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A Long-Term Blockade of L-Type Calcium Currents Upregulates the Number of Ca^{2+} Channels in Skeletal Muscle

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Abstract. The effects of a long-term blockade of L-type Ca^{2+} channels on membrane currents and on the number of dihydropyridine binding sites were investigated in skeletal muscle fibers. Ca^{2+} currents (I_{Ca}) and intramembrane charge movement were monitored using a voltage-clamp technique. The peak amplitude of I_{Ca} increased by more than 40% in fibers that were previously incubated for 24 hr in solutions containing the organic Ca^{2+} channel blocker nifedipine or in Ca^{2+}-free conditions. A similar incubation period with Cd^{2+}, an inorganic blocker, produced a moderate increase of 20% in peak I_{Ca}. The maximum mobilized charge (Q_{max}) increased by 50% in fibers preincubated in Ca^{2+}-free solutions or in the presence of Cd^{2+}.

Microsomal preparations from frog skeletal muscle were isolated by differential centrifugation. Preincubation with Cd^{2+} prior to the isolation of the microsomal fraction doubled the number of ^{3}H-PN200-110 binding sites and produced a similar increase in the values of the dissociation constant. The increase in the number of binding sites is consistent with the increase in the peak amplitude of I_{Ca} as well as with the increase in Q_{max}.

Key words: Ca^{2+} channels — DHP receptors — Upregulation — Skeletal muscle — Channel blockers

Introduction

In skeletal muscle, Ca^{2+} is released from the sarcoplasmic reticulum when a signal is transmitted across the triad as a result of depolarization of the transverse tubular system. In this process, the dihydropyridine receptors (DHPR) of the transverse tubule play an essential role. DHPR are the voltage sensors of excitation-contraction coupling that produce the charge movement recorded in electrophysiological experiments (for reviews *see* Lamb, 1992; Melzer, Frank & Lüttgau, 1995). In addition to their role as voltage sensors, DHPR are also permeant to Ca^{2+}, giving rise to very slowly activated L-type Ca^{2+} currents (for a review *see* Melzer et al., 1995).

Short-term regulation of L-type Ca^{2+} channel activity in skeletal muscle by ions, second messengers and G proteins has been extensively described (for reviews *see* Hille (1992), Huang (1992) and Melzer et al., 1995). These agents change the amplitude or the voltage-dependence of Ca^{2+} channel activity within a time frame of seconds or minutes. However, little information is available regarding the long-term regulation of L-type Ca^{2+} channels in skeletal muscle. In the present experiments, we describe the long-term effects of the Ca^{2+} channel blockade leading to upregulation of the Ca^{2+} channel activity itself and to an increase in the number of DHP receptors. A role for the Ca^{2+} current in this process was suggested by electrophysiological experiments performed in Ca^{2+}-free conditions.

Preliminary results have been published in abstract form (Escamilla, García & Sánchez, 1996).

Materials and Methods

General Procedure

The experiments were performed in frog skeletal muscle fibers isolated from adult specimens of *Rana montezumae.* The frogs were sacrificed by decapitation. A long-term blockade of Ca^{2+} channels was achieved by incubating the muscle fibers in a solution containing 10 μM nifedipine or 1 mM Cd^{2+}, mixed as detailed below. Fibers were maintained at 4°C during 24 hr in the control or in the test solution, and afterwards

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Table 1. Composition of solutions

	Na^+	K^+	TEA^+	Ca^{2+}	Mg^{2+}	$CH_3SO_3^-$	Cl^-	EGTA
A	117	2.5	—	1.8	—	—	123.1	—
B	—	—	110	10	—	130	—	—
C	—	—	110	—	10	130	—	—
D	—	—	140	—	—	—	—	70

the experiments were continued at room temperature (20–25°C) in standard Ringer's, without Ca^{2+} channel blockers.

Solutions

The standard Ringer's (solution *A*) and the solutions employed for measurements of I_{Ca} and charge movement were composed as shown in Table 1. An external solution containing Ca^{2+} (solution *B*) was used in Ca^{2+} current experiments and a Ca^{2+}-free solution (solution *C*) for charge movement experiments. Extracellular and intracellular solutions were buffered with 3-*N*-morpholino propanesulphonic acid (MOPS) at pH = 7.2 and 7.1, respectively. Nifedipine solutions (10 μM) were prepared from a concentrated DMSO stock solution. The nifedipine experiments were conducted under dim red light conditions. The Cd^{2+}-containing solution was prepared by adding $CdCl_2$ (1 mM) to solution *A* in Table 1. Extensive washing with standard Ringer's solution ensured the removal of Ca^{2+} channel blockers. In the nominally Ca^{2+}-free solution, Ca^{2+} ions were replaced by Mg^{2+} (3 mM) in the standard Ringer solution to preserve the electrical properties of muscle membranes (Arreola et al. 1987). The chemicals used were purchased from Sigma or Aldrich. (+)-[5-Methyl ^{3}H]-PN200-110 (isopropyl 4-(2,1,3 benzodiazol-4-yl)-1,4-dihydro-5-methoxycarbonyl-2,6-dimethyl-3-pyridine carboxylate) specific activity 83 Ci/mmol (3.07 TBq/mmol) was obtained from Amersham.

