Identification of putative calcium channels in skeletal muscle microsomes

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Received 26 September 1982

Saturable binding sites for the labelled calcium antagonist (±)[^{3}H]nimodipine were found in guinea-pig hind limb skeletal muscle homogenates. Binding sites were enriched in a microsomal pellet by differential centrifugation of the homogenate. [^{3}H]Nimodipine binding (K_d = 1.5 ± 0.03 nM, B_{max} = 2.1 ± 0.25 pmol/protein, at 37°C) copurified (6-fold) in this fraction with [^{3}H]ouabain binding (6.6-fold) and ^{125}I-α-bungarotoxin binding (5-fold). d-*cis*-Diltiazem (but not l-*cis*-diltiazem) stimulated (±) [^{3}H]nimodipine binding (ED_{50} 1 μM) by increasing the B_{max}. Binding sites discriminated between the optical enantiomers of 1,4-dihydropyridine calcium antagonists and the optically pure enantiomers of D-600. The data confirm, with biochemical techniques, the presence of 1,4-dihydropyridine and (±)D-600 inhibitable calcium channels in skeletal muscle, previously found with electrophysiological techniques.

Skeletal muscle *Calcium channel* *Antagonist* *Receptor* *Allosterism* *Nimodipine*

1. INTRODUCTION

Putative Ca^{2+} channels have been directly identified by the utilization of radiolabelled 1,4-dihydropyridine Ca^{2+}-antagonists in mammalian heart [1–4], brain [2–6] and duodenal membranes [7]. The potent 1,4-dihydropyridine Ca^{2+}-antagonist nimodipine [8] when radiolabelled with tritium binds to a homogeneous population of high affinity binding sites (K_d = 0.6 nM; 37°C) in guinea-pig brain membranes [5]. The (±)[^{3}H]-nimodipine binding site interacts with organic and inorganic Ca^{2+}-antagonists [6] suggesting that (±)[^{3}H]nimodipine binds to physiologically relevant Ca^{2+} channels.

Besides the above tissues, skeletal muscle has Ca^{2+} channels, which have been identified by electrophysiological studies (review [9]). Therefore, we have performed ligand binding experiments and report on the direct identification of putative Ca^{2+} channels and their regulation by organic and inorganic Ca^{2+}-antagonists in a partially purified skeletal muscle membrane fraction derived from guinea-pig hind limb.

+ This is part of the thesis of D.R.F. to be presented to the Medical Faculty of the Justus-Liebig-Universität Gießen

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2. MATERIALS AND METHODS

Male guinea-pigs (250–350 g body wt) were stunned and bled. The skin around the hind limbs was removed and the muscles (15 g/animal) rapidly excised and placed in ice-cold 20 mM $NaHCO_3$ supplemented with 0.1 mM phenylmethylsulphonylfluoride (PMSF). The muscle was trimmed free from fat and connective tissue and homogenized by two 30 s disruptions with an ultraturrax (three quarters of maximal speed). The wet weight to volume ratio was 1:5. The crude homogenate was filtered through 2 layers of cheesecloth, and the resultant filtrate was centrifuged at 3500 × *g* (15 min). The pellet was discarded, and the supernatant spun at 45 000 × *g* for 15 min. The resultant pellet is resuspended in 50 mM Tris–HCl (pH 7.4) and centrifuged as above. The final crude microsomal pellet was suspended in Tris–HCl buffer (50 mM, pH 7.4) and stored in liquid nitrogen until use.

(±)[^{3}H]Nimodipine (160 Ci/mmol, Bayer AG, >95% radiochemical purity) binding assays were performed under sodium vapour light as in [5]. In

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00145793/82/0000–0000/$2.75

brief: (±)[^{3}H]nimodipine was incubated with tissue fractions and various additions in 50 mM Tris–HCl buffer (pH 7.4 at 37°C) and bound and unbound ligand separated by rapid filtration through Whatman GF/C filters. Non-specific binding was defined by 1 μM unlabelled (±)nimodipine. Specific binding represents ≥85% of bound radioactivity in all (±)[^{3}H]nimodipine binding experiments.

[^{3}H]Ouabain (18 Ci/mmol, NEN, Dreieich) binding was performed as in [10] except that Tris–HCl (50 mM,pH 7.4) replaced the imidazol buffer. ^{125}I-Labelled hydroxyphenylisopropyladenosine (2175 Ci/mmol, prepared and purified in our laboratory) binding was done as in [11].

