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ENHANCEMENT OF BINDING OF THE DIHYDROPYRIDINE CALCIUM ANTAGONIST PN200-110 TO HUMAN MYOMETRIAL SARCOLEMMA BY THE HETEROLOGOUS CALCIUM ANTAGONIST DILTIAZEM

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Abstract—Binding of the dihydropyridine calcium antagonist PN200-110 was studied in human myometrial membranes. PN200-110 bound reversibly and with high affinity to membrane fragments. The highest concentration of binding sites was found in the sarcolemma. The benzothiazepine calcium antagonist diltiazem stimulated PN200-110 binding by increasing the amount bound at equilibrium. Kinetic studies detected a fast and slow rate of dissociation in the presence of diltiazem.

Ca^{2+} channel antagonists, which have found widespread usefulness in the treatment of angina, hypertension, and cardiac arrhythmias [1], also show considerable promise as uterine smooth muscle relaxing agents for the treatment of premature labor [2, 3]. Recently, understanding of the mechanism and sites of action of these agents has been aided by ligand binding studies using radiolabeled 1,4-dihydropyridines [4]. Specific, reversible, high-affinity binding of dihydropyridines has been quantitated in several different tissues including nervous system, cardiac muscle, skeletal muscle and vascular and nonvascular smooth muscle [4]. As expected, chemically similar dihydropyridines act as competitive inhibitors of labeled dihydropyridine binding. Structurally unrelated calcium antagonists, on the other hand, generally do not act as pure competitive inhibitors. In fact, stimulation of dihydropyridine binding by the benzothiazipine calcium antagonist diltiazem has been noted in several tissues [5–17]. The manner in which this agent influences dihydropyridine binding remains in dispute, however. Several studies have found that increased binding is caused by an apparent increase in the total number of binding sites (B_{max}) [7, 9, 13, 14, 16], whereas others have reported that greater binding results from a decrease in the kinetic dissociation rate constant, k_{-1}, and thus an apparent decrease in the equilibrium dissociation constant, K_d [6, 8, 15].

Since diltiazem stimulation of dihydropyridine interaction within uterine smooth muscle membrane may lead to improved effectiveness of the dihydropyridine as a tocolytic agent, we were interested in quantitating any effect of diltiazem on dihydropyridine binding to uterine smooth muscle sarcolemma vesicles. The tritiated dihydropyridine [^{3}H]PN200-110 was used in these studies because preliminary experiments had shown that it was associated with a lower degree of "nonspecific" binding than the more commonly studied [^{3}H]nitrendipine.

We report that diltiazem did indeed increase the amount of PN200-110 bound to uterine plasmalemma and moreover that stimulation was not due to a change in K_d but rather to an increase in B_{max}.

MATERIALS AND METHODS

(+)-[*Methyl*-^{3}H]PN200-110 (80 μCi/mmole) was obtained from Amersham, Arlington Heights, IL, and levo[ring,*propyl*-^{3}H(N)]dihydroalprenolol (104.8 Ci/mmole) from New England Nuclear, North Villerica, MA. Nifedipine was provided by Pfizer, Inc., Brooklyn, NY, and *d-cis*-diltiazem by Marion Laboratories, Kansas City, MO. The remaining chemicals were products of the Sigma Chemical Co., St. Louis, MO, or were the highest purity available commercially.

Membrane preparation. Samples of myometrium were obtained from uteri removed for benign indications from premenopausal women, trimmed free of larger blood vessels, minced, and homogenized in 5 vol. of 0.25 M sucrose, 10 mM histidine (pH 7.4) at 0–4° with a Polytron PE20 for three 30-sec bursts at half-maximal speed. The homogenate, designated fraction F_1, was centrifuged for 20 min at 14,000 *g* and the decanted supernatant fraction was centrifuged again for 20 min at 14,000 *g*. The resulting supernatant was centrifuged at 105,000 *g* for 30 min. The pellet (F_2) was suspended in 0.25 M sucrose, 30 mM histidine, 0.6 M KCl (pH 7.4) in order to extract contractile protein. The suspension was then centrifuged at 17,000 *g* to sediment additional mitochondrial fragments (F_3), and the supernatant fraction was centrifuged at 105,000 *g* for 30 min.