Electrophysiological Techniques

Membrane currents were measured using the triple-Vaseline gap voltage-clamp technique developed by Hille and Campbell (1976), with minor modifications described in Arreola et al. (1987). A long segment of a single muscle fiber was dissected in frog Ringer's solution (solution *A* in Table 1). The fiber was then laid in a chamber across three Vaseline seals, which divided the chamber into four pools. The ends of the fiber were cut while they lay in the end pools that contained the internal solution (solution *D*). The segment of the fiber lying in a particular inner pool was voltage-clamped; the change of the external solution for this segment was ensured by steadily flowing new solution through that pool, effectively changing the volume of the pool 40 times at least. Movement artifacts consequent to stimulation were suppressed by a high concentration of intracellular EGTA.

Intracellular recordings of the resting and action potentials were done with standard microelectrode techniques. Pipette resistances ranged 30–35 MΩ and were filled with 3 M KCl. The membrane potential was recorded with an Axoclamp amplifier (Axon Instruments, Foster City, CA) and was monitored with a digital voltmeter.

Data Collection

Analog signals were digitized to a resolution of 12 bits using a Scientific Solutions LabMaster interface (TL-1-125 DMA interface, Axon Instruments) that also generated the command pulses in the voltage-clamp experiments. Data were analyzed using pCLAMP (ver. 6.0, Axon Instruments). The membrane current (I_m) was sampled at 10,000 samples/sec when short pulses were delivered and at 450 samples/sec for long depolarizations. Analog signals were amplified and filtered with an active four-pole lowpass Bessel filter set at a corner frequency of no more than half the sampling frequency.

To measure I_{Ca}, sixteen command pulses of 1,000 msec duration and of increasing amplitude in 10-mV steps were delivered. The whole sequence was bracketed by four consecutive hyperpolarizing control pulses, −20 mV from the holding potential (E_h). The currents generated during the hyperpolarizing pulses were used to calculate the linear membrane capacitance and to measure the leakage current during the experiment. To measure nonlinear charge movement, step depolarizations lasting 25 msec to preselected potentials were delivered. Linear membrane currents were subtracted off-line by appropriate scaling of membrane currents generated by control pulses. E_h was −100 mV.

Preparation of Microsomal Membranes

Skeletal muscles dissected from the hind legs of *Rana montezumae* were incubated during 24 hr in a shaker water bath with gentle agitation at 4°C. The incubation solution in control experiments was standard Ringer's (solution *A* in Table 1), while the solution used to test the effects of the long term blockade of Ca^{2+} channels also contained Cd^{2+} (1 mM). The solutions in the incubation media were renewed every 4 hr. After the incubation period, the muscles in both groups were washed several times with fresh media and samples with a wet weight between 12 and 15 g were selected. A crude microsomal fraction was prepared as described by Hidalgo et al. (1986), with some modifications. In brief, the muscles were minced with scissors and homogenized at 2,300 rpm in four volumes of 0.1 M KCl-20 mM Tris-maleate buffer, pH 7.2 at 4°C in a glass-Teflon homogenizer. The homogenate was transferred to a beaker and homogenized again in a polytron (Teckmar TR-10) at a setting of 7, during 1.5 min. Three rest periods lasting 30 sec each were allowed to prevent the heating of the sample. After that, the samples were filtered through three layers of cheesecloth to remove floating material. The supernatant was centrifuged at 1,000 × *g* for 20 min at 4°C, the pellet was discarded, and the supernatant was collected. This fraction was used in initial binding experiments from muscles preincubated under control conditions, but the results were inconsistent. Therefore, we further purified this fraction by centrifugating the supernatant at 10,000 × *g* during 20 min at 4°C. To remove contractile proteins, the resulting suspension was saved and solid KCl was added to obtain a final potassium concentration of 0.6 M. Once the added KCl was dissolved, the supernatant was centrifuged at 100,000 × *g* during 45 min at 4°C. The microsomal pellet was washed twice. First with 0.1 M KCl-20 mM Tris-maleate and then with 0.32 M sucrose-20 mM Tris-maleate buffer solution. The final pellet was resuspended in 3 ml of Tris-20 mM-buffer solution.