^{125}I-Labelled α-bungarotoxin (12.2 μCi/μg, NEN, Dreieich) binding was performed as in [12], except that non-specific binding was defined by 0.2 μM unlabelled α-bungarotoxin (Sigma, München).

(+)PN 205-033 and (−)PN 205-034 are the optically pure enantiomers of the potent 1,4-dihydropyridine Ca^{2+}-antagonist (±)PN 200-110 (isopropyl-4-(2,1,3-benzoxadiazol-4-yl)-1,4-dihydro-2,6-dimethyl-5-methoxycarbonyl-pyridine-3-carboxylate) and were provided by Sandoz AG (Basel). (±)Fendiline was from Thiemann (Lünen), Tiapamil, (*N*-(3,4-Dimethoxyphenethyl)-2-(3,4-dimethoxyphenyl)-*N*-methyl-*m*-dithiane-2-propylamine-1,1,3,3-tetraoxide) was a gift from Professor Häusler (Basel). The optically pure enantiomers of D-600 were a gift from Professor Flohe (Aachen-Eilendorf). (±)Nimodipine (Bay e 9736) and Nifedipine were gifts from Professor Hoffmeister (Wuppertal). Optically pure d-*cis* and l-*cis*-diltiazem were from Goedecke AG (Freiburg).

Statistics: All means are presented with standard errors for *n* independent experiments. Comparison of data sets for significant differences was done with Student's two-tailed *t*-test. $p \leq 0.05$ is as usual taken to be the acceptable level of significance between 2 means.

3. RESULTS

3.1. *General properties of (±)[^{3}H]nimodipine binding in skeletal muscle*

Saturable binding sites for the labelled calcium antagonist were found in filtered homogenates from guinea-pig hind limb skeletal muscle. The binding was time and temperature dependent and fully reversible. A crude microsomal fraction was prepared from the filtered homogenate as in section 2. Compared to the starting material [^{3}H]-ouabain binding sites were enriched by a factor of 6.6 ± 1.6 (n=5), ^{125}I-α-bungarotoxin binding by a factor of 5.02 ± 1.0 (n=4) whereas specific ^{125}I-HPIA binding was not detectable, indicating the absence of contamination by fat cell membranes.

(±)[^{3}H]Nimodipine binding was enriched by a factor of 6.67 ± 0.8 (n=5). All further studies were performed with this partially purified microsomal preparation. Binding of (±)[^{3}H]nimodipine to this fraction was stimulated by d-*cis*-diltiazem (ED_{50} = 1020 ± 130 nM, n=3) but not by l-*cis*-diltiazem (fig.1). In the presence of 10 μM d-*cis*-diltiazem (±)[^{3}H]nimodipine binding was enriched 7.57 ± 1.7 (n=5)-fold relative to the homogenate in the microsomal fraction.

3.2. *Kinetics of (±)[^{3}H]nimodipine binding*

At 37°C the kinetics were rapid and binding was fully reversible upon addition of 1 μM unlabelled (±)-nimodipine. Data from an experiment which was performed in the absence and presence of d-*cis*-diltiazem (10 μM) are shown in fig.1. The dissociation rate constant (k_{-1}) in the presence of 10 μM d-*cis*-diltiazem was 0.343 min^{-1} and in the absence 1.5 min^{-1}. The K_d derived from the kinetic constants was 1.6 nM in the presence of 10 μM diltiazem. The association rate of (±)[^{3}H]-nimodipine at 37°C was too rapid to accurately compute k_{obs} in the absence of diltiazem.

