]_o$) or depolarizing (50 mM $[K^+]_o$) Hanks' solution containing $^{45}Ca^{2+}$ were added to each dish. Incubation was then continued for 20 sec. At the end of the incubation, $^{45}Ca^{2+}$-containing buffer was aspirated and the cultures were washed three times in <15 sec with ice-cold Hanks' buffer containing 5 mM $LaCl_3$. Cells were solubilized in 0.5 N NaOH, and radioactivity was measured by liquid scintillation counting. Under these experimental conditions, $^{45}Ca^{2+}$ uptake through Na^+ channels or the Na^+/Ca^{2+} exchange processes was negligible (29).

Membrane potential measurements. Membrane potential was estimated from the equilibrium distribution of [^{3}H]TPP^+ between the intracellular and extracellular spaces (18, 29). Cells in monolayer cultures were washed with Hanks' solution and incubated at 37° for 30 min in 2 ml of Hanks' solution containing different concentrations of K^+ and 2 nM [^{3}H]TPP^+. The uptake of [^{3}H]TPP^+ was terminated by washing of the cells three times with ice-cold Hanks' solution. Cells

were solubilized and radioactivity was determined. The distribution of TPP^+ is according to the Nernst equation, and the transmembrane potential V_m could be estimated by the relationship:

$$V_m = (-RT/F)\ln([TPP]_{in}/[TPP]_{out}) = -61 \log([TPP]_{in}/[TPP]_{out})$$

Intracellular volume was estimated using [^{14}C]inulin as an extracellular marker and [^{3}H]H_2O to measure total volume (18, 29, 30).

LDH assay. LDH is a cytosolic enzyme and serves as an indicator of cell integrity. Cells were solubilized with phosphate-buffered saline containing 0.5% Triton X-100, pH 7.4, and were centrifuged to remove debris. The enzymatic activity was measured spectrophotometrically by following the conversion of NADH to NAD in the presence of pyruvate and cell extract. One unit of LDH activity is defined as a decrease of 1 absorbance unit/min in the absorption of light at 340 nm, using a pathlength of 1 cm (31).

Materials. [^{3}H]PN200–110 [isopropyl-4-(2,1,3-benzoxadiazol-4-yl)-1,4-dihydro-5-methoxycarbonyl-2 ,6-dimethyl-3-pyridinecarboxylate] (specific activity, 87.0 Ci/mmol), [^{3}H]TPP^+ (specific activity, 39.4 Ci/mmol), [^{14}C]inulin (3 mCi/g), and $^{45}Ca^{2+}$ (in the form of $CaCl_2$; specific activity, 23.8 mCi/mg) were purchased from DuPont-New England Nuclear (Boston, MA). Tissue culture medium, serum, and antibiotics were obtained from GIBCO (Grand Island, NY). SDZ 207-180, a permanently charged dihydropyridine, was a gift from Sandoz (Basel, Switzerland).

Results

Dependence of membrane potential on $[K^+]_o$. The membrane potential (V_m) of GH_4C_1 cells was varied by changes in $[K^+]_o$ and was measured by the [^{3}H]TPP^+ distribution procedure (Fig. 1). The resting V_m determined at $[K^+]_o$ of 5 mM was −50 mV, in close agreement with literature values of −51 to −48 mV (28, 32, 33). At $[K^+]_o$ of >30 mM, the relation between V_m and $[K^+]_o$ agreed well with the Nernst equation. Between 5 and 20 mM $[K^+]_o$, V_m deviated from the line, as would be expected if the GH_4C_1 cells were also permeable to other ions such as Na^+ and Cl^- (34). At $[K^+]_o$ of 50 mM the membrane was depolarized to −31 mV; this new V_m could be maintained for at least 2 days at this $[K^+]_o$.

Dependence of equilibrium [^{3}H]PN200–110 binding on membrane potential. Binding of [^{3}H]PN200–110 was measured as a function of different membrane potentials, achieved by varying K^+ concentrations in the buffer. Fig. 2 shows the saturation curve of [^{3}H]PN200–110 binding in intact GH_4C_1 cells under resting (5 mM $[K^+]_o$) and depolarizing (50 mM $[K^+]_o$) conditions, corresponding to membrane potentials of −50 mV and −31 mV. As summarized in Table 1, the number of binding sites and the binding affinity were both dependent

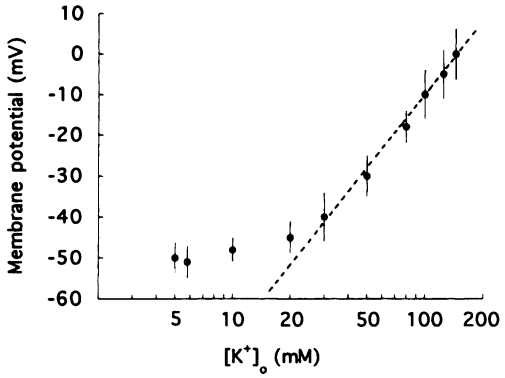

Fig. 1. Estimated membrane potential (V_m) as a function of increasing $[K^+]_o$, measured by [^{3}H]TPP^+ distribution. *Dashed line*, theoretical values based on Nernst equation. *Bars*, standard error (eight experiments).