Radioligand Binding Assay

Triplicate aliquots of 50 μl of the crude microsomal fraction (containing 100–150 μg of protein), 20 μl ^{3}H-PN200-110 (range 0.05 to 5 nM), and 180 μl of 10 mM Tris-HCl buffer, pH 7.2 at 25°C, were incubated in saturation experiments at 25°C during 90 min. Nonspecific binding was determined using 1 μM nifedipine and it was found to represent 5–10% of total binding at low ligand concentrations. After incubation, binding was ended by a 16-fold dilution immediately followed by rapid filtration through Whatman GF/C filters. The filters were rinsed three times with 3.5 ml of ice-cold Tris-HCl buffer and the ^{3}H present on the filters were detected by liquid scintillation spectroscopy. The dissocia-

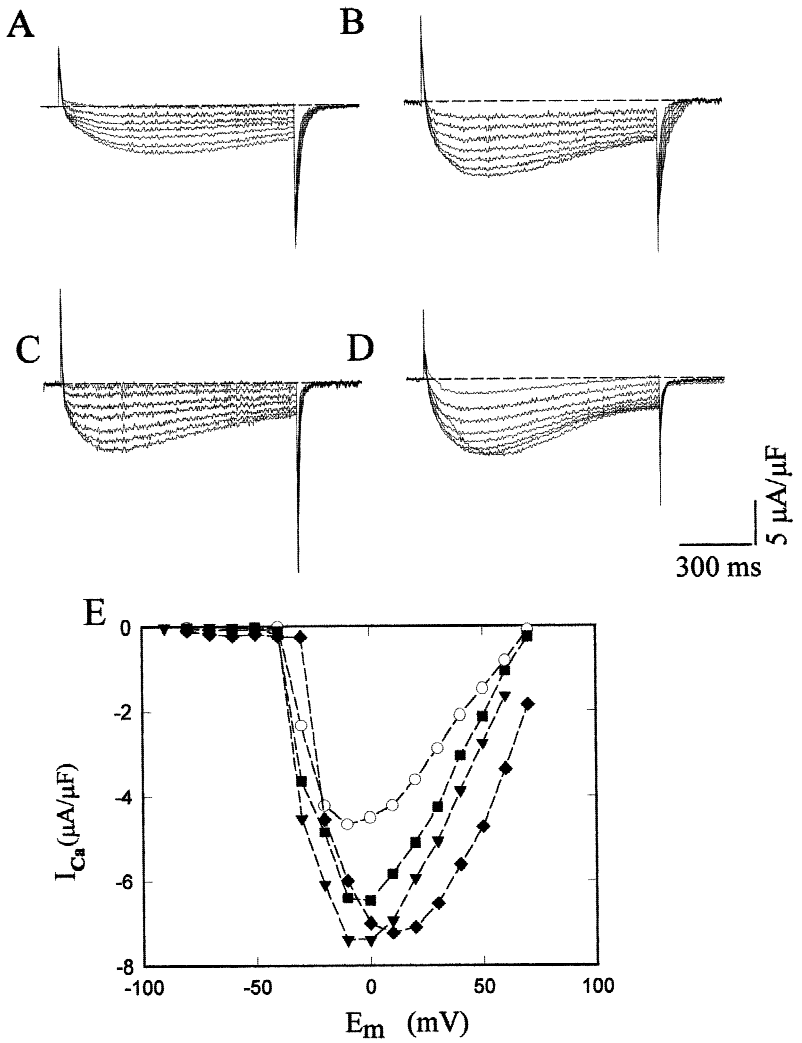

Fig. 1. The increase in the amplitude of Ca^{2+} currents after a long-term blockade. Panel *A* shows representative Ca^{2+} currents after an incubation period of 24 hr in control solution. Panels *B* and *C* show the effects on I_{Ca} of long-term preincubation (24 hr) in nifedipine (10 μM) and Cd^{2+} (1 mM), respectively. Panel *D* shows records after the cell was long-term incubated (24 hr) in nominally Ca^{2+}-free conditions. Ca^{2+} currents were generated by step depolarizations that clamped the membrane potential from −80 to +70 mV in 10 mV steps in panels *A, C* and *D*, and from −90 to +60 mV in panel *B*. E_h = −100 mV. Panel *E* shows the current-voltage relation of the experiments shown in *A–D* (○) represent the peak values of I_{Ca} of the control experiment; (▼), the corresponding values in the fiber preincubated in nifedipine; (■) the values in the fiber preincubated in Cd^{2+}, and (◆) in the fiber preincubated in Ca^{2+}-free conditions.

tion constants and the maximum number of binding sites were calculated by a nonlinear fit of the experimental data to the Eq. (1):

$$B = B_{max} * F/(K_d + F) \quad (1)$$

where B is the radioligand-receptor complex, B_{max} is the maximum number of binding sites, F is the free ligand concentration, and K_d is the dissociation constant.

