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Depolarization of Chick Myotubes Triggers the Appearance of (+)-[^{3}H]PN 200-110-Binding Sites

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SUMMARY

Regulation of the appearance of dihydropyridine-binding sites was studied in primary cultures of chick myotubes. Labeling of surface dihydropyridine-binding sites on intact myotubes at 37° was achieved with (+)-[^{3}H]PN 200-110. The appearance of the sites was prevented in a calcium-free medium using 1.8 mM EGTA, in accordance with the presumed Ca^{2+} dependency of the appearance of dihydropyridine-binding sites in skeletal muscle cells. Chronic treatment of myotubes with isoproterenol or various other drugs did not modulate either the B_{max} or K_d value of the (+)-[^{3}H]PN 200-110 binding to the membrane preparation of treated myotubes (control values were: K_d = 0.16 nM, B_{max} = 556 fmol/mg of protein), suggesting a lack of heterologous regulation via β-adrenergic receptor stimulation. However, depolarization of intact myotubes, either by 47 mM K^+ or 10^{-5} M veratridine, provoked a 3-fold increase in the B_{max} value of (+)-[^{3}H]PN 200-110 binding measured on the intact muscle cells without affecting the K_d value. The effect was reversed upon repolarization of the cells. Depolarizing conditions did not affect the binding on a membrane preparation of the myotubes. Hence, depolarization appeared to specifically trigger the appearance of (+)-[^{3}H]PN 200-110-binding sites on intact myotubes; several hypotheses which could explain the involved mechanism are discussed.

Like other excitable cells, skeletal muscle fibers have voltage-dependent Ca^{2+} channels. The skeletal muscle Ca^{2+} channel appears to be localized primarily on the transversal-tubular system of the cell membrane (1–4). This membrane system shows an unusually high density of Ca^{2+} ligand-binding sites. For example, for the transversal-tubular membrane preparation from rabbit muscle, the maximum [^{3}H]nitrendipine-binding capacity (B_{max}) is 50 pmol/mg of protein (5). These binding sites are presumably associated with voltage-dependent Ca^{2+} channels.

The physiological role of this elevated amount of binding sites is less clear (6). One way to elucidate its role could be the investigation of how the appearance of these binding sites is regulated. Chick myotubes were chosen because Schmid *et al.* (7) had shown that physiological stimulation of β-adrenergic receptors regulates both the number of [^{3}H]nitrendipine-binding sites and the affinity of such binding sites for dihydropyridines. In this paper, we characterized these binding sites with (+)-[^{3}H]PN 200-110. This radioligand was found to be suitable for selective labeling of surface binding sites on intact skeletal muscle cultures. We observed that isoproterenol and various other drugs did not modulate the appearance of (+)-[^{3}H]PN 200-110-binding sites. Modulation of these binding sites was well obtained by depolarization of myotubes. The observations indicate that depolarization promotes the increase in the number of (+)-[^{3}H]PN 200-110-binding sites. Some of these results were presented at the Joint Meeting of the Belgian/British Biochemical Societies (Genth, May 22–23, 1987).

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Materials and Methods

Cell culture. Primary cultures of skeletal muscle cells from chick embryos were prepared according to the method described by Schmid *et al.* (8). Breast and leg muscles from 10- to 11-day-old chick embryos were collected in Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution and mechanically dissociated by repetitive suction through a pipette. The resulting suspension was placed in a nylon bag, 200 μm in pore diameter, and filtered by gravity flow through a layer of nylon, 80 μm in pore diameter. Cells were collected by centrifugation (10 min at 400 × *g*) and suspended in Dulbecco's modified Eagle's minimal essential medium and M 199 medium (3:1, v/v), supplemented with 5% fetal calf serum, 200 units/ml penicillin, and 200 μg/ml streptomycin. Viability tests performed by the trypan blue exclusion method generally yielded viability values >95%. Cells were plated either in gelatin-coated (2%, w/v) 50-mm-diameter dishes (Falcon) with 8 ml of medium or in 24-well tissue culture plates (Nunc) with 1.0 ml of medium/well at a density of 2.4 × 10^5 cells/cm^2. In general, 60 eggs yielded 6 × 10^8 cells (10-day-old embryos) to 8.4 × 10^8 cells (11-day-old embryos). Cultures

ABBREVIATIONS: EDTA, ethylenediaminetetraacetate; Hepes, 4-(2-hydroxyethyl)-1piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; TPP, tetraphenylphosphonium.

were maintained at 37° in an air/CO_2 (95/5%), water-saturated atmosphere.

Chronic drug treatment. Drugs were added to cultures 3–4 days after plating, at the time when the maximum extent of fusion of myoblasts surpassed 80%. The drug treatment was repeated every 12 hr. Then, the myotubes were washed twice in a 20 mM Tris buffer, pH 7.4, containing 0.25 M sucrose and 1 mM EDTA and assayed for (+)-[^{3}H]PN 200-110 binding using a cell membrane preparation.