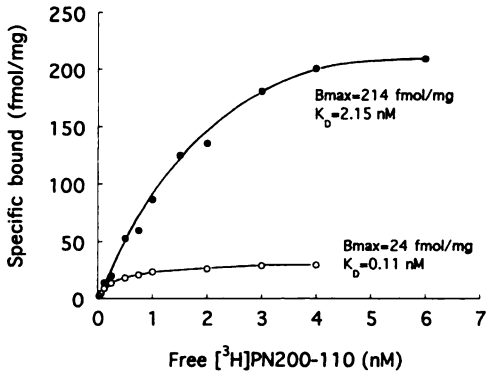

Fig. 2. Specific [^{3}H]PN200–110 binding to intact GH_4C_1 cells in the presence of 5 mM (●) or 50 mM (○) $[K^+]_o$. Depicted is one representative plot from 12 separate experiments. Data are summarized in Table 1.

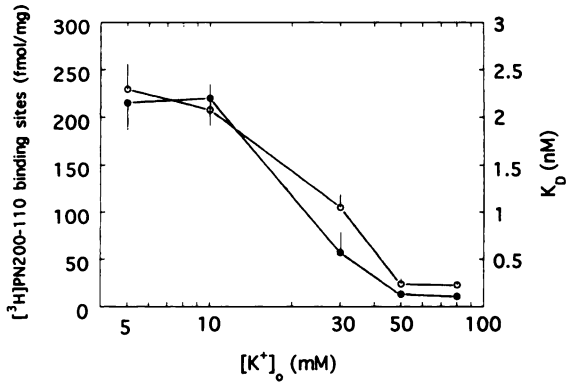

Fig. 3. Changes in the dissociation constant (K_d) (○) and the number of [^{3}H]PN200–110 binding sites (●) as a function of increasing $[K^+]_o$. *Bars*, standard error (five experiments).

on the membrane potential. During depolarization, the binding affinity increased approximately 20-fold, as reflected by the change of dissociation constant (K_d) from 2.15 to 0.11 nM, and the number of binding sites (B_{max}) decreased by approximately 90%. No significant changes in binding properties were seen at 10 mM $[K^+]_o$ and the changes were complete between 30 mM and 50 mM $[K^+]_o$ (Fig. 3), corresponding to the $[K^+]_o$-dependent curve of $^{45}Ca^{2+}$ uptake (see Fig. 6, *upper*). Similar changes in the binding affinity and the number of binding sites were also seen in rat CGC when these cells were exposed to depolarizing conditions (Table 1).

Dependence of kinetics of [^{3}H]PN200–110 binding on membrane potential. The association rate constant (k_1) and dissociation rate constant (k_{-1}) for [^{3}H]PN200–110 binding to intact GH_4C_1 cells under resting (5 mM $[K^+]_o$) and depolarizing (50 mM [$[K^+]_o$) conditions were determined by kinetic analysis (Table 1). There was no significant difference in the dissociation rate, k_{-1}, under the two conditions (0.032 min^{-1}), but the association rate, k_1, was 20 times higher in depolarized cells (V_m = −31 mV) than in polarized cells (V_m = −51 mV). The change in k_1 value corresponded to the change in K_d value, indicating that the increase in binding affinity arose from an increased association rate.

Membrane localization of Ca^{2+} channels regulated by depolarization. After exposure of GH_4C_1 cells to depolarizing medium, [^{3}H]PN200–110 binding was measured in both the heavy membrane preparation (30,000 × *g*) and the total cell homogenate. As shown in Fig. 4, after 2 hr of depolarization in 50 mM $[K^+]_o$ the number of Ca^{2+} channel binding sites on the

TABLE 1

Binding of [^{3}H]PN200-110 to intact GH_4C_1 cells and CGC in resting (5 mM K^+) or depolarizing (50 mM K^+) Hanks' solution

K_d and B_{max} values were determined by Scatchard analysis of saturation binding; k_1 and k_{-1} values were determined by kinetic analysis. Values are (mean ± standard error from six to 12 separate experiments.