The protein content was determined by the method of Lowry et al. (1951) using bovine serum albumin as the standard.

Parameter values given in the tables and text are expressed as mean ± SE. The binding results represent the mean of five independent preparations with determinations made in triplicate for each concentration tested. Unless otherwise noted, Student's *t*-test for independent samples was used to calculate statistical significance. The significance level was $P < 0.05$.

Results

Effects of Long-Term Blockade On I_{Ca}

The main novel result of the present study is that long-term preincubation of skeletal muscle fibers in the presence of Ca^{2+} channel blockers upregulates the activity and the number of Ca^{2+} channels. The upregulation is manifested, after the solution containing the Ca^{2+}-channel blockers has been washed, by an increase in the amplitude of Ca^{2+} currents and in the number of DHP binding sites (*see below*). Figure 1*A–D* illustrate superimposed records of I_{Ca} obtained after a preincubation period of 24 hr in different experimental solutions. This period of incubation was chosen because relatively short times (2 hr) did not produce changes in the amplitude of Ca^{2+} currents that were statistically significant (*data not shown*). In Fig. 1*A*, traces of I_{Ca} recorded after preincubation in control Ringer's are illustrated. In Fig. 1*B*, the preincubation solution contained nifedipine (10 μM) and the increase in the amplitude of I_{Ca} after a 24-hr period of blockade is apparent. In Figure 1*C*, currents were recorded from a fiber that was preincubated for 24 hr in a Cd^{2+}-containing solution. Clearly, the amplitude of I_{Ca} was larger than in the control experiment. Figure 1*E* shows the relationship between peak I_{Ca} and membrane

Table 2. Long-term effects of channel blockers on Ca^{2+} currents

Solution	I_{Ca} (μA/μF)
Control (t = 0 hr)	6.8 ± 0.9 (16)
Control (t = 24 hr)	5.7 ± 0.3 (47)
Cd^{2+}	7.5 ± 0.8 (12)*
Nifedipine	9.1 ± 0.8 (19)*
Ca^{2+}-free	9.0 ± 0.8 (13)*

* $P < 0.05$

potential from the experiments shown in Fig. 1*A–D*. Open symbols represent results from the control experiment and filled triangles and filled squares, results from the fibers preincubated in nifedipine and Cd^{2+}, respectively. The peak amplitude of I_{Ca} increased at all potentials by the presence of Ca^{2+} channel blockers in the preincubation solution, without major shifts in the current-voltage relation

The actions of Ca^{2+} channel blockers described above suggest that a Ca^{2+} influx through nifedipine-sensitive Ca^{2+} channels could be involved in the upregulation of the currents. This is supported by the results obtained in Ca^{2+}-free conditions. Similarly to the experiments where cells were preincubated in the presence of Ca^{2+} channel blockers, the amplitude of the currents (Fig. 1*D*) increased when compared to the control experiment (Fig. 1*A*). Also, the current-voltage relation showed no major shifts (Fig. 1*E*, ♦). The increase in the amplitude of I_{Ca} by the long-term blockade of Ca^{2+} channels or by preincubation in Ca^{2+}-free solutions was consistently observed. Table 2 summarizes results from several experiments. To compare data from different fibers, currents were normalized per unit capacitance. A long-term preincubation in the presence of Ca^{2+} channel blockers or in the absence of external Ca^{2+} produced a significant increase in peak I_{Ca} values, suggesting an upregulation of Ca^{2+} channels. It could be argued that a long-term preincubation of muscle fibers with Ca^{2+} channel blockers or in Ca^{2+}-free solutions does not genuinely increase the amplitude of I_{Ca} but instead prevents their rundown. This possibility was ruled out when we compared the values of peak I_{Ca} density of freshly dissected fibers (t = 0) with those of fibers preincubated during 24 hr in control Ringer's. The mean Ca^{2+} current density was somewhat smaller in fibers incubated during 24 hr, but the difference was not statistically significant (Table 2). This indicates that Ca^{2+} channels of skeletal muscle fibers do not greatly rundown under our experimental conditions.