(+)-[^{3}H]PN 200-110 binding assay on membrane preparation. Cells were scraped off in 50 mM Tris-HCl, pH 7.4, and homogenates were prepared with an Ultra Turrax homogenizer. A membrane preparation was obtained by centrifugation at 26,000 × *g* for 10 min. Binding assays were carried out as follows. The equivalent of membrane fraction from 5×10^5 cells was suspended in 1.1 ml of 50 mM Tris-HCl, pH 7.4, containing 0.25 nM (+)-[^{3}H]PN 200-110 in the absence (total binding) or in the presence (nondisplaceable binding) of 1 μM unlabeled nitrendipine. The mixture was incubated for 30 min at 37°. Incubation was stopped by filtering through Whatman glass fiber filters (GF/B). Filters were immediately washed twice with 5 ml of ice-cold 25 mM Hepes/Tris-HCl, pH 7.4, containing 140 mM choline chloride and 0.1 mM EGTA. The filters were extracted in 8 ml of Insta-Gel II, and the radioactivity was counted in a Packard Tri-carb 4530 liquid scintillation counter. Displaceable binding of (+)-[^{3}H]PN 200-110 was defined as the portion of total binding which was inhibited by 1 μM nitrendipine. To investigate concentration binding curves, the (+)-[^{3}H]PN 200-110 concentration ranged from 25 to 500 pM. Data were analyzed in Scatchard plots. The line of best fit was calculated by linear regression using the method of least squares.

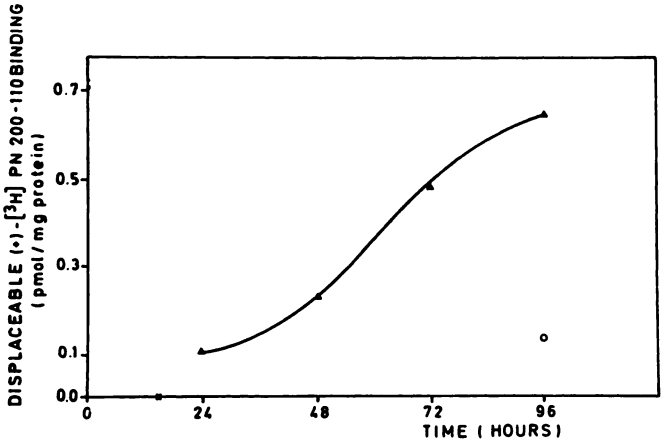

Fig. 1. Time course of appearance of total number of (+)-[^{3}H]PN 200-110-binding sites into isolated membranes of skeletal muscle cultures. The total number of (+)-[^{3}H]PN 200-110-binding sites was measured at 0.25 nM (+)-[^{3}H]PN 200-110 on a membrane preparation at the indicated times. (+)-[^{3}H]PN 200-110 binding was carried out as described under Materials and Methods. EGTA treatment (1.8 mM) was started 16 hr after plating (*x*). Each value is the mean of two observations: control cultures (▲) and EGTA-treated cultures (○).

TABLE 1

K_d and B_{max} values for (+)-[^{3}H]PN 200-110 binding to membrane preparation from myotubes chronically treated with drugs or toxins

Three or 4 days after plating, cultures were treated with drugs or toxins. After a period of 24 hr, (+)-[^{3}H]PN 200–110 saturation experiments were carried out on a membrane preparation, as described under Materials and Methods. Binding results were analyzed by means of a Scatchard plot. Results are given as the mean (n = 1) or as the mean ± standard deviation ($n \geq 2$).

Treatment	Concentration	n^a	K_d	B_{max}
	M		*nM*	*fmol/mg protein*
Control		15	0.16 ± 0.05	556 ± 155
BAY K 8644	10^{-6}	2	0.19 ± 0.01	559 ± 17
Tetrodotoxin	10^{-6}	2	0.13 ± 0.02	545 ± 16
Veratridine	10^{-5}	2	0.21 ± 0.02	445 ± 64
Transcainide	10^{-6}	2	0.12 ± 0.01	488 ± 52
Ouabain	10^{-6}	2	0.17 ± 0.01	529 ± 10
Calcimycin	10^{-7}	2	0.13 ± 0.01	463 ± 54
Reserpine	10^{-6}	1	0.15	480
Carbachol	10^{-6}	2	0.15 ± 0.02	623 ± 74
Isoproterenol	5.10^{-5}	7	0.15 ± 0.06	660 ± 132
Methylisobutylxanthine	10^{-6}	2	0.18 ± 0.05	461 ± 43
Aminophylline	10^{-6}	2	0.17 ± 0.04	407 ± 81
Nebivolol	10^{-7}	2	0.17 ± 0.06	520 ± 35

[a] n = number of determinations.

TABLE 2

Displaceable (+)-[^{3}H]PN 200-110 binding to intact myotubes under low and high K^+ conditions

Binding on intact cells from 6-day-old cultures was carried out under low (5 mM) and high (47 mM) K^+ conditions at 4° and 37°, as described under Materials and Methods. Displaceable binding was expressed as the difference between total binding and binding in the presence of 1 μM nitrendipine. All values are the mean of six determinations. The standard deviation was less than 10%.