Cells	Conditions	$[K^+]_o$	K_d	B_{max}	k_1	k_{-1}
		mM	nM	fmol/mg	$\times 10^8\ M^{-1}\ min^{-1}$	$\times 10^{-2}\ min^{-1}$
GH_4C_1	Resting	5	2.15 ± 0.42	214 ± 48	0.175 ± 0.035	3.28 ± 0.23
	Depolarizing	50	0.11 ± 0.02	24 ± 6	3.08 ± 0.39	3.34 ± 0.31
CGC	Resting	5.8	3.75 ± 0.62	364.2 ± 95.5		
	Depolarizing	50	0.25 ± 0.05	70.6 ± 14.4		

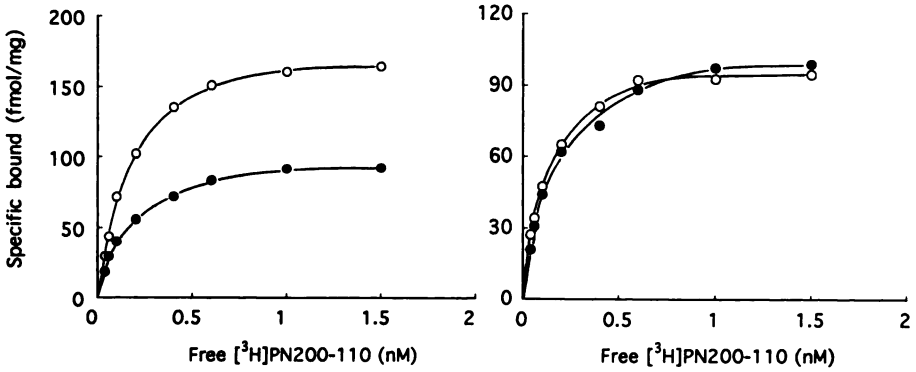

Fig. 4. Specific binding of [^{3}H]PN200–110 in membrane preparations (*left*) and cell homogenates (*right*) from cells that had been preincubated in 5 mM (○) or 50 mM (●) K^+-containing Hanks' buffer for 2 hr. Depicted is one representative plot from four separate experiments.

cell surface was decreased by 43%, whereas the total number of binding sites measured in the homogenate remained constant. Concanavalin A and phenylarsine oxide, agents that block receptor internalization (35, 36), blocked the down-regulation of L-type Ca^{2+} channels from the plasma membrane (Table 2).

Time course of down-regulation of L-type Ca^{2+} channels. When exposed to 50 mM $[K^+]_o$ for increasing times, GH_4C_1 cells showed a progressive reduction in [^{3}H]PN200–110 binding sites, as revealed by whole-cell binding and membrane binding studies (Fig. 5). In the first 45 min [^{3}H]PN200–110 binding sites decreased rapidly, but further decline was quite slow. When returned to the resting condition after 2 hr of exposure to 50 mM $[K^+]_o$, the down-regulated Ca^{2+} channels recovered rapidly. Within 2 hr, the recovery was 80% (Fig. 5, *insets*). Preincubation of the cells with the protein synthesis inhibitor cycloheximide (50 nM) did not prevent this recovery (data not shown).

Modulation of Ca^{2+} channel function by depolarization. $^{45}Ca^{2+}$ uptake into GH_4C_1 cells was measured to determine whether functional changes in Ca^{2+} channels accompanied the reduction in [^{3}H]PN200–110 binding sites. Stimulated $^{45}Ca^{2+}$ uptake was defined as the difference in $^{45}Ca^{2+}$ uptake between 5 mM and 50 mM $[K^+]_o$. Fig. 6, *upper*, shows the dependence of $^{45}Ca^{2+}$ uptake on membrane potential. At 5, 10, and 20 mM $[K^+]_o$ (V_m greater than −45 mV) there was no increase of $^{45}Ca^{2+}$ uptake above basal levels. $^{45}Ca^{2+}$ uptake was significantly stimulated when $[K^+]_o$ was raised to or above 30 mM, which depolarized the membrane potential to −38 mV or less. At 50 mM $[K^+]_o$, $^{45}Ca^{2+}$ uptake was maximal.

Fig. 6, *lower*, shows that, when the cells were exposed to increasing $[K^+]_o$ for 60 min, the stimulated $^{45}Ca^{2+}$ uptake decreased in a concentration-dependent manner. The decrease was evident even at 20 mM $[K^+]_o$. At $[K^+]_o$ of >30 mM, the stimulated $^{45}Ca^{2+}$ uptake was decreased to approximately 10% of the initial value. The decrease of the stimulated $^{45}Ca^{2+}$ uptake occurred very rapidly. Within 10 min of exposure to 50 mM $[K^+]_o$, the stimulated $^{45}Ca^{2+}$ uptake dropped to about 27% of

TABLE 2

Effects of pharmacological agents on the down-regulation of L-type Ca^{2+} channels induced by exposure to 50 mM K^+ for 2 hr, measured by PN200-110 binding in the plasma membranes of GH_4C_1 cells

Values are from at least three experiments.