Actions of the Long-Term Blockade on Charge Movement

The fact that a long-term blockade of Ca^{2+} channels increases the amplitude of I_{Ca}, raises the possibility that charge movement might also be affected. To test this possibility, charge movement was measured in Ca^{2+}-free conditions (*see* Materials and Methods). Charge movement was measured in these ionic conditions to avoid contamination of the records by the presence of I_{Ca}. Figure 2*A* shows the movement of charge in the control experiment, after the fiber had been preincubated for 24 hr in standard Ringer's. As expected, the amplitude of nonlinear capacitive currents increases by membrane depolarization. The corresponding results from a separate experiment after an incubation period of similar duration but in the presence of Cd^{2+}, are shown in Fig. 2*B*. Charge movement activates at the same potentials in fibers that were previously incubated in Cd^{2+}, but "on" and "off" charges are larger, especially at very positive depolarizations. Figure 2*C* shows the charge moved at several potentials from the experiments illustrated in Fig. 2*A* and *B*. Each symbol is the average value of "on" and "off" charges at each potential. Open symbols represent control results and filled symbols results from the long-term blockade with Cd^{2+}. The points were fitted to a two-state Boltzmann distribution:

$$Q = Q_{max}/[1 + \exp((-V+\overline{V})/k)] \quad (2)$$

where Q_{max} is the maximum charge per unit linear capacitance, $\overline{V}$ is the mean point, and k a measure of the steepness. The primary action of Cd^{2+} in the preincubation solution was to increase the maximum charge. Table 3 summarizes results from several experiments. The long-term blockade of Ca^{2+}-channels by Cd^{2+} increased by 50% the maximum charge mobilized by large depolarizations. A similar increase was observed in fibers that were preincubated in Ca^{2+}-free solutions. However, preincubation with nifedipine did not significantly increase the values of Q_{max} (Table 3). This observation may be puzzling considering the fact that long-term preincubation with this organic blocker increased the amplitude of I_{Ca}, as shown in Table 2. However, as discussed elsewhere (Delay, Garcia & Sanchez, 1990), differences in the actions of nifedipine on I_{Ca} and charge movement have been previously described in short-term experiments performed in skeletal muscle fibers.

The Long-Term Effects of Cd^{2+} on the Resting and Action Potentials

Permeation of Cd^{2+} through DHP-sensitive channels has been described in other excitable cells such as pituitary cells (Hinkle, Shanshala & Nelson, 1992). This fact raises the possibility that a long-term preincubation with Cd^{2+} may produce deleterious effects on muscle fibers. To test this possibility, we recorded resting and action potentials during and after incubation of muscle fibers in 1 mM Cd^{2+}. We found that the resting potential progressively declined over a time course of several hours reach-