Conditions	Displaceable (+)-[^{3}H]PN 200-110 binding at	
	4°	37°
	fmol/mg protein	
5 mM K^+	14.6	66.3
47 mM K^+	15.3	154.2

Depolarization and binding experiments on intact myotubes. To each well of a 24-well tissue culture plate was added either 0.5 ml of 5 mM K^+ solution (5 mM KCl, 130 mM NaCl, 2 mM $CaCl_2$, 5 mM glucose, 50 mM Hepes/Tris-HCl, pH 7.4) or 130 mM K^+ solution (the concentration of KCl was increased to 130 mM, while the concentration of NaCl was decreased to keep osmolarity equal) to give a final K^+ concentration of 5 or 47 mM. At the same time, 0.25 nM (+)-[^{3}H]PN 200-110 in the absence (total binding) or in the presence (nondisplaceable binding) of 1 μM unlabeled nitrendipine was added. Incubation was run for 30 min at 37° and was stopped by washing twice with 1.0 ml of ice-cold Ca^{2+} wash buffer. Cells were digested into 2.0 ml of 0.1 N NaOH and 1.0 ml was counted as described above. Concentration binding curves were carried out with (+)-[^{3}H]PN 200-110 between 50 and 700 pM.

Accumulation of TPP ions. This was measured as previously described (9). Cells containing 1 nM [phenyl-^{3}H]TPP^+ in 5 mM K^+ solution or at various K^+ concentrations were incubated for 30 min at 37°. The reaction was stopped by washing twice with ice-cold Ca^{2+} wash buffer. Cells were digested into 0.1 N NaOH as described above.

Protein content. Protein was estimated using the Bio-Rad kit (10). Bovine serum albumin was taken as a standard.

Materials. All media, sera, and 24-well tissue culture plates were obtained from Gibco/Biocult Laboratories, Scotland. Tissue culture dishes were purchased from Falcon, Becton-Dickinson, Oxnard, CA. (+)-[*methyl*-^{3}H]PN 200-110 (71 Ci/mmol) was from the Radiochemical Centre, Amersham, UK. Eggs were obtained from a local breeding center. Drugs were kindly provided by the companies of origin. The stock solutions of the drugs were prepared in 100% ethanol. Dilutions were made in 10% ethanol. The maximal concentration of ethanol allowed in the incubation medium was 0.2%.

Results

Fusion of mononucleated myoblasts to polynucleated myotubes was accompanied by the appearance of (+)-[^{3}H]PN 200-110-binding sites. Fig. 1 shows the time course of the appearance of the total number of (+)-[^{3}H]PN 200-110-binding sites measured on a membrane preparation of the cells. The appearance of these binding sites was dependent on Ca^{2+}. Addition of 1.8 mM EGTA, 16 hr after plating, blocked the fusion of myoblasts to myotubes as well as the appearance of (+)-[^{3}H]PN 200-110-binding sites measured on a membrane prepara-

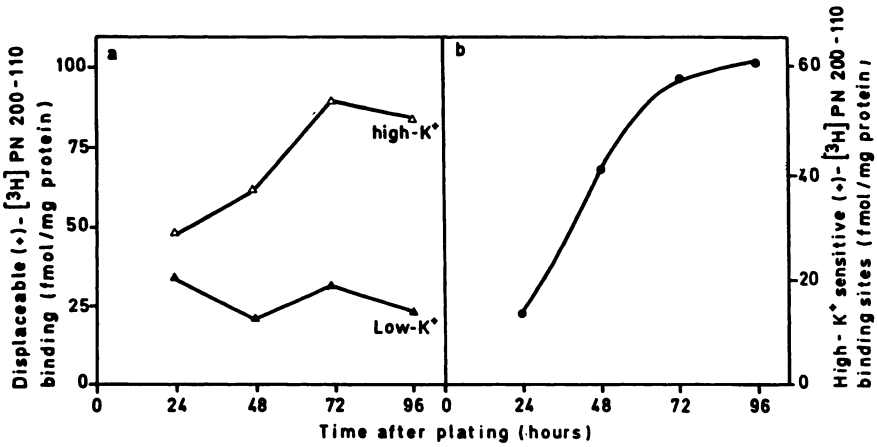

Fig. 2. Time course of appearance of depolarization-sensitive (+)-[^{3}H]PN 200-110-binding sites on intact skeletal muscle cultures. At the times indicated after plating, (+)-[^{3}H]PN 200-110 binding was carried out at 0.25 nM (+)-[^{3}H]PN 200-110 on intact cells under low K^+ and high K^+ conditions for 30 min as described under Materials and Methods. a. Results are given as displaceable (+)-[^{3}H]PN 200-110 binding under low K^+ (▲) and high K^+ (△) conditions. b. Results are given as the difference between displaceable (+)-[^{3}H]PN 200-110 binding under high K^+ and low K^+ conditions (●). Each value is the mean of six determinations. The standard deviation was less than 10%.

TABLE 3

The effect of a lysomotropic drug and cell depolarization on (+)-[^{3}H]PN 200-110 binding to intact myotubes

Drug treatment was performed on intact cells from 4-day-old cultures. Binding was carried out at 0.25 nM (+)-[^{3}H]PN 200-110. Nondisplaceable binding was measured in the presence of 1 μM nitrendipine. The results are given as the mean ± standard deviation of six determinations.