Agents	Concentration	Target	Inhibition
			%
Staurosporine	50 nM	Protein kinase C	None
8-Bromo-cAMP	100 μM	Protein kinase A	None
IBMX	500 μM	Phosphodiesterase	None
Trifluoperazine	10 μM	Calmodulin	40
Calmidazolium	50 nM	Calmodulin	50
Concanavalin A	0.5 mg/ml	Internalization	70
Succinyl-concanavalin A	0.25 mg/ml	?	None
Wheat germ agglutinin	0.25 mg/ml	?	None
Phenylarsine oxide	50 μM	Internalization	64
NH_4Cl	20 mM	Intracellular pH	None
Sodium acetate	20 mM	Intracellular pH	None
D600	1–5 μM	Ca^{2+} channel	100

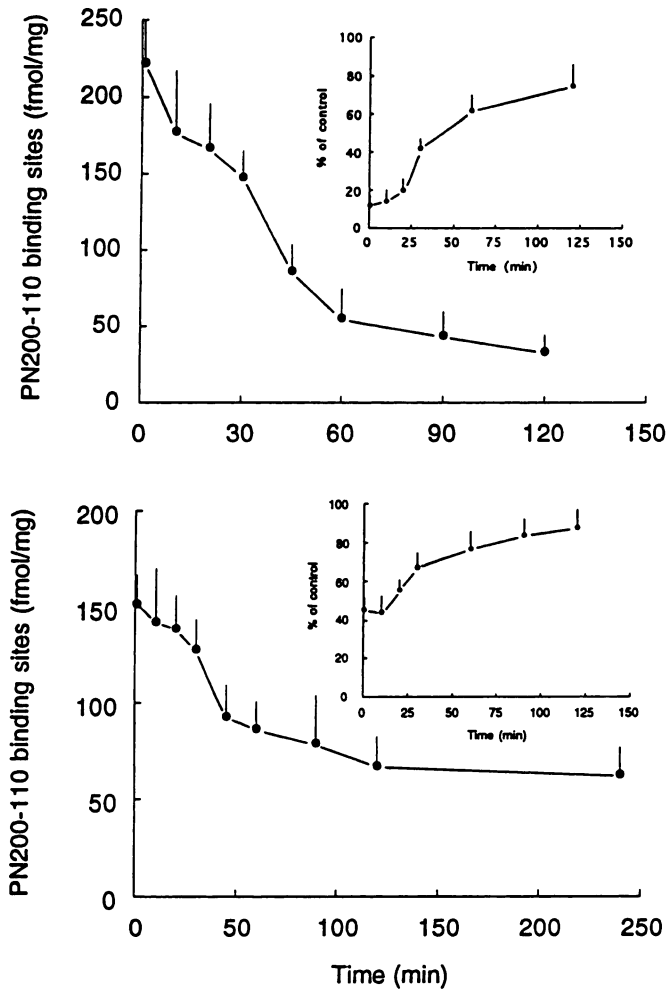

Fig. 5. Time course of down-regulation of [^{3}H]PN200–110 binding sites by membrane depolarization (50 mM $[K^+]_o$), as measured in intact cells (*upper*) and membranes (*lower*). *Insets*, recovery from 2-hr depolarization. *Bars*, standard error (five experiments).

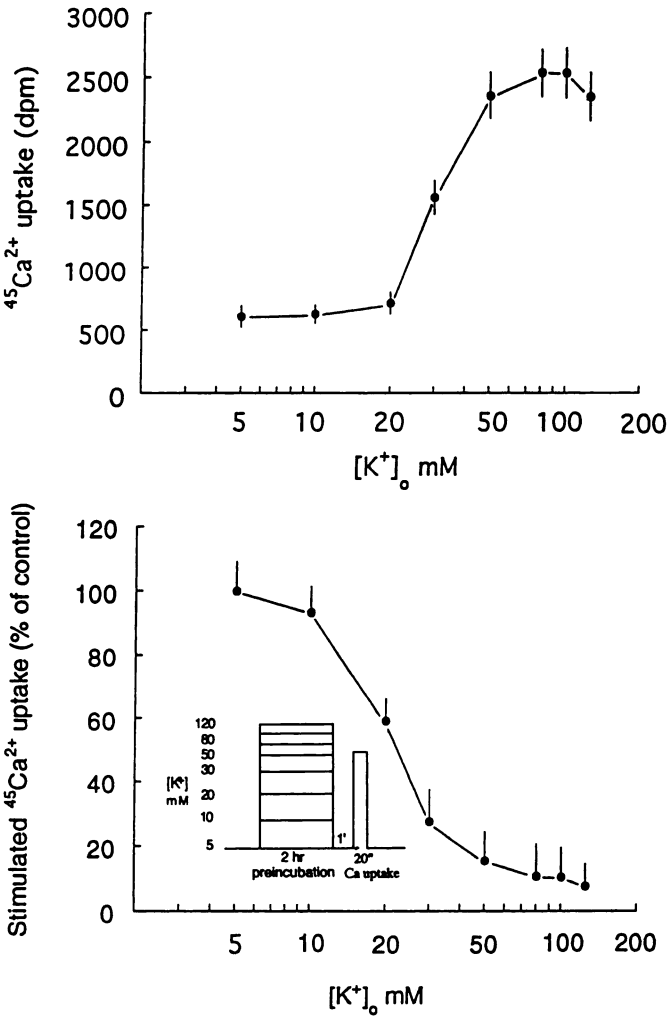

Fig. 6. $^{45}Ca^{2+}$ uptake in the presence of the indicated $[K^+]_o$, in a period of 20 sec (*upper*), and decrease in stimulated $^{45}Ca^{2+}$ uptake (50 mM $[K^+]_o$, 20 sec) measured after incubation at the indicated $[K^+]_o$ for 1 hr (*lower*). *Inset*, procedure. *Bars*, standard error (four experiments).