3.3. *Saturation analysis of (±)[^{3}H]nimodipine binding*

To determine the density of binding sites and the equilibrium binding K_d of (±)[^{3}H]nimodipine, saturation analysis with varying ligand concentrations was performed. Fig.2 shows that in the presence of 10 μM diltiazem the K_d is essentially independent from the receptor concentration (10–90 pM) and that transformation of the data according to Scatchard yields linear plots. The B_{max}-values (density of (±)[^{3}H]nimodipine binding sites/mg microsomal protein) obtained were: 2.1 ± 0.25 pmol/mg protein (n=3) and in the presence of diltiazem (10 μM) 10 ± 2 pmol/mg protein (n=5). Clearly, d-*cis*-diltiazem increased the con-

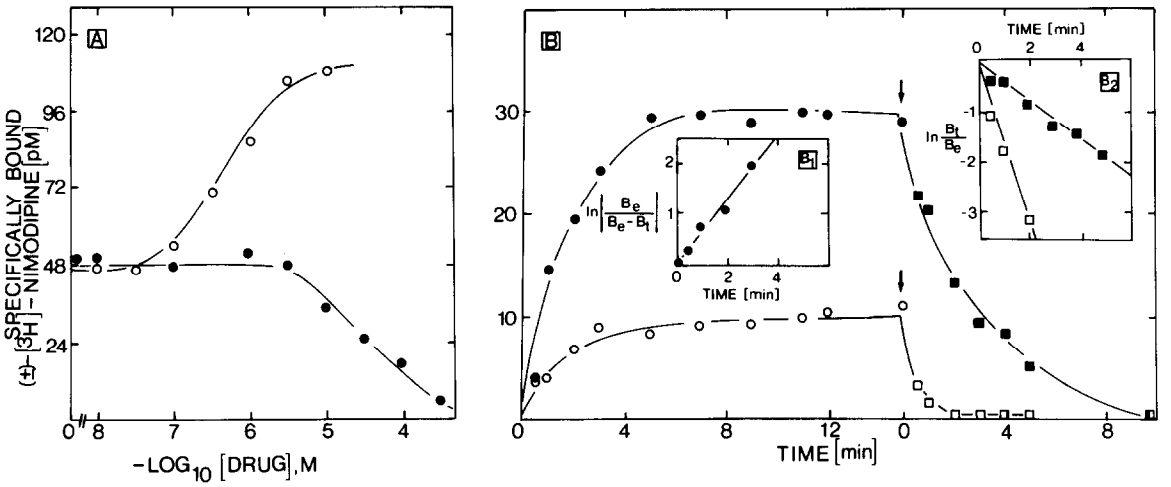

Fig.1. Stimulation of (±)[^{3}H]nimodipine binding by d-*cis*-diltiazem and kinetics of (±)[^{3}H]nimodipine binding. (A) Skeletal muscle microsomal membrane (48 μg protein/ml) were incubated for 30 min at 37°C in 0.25 ml with 1.02 nM (±)[^{3}H]nimodipine. The filled symbols (•) represent data in the presence of increasing concentrations of l-*cis*-diltiazem (IC_{50} = 35 μM). The open symbols (○) represent d-*cis*-diltiazem; the ED_{50} for stimulation was 0.79 μM. In the absence of additions the specific (±)[^{3}H]nimodipine binding was 47 pM. (B) Kinetics of (±)[^{3}H]nimodipine binding to skeletal muscle microsomes. The experiment was performed in the absence (○) and presence of 10 μM d-*cis*-diltiazem (•). Dissociation of specifically bound radioligand was induced by the addition of 1 μM unlabelled (±)-nimodipine (arrows). Insert (B_1): B_e is specifically bound (±)[^{3}H]nimodipine at equilibrium and B_t the specifically bound (±)[^{3}H]-nimodipine at a given time. K_{obs} in the presence of d-*cis*-diltiazem is 0.631 min^{-1}. In the absence of diltiazem the on reaction was too rapid to accurately compute. Insert (B_2): Dissociation of specifically bound (±)[^{3}H]nimodipine. The calculated dissociation rate constant, k_{-1} in the presence of 10 μM d-*cis*-diltiazem, was 0.343 min^{-1}, and in the absence of d-*cis*-diltiazem 1.5 min^{-1}.

centration of sites which bound (±)[^{3}H]nimodipine with high affinity ($p < 0.01$).

The K_d-value of (±)[^{3}H]nimodipine was decreased from 1.5±0.03 (n=3) to 0.99±0.15 (n=5) ($p < 0.05$) by 10 μM d-*cis*-diltiazem.

In order to get another estimate for the K_d, saturation analysis of (±)[^{3}H]nimodipine by receptors as in [16] was performed. The K_d in the presence of d-*cis*-diltiazem was 0.58 nM, in the absence 1.06 nM. The saturation analysis also gives the bindability fraction of the labelled ligand. As expected (since the ligand is a radiochemically pure racemate) the bindability was 40–50%.