Treatment	Total binding	Nondisplaceable binding	Displaceable binding
	fmol/well	fmol/well	fmol/well
5 mM Potassium (LK)[a]	19.8 ± 1.0	13.9 ± 0.8	5.9 ± 0.7
47 mM potassium (HK)	30.3 ± 2.4	14.2 ± 0.6	16.1 ± 1.8
LK + 10^{-6} M chloroquine	19.1 ± 1.2	13.3 ± 0.8	5.8 ± 0.5
HK + 10^{-6} M chloroquine	26.0 ± 0.9	11.0 ± 1.4	15.0 ± 0.5
LK + 10^{-6} M veratridine	25.6 ± 1.2	13.6 ± 0.9	12.0 ± 0.7
HK + 10^{-6} M veratridine	26.0 ± 1.8	11.4 ± 0.6	14.6 ± 1.2
LK + 10^{-6} M TTX	17.1 ± 0.9	13.3 ± 0.7	3.8 ± 0.6
HK + 10^{-6} M TTX	27.6 ± 0.4	13.1 ± 0.2	14.5 ± 0.4
LK + 10^{-6} M veratridine + 10^{-6} M TTX	16.9 ± 1.1	11.6 ± 0.7	5.3 ± 0.5
HK + 10^{-6} M veratridine + 10^{-6} M TTX	26.8 ± 1.3	13.4 ± 0.2	13.4 ± 1.2

[a] LK, low K^+ treatment; HK, high K^+ treatment; TTX, tetrodotoxin.

tion. The latter were decreased to 20% of cultures without EGTA treatment after 4 days of culture.

Three to 4 days after plating, at the time when the extent of fusion reached a plateau, cultures were treated chronically with a number of toxins or drugs shown in Table 1. With the exception of BAY K 8644, toxins or drugs were tested at concentrations which did not compete with (+)-[^{3}H]PN 200-110 binding in a membrane preparation. Drug-treated myotubes did not show any detectable morphological change as compared to control, untreated cells. A summary of the binding experiments is given in Table 1. Membranes from control myotubes had a K_d value of 0.16 nM and a binding capacity of 556 fmol/mg of protein. In myotubes chronically treated with a dihydropyridine agonist (BAY K 8644) or an Na^+ channel blocker (tetrodotoxin), a partial Na^+ channel agonist (veratridine), an Na^+,K^+-ATPase inhibitor (ouabain), a Ca^{2+} ionophore (calcimycin), a monoamine depletor (reserpine), a muscarinic cholinergic agonist (carbachol), a β_1-adrenergic antagonist (nebivolol), and phosphodiesterase blockers (aminophylline and methylisobutylxanthine), no change was found in the maximum binding capacity and K_d value of (+)-[^{3}H]PN 200-110 binding on cell membrane preparations. For all of the different treatment conditions, concentration binding curves of (+)-[^{3}H]PN 200-110 were performed, and the data were analyzed in Scatchard plots. The plots were found to be rectilinear as revealed by the high correlation coefficient of the lines ($r \geq 90$, method of least squares).

The observations with aminophylline, methylisobutylxanthine, and isoproterenol were surprising. Schmid *et al.* (7) had observed an increase in the number of [^{3}H]nitrendipine-binding sites in myotubes treated with these drugs. This increase was

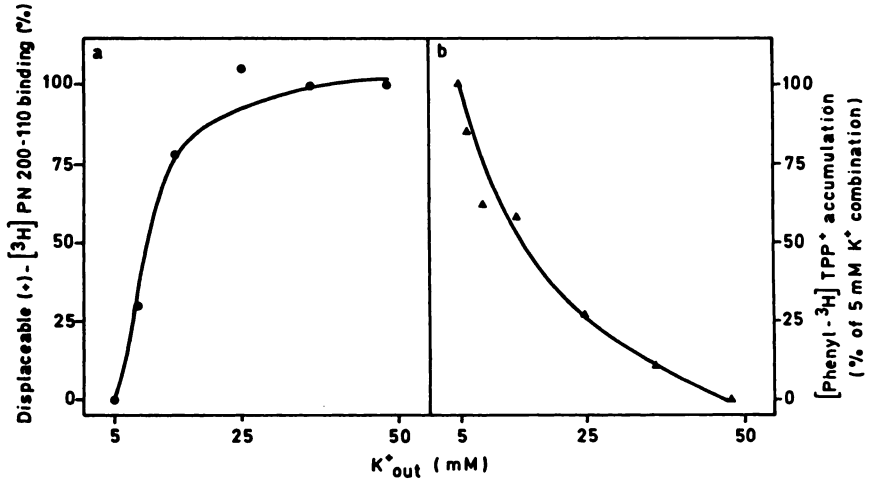

Fig. 3. Effect of K^+ concentration on (+)-[^{3}H]PN 200-110 binding and [phenyl-^{3}H]TPP^+ accumulation in skeletal muscle cultures. a. Cultures were incubated with 0.25 nM (+)-[^{3}H]PN 200-110 for 30 min at 37° as described under Materials and Methods at different K^+ concentrations. [Na^+]+[K^+] was kept constant at 135 mM. Displaceable (+)-[^{3}H]PN 200-110 binding was expressed as a per cent of the difference obtained between the values of 47 mM and 5 mM K^+. The 5 mM K^+ value was substracted from each value. The half-maximal increase of displaceable (+)-[^{3}H]PN 200-110 binding was obtained at 10 mM K^+. b. Cultures were incubated with 1 nM [phenyl-^{3}H]TPP^+ for 30 min at 37° at different K^+ concentrations as described under Materials and Methods. The [phenyl-^{3}H]TPP^+ accumulation was expressed as a per cent of the value obtained with 5 mM K^+ (15,096 ± 246 dpm, $n = 6$ at 1 nM [phenyl-^{3}H]TPP^+). It was assumed that at 47 mM K^+, maximal depolarization was achieved. Half-maximal [phenyl-^{3}H]TPP^+ accumulation was measured at 14.5 mM K^+. Three-day-old cultures were used. The mean protein constant was 147 μg/well.

