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Dihydropyridine receptor in rat brain labeled with [^{3}H]nimodipine*

(calcium antagonists/binding site/structural specificity/stereoselectivity/cerebrovascular diseases)

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ABSTRACT **Receptor binding sites for 1,4-dihydropyridine (DHP) calcium antagonists have been characterized by using [^{3}H]-nimodipine, a potent analogue of nifedipine with cerebrovascular and neuro- and psychopharmacological properties. [^{3}H]Nimodipine exhibited reversible and saturable binding to partially purified brain membranes. The equilibrium dissociation constant, K_d, was 1.11 nM and the maximal binding capacity, B_{max}, was 0.50 pmol/mg of protein. The DHP receptor proved to be highly specific for various potently displacing DHP derivatives and discriminated between their optical isomers (stereoselectivity) with inhibition constants (K_i) in the nanomolar or even subnanomolar range. Structurally different calcium antagonists such as gallopamil (D-600), on the other hand, displayed much lower affinities, further substantiating the specificity of the receptor for DHP structures. Furthermore, the displacement potency of a series of DHP derivatives correlated well with that determined for inhibition of mechanical response in the intact smooth muscle over 5 orders of magnitude. [^{3}H]Nimodipine binding thus may provide a molecular probe to elucidate the nature of the interaction of calcium entry blockers with specific membrane-located receptor sites that may be associated with the putative calcium channel. These receptor sites might well represent the loci of signaling events where the potent DHPs exert their pharmacological action.**

Transmembrane fluxes of cations or nutrients—e.g., K^+ or amino acids (1)—are known to cause intracellular reactions of enormous potency. Ca^{2+} plays important multiple roles in various regulatory and signaling processes in cellular activity, and it is generally accepted that Ca^{2+} influxes can be either enhanced by various cardiostimulatory drugs—e.g., epinephrine (2) or theophylline (for further refs. see ref. 3)—or inhibited by calcium entry blockers. Ca^{2+} enter the cell via proposed calcium channels that are mainly controlled by transmembrane signals of either chemical or electrical origin. Drugs that block the calcium entry have been termed "calcium antagonists" (4) and are of considerable potential in the therapy of angina pectoris, hypertension, and many cardiovascular disorders (for further refs. see ref. 5). They belong to a pharmacologically potent group of compounds whose mechanism of action is postulated to inhibit the slow inward current of Ca^{2+} in several tissues (4, 6), particularly in the cardiac (7), peripheral (8), and cerebral (9, 10) smooth muscle. Examples of these structurally heterogeneous drugs are verapamil, diltiazem, and the dihydropyridines (11), of which nifedipine is increasingly being used as a tool to investigate the properties of the proposed calcium channels.

In 1981, a high-affinity binding site for dihydropyridines (DHPs) was identified in cardiac membranes (12) and later was found also in other tissues (13, 14). The present report demonstrates characteristics of a receptor for DHP calcium antagonists in brain membranes by use of ^{3}H-labeled nimodipine, a potent analogue of nifedipine with cerebrovascular and neuro- and psychopharmacological actions (15, 16).

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MATERIALS AND METHODS

Materials. [^{3}H]Nimodipine (New England Nuclear) had a specific activity of 160–180 Ci/mmol (1 Ci = 3.7×10^{10} Bq) and its purity was continuously monitored by thin-layer radiochromatography. The ligand was stored light-protected (−30°C) under nitrogen gas to prevent radiolysis and oxidation.

The DHP derivatives nifedipine, nimodipine, niludipine, nisoldipine, nitrendipine, and BAY E 6927, the stereoisomers (Bayer AG, Wuppertal, Federal Republic of Germany), and calcium entry blockers or vasodilators without DHP structure were dissolved first in dimethyl sulfoxide to make 10 mM stock solutions, and then diluted to the appropriate concentrations with 50 mM Tris·HCl at pH 7.4; the concentration of dimethyl sulfoxide in the assay never exceeded 4% at a final DHP concentration of 10 μM. All other chemicals used were of the highest grade commercially available.