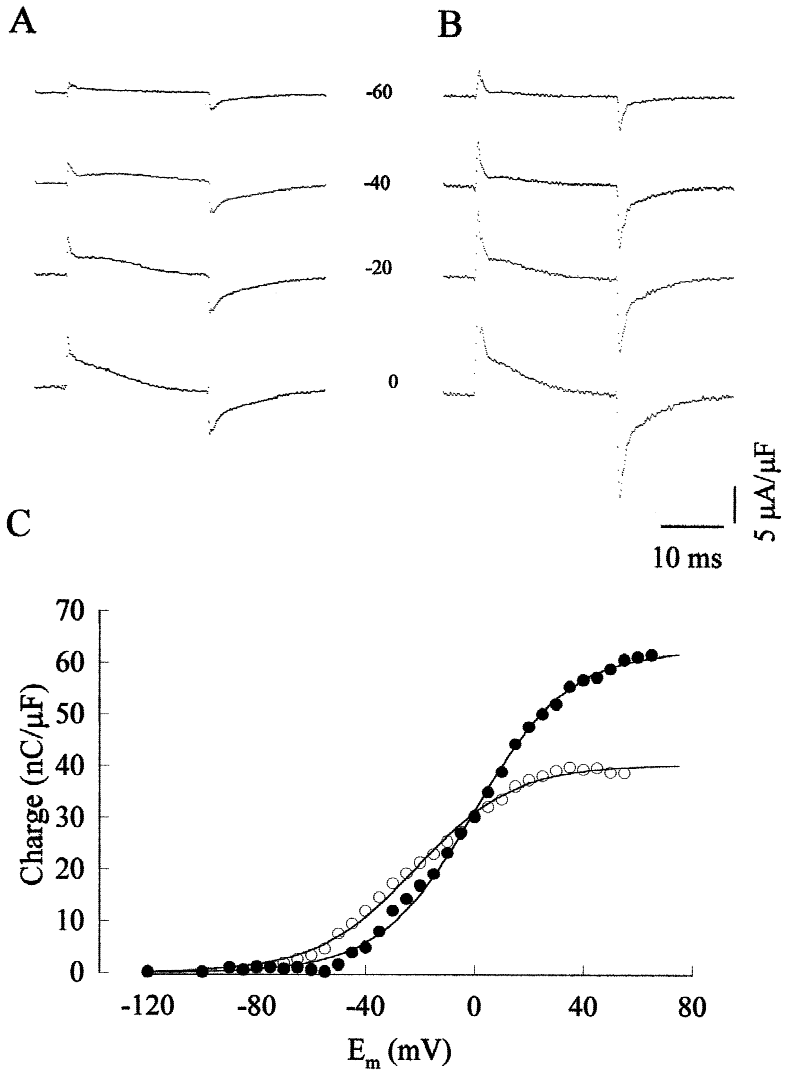

Fig. 2. The effect of a long-term blockade of Ca^{2+} channels upon charge movement. The records show membrane currents during voltage steps to the potentials indicated, after a preincubation period of 24 hr in control conditions (panel *A*) and after preincubation in Cd^{2+} during a similar period (panel *B*). Records were subtracted from the currents generated by a −20 mV pulse from E_h = −100 mV. In panel *C*, ○ and ● represent the averaged values of charge displaced by "on" and "off" transients from the experiments shown in *A* and *B*, respectively. Smooth curves are best fits of Eq. (2) with Q_{max} = 40.4 nC/μF, $\overline{V}$ = −21.6 mV and k = 18.2 mV for ○, and Q_{max} = 62.9 nC/μF, $\overline{V}$ = 0.2 mV and k = 18.0 mV for ●.

Table 3. Long-term effects of channel blockers on charge movement

	Q_{max} (nC/μF)	$\overline{V}$ (mV)	k (mV)
Control	33.1 ± 2.4 (36)	−28.1 ± 1.6 (36)	9.4 ± 0.6 (36)
Cd^{2+}	50.3 ± 5.9 (5)*	−14.8 ± 7.9 (5)	14.1 ± 1.0 (5)
Ca^{2+}-free	51.6 ± 1.3 (6)*	−21.4 ± 2.8 (6)	10.0 ± 0.6 (6)
Nifedipine	39.8 ± 3.8 (13)*	−27.7 ± 2.5 (13)	10.7 ± 0.8 (13)

* $P < 0.05$

ing a value of −45 mV after 24 hr (Table 4). At this time, fibers could not generate action potentials probably because of inactivation of sodium channels produced by this depolarized resting potential. Although we did not investigate the underlying mechanism of membrane depolarization by Cd^{2+}, a likely target is the inward rectifier K^+ channel. Previous work has shown that Cd^{2+} changes the voltage dependence of delayed rectifier K^+ channels (Follmer et al., 1992) and it may affect other K^+ channels as well. The action of Cd^{2+} was completely reversible and fibers recovered their normal resting potentials after wash (Table 4). This suggests that the protracted incubation in Cd^{2+} produced no major noxious effects on muscle fibers. Furthermore, these fibers readily produced action potentials when stimulated, whose time course (measured as the half-width) did not change in any significant way when compared with action potentials recorded from fibers preincubated in control Ringer's during 24 hr. There was however, a small but significant increase in their amplitude by preincubation in Cd^{2+} (Table 5). The increase in the amplitude of action potentials could also be observed after preincubation in Ca^{2+}-free conditions, and in this case, a decrease in the values of half-width was observed. On the other hand, action potentials recorded from fibers preincubated in either control Ringer's, Cd^{2+} or in Ca^{2+}-free solutions, were distinctly smaller and broader than action potentials recorded from freshly dissected fibers (Table 5).

Table 4. Long-term effects of cadmium (1 mM) on the resting potential

Time (hr)	Resting potential (mV)
0.0	−89.2 ± 0.5 (94)
0.5	−87.4 ± 1.2 (53)
1.5	−74.1 ± 2.0 (45)
2.5	−67.7 ± 3.3 (28)
4.5	−50.5 ± 3.4 (7)
5.5	−45.1 ± 2.8 (7)
6.5	−48.6 ± 3.4 (9)
24	−45.3 ± 1.3 (6)
Wash	−87.1 ± 0.7 (77)

Table 5. Long-term effects of Cd^{2+} (1 mM) and Ca^{2+}-free solutions on action potentials

	Amplitude (mV)	Half-width (msec)
Control ($t = 0$ hr)	124.0 ± 1.3 (94)	0.98 ± 0.02 (94)
Control ($t = 24$ hr)	97.2 ± 2.1 (70)	1.49 ± 0.05 (70)
Cd^{2+}	112.6 ± 1.6 (77)*	1.40 ± 0.05 (77)
Ca^{2+}-free	107.5 ± 1.7 (31)*	1.18 ± 0.02 (31)*