TABLE 4

The effect of depolarizing stimuli on (+)-[^{3}H]PN 200-110 binding to a membrane fraction of myotubes

A membrane fraction was prepared as described under Materials and Methods. Membranes were incubated at 37° under low K^+, low K^+ + 10^{-5} M veratridine, or high K^+ conditions for 30 min in the presence of 0.25 nM (+)-[^{3}H]PN 200-110. Values are given as displaceable binding.

Conditions	Displaceable binding
	fmol/mg protein
5 mM K^+	587
5 mM K^+ + 10^{-5} M veratridine	531
47 mM K^+	521

TABLE 5

Comparison between (+)-[^{3}H]PN 200-110 binding to intact and broken cell preparations from low and high K^+-treated myotubes

Three-day-old cultures were incubated under low and high K^+ conditions. Binding assays on intact muscle cells were performed as described under Materials and Methods. A broken cell preparation was prepared in 10 mM Tris-HCl, pH 7.4, and binding was carried out as described in Table 4. The standard deviation was less than 10%.

Cell preparation	Displaceable (+)-[^{3}H]PN 200-110 binding	
	Low K^+	High K^+
	fmol/mg protein	
Intact	43.2	169
Broken	46.1	46.8

accompanied by a 4- to 10-fold decrease in the affinity of the binding site for [^{3}H]nitrendipine. We repeated our experiments after a 48-hr drug treatment. However, no changes could be detected. We also determined the maximal number of binding sites in an isolated membrane preparation from untreated cells with [^{3}H]nitrendipine at 4° as described by Schmid *et al.* (7). A B_{max} value of 486 fmol/mg of protein was obtained. To exclude the possibility that components in the fetal calf serum were responsible for the induction of a maximal expression of (+)-[^{3}H]PN 200-110-binding sites, the fetal calf serum was dialyzed to remove substances with a molecular weight of 15,000 or less. Using dialyzed serum, there were no significant differences in the expression of (+)-[^{3}H]PN 200-110-binding sites, nor between control cultures in the presence of nondialyzed and dialyzed serum, nor between control and isoproterenol-treated cells.

Since chronic drug treatment did not affect the expression of (+)-[^{3}H]PN 200-110-binding sites, another approach was undertaken. Binding sites for Ca^{2+} ligands are presumably associated with voltage-dependent Ca^{2+} channels. Therefore, we checked the effect of polarization on (+)-[^{3}H]PN 200-110 binding. Intact myotubes were incubated in low and high K^+ conditions. Addition of low K^+ did not influence (+)-[^{3}H]PN 200-110 binding. By contrast, addition of high K^+ (final concentration, 47 mM) induced after a 30-min incubation at 37° a significant increase in total (+)-[^{3}H]PN 200-110 binding measured on intact cells, whereas the nondisplaceable binding did not change. In an experiment carried out at 4°, depolarization did not affect the (+)-[^{3}H]PN 200-110 binding on the intact cells. Data are shown in Table 2. Fig. 2 shows the time course of the high K^+-sensitive (+)-[^{3}H]PN 200-110-binding sites on intact myotubes. The displaceable (+)-[^{3}H]PN 200-110 binding on intact cells under high K^+ conditions was increased with time up to 3 days after the start of cultivation. A plateau in the number of binding sites was reached after 4 days. By contrast, displaceable (+)-[^{3}H]PN 200-110 binding on intact myotubes under low K^+ conditions had a constant value of 27 ± 5 fmol/mg of protein between 24 and 96 hr of cultivation.

The observed increase in (+)-[^{3}H]PN 200-110 binding under high K^+ conditions was not due to a higher rate of (+)-[^{3}H]PN 200-110 trapping. Table 3 shows that (+)-[^{3}H]PN 200-110 binding in the presence of chloroquine, a lysomotropic drug which prevents trapping, was not significantly changed.

Experiments set up at various K^+ concentrations revealed that a half-maximal increase of displaceable (+)-[^{3}H]PN 200-110 binding was obtained at 10 mM K^+ (Fig. 3). The membrane potential of myotubes was measured by [phenyl-^{3}H]TTP^+ accumulation, to define the K^+concentration at which half-maximal depolarization was achieved. The latter was obtained at 14.5 mM K^+ (Fig. 3a). Hence, the half-maximal K^+ effect on expression of (+)-[^{3}H]PN 200-110-binding sites was obtained in the range of half-maximal depolarization.

Veratridine was used as a depolarizing stimulus to exclude the possibility of measuring an ion effect, as opposed to a depolarizing effect. Table 3 clearly shows that 10^{-5} M veratridine increased total as well as displaceable (+)-[^{3}H]PN 200-110 binding under low K^+ conditions. This could be totally blocked by the Na^+ channel blocker, tetrodotoxin.