Methods. Adult male Wistar rats (250–280 g; Lippische Versuchstierzucht, Extertal, Federal Republic of Germany) were killed by cervical dislocation. The brains were rapidly removed, dissected into distinct anatomical regions, and gently homogenized (Potter–Elvehjem homogenizer) in 10 vol of ice-cold 0.32 M sucrose supplemented with 1 mM phenylmethanesulfonyl fluoride prior to homogenization for 10 sec in the same medium with an Ultra-Turrax (Janke & Kunkel, Staufen i. Br., Federal Republic of Germany). The homogenate was centrifuged (4°C) at 1,000 × *g* for 10 min, the supernatant was then recentrifuged at 40,000 × *g* (20 min), and the pellet was washed three times with ice-cold 50 mM Tris·HCl at pH 7.4. The membrane fraction was stored under liquid nitrogen at −196°C.

Binding assays were performed essentially as described (12) under strict sodium light to prevent breakdown of DHPs which can occur at shorter wavelengths. In short, in a final volume of 0.25 ml, membrane protein (50–80 μg per assay) was incubated at 37°C in 50 mM Tris·HCl, pH 7.4/150 mM NaCl/1 mM $CaCl_2$ containing the indicated concentrations of radioligand and various additives—e.g., calcium antagonists as presented in Table 1. After the indicated time intervals, the reaction was terminated by dilution with 3.5 ml of ice-cold Tris·HCl at pH 7.4. Particle-bound and free ^{3}H-labeled ligand were then separated by rapid vacuum filtration through GF/C glass fiber filters (Whatman), and the precipitate was washed twice (3.5 ml) with ice-cold Tris·HCl at pH 7.4.

Abbreviations: DHP, 1,4-dihydropyridine; NE, norepinephrine.

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Nonspecific binding was determined by addition of 10 μM unlabeled nimodipine and was subtracted from the total binding to yield what will be called "specific binding." For saturation kinetics [^{3}H]nimodipine concentration was varied between 0.11 and 6.25 nM, the binding experiments (displacement) were performed with 1–1.5 nM radioligand; equilibrium was reached after 30 min at 37°C (see below). Assays were performed in duplicate or triplicate with at least three different protein preparations. Protein was measured by using Bradford's method (17) with bovine serum albumin as the standard.

Data Analysis. Bound [^{3}H]nimodipine was measured by conventional liquid scintillation spectrophotometry. Data were calculated and plotted according to Scatchard (18), and displacement experiments were analyzed with computer programs.

Pharmacological Experiments. The pharmacological activity of the DHPs was examined by using the norepinephrine (NE)- or K^+-stimulated isolated rabbit aortic strip preparation (for details see refs. 16 and 19).

RESULTS

[^{3}H]Nimodipine bound reversibly and saturably to membrane fractions from various organs of the rat. Brain, heart, ileum, liver, kidney, and several endocrine organs contained binding sites that interact with labeled DHP calcium antagonists. Rat brain membranes were chosen to evaluate the binding characteristics of the centrally active nimodipine.

[^{3}H]Nimodipine has been shown by thin-layer chromatography to remain stable under all experimental conditions reported here. Specific binding of 1.5 nM [^{3}H]nimodipine to cortical membranes was linearly proportional to concentration of protein in the binding assay up to 2 mg/ml, and thus all binding studies were carried out in the linear range. Denaturation of the membranes by boiling elicited heat sensitivity of the receptor site: upon heating membranes to 65°C for 10 min, virtually all specific binding was lost. The amount of nonspecific binding, defined as binding in the presence of excess unlabeled ligand, was quite low. Addition of 10 μM nimodipine to the binding assays displaced 85–93% of the total [^{3}H]nimodipine binding. Binding of the radioligand to glass fiber filters ("filter blank") was negligible.