3.4. *Pharmacological profile of the (±)[^{3}H]nimodipine binding sites in the skeletal muscle microsomes*

In guinea-pig brain membranes [5] and bovine sarcolemma [6] 3 distinct classes of Ca^{2+} antagonists can be discerned on the basis of their interaction with (±)[^{3}H]nimodipine binding sites. Class I Ca^{2+} antagonists (to which all 1,4-dihydropyridine derivatives we have examined, belong) compete for (±)[^{3}H]nimodipine binding with a Hill slope of 1.0. Class II Ca^{2+} antagonists are the optically pure enantiomers of verapamil and D-600 which compete with Hill slopes of < 1.0. The binding-inhibition data of class II Ca^{2+}-antagonists fit significantly better to a two site model than to a one site model [5]. Class III Ca^{2+}-antagonists stimulate (±)[^{3}H]nimodipine binding by acting as allosteric heterotropic regulators. d-*cis*-Diltiazem is the most potent class III Ca^{2+}-antagonist (review [13]).

In guinea-pig skeletal muscle microsomes nifedipine had an IC_{50} of 240 nM (n=2). Table 1 summarizes the data obtained from binding-inhibition experiments with various unlabelled calcium antagonists, performed in the absence and presence of 10 μM d-*cis*-diltiazem.

The 1,4-dihydropyridine enantiomers of (±)PN 200-110, (+)PN 205-033 and (–)PN 205–034 exhibit a eudismic ratio of 94 in the absence of d-*cis*-diltiazem, and of 125 in the presence of 10 μM d-*cis*-diltiazem. The pharmacologically more po-

Table 1

Binding-inhibition constants of (±)[^{3}H]nimodipine binding by various Ca^{2+}-antagonists

Drug	Control			With 10 μM d-*cis*-diltiazem present			
	IC_{50} (nM)	n_H	n	IC_{50} (nM)	n_H	n	shift factor
(+)PN 205-033	2.2± 0.55	1.04±0.08	3	3.5± 1.6	1.09±0.05	3	1.6
(−)PN 205-034	206 ± 42[e]	0.89±0.07	3	333 ± 88[e]	1.18±0.06	3	1.6
(±)Fendiline	540 ±125	0.98±0.10	3	1209 ± 205[a]	1.23±0.12	3	2.2
Tiapamil	292 ± 74	1.08±0.15	3	12 230 ±4260[b]	0.89±0.09	3	42.0
(+)D-600	1002 ±267	0.78±0.04	4	5745 ± 764[d]	0.95±0.14	3	5.7
(−)D-600	$>10^7$	–	3	1410 ± 309[c]	0.90±0.14	3	–
La^{3+}	200×10^3	1.3	2	450×10^3	1.2	2	1.2
Tetrodotoxin	$>10^7$	–	2	$>10^7$	–	2	–

Significance: [a] $p<0.10$ with respect to control; [b] $p<0.025$ with respect to control; [c] $p<0.01$ with respect to (+)D-600; [d] $p<0.001$ with respect to control; [e] $p<0.01$ with respect to (+)PN 205-033

Average data with standard errors from n separate experiments, each performed in duplicate with 5–10 concentrations of unlabelled drugs. n_H is the Hill slope and IC_{50} the concentration of drug causing 50% inhibition of specific (±)[^{3}H]nimodipine binding, as calculated from linear regression analysis of data transformed into the Hill equation. Shift factor is the ratio of IC_{50} in the presence 10 μM d-*cis*diltiazem: IC_{50} in the control experiment. The microsomal protein concentration was between 10–25 μg/ml and the concentration of (±)[^{3}H]nimodipine between 0.8–1.5 nM Examples of binding-inhibition experiments are shown in fig.3

tent (+)PN 205–033 is the eutomer under both experimental conditions.

(±)Fendiline becomes a weaker competitor of (±)[^{3}H]nimodipine binding in the presence of 10 μM d-*cis*-diltiazem, the IC_{50} value being shifted by a factor of 2.2-fold ($p<0.1$). Tiapamil exhibits an even greater d-*cis*-diltiazem-induced shift of the IC_{50} value, namely of 42-fold ($p<0.025$).