Intact cells were required to observe the depolarization effect. Table 4 shows that, on a membrane preparation (although still polarized) from myotubes in low K^+ + veratridine, and under high K^+ conditions, no effect was observed. (+)-[^{3}H]PN 200-110 binding to a homogenate or a membrane preparation from low or high K^+-treated myotubes was also without effect. Table 5 shows that (+)-[^{3}H]PN 200-110 binding on a broken cell preparation never exceeded 43 fmol, the low K^+ (+)-[^{3}H]PN 200-110 value of intact cells.

A (+)-[^{3}H]PN 200-110 saturation experiment on intact myotubes under low and high K^+ conditions is shown in Fig. 4. Scatchard analysis of the binding data shows that there was no significant difference between the K_d values but that the B_{max} value of (+)-[^{3}H]PN 200-110 binding under high K^+ conditions was increased about 4 times. Nitrendipine displaced 50% of displaceable (+)-[^{3}H]PN 200-110 binding on intact myotubes under high K^+ conditions at 8×10^{-9} M. This value was good compared with the IC_{50} value obtained with a membrane preparation: 5×10^{-9} M (not shown).

Finally, an experiment was performed to check the reversibility of this effect. From Fig. 5 it is apparent that displaceable (+)-[^{3}H]PN 200-110 binding varied from low to high values or from high to low values, once myotubes were switched from low to high K^+ or from high to low K^+.

Discussion

There was a good correlation between the appearance of (+)-[^{3}H]PN 200-110-binding sites in skeletal muscle cultures and the degree of fusion of myoblasts to myotubes. At the same time, it was shown that Ca^{2+} was necessary for the fusion process as well as the appearance of (+)-[^{3}H]PN 200-110-binding sites. The number of (+)-[^{3}H]PN 200-110-binding sites on intact polarized myotubes was already maximally present at 1 day. There was still an increase in the number of (+)-[^{3}H]PN 200-110-binding sites after depolarization.

Chronic drug treatment. The expression of the total number of (+)-[^{3}H]PN 200-110-binding sites seems to be insensitive to chronic treatment with toxins or drugs (Table 1). This is not the case with Na^+ channels. Calcimycin and tetrodotoxin