The time taken for the specific binding of [^{3}H]nimodipine to cortical membranes rapidly leveled out and showed maximal binding after about 8 min. The addition of unlabeled nimodipine resulted in a monophasic dissociation of [^{3}H]nimodipine (k_{-1}, the dissociation rate constant, was 0.0989 min^{-1}, $r = 0.956$). The time course of association yielded a rate constant k_{obs} (20) of 0.4732 min^{-1} ($r = 0.952$). The kinetic dissociation constant (K_d) was determined to be 0.4 nM from the measured association and dissociation rates (Fig. 1) and, to a great extent, agrees with the equilibrium binding data.

The specific [^{3}H]nimodipine binding was saturable and steady-state levels were achieved between 3 and 4 nM radioligand (Fig. 2). Scatchard analyses of the saturation isotherm (Fig. 2, *Inset*) revealed a single straight line in the concentration range <6 nM, indicating the presence of a single binding site. The equilibrium dissociation constant K_d was 1.11 ± 0.15 nM ($r > 0.95$; $n = 8$), with the total number of binding sites (density), B_{max}, equivalent to 0.50 ± 0.12 pmol/mg of protein ($r > 0.95$; $n = 8$). Hill plots of the [^{3}H]nimodipine saturation isotherm (21) gave a K_d of 1.04 nM ($r = 0.98$) and slopes of 0.91–1.02 ($r = 0.958$–0.996; $n = 8$), indicating absence of cooperativity.

Distribution of DHP receptor in seven rat brain regions demonstrated only one population of binding sites in each brain area tested, with no major regional differences in the dissociation constant and density: cortex, K_d = 1.11 nM, B_{max} = 0.50 pmol/mg of protein; cerebellum, 1.17, 0.18; mesencephalon, 1.08, 0.16; hypothalamus, 0.69, 0.26; hippocampus, 0.60, 0.54; septum/basal ganglia, 0.76, 0.36; pons/oblongata, 1,86, 0.16 (n = 6–8 experiments; $r > 0.95$).

The high selectivity of the DHP receptor is apparent in the lack of effect of nimodipine on other receptors in brain and heart membranes. Interaction of nimodipine, nifedipine, and certain other DHP derivatives with 10 different receptors—muscarinic cholinergic-, α_1-, α_2-, and β-adrenergic, benzodiazepine, dopamine, γ-aminobutyric acid, histamine, opiate, and serotonin receptors—revealed low displacement activities—e.g., for α_1-adrenergic receptor ([^{3}H]prazosin) versus nimodipine, IC_{50} = 5 μM; or for opiate receptor ([^{3}H]naloxone) versus nifedipine, IC_{50} = 9.9 μM.

In addition, the high specificity of the receptor for DHP structures was well confirmed, and binding sites discriminated between optical isomers. Several pharmacologically active DHP analogues potently displaced [^{3}H]nimodipine binding with inhibition constants (K_i) in the nanomolar or even subnanomolar