the initial level (Fig. 7). The time course of the decrease had two exponential components, a fast component with a time constant of 2.5 min and a slow component with a time constant of 54 min (Fig. 7, *inset*). Returning the depolarized cells to the resting condition produced rapid recovery of stimulated $^{45}Ca^{2+}$ uptake. The decrease caused by short periods of exposure (<10 min) recovered completely in 10 min. As the exposure time increased, a smaller percentage of stimulated $^{45}Ca^{2+}$ uptake recovered (Fig. 7).

Membrane depolarization linked to Ca^{2+} channel regulation. To confirm that the effects of elevated $[K^+]_o$ on Ca^{2+} channels were due to membrane depolarization rather than to ion substitution, two experiments were done. [^{3}H]PN200–110 binding was measured with broken cell preparations (homogenates) in resting and depolarizing buffers and showed no significant difference in K_d and B_{max} values (data not shown). [^{3}H]PN200–110 binding and $^{45}Ca^{2+}$ uptake were also measured in the presence of veratridine. Fig. 8 shows that depolarization of cells by the Na^+ channel activator veratridine (50 μM) had an effect on L-type Ca^{2+} channels similar to that of K^+ depolarization.

Within the period of exposure to 50 mM K^+-containing medium, GH_4C_1 cells showed no significant change in cell morphology (size and shape), total protein content, or LDH activity (data not shown), suggesting that this treatment did not cause changes in cell integrity.

Ca^{2+} dependence of down-regulation. To examine the role of Ca^{2+} ions in the down-regulation process, the cells were exposed to depolarizing medium containing different concentrations of Ca^{2+}. As shown in Fig. 9, stimulated Ca^{2+} uptake and [^{3}H]PN200–110 binding sites on the plasma membrane exhibited a Ca^{2+}-dependent reduction. Cells treated with the Ca^{2+} ionophore A23187 (1.0 μM) in resting buffer showed a decrease in stimulated $^{45}Ca^{2+}$ uptake and [^{3}H]PN200–110 binding sites in the presence of 1.25 mM Ca^{2+}. Removal of Ca^{2+} ions from the buffer diminished the effect of the Ca^{2+} ionophore (Fig. 8). This result also indicates a requirement for Ca^{2+} in the down-regulation process. Experiments with Ba^{2+} replacing Ca^{2+} showed similar down-regulation induced by depolarization. When Ca^{2+} was replaced with 1.25 mM Ba^{2+}, a 120-min period of depolarization by 50 mM $[K^+]_o$ produced 82 ± 10% loss of [^{3}H]PN200–110 binding sites in the whole-cell GH_4C_1 binding assay.

Blockade of the Ca^{2+} channel down-regulation process. To identify possible mechanisms, several pharmacological agents were tested for blocking of the down-regulation process (Table 2). Staurosporine is a potent inhibitor of protein kinase

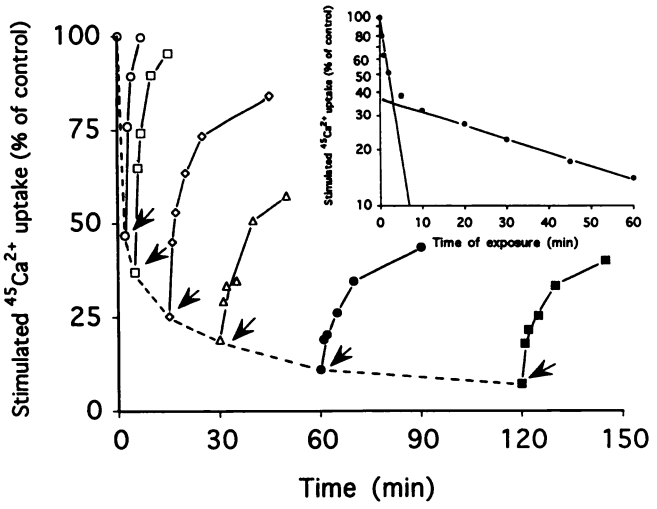

Fig. 7. Time course of reduction (– – –) and recovery (——) of stimulated $^{45}Ca^{2+}$ uptake (50 mM $[K^+]_o$, 20 sec) after exposure to the depolarizing medium for the indicated times. *Arrows*, times of changes from depolarizing to resting buffer. *Inset*, two exponential components of the down-regulation of stimulated $^{45}Ca^{2+}$ uptake. The data are the mean of six replicates, and the experiment was repeated three times.