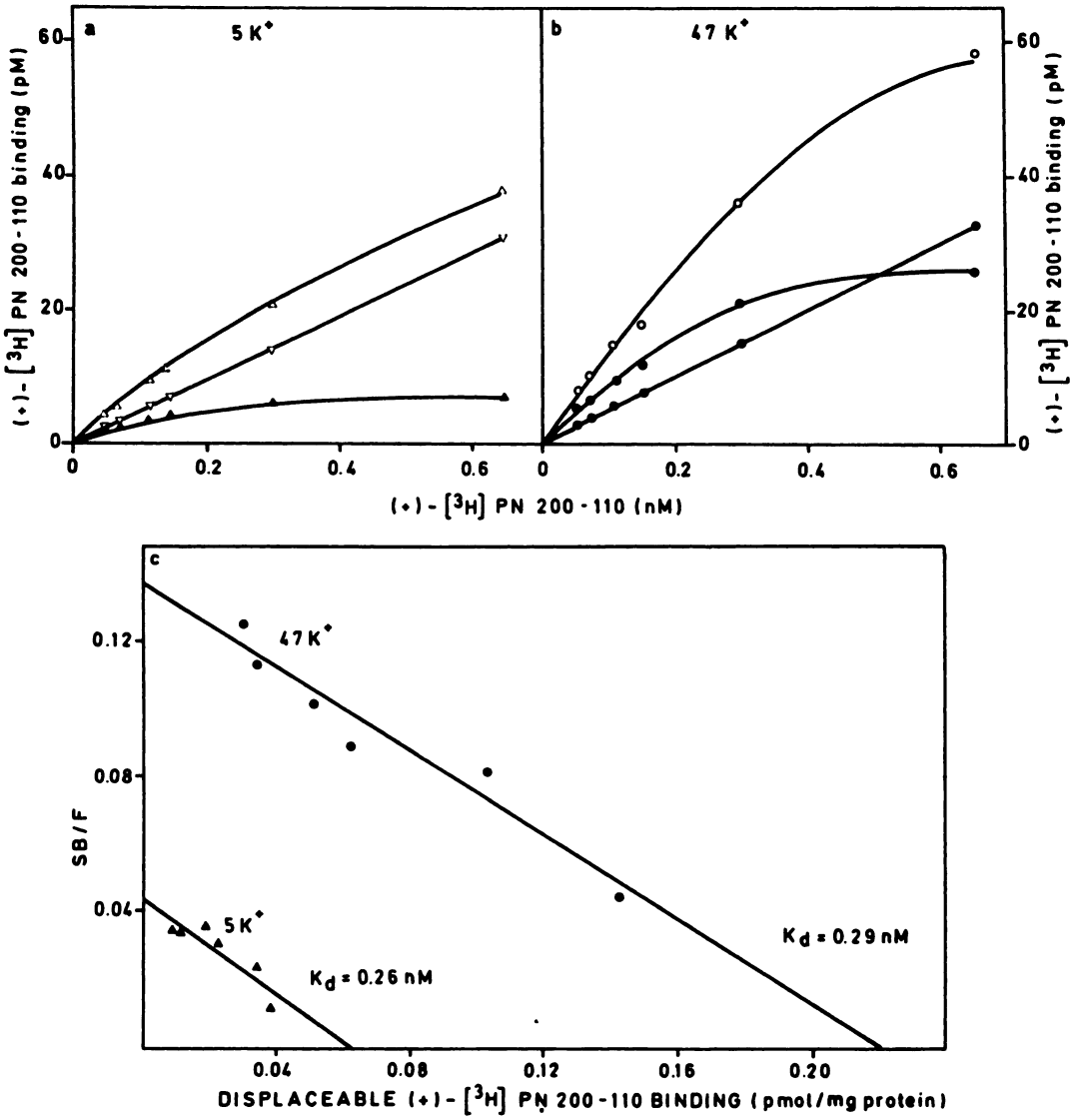

Fig. 4. Saturation curves and Scatchard plots of (+)-[³H]PN 200-110 binding to intact myotubes under low K^+ and high K^+ conditions. Binding was carried out on 4-day-old cultures as described under Materials and Methods. a. Saturation curve for (+)-[³H]PN 200-110 binding under low K^+ conditions, showing total (△), nondisplaceable (▽), and displaceable (▲) (+)-[³H]PN 200-110 binding. b. Saturation curve for (+)-[³H]PN 200-110 binding under high K^+ conditions, showing total (○), nondisplaceable (◑), and displaceable (●) binding. c. Scatchard plots of displaceable (+)-[³H]PN 200-110 binding at 5 mM K^+ (▲), B_{max} = 62 fmol/mg of protein and 47 mM K^+ (●), B_{max} = 220 fmol/mg of protein. Each value is the mean of six determinations. *SB*, displaceable (+)-[³H]PN 200-110 binding; *F*, free (+)-[³H]PN 200-110 concentration: added concentration of (+)-[³H]PN 200-110 minus the concentration totally bound. K_d values were given by the reciprocal value of the slope of the line. B_{max} values were given by the intersection point with the *abscissa* (in pmol/mg of protein). *Lines* were calculated using the method of least squares. The mean protein content was 185 μg/well.

reportedly modulate the expression of [³H]saxitoxin-binding sites (11, 12). The observation that aminophylline, methylisobutylxanthine, and isoproterenol did not induce an effect on (+)-[³H]PN 200-110 binding is at variance with the results of Schmid *et al.* (7). These authors observed an increase in the total number of [³H]nitrendipine-binding sites. Ligand specificity might account for this discrepancy. (+)-[³H]PN 200-110 and [³H]nitrendipine would bind to a different population of binding sites. This is probably not the case. The B_{max} value for [³H]nitrendipine was similar to the value obtained with (+)-[³H]PN 200-110. This may be an indication that it concerns the same population of binding sites. It is possible that our culture method induces a maximal appearance of (+)-[³H]PN 200-110-binding sites. Under these conditions, further stimulation with isoproterenol would be impossible. We tried an experiment with dialyzed serum, however, without result. Substances with a molecular weight >15,000—which were not separated by dialysis—might have been responsible for induction of the maximal appearance of (+)-[³H]PN 200-110-binding sites.

Binding of (+)-[³H]PN 200-110 to intact skeletal muscle cultures. (+)-[³H]PN 200-110 seems to be a valuable ligand for binding experiments on intact cells. The (+)-[³H]PN 200-110 binding to intact skeletal muscle cultures was characterized by a B_{max} value of 62 fmol/mg of protein and a K_d value of 0.26 nM. This is comparable with the data obtained by Navarro (13), who reported labeling of (+)-[³H]PN 200-110-binding sites on intact cells. Binding to intact cells in the presence of chloroquine indicated that this ligand is not trapped in lysosomes. The physiochemical properties of (+)-[³H]PN 200-110 ap-

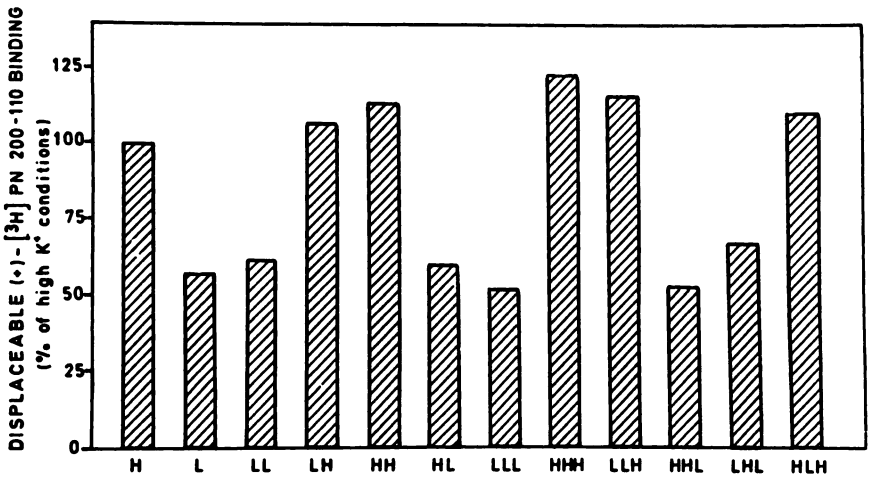

Fig. 5. Reversibility of (+)-[^{3}H]PN 200-110 binding to low or high K^+-treated myotubes. Two-day-old myotubes were chosen so that they could be treated three times with low or high K^+ at 37°. Each treatment lasted 30 min. The last treatment was performed with 0.25 nM (+)-[^{3}H]PN 200-110, in the absence or presence of 1 μM nitrendipine. The reaction was stopped, as described under Materials and Methods. Each value is the mean of six determinations. *L*, low K^+; *H*, high K^+ treatment. The *symbols* indicate the subsequent treatments.

peared to allow selective labeling of the surface binding sites on intact skeletal muscle cells. This was of particular interest for the depolarization experiments.