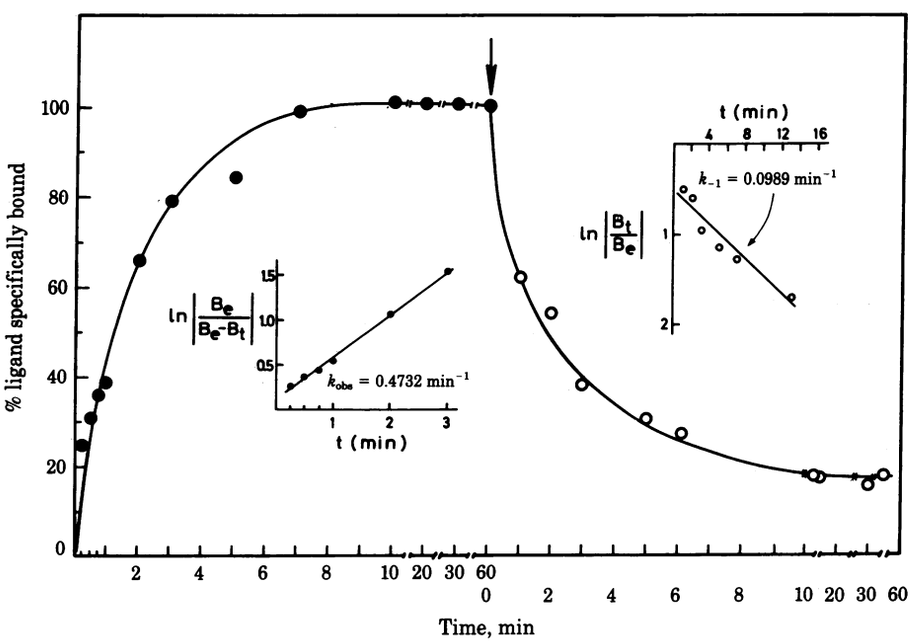

FIG. 1. Time course of [^{3}H]nimodipine specific binding demonstrating saturability and reversibility in pulse–chase experiments. The figure shows a representative experiment carried out with partially purified membranes from rat cortex at 37°C in 50 mM Tris·HCl, pH 7.4/150 mM NaCl/1 mM $CaCl_2$. The association reaction (●) had a half-life of approximately 1.6 min, and the dissociation reaction (arrow) had a half-life of 1.7 min after addition of excess (10 μM) unlabeled nimodipine (○). From the time course of association k_{+1} values were estimated by the k_{obs} method (20); the kinetic K_d value was then calculated by $K_d = k_{-1}/k_{+1}$. (*Insets*) Linear transformation of the data and the kinetic constants (correlation coefficient $r > 0.95$).

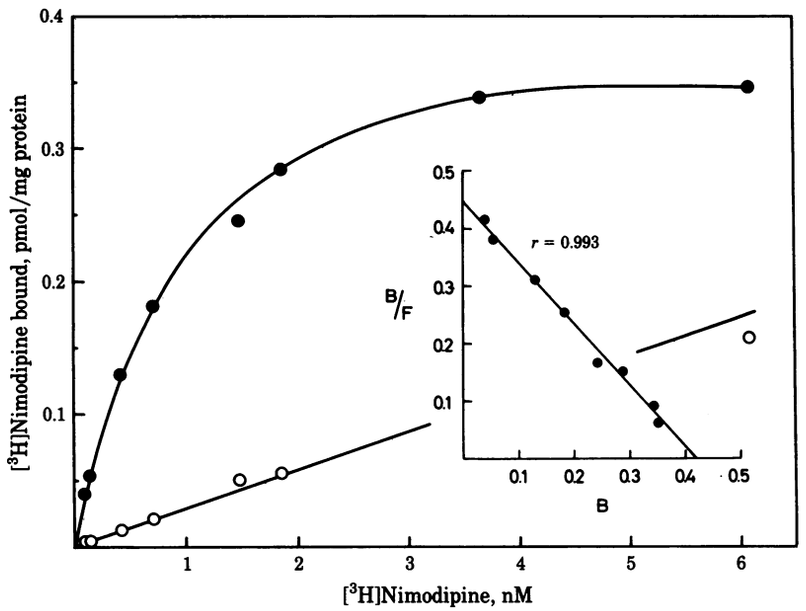

FIG. 2. Saturation isotherm of [^{3}H]nimodipine binding to partially purified membranes of rat cortex. [^{3}H]Nimodipine, 0.11–6.25 nM, was incubated in triplicate with or without excess unlabeled nimodipine. Specific binding (●), defined as conditions under which nonspecific binding remains linear, was calculated as the difference between total binding and that not displaced by excess (10 μM) nimodipine (○). (*Inset*) Scatchard analysis of the specific binding data (B, bound ligand; F, free ligand) revealed linearity and indicated one binding site with an equilibrium dissociation constant K_d = 0.94 nM (reciprocal of the slope value) and maximal binding (B_{max}) of 0.42 pmol/mg of protein (r = 0.993; intercept on abscissa). The experiment was repeated several times with different protein preparations and yielded similar results (r = 0.95–0.993).