The optically pure enantiomers of D-600 competed in a stereoselective manner with specific (±)[^{3}H]nimodipine binding. (+)D-600 behaved as (±)fendiline and tiapamil becoming a weaker inhibitor of (±)[^{3}H]nimodipine binding in the presence of 10 μM d-*cis*-diltiazem ($p<0.001$). Remarkably, (−)D-600 only inhibited labelled calcium antagonist binding stimulated by d-*cis*-diltiazem, and then incompletely to the level of control binding in the absence of d-*cis*-diltiazem (fig.3). Specific (±)[^{3}H]nimodipine binding was inhibited by La^{3+} in the presence and absence of d-*cis*-diltiazem with IC_{50}-values of 210 μM and 460 μM, respectively. However, the divalent cations Ca^{2+} and Mg^{2+} at up to 10 mM and the monovalent cation Na^+ at up to 100 mM had no effect on (±)[^{3}H]nimodipine binding.

4. DISCUSSION

Our results demonstrate that the potent 1,4-dihydropyridine calcium antagonist nimodipine [14] when radiolabelled binds in a time-dependent, fully reversible manner to crude skeletal muscle microsomes. Equilibrium binding experiments reveal that (±)[^{3}H]nimodipine binding is of high affinity ($K_d=1-1.5$ nM) and to an apparently homogeneous population of receptors.

The binding site was stereoselective for the optically pure enantiomers of the 1,4-dihydropyridine calcium antagonist (±)PN 200–110. 40–50% of (±)[^{3}H]nimodipine binds to an excess of empty receptor sites although the radiolabel is of >95% radiochemical purity. This is most likely because only the more potent 4 (*S*) enantiomer (which in biological test systems is the eutomer [15]) is able to interact with the binding site with high affinity.

As is expected, ligand saturation by receptors gave the true K_d of the bindable enantiomer for

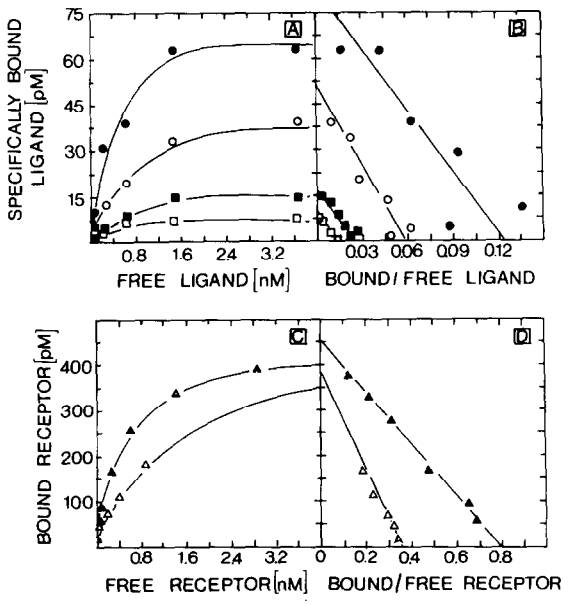

Fig.2. Saturation analysis of equilibrium binding. (A) Skeletal muscle microsomal membranes at 7.2 μg/ml (•), 2.9 μg/ml (○), 1.4 μg/ml (▪) and 0.7 μg/ml (□) protein was incubated with 0.06–3.6 nM (±)[^{3}H]nimodipine at 37°C in 50 mM Tris–HCl (pH 7.4) with 10 μM d-*cis*-diltiazem in 1.0 ml for 45 min. At 7.2 μg protein/ml the non-specific binding was linear with respect to (±)[^{3}H]nimodipine concentration (r=0.999) with a slope of 2.84 pM/nM radioligand. The bound axis intercept was 0.2 pM (=40 cpm, which is the counter background radioactivity). (B) Hofstee transformation of the data shown in (A). The symbols correspond to those in (A). The K_d and B_{max} of (±)[^{3}H]nimodipine, calculated by linear regression analysis, were: at 7.2 μg membrane protein/ml, 694 pM, 11.94 pmol/mg; at 2.9 μg membrane protein/ml, 898 pM, 17.1 pmol/mg; at 1.4 μg membrane protein/ml, 602 pM, 10.93 pmol/mg; and at 0.7 g of membrane protein/ml, 758 pM, 14.3 pmol/mg. (C) Plot of specifically bound (±)[^{3}H]nimodipine against the free receptor concentration in the presence (•) and absence (○) of 10 μM d-*cis*-diltiazem. The experiment was performed at 37°C in 0.25 ml with 1.05 nM total (±)[^{3}H]nimodipine, allowing 30 min to reach equilibrium prior to the separation of bound and free radioligand. The highest protein concentration was 440 μg/ml and the lowest 16 μg/ml. (D) Hofstee plot of the data in (C). In the presence of 10 μM d-*cis*-diltiazem the K_d of the receptor for the bindable fraction of radioligand (calculated by linear regression analysis) is 580 pM whereas in the absence of d-*cis*-diltiazem the K_d of the receptor for the bindable (±)[^{3}H]nimodipine is 1060 pM.