* $P < 0.05$

Actions of the Long-Term Blockade on Dihydropyridine Receptor Binding

We estimated the ^{3}H-PN200-110 binding capacity (B_{max}) and the dissociation constant (K_d) of the microsomal preparation isolated from muscles incubated in control Ringer's during 24 hr. In five independent determinations these values were 1.50 ± 0.08 pmoles/mg protein, and of 0.92 ± 0.07 nM, respectively. These numbers are in good agreement with those previously described for the dihydropyridine receptor binding in rabbit (Fosset et al., 1983), in frog (Margreth, Damiani & Tobaldin, 1993), and in guinea-pig skeletal muscle microsomes (Ferry, Goll & Glosmann, 1983). Muscles that were preincubated during 24 hr in a Cd^{2+}-containing solution (1 mM) prior to the isolation of the microsomal fraction, showed a 2.3-fold increase in the maximum number of ^{3}H-PN200-110 binding sites (3.36 ± 0.65 pmol/mg protein, $n = 5$). The values of K_d were also significantly higher (2.90 ± 0.75 nM, $n = 5$). This suggests that Cd^{2+} promotes the incorporation of new receptors into the cellular membrane displaying a lower affinity for dihydropyridines. Figure 3 shows data from a representative experiment, open symbols correspond to the control experiment and filled symbols represent results after preincubation in Cd^{2+}. Taken together, our results are consistent with a net increase in the number of functional DHP-sensitive Ca^{2+} channels after the long-term blockade.

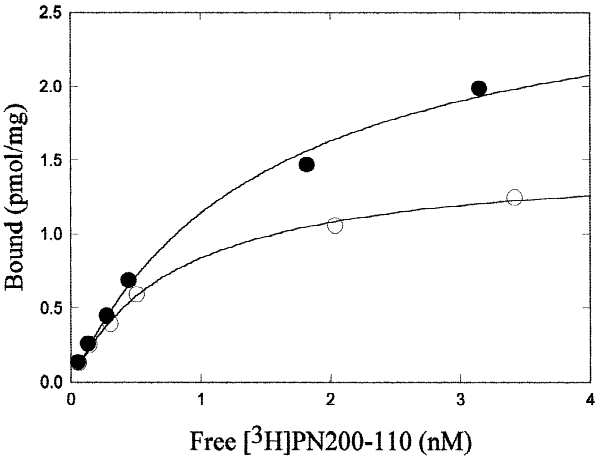

Fig. 3. The effect of a long-term blockade of Ca^{2+} channels on specific binding. ^{3}H-PN200-110 specific binding to frog skeletal muscle microsomal membranes was measured as described in the text. Whole muscles were incubated during 24 hr in the absence (○) or in the presence (●) of 1 mM Cd^{2+} prior to the isolation of the microsomal membranes. Smooth curves are best fits of Eq. (1) with $B_{max} = 1.51$ pmoles/mg protein and $K_d = 0.81$ nM for ○, and $B_{max} = 2.85$ pmoles/mg protein and $K_d = 1.49$ nM for ●. Each point is the average of three determinations.

Discussion

Comparison with Previous Work

The electrical activity of excitable cells is greatly influenced by second messengers and other modulators that act within a period of seconds or minutes. In many cases, ion channels change their unitary behavior as a result of the interaction with modulatory influences. However, slower and long-lasting responses usually involve changes in the density of ion channels in the cell membrane. A classical example in muscle cells is the dramatic increase in the density of Ach receptors that follows denervation (Fambrough, 1979; Laufer & Changeux, 1989). Depolarization has opposite effects, inducing a downregulation of the Ach receptors, an effect mediated by Ca^{2+} ions that flow across L-type channels (Huang et al., 1994). Thus, besides their well-known role in excitation-contraction coupling, DHP-sensitive channels also play a significant role in the long-term regulation of Ach receptors and possibly of other ion channels as well. Our present experiments suggest that the DHP-sensitive channel is involved in its own regulation. We found that the long-term blockade of these channels results in an increase in the density of DHP receptors and in the amplitude of L-type Ca^{2+} currents and charge movement.

The skeletal muscle calcium channel is a complex of five subunits: α_1, β, γ and α_2–δ. The α_1 subunit alone can function as a voltage-gated calcium channel and con-

tains the receptor sites for calcium channel antagonists and channel modulators (for a review *see* Catterall et al., 1993). Since we found upregulation of calcium channel function and the number of dihydropyridine-binding sites by prolonged blockade, our data suggest that it is the α_1 subunit that is being upregulated. This does not rule out the possibility that other subunits of the channel may be upregulated or play a role in the upregulation of the α_1 subunit. For example, it has been previously shown that the β subunit has dramatic effects on calcium channel function. Thus, the amplitude of calcium currents and their time course is substantially accelerated by the β subunit. Furthermore, the voltage dependence of activation and inactivation is altered by this subunit (for a review *see* Catterall, 1995). Since we observed an increase in the values of the dissociation constant of ^{3}H-PN200-110 binding sites, it is possible that upregulation of DHP receptors involves the incorporation of new α_1 subunits to the muscle membrane with different properties.