range (Table 1). On the other hand, structurally different calcium antagonists or vasodilators (e.g., bencyclane, bepridil, cinnarizine, D-600, fendiline, perhexiline, or suloctidil) were much less potent (Fig. 3) and exhibited displacement only at concentrations >0.1 μM (Table 1). Diltiazem in low concentrations did not displace [^{3}H]nimodipine but, in contrast, appeared to increase the amount of [^{3}H]-labeled ligand bound to the receptor.

The stereoselectivity of the receptor, an additional criterion of specificity of action, was demonstrated with the enantiomers of nimodipine (22), nitrendipine (21, 23), and BAY E 6927 (Table 1). In each case—e.g., BAY E 6927 (Fig. 4)—the (−) stereoisomer displayed much higher affinity for [^{3}H]nimodipine binding than did the racemate which in turn was more potent than the (+) isomer. It was of interest to note that the difference in displacement potency between the enantiomers was most prominent between (−) and (+) BAY E 6927 (≈300-fold). Differences in IC_{50} values between (−) and (+) stereoisomers of nitrendipine (10- to 12-fold), and nimodipine (3- to 4-fold) were less pronounced, suggesting that the (−) enantiomer of the racemic radioligand actually contributes to the DHP specific action.

Saturability, reversibility, and pronounced selective displacement activities as reported above are attributes of binding properties that ought to correlate with pharmacological data. Excellent correlation (r = 0.954) over 5 orders of magnitude was achieved between [^{3}H]nimodipine displacement potency of various DHP derivatives, including the enantiomers, and their inhibitory effects of K^+-stimulated contraction of rabbit aortic strips (Fig. 5), or Ba^{2+}-induced contraction of guinea pig ileum (24). The DHP derivatives tested exhibited no inhibitory effects on the contraction of the isolated rabbit aortic strips induced by NE. However, their specific inhibition of K^+-depolarization-induced contraction is characteristic of calcium antagonists (19, 25) and thus indicates interaction with the potential sensitive calcium channels.

DISCUSSION

The neuro- and psychopharmacologically active DHP (15) nimodipine has been demonstrated to bind with high affinity to a limited number of binding sites in brain that seem to be the loci where the potent DHP calcium antagonists exert their

Table 1. Inhibition of [^{3}H]nimodipine specific binding to rat cortical membranes by various pharmacologically potent calcium antagonists and vasodilators with and without DHP structure

Compound	K_i, nM
With DHP structure:	
(±)-BAY E 6927	0.22
BAY K 5552 (nisoldipine)	0.24
BAY A 7168 (niludipine)	0.33
(±)-BAY E 5009 (nitrendipine)	0.93
(±)-BAY E 9736 (nimodipine)	1.44
BAY A 1040 (nifedipine)	7.0
BAY K 7721	73
BAY M 5579	800
Enantiomers:	
(−)-BAY E 9736	1.04
(+)-BAY E 9736	2.4
(−)-BAY E 5009	0.43
(+)-BAY E 5009	8.8
(−)-BAY E 6927	0.09
(+)-BAY E 6927	27.5
Without DHP structure:	
Gallopamil (D-600, Knoll, Fed. Rep. of Germany)	400
Cinnarizine (Janssen, Belgium)	750
Prenylamine (Hoechst AG, Fed. Rep. of Germany)	975
Flunarizine (Janssen, Belgium)	1,030
Tiapamil (Hoffmann–La Roche, Switzerland)	1,300
Suloctidil (Searle, Fed. Rep. of Germany)	2,170
Fendiline (Thiemann, Fed. Rep. of Germany)	2,450
Tinofedrine (Chemiewerke Homburg, Fed. Rep. of Germany)	7,500
Perhexiline (Merell, Fed. Rep. of Germany)	10,000
SG 75 (Chugai, Japan)	10,000
Bepridil (Organon, Fed. Rep. of Germany)	15,000
Mixidine (McNeil, Ft. Washington, PA)	20,000
P 1134 (Leo, Denmark)	25,000
Bencyclane (Thiemann, Fed. Rep. of Germany)	72,000
AQA-39 (Thomae, Fed. Rep. of Germany)	98,000