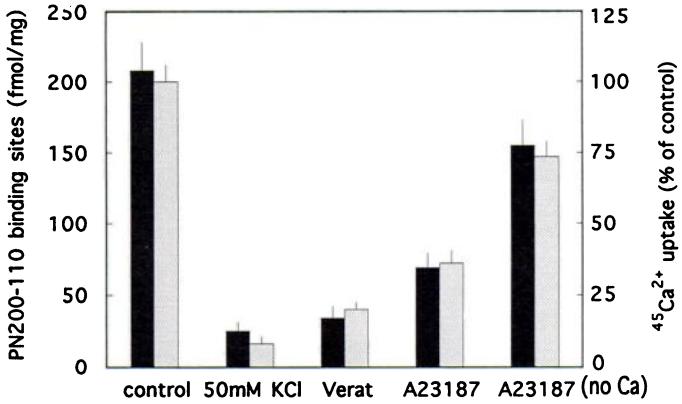

Fig. 8. Changes in [^{3}H]PN200–110 binding site density (■) and stimulated $^{45}Ca^{2+}$ uptake (■) induced by 2-hr incubation of the cells with 50 mM $[K^+]_o$, 50 μM veratridine (*Verat*), and 10 μM Ca^{2+} ionophore A23187, with or without extracellular Ca^{2+}. *Bars*, standard error (six experiments).

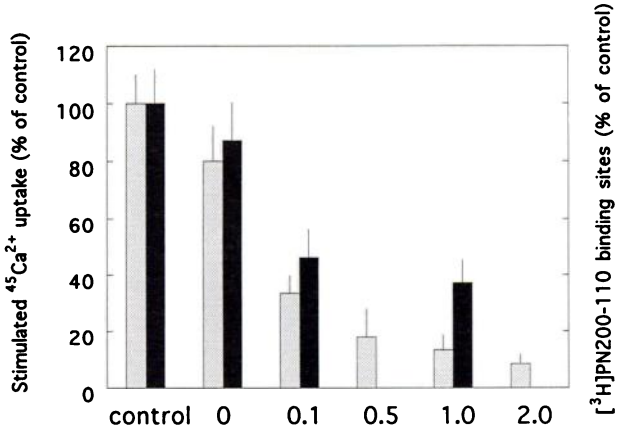

Fig. 9. Ca^{2+} dependence of the down-regulation of stimulated $^{45}Ca^{2+}$ uptake (■) and [^{3}H]PN200–110 binding site density in cell membranes (■). The cells were incubated for 2 hr in resting medium (control) or depolarizing medium, in the presence of the indicated concentrations of Ca^{2+}. *Bars*, standard error (five experiments).

C (37). Staurosporine (50 nM), when included in the depolarizing buffer, had no significant effect on the down-regulation of L-type Ca^{2+} channels. Bromo-cAMP and IBMX, an inhibitor of cyclic nucleotide phosphodiesterases (38), included in the depolarizing buffer did not block the down-regulation of Ca^{2+} channels. Trifluoperazine (10 μM) and calmidazolium (50 nM), calmodulin inhibitors (39), inhibited the down-regulation significantly. At the same concentrations, these inhibitors only slightly inhibited (<10%) (data not shown) the rapid stimulated $^{45}Ca^{2+}$ uptake. Concanavalin A and phenylarsine oxide, blockers of membrane internalization processes (35, 36), greatly inhibited the down-regulation of Ca^{2+} channels induced by membrane depolarization. Changes in intracellular pH produced by NH_4Cl and sodium acetate did not affect the down-regulation process significantly.

The L-type Ca^{2+} channel antagonist D600, at concentrations of 1–5 mM, completely blocked depolarization-induced reduction of [^{3}H]PN200–110 binding sites on the plasma membrane. The use of 1,4-dihydropyridines to generate a more comprehensive pharmacological characterization of the down-regulation is difficult, because their blocking effects are difficult to rapidly wash out of the cellular preparations used here.

Discussion

Membrane depolarization is both a physiological and a pathological stimulus for VGCC. Under physiological conditions depolarization serves to permit Ca^{2+} entry for stimulus-response coupling processes. Under pathological conditions prolonged channel opening may cause excess Ca^{2+} entry, cellular Ca^{2+} overload, and cell damage and death (40, 41). This study has characterized the regulation of 1,4-dihydropyridine-sensitive L-type Ca^{2+} channels by membrane depolarization. We have shown that the binding of the 1,4-dihydropyridine [^{3}H]PN200–110 to L-type channels in GH_4C_1 cells and CGC is regulated by membrane potential; the binding affinity increases approximately 20-fold when cells are depolarized by 50 mM $[K^+]_o$. Additionally, we show that depolarization also results in a reversible loss of 1,4-dihydropyridine binding sites and function in intact cells.