Depolarizing effect on (+)-[^{3}H]PN 200-110-binding sites. A remarkable observation was that more (+)-[^{3}H]PN 200-110-binding sites could be measured on intact myotubes under depolarizing conditions. This was only true at 37° for high K^+ as well as low K^+ plus veratridine. Similar observations have been reported (14–16). Although all have shown augmentation of binding under depolarizing conditions, the basis of this effect has varied. Depolarization increased the number of (+)-[^{3}H]PN 200-110-binding sites in sartorius muscle (14) and [^{3}H]nitrendipine sites in cardiac myocytes (15). In contrast, the depolarization-induced increase in (+)-[^{3}H]PN 200-110 binding to pheochromocytoma cells (16) was the result of enhanced affinity. In the present study, we found that there was a voltage-dependent effect on the maximal binding capacity for (+)-[^{3}H]PN 200-110 to intact chick myotubes but not on the dissociation constant. Veratridine-induced depolarization was inhibited under low K^+ conditions in the presence of 1 μM tetrodotoxin. This suggests the presence of tetrodotoxin-sensitive Na^+ channels.

There are several reasons to believe that we deal with a depolarizing effect as opposed to an ion effect. First, chemical depolarization of myotubes by veratridine was effective. Second, there was a lack of effect of depolarizing stimuli on a membrane preparation from myotubes. Third, there was an absolute requirement of intact cells to measure the enhancement of (+)-[^{3}H]PN 200-110 binding.

To explain the depolarizing effect on (+)-[^{3}H]PN 200-110 binding, several hypotheses can be put forward. 1) Depolarization induces the synthesis of (+)-[^{3}H]PN 200-110-binding macromolecules; however, this is unlikely to occur during the short incubation time of 30 min. Moreover, the number of binding sites in membrane preparations derived from low and high K^+-treated myotubes was not different. This suggests that the binding sites are always present, but that they cannot be measured on intact polarized cells. 2) Depolarization increases externalization of (+)-[^{3}H]PN 200-110-binding sites. This supposes the presence of (+)-[^{3}H]PN 200-110-binding sites in intracellular vesicles. This is probably not the case since, in a broken cell preparation, the number of sites determined was not higher than the number of sites measured on intact muscle cells under low K^+ conditions (Table 5). It cannot be excluded that a part of the binding sites in a broken cell preparation is inaccessible to (+)-[^{3}H]PN 200-110 due to the formation of vesicles during homogenization. 3) Depolarization activates masked (+)-[^{3}H]PN 200-110-binding sites. Depolarization would change the conformation of the latent binding site so that it could bind (+)-[^{3}H]PN 200-110. 4) Depolarization induces a conformational change in the transversal tubuli. Depolarization would change the charge partition of the membrane, in such a way that its conformation would be more favorable for access of (+)-[^{3}H]PN 200-110 to the binding site. One would expect that this process is reversible in a short time. Our experiments indicated that the (+)-[^{3}H]PN 200-110 binding varied from low to high values or vice versa, once myotubes were switched from low to high K^+ or from high to low K^+. 5) Polarization induces a conversion of high affinity (+)-[^{3}H]PN 200-110-binding sites into low affinity (+)-[^{3}H]PN 200-110-binding sites. The decrease in the maximal number of high affinity (+)-[^{3}H]PN 200-110-binding sites by passing from high K^+ to low K^+ would reflect the conversion of part of them (approximately 75% under our conditions) into low affinity binding sites. We were not able to detect low affinity binding sites. This may be difficult because of the existence of a large component of a nonspecific binding of (+)-[^{3}H]PN 200-110 to intact skeletal muscle cells. Electrophysiological results favor this hypothesis (17). PN 200-110 is a very potent Ca^{2+} channel blocker when associated with myoballs depolarized at −65 mV ($K_{0.5}$ = 0.15 nM). When (+)-[^{3}H]PN 200-110 is associated with myoballs at a holding potential of −90 mV, a $K_{0.5}$ value of 13 nM is obtained. These results suggest that the affinity of (+)-[^{3}H]PN 200-110 for the Ca^{2+} channel depends on the state of the channel such that binding to the resting state is less potent by a factor of about 100 than binding to the inactivated state. Similar observations have been reported for cardiac Ca^{2+} channels (18, 19).

These depolarization-sensitive (+)-[^{3}H]PN 200-110-binding sites could have a role in the following process. Contraction of skeletal muscle is dependent on Ca^{2+}. The release of Ca^{2+} from the sarcoplasmic reticulum starts the contraction. The intracellular Ca^{2+} store is probably not sufficient to maintain the activated state. By contrast, the extracellular Ca^{2+} concentration is sufficiently high. However, Ca^{2+} must be transported. An increase in the number of (+)-[^{3}H]PN 200-110-binding sites

by depolarization would probably accelerate this transport process.

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