Values given are the means from four to eight experiments (in triplicate) with at least three different protein preparations for displacements. The chemical structures are given in the references cited in the text. $K_i = IC_{50}/(1 + LC/K_d)$, in which LC is ligand concentration, and IC_{50} is concentration causing 50% inhibition of [^{3}H]nimodipine specific binding.

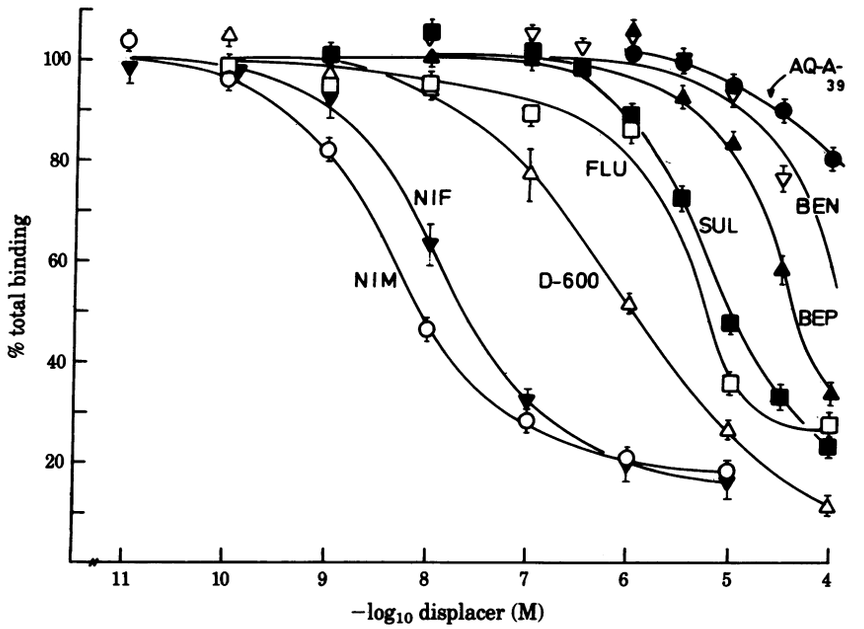

FIG. 3. Displacement experiments using [^{3}H]nimodipine (<1.5 nM) and various pharmacologically potent calcium antagonists and vasodilators with or without DHP structure. Total binding is plotted against $-\log_{10}$ of displacer concentration (M). With DHP structure: ○, NIM, nimodipine; ▼, NIF, nifedipine. Without DHP structure: △, gallopamil, D-600; □, FLU, flunarizine; ■, SUL, suloctidil; ▲, BEP, bepridil; ▽, BEN, bencyclane; ●, AQA-39. The results demonstrate the high specificity of the binding sites for DHP derivatives. Data are the mean ± SEM of three to eight experiments using at least three different protein preparations.

pharmacological action. Reversibility and high structural specificity were found to be characteristics of these binding sites because various pharmacologically active DHP derivatives potently displaced [^{3}H]nimodipine binding with K_i values in the nanomolar or even subnanomolar range. According to the results of the binding experiments shown, potent DHP analogues might also typically be the most active inhibitors of excitation-contraction coupling. DHP derivatives of weaker affinity—e.g., BAY K 7721 or BAY M 5579 (Table 1)—also exhibited less-potent calcium antagonistic action (Fig. 5). In contrast to the high potencies of the DHP members, the structurally dissimilar but pharmacologically effective calcium antagonists or vasodilators D-600 and cinnarizine analogues, bencyclane, fendiline, and others elicited weak displacement, presumably caused by an antagonism of different origin in that they may act preferentially via voltage dependency (4, 26–28) ("potential-operated site"); this further substantiates the specificity of the DHP receptor site for the dihydropyridine structure ("receptor-operated site").