the racemic radioligand. This value is ~½ of the K_d derived from conventional receptor saturation experiments.

d-*cis*-Diltiazem, but not l-*cis*-diltiazem stimulates (±)[^{3}H]nimodipine binding to guinea-pig brain membranes [5]. l-*cis*-Diltiazem is the biologically inactive enantiomer with respect to Ca^{2+} antagonism [17]. In guinea-pig brain membranes d-*cis*-diltiazem stimulates (±)[^{3}H]nimodipine binding by decreasing the K_d 3-fold, due to a decrease in the k_{-1} of the (±)[^{3}H]nimodipine receptor complex. No increase in B_{max} is observed [5]. However, in skeletal muscle microsomes d-*cis*-diltiazem stimulates (±)[^{3}H]nimodipine binding by reducing the K_d of a sub-population of channels from >50 nM to ~1 nM with respect to the bindable enantiomer of (±)[^{3}H]nimodipine. We observe an increase in B_{max} of (±)[^{3}H]nimodipine binding sites with a K_d of 1 nM. As in guinea-pig brain membranes stimulation of (±)[^{3}H]nimodipine is only seen with biologically active Ca^{2+}-antagonistic enantiomer of diltiazem. l-*cis*-Diltiazem only inhibits (±)[^{3}H]nimodipine binding with an IC_{50} of 40±18 μM (n=3).

This stereoselectivity of the binding site underlines its physiological and biochemical significance. Further evidence that the binding of (±)[^{3}H]nimodipine is to biologically relevant Ca^{2+} channels came from binding-inhibition experiments.

In [18] racemic D-600, nifedipine and inorganic Ca^{2+}-antagonists blocked Ca^{2+} channels in skeletal muscle. Appropriately we found that the inorganic Ca^{2+}-antagonist La^{3+}, nifedipine and the optically pure enantiomers of D-600 exhibited affinity for the (±)[^{3}H]nimodipine binding sites. Remarkably (−)D-600 only inhibited (±)[^{3}H]nimodipine binding stimulated by 10 μM d-*cis*-diltiazem, whereas (+)D-600 was inhibitory under both experimental conditions, losing binding-inhibition potency by 5-fold (p<0.001) in the presence of 10 μM d-*cis*-diltiazem.

The inhibition curves of (+)D-600 exhibited Hill slopes of <1 in the absence of d-*cis*-diltiazem, suggesting heterogeneity of the binding sites on the putative Ca^{2+} channels. Tiapamil lost binding inhibition potency in the presence of 10 μM d-*cis*-diltiazem by 42-fold and exhibited a Hill slope of <1 under this experimental condition.