According to the modulated receptor hypothesis (42, 43), drugs may selectively interact with binding sites in the resting, open, or inactivated states of the channel. Accordingly, the apparent affinity of a drug varies according to its affinity for specific states and the time- and voltage-dependent equilibria between these states. Electrophysiological evidence suggests that the 1,4-dihydropyridine antagonists bind to the open and/or inactivated states of the channel with high affinity, whereas they bind to the closed (resting) states with significantly lower affinity (13, 20, 44, 45). Thus, the measured K_d values are determined by the fractional availability of the open/inactivated channel state, according to membrane potential. Radioligand binding studies in cardiac myocytes and smooth muscle have also shown voltage-dependent interactions of 1,4-dihydropyridines (16, 18, 19, 46–48).

Our kinetic study shows that the increased binding affinity of [^{3}H]PN200–110 for GH_4C_1 cells with depolarization results from an increased association rate; the dissociation rate is independent of membrane potential. This contrasts with previous studies of the voltage-dependent binding of this 1,4-dihydropyridine to cardiac myocytes, where the increased affinity is derived from a decreased dissociation rate (16, 18). These observations suggest different modes of interaction of 1,4-

dihydropyridines with L-type Ca^{2+} channels in GH_4C_1 cells and cardiac myocytes. Our observations on GH_4C_1 cells accord with those of Cohen and McCarthy (20), whose electrophysiological studies showed that the rate of onset of block by the 1,4-dihydropyridine nimodipine increased with increasing depolarization. Because the voltage dependence of nimodipine block accorded with the voltage dependence of channel activation, Cohen and McCarthy (20) proposed an "open channel" block by nimodipine. In contrast, previous electrophysiological studies in cardiac preparations have generally attributed the voltage dependence of 1,4-dihydropyridine binding to preferential interaction with the inactivated channel state (13, 14, 48, 49).

An explanation for the different modes of interaction of 1,4-dihydropyridines with L-type channels in cardiac and pituitary cells likely resides in structural features that determine differences in channel gating or 1,4-dihydropyridine receptors. Details of such differences are not yet available, but at least four distinct genes code for the $\alpha 1$ subunit of 1,4-dihydropyridine-sensitive L-type channels, with variations being available through alternative splicing (reviewed in Refs. 50 and 51). The products of one gene, *CaCh2*, code for cardiac and smooth muscle channels (52–54), whereas the products of *CaCh3* code for neural and endocrine channels (55–57).

Hypotheses alternative to those presented must also be noted. One such interpretation is the "guarded receptor" hypothesis (58, 59), according to which membrane potential modulates ligand access to a constant affinity state and access increases with increasing depolarization. Our data presented here and previously (18) suggest that this model could apply to the channel in GH_4C_1 cells but not that in ventricular myocytes. This difference would presumably have a structural basis. As an additional alternative, a dependence of ligand affinity upon association rate may reflect a multistep reaction pathway in which the formation of the complex is described by a rapid preequilibrium association with at least one less stable intermediate

$$D + R \rightleftarrows [DR]_1 \rightleftarrows [DR]_2$$

The initial complex may represent a weak interaction with the 1,4-dihydropyridine binding site or may represent a hydrophobic partitioning of the ligand into the membrane, from which two-dimensional diffusion can generate a high affinity complex (60). A two-step reaction was earlier postulated to describe the interactions of sulfonamides with carbonic anhydrase (61). With the low concentrations used to measure association rates in radioligand binding protocols, the concentration of $[DR]_1$, generated by a bimolecular process, is low and only the formation of the final complex by an effectively unimolecular interaction is measured, without observation of biphasic reaction kinetics. However, the availability of these pathways must differ between cardiac myocytes and GH_4C_1 cells and may reflect structural differences in the channel or differences in the membrane environment.

The state-dependent interactions of drugs active at voltage-gated ion channels are clearly important for the determination of both drug efficacy and selectivity. The efficacy of a drug at a Ca^{2+} channel depends upon its ability to interact with high affinity at a particular state or states. If this high affinity state becomes available only during channel use, then the drug exhibits selectivity for those tissues or cells where the channels are in use. This is manifestly the case for the clinically available Ca^{2+} channel antagonists and underscores both their therapeutic heterogeneity and the ability of a single class of molecules, the 1,4-dihydropyridines, to exhibit differential cardiovascular selectivity, regional vascular selectivity, and a general absence of neuronal effects under clinically physiological conditions (45, 62, 63).

Depolarization has also been shown to modulate the number of L-type Ca^{2+} channels in some excitable cells. Chronic depolarization reduces 1,4-dihydropyridine binding sites and Ca^{2+} influx in PC-12 cells (8, 9) and chick retinal neurons (10), reduces neuronal Ca^{2+} currents in cultured rat myenteric neurons (11), and reduces both low- and high-voltage-activated Ca^{2+} currents in molluscan neurons (64). Although these studies involved depolarization times of several days, it was observed that down-regulation in chick retinal neurons was apparent as early as 4 hr after depolarization (10). This down-regulation in chick retinal neurons was a Ca^{2+}-dependent process and was prevented by the presence of a Ca^{2+} channel antagonist during depolarization (10). The present study with GH_4C_1 cells used brief periods of depolarization (up to 120 min), under which conditions a 90% reduction in 1,4-dihydropyridine binding sites was observed. This protocol in CGC produced a similar reduction in binding site density. However, the CGC were cultured with 25 mM $[K^+]_o$, under which conditions partial depolarization is expected. Thus, the channels may have been partially down-regulated in culture. Nonetheless, an additional large reduction was observed when $[K^+]_o$ was raised to 50 mM.