Modulation of [^{3}H]nimodipine binding by mono-, bi-, and trivalent cations (22) generally agrees with electrophysiological findings (29, 30); it seems to be related to the ionic crystal radii (31) whereas tissue specificity may correspond to effects of anions and ionic strength (32).

The high specificity of the binding sites for DHPs is further substantiated by the stereoselectivity. The introduction of nonidentical ester groups in the 3,5 positions of the heterocyclic ring (23) makes the molecule chiral. The (−) isomers of nimodipine, nitrendipine, and BAY E 6927 generally displayed greater affinities than the racemates, or the even less-potent (+) enantiomers (Table 1). Results presented here correspond well with those found for smooth muscle contraction in the intact preparation (6, 24). The differences in potencies between the individual enantiomers are more pronounced with compounds containing both methyl and isopropyl ester groups. They are likely to be sterically "more" unsymmetric than the isomers of nitrendipine or nimodipine which have ethyl and methyl, or isopropyl and methoxyethyl groups, respectively, in their chemical structure (23, 24).

It is of interest that in all cases the displacement data and curves of the racemic nimodipine (22), nitrendipine (21, 23),

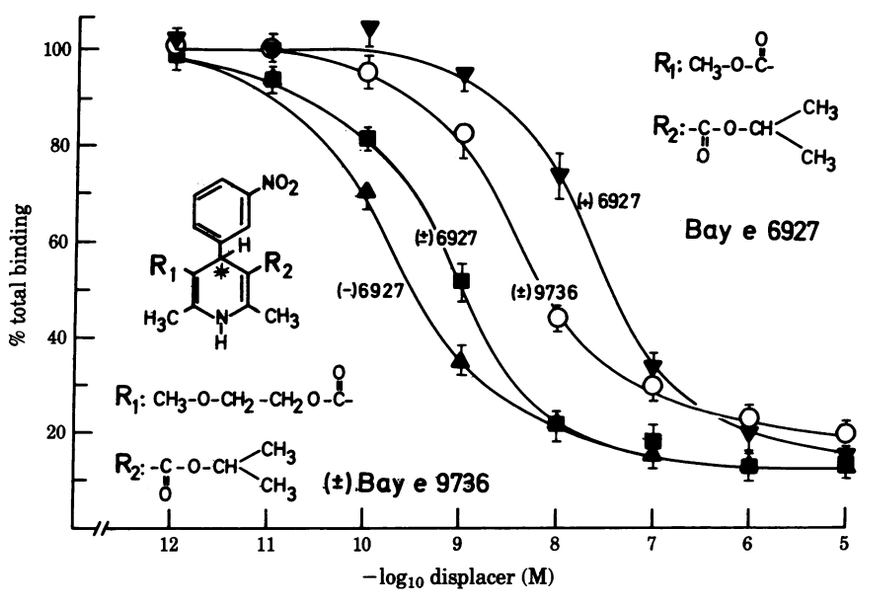

FIG. 4. Displacement experiments using [^{3}H]nimodipine (<1.5 nM) and the enantiomers of BAY E 6927. Total binding is plotted against $-\log_{10}$ of displacer concentration (M). ○, (±)-BAY E 9736, nimodipine; ▲, (−)-BAY E 6927; ■, (±)-BAY E 6927; ▼, (+)-BAY E 6927. Structures of DHP derivatives are shown. The optically active center of the molecule is marked by an asterisk. Data substantiating stereoselectivity are the means ± SEM of five to nine experiments using different protein preparations.