Restoration of excitation-contraction coupling and Ca^{2+} currents takes place after the introduction of the cDNA of the α_1 subunit of the DHP receptors into the nucleus of myotubes of dysgenic mice (for a review *see* Melzer et al., 1995). This suggests that a single gene encodes the protein (or proteins) that performs both functions. However, it is not yet known whether two separate receptors coexist in skeletal muscle, one acting as a voltage sensor and the other as a Ca^{2+} channel, or whether the same receptor can perform both functions (for a review *see* Melzer et al., 1995). If one receptor is involved in both functions, upregulation of DHP receptors would produce the corresponding changes in Ca^{2+} currents and charge movement, as our electrophysiological data show. Alternatively, if two types of DHP receptors coexist in skeletal muscle, then our experiments suggest that both are susceptible of upregulation by a long-term blockade of Ca^{2+} channels. The presence of two DHP receptors would make easier to explain the differences of long-term blockade by nifedipine on charge movement and Ca^{2+} currents described in the present paper. It has been previously shown that under certain experimental conditions nifedipine blocks Ca^{2+} currents with very little effects on charge movement (for a review *see* Melzer et al., 1995).

To the best of our knowledge, this is the first demonstration of upregulation of L-type Ca^{2+} channels by a long-term blockade. This upregulation could be due to an increase in the synthesis of new receptors that are incorporated to the muscle membrane, however it is also possible that a slow down in the turnover of the channel could also play a role. Previous work has shown that N-type Ca^{2+} channels can also be upregulated in neuroblastoma cells. Passafaro et al. (1994) reported that a long-term blockade of N-type Ca^{2+} channels by ω-conotoxin or Cd^{2+} ions, increases the density of ω-conotoxin binding sites and the amplitude of Ba^{2+} currents with a time course of hours.

The Long-Term Regulation of DHP-Sensitive Channels

The increase in the number of dihydropyridine binding sites and in the amplitude of Ca^{2+} currents after a preincubation period with Ca^{2+} channel blockers or in Ca^{2+}-free solutions, shown in the present paper, suggests that a Ca^{2+} influx plays a role in the upregulation of DHP-sensitive channels. Therefore, it is expected that agents that promote an increase in Ca^{2+} inflow through DHP-sensitive channels would have opposite effects to those reported here. In agreement with this possibility, downregulation of L-type Ca^{2+} channels by prolonged depolarization has been observed in other excitable cells such as rat GH_4C_1 pituitary cells. A significant reduction in the number of [^{3}H]PN200-110 binding sites and in Ca^{2+} uptake is observed after incubation in a high KCl depolarizing solution (Liu et al., 1994). A long-term regulation of Ca^{2+} channels has also been studied in myenteric neurons. Electrophysiological experiments have revealed that depolarization with KCl downregulates a sustained component of Ca^{2+} currents. The effect develops with a relatively fast time course and Ca^{2+} currents decline by 50% after 16 hr. The reduction in the amplitude of the Ca^{2+} currents produced by depolarization is blocked by nitrendipine suggesting a role for a Ca^{2+} influx (Franklin, Fickohm & Willard, 1992). DeLorme et al. (1988) described that protracted incubation of PC12 pheochromocytoma cells in a high K^+ depolarizing solution decreases the number of DHP receptors, estimated by the binding of [^{3}H]nitrendipine. The reduction in the number of receptors and the associated decline in $^{45}Ca^{2+}$ uptake is significant only after a lag period of one day and it takes several days to recover.

Although the hallmark of downregulation of Ca^{2+} channels in these examples is a decrease in the number of binding sites and in the Ca^{2+} current density, the underlying mechanisms that have been proposed differ widely and include internalization of the protein (Liu et al., 1994, 1995) and changes in the expression of the α_1 subunit mRNA (Feron & Godfraind, 1995). On the other hand, upregulation of N-type Ca^{2+} channels involves the recruitment to the cell membrane of an intracellular pool of channels (Passafaro et al., 1994). In skeletal muscle, the nature of the events linking a blockade of Ca^{2+} entry to upregulation of DHP-sensitive channels is presently unknown. It is plausible that, when the channel is unblocked, a Ca^{2+} influx prevents upregulation of the channel by hitting a target that is located in the microdomain of the DHP-sensitive channel. This is because the myoplasmic Ca^{2+} is well buffered in skeletal muscle by the

sarcoplasmic reticulum and by Ca^{2+}-binding proteins that would prevent the free-diffusion of this cation far from the plasma membrane.

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