The down-regulation process is Ca^{2+} dependent in GH_4C_1 cells, as previously reported in chronic regulation studies in PC-12 cells (8) and chick retinal neurons (10). Intracellular Ca^{2+} levels in GH_4C_1 cells rise rapidly and remain elevated after K^+-induced depolarization (65). Staurosporine and IBMX failed to alter the down-regulation, thus excluding the involvement of protein kinase C and phosphodiesterase. However, the calmodulin inhibitors trifluoperazine and calmidazolium did block down-regulation, suggesting the operation of a Ca^{2+}/calmodulin-dependent process. Calmodulin inhibitors, including trifluoperazine and calmidazolium, have been reported to interact with L-type VGCC (66, 67). However, at the concentrations used in this study they only slightly inhibit K^+-stimulated $^{45}Ca^{2+}$ uptake. The finding that D600 afforded complete protection against down-regulation in GH_4C_1 cells is also consistent with a Ca^{2+}-dependent process mediated through L-type Ca^{2+} channels. However, because a similar down-regulation is observed when Ca^{2+} is nominally replaced by Ba^{2+}, a charge carrier and calmodulin activator, it is likely that the Ca^{2+}-dependent channel inactivation does not underlie the observed down-regulation.

The depolarization-induced loss of 1,4-dihydropyridine sites in GH_4C_1 cells must result from sequestration or internalization processes, rather than decreased synthesis or increased degradation, because the number of binding sites remains constant in the total cell homogenate preparation, whereas the number of binding sites in the plasma membrane decreases by approximately 40–60%. The difference between the reductions in binding sites observed in the whole-cell and membrane preparations likely arises from incomplete separation of plasma membrane and internalized light vesicle fractions. Because the loss of 1,4-dihydropyridine binding sites is accompanied by a loss of pre-depolarization-induced $^{45}Ca^{2+}$ uptake, the depolarization process must involve a loss of functional L-type VGCC. Furthermore, the recovery of binding sites after reversal of depolarization was not dependent on protein synthesis.

Additional support for an internalization process underlying the loss of 1,4-dihydropyridine binding sites and channel function during depolarization is provided by the inhibitory effects of concanavalin A and phenylarsine oxide. Concanavalin A prevents the internalization and redistribution of a number of receptors, including β-adrenergic receptors (35, 68, 69). Because succinyl-concanavalin A was ineffective in GH_4C_1 cells, it is likely that concanavalin A functions through cross-linking of the glycoprotein components of the Ca^{2+} channel. The α2 subunit is heavily glycosylated and may represent the target (70–72). However, concanavalin A is known to have a number of effects in neuronal cells, including enhancement of neurite outgrowth (73, 74) and elevation of intracellular pH and activation of high-threshold current in chick dorsal root ganglia (75). Thus, it is possible that the inhibitory effect of concanavalin A on channel down-regulation represents an increased expression of membrane channels. Support for this view comes from observations that in PC-12 cells concanavalin A, but not the succinyl derivative, increased depolarization-induced $^{45}Ca^{2+}$ uptake and the number of 1,4-dihydropyridine sites (76). Similarly, leech retzius neurons grown on a concanavalin A substrate showed increased Ca^{2+} current in the cell body (77). Phenylarsine oxide, via reaction with sulfhydryl groups, blocks internalization of a number of proteins, including the epidermal growth factor receptor and the β-adrenoceptor (36, 68, 78). Its effectiveness in blocking the depolarization-induced down-regulation of 1,4-dihydropyridine sites provides further evidence for the internalization and sequestration route.

Ion channels are increasingly recognized to be modulated by multiple factors, and in many respects their behavior is analogous to that of conventional ligand-activated receptors, where both short and long term influences control receptor function and number (7). VGCC are modulated rapidly through both electrical and chemical signaling systems and these processes, including channel phosphorylation, are responsible for the rapid channel gating events (79–81). However, the channels are also subject to long term regulation by chemical, hormonal, electrical, and developmental pathways (7, 82, 83). The down-regulation observed during short term and long term depolarization likely reflects different mechanisms but may serve to protect cells against the detrimental effects of Ca^{2+} overload during a number of pathological processes. Additionally, because we observe this process in neurons, but not in cardiac cells, it may also contribute to neuronal plasticity phenomena.

Several issues remain, however, to be resolved. In particular, the relevance to physiological events of the maintained depolarization employed needs to be established. Additionally, it is likely that other types of VGCC are regulated similarly and that these channels also contribute to depolarization-induced regulation in neurons.

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