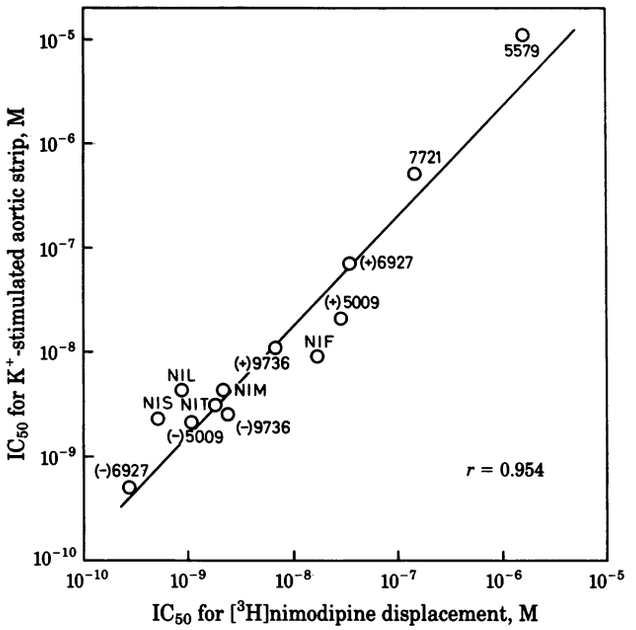

FIG. 5. Correlation between IC_{50} values for [^{3}H]nimodipine displacement (binding data) by various DHP derivatives (from Table 1) and inhibition of K^+-stimulated contraction of strips of rabbit aorta (functional response). The best curve fit was assessed by linear regression analysis ($r = 0.954$; $P < 0.005$). Numbers on graph indicate Bayer code numbers. NIM, nimodipine [(±)-BAY E 9736]; NIT, nitrendipine [(±)-BAY E 5009]; NIL, niludipine; NIS, nisoldipine; NIF, nifedipine.

and BAY E 6927 (Fig. 4) is shifted toward the inhibitory characteristics (K_i) of the (−) enantiomers. These properties may either indicate an uneven distribution of the (−) and (+) isomer in the racemic compound or suggest that the protein structure of the receptor contains a pharmacophore group that recognizes preferentially the (−) isomer. Thus, in binding studies with racemic [^{3}H]nimodipine it seems reasonable to assume that the (−) enantiomer is the major determinant of the DHP specific activity.

Reversibility, saturability, and marked stereoselectivity are attributes of binding properties of [^{3}H]nimodipine that may not even occur at meaningful recognition sites, and thus the binding data should correspond to the pharmacological response in order to be able to term the binding site for DHPs a "receptor." Indeed, the binding characteristics of the DHP receptor presented here correlate well with the pharmacological activities in vascular smooth muscle (Fig. 5), substantiating that the DHP receptor is physiologically significant and seems to mediate the pharmacological action of the DHP calcium antagonists.

As reported for some individual compounds the DHP derivatives tested inhibited only the K^+-induced aortic contractions, not those induced by NE (16, 19, 33). This characteristic behavior has been reported frequently for calcium antagonists and is regarded as demonstrating that the calcium antagonists act by inhibiting calcium influx through potential sensitive calcium channels (see refs. 34 and 35). Several authors have proposed other mechanisms for the vasodilator action of "calcium antagonists,"—e.g., inhibition of phosphodiesterase (36) or interaction with calmodulin (37)—but these latter mechanisms would also affect the NE-induced contractions (37).

In addition, we have found that some of the DHP derivatives tested competitively inhibit contractions of the depolarized aorta induced by addition of Ca^{2+} (unpublished data). The potent pharmacological activity of the DHP derivatives reported here therefore may be ascribed to inhibition of influx of Ca^{2+} through voltage-sensitive calcium channels.

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