The specific binding of (±)[^{3}H]nimodipine is

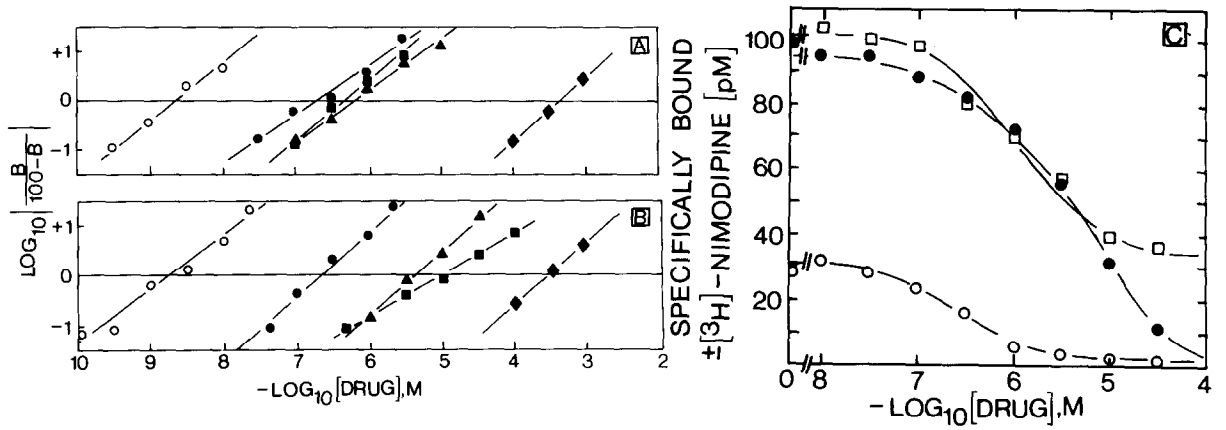

Fig.3. Inhibition of saturable (±)[^{3}H]nimodipine binding in skeletal muscle microsomes by Ca^{2+}-antagonists. All experiments were incubated for 30–40 min at 37°C with 1–2 nM (±)[^{3}H]nimodipine, 16–25 μg skeletal muscle microsomal protein/ml and unlabelled Ca^{2+}-antagonists present as indicated. (A) Binding-inhibition data in the absence of d-*cis*-diltiazem transformed into Hill plots. *B* is % inhibition at a given concentration of unlabelled Ca^{2+}-antagonist. Representive experiments are shown. The IC_{50} and Hill slopes computed by linear regression analysis for the individual experiments are: (○) (+)PN 205-033, 2.1 nM, 1.13; (●) (−)PN 205-034, 200 nM, 1.04; (■) Tiapamil, 300 nM, 1.2; (▲) (±)Fendiline, 600 nM, 1.18; (*) La^{3+}, 250 μM, 1.16. (B) As for (A) except that the experiments were performed in the presence of 10 μM d-*cis*-diltiazem. The IC_{50} values and Hill slopes are: (○) (+)PN 205-033, 1.7 nM, 1.01; (●) (−)PN 205-034, 200 nM, 1.17; (▲) (±)Fendiline, 1400 nM, 0.991; (■) Tiapamil, 10 500 nM, 0.760; (*) La^{3+}, 464 μM, 1.21. (C) Direct plot of specifically bound (±)[^{3}H]nimodipine in the absence of d-*cis*-diltiazem (●) and presence of d-*cis*-diltiazem at 10 μM (○, □). The 3 binding-inhibition curves shown were run in parallel at 1.16 nM (±)[^{3}H]nimodipine and 34 μg protein/ml: (●) (+)D-600 in the absence of d-*cis*-diltiazem; IC_{50} = 700 nM, n_H = 0.748; (○) (+)D-600 in the presence of d-*cis*-diltiazem; IC_{50} = 5750 nM, n_H = 1.06. The IC_{50} of (−)D-600 in the presence of d-*cis*-diltiazem (□) was calculated by subtracting control binding in the absence of d-*cis*-diltiazem as blank. The IC_{50} for (−)D-600 in this experiment is 880 nM and n_H = 1.19.

enriched in a microsomal fraction of skeletal muscle, as do other accepted plasma membrane markers. Evidence suggesting that the Ca^{2+} current in skeletal muscle originates within the transverse tubular system was presented in [18]. We have shown that the binding sites are located on particles of low buoyant density, can be separated from elements of the sarcoplasmic reticulum, and copurify with [^{3}H]ouabain binding, an accepted marker for transverse tubules in skeletal muscle [19].

We have identified high-affinity binding sites for a labelled 1,4-dihydropyridine calcium antagonist in skeletal muscle microsomes. Stereoselective inhibition by 1,4-dihydropyridine derivatives, (−)D-600 and (+)D-600, and allosteric heterotropic regulation by the biologically potent enantiomer of diltiazem suggest that the putative Ca^{2+} channels in skeletal muscle can now be studied with biochemical techniques.

ACKNOWLEDGEMENTS

This work was supported by a grant from the DFG to H.G. We are grateful to Cornelia Auriga, Barbara Habermann and Inge Seidel for technical assistance and Drs Hoffmeister, Kazda and Meyer of the Bayer company for their generous support.

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