The 105,000 *g* pellet (F_4) was suspended in 0.25 M sucrose containing 300 mM KCl, 50 mM sodium pyrophosphate, and 180 mM Tris–HCl (pH 7.4) at a protein concentration of 1.5 mg/ml. Samples of 8 ml were placed on discontinuous sucrose gradients consisting of 15 ml of 0.7 M (24%, w/v), 10 ml of 0.9 M (30%, w/v) and 5 ml of 1.5 M (50%, w/v) sucrose

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dissolved in the same buffer solution and centrifuged at 28,000 rpm for 3 hr at 4°. Four fractions were collected: the opalescent bands at the interfaces between 0.25 M and 0.7 M (S_1), 0.7 M and 0.9 M (S_2), and 0.9 M and 1.5 M (S_3), and the pellet (S_4). Fractions were diluted with water and centrifuged for 1 hr at 105,000 *g* to recover the protein and stored at −20°.

Enzyme assays. All enzyme activities were measured at 37° and were linear with time and amount of protein added. (Na^+,K^+)-ATPase assay was performed in a medium containing 40 mM histidine, 3 mM $MgCl_2$, 1 mM Tris-ethyleneglycolbis(amino-ethylether)tetra-acetate (Tris-EGTA), 100 mM NaCl, 10 mM KCl, 3 mM disodium ATP and 20 μg/ml protein (pH 7.4) [18]. (Na^+,K^+)-ATPase was considered that activity inhibitable by 1 mM ouabain. 5′-Nucleotidase was determined in a mixture containing 30 μg/ml protein, 0.25 M sucrose, 19 mM $MgCl_2$, 2 mM disodium 5′-AMP, and 100 mM glycine (pH 8.0) [19].

Succinic dehydrogenase was measured by the method of Pennington [20] with the modifications that the final reaction mixture (0.5 ml) contained 30 μg of membrane protein, 25 mM sucrose, 0.50 mg iodonitrotetrazolium and 40 mM sodium succinate in 15 mM potassium phosphate buffer (pH 7.4). NADPH–cytochrome *c* reductase was measured in a medium containing 0.1 mM NADPH, 0.1 mM cytochrome *c* and 0.3 mM KCN in 50 mM sodium phosphate (pH 7.4) [21]. Protein was measured by the method of Lowry *et al.* [22].

Binding assays. β-Adrenergic receptor content of membrane fractions was estimated by [^{3}H]dihydroalprenolol binding [23] which was performed in reaction volumes of 0.50 ml containing 25 mM $MgCl_2$, 50 mM Tris–HCl (pH 7.4), 0.6 mM [^{3}H]dihydroalprenolol, and 100 μg/ml protein. After incubation at 37° for 60 min, the reaction was terminated by the addition of 5 ml of unlabeled incubation buffer and immediately filtered through Whatman GF/C filters. Filters were washed with two 5-ml volumes of the same buffer, dried in air, and counted in toluene based scintillation fluid at 37% efficiency. Specific binding was considered that displaceable by 2 μM (−)-propranolol.

[^{3}H]PN200-110 binding was also measured in 50 mM Tris–HCl and 25 mM $MgCl_2$ (pH 7.4). Filtration and counting techniques were identical to those used for [^{3}H]dihydroalprenolol binding studies. Incubation was carried out in the dark for 60 min at 37°, for 2 hr at 25° and overnight at 0–4° unless otherwise noted. Nonspecific binding was estimated by comparing [^{3}H]PN200-110 binding in the presence and absence of a 100-fold excess of the unlabeled nifedipine, or, in the case of saturation studies, by the LIGAND computer programs of Munson and Rodbard [24] converted to Applesoft Basic by Martin Teicher, Department of Psychiatry, Harvard Medical School, and made available by the Biochemical Computing Technology Information Center, Vanderbilt Medical Center, TN. The equilibrium dissociation constant, K_d, and the maximal binding capacity, B_{max}, were also determined from saturation isotherms using the LIGAND program.

Determinations of the kinetic association and dissociation constants k_1 and k_{-1} were performed with a [^{3}H]PN200-110 concentration of approximately 0.6 nM. Dissociation of bound [^{3}H]PN200-110 was initiated by the addition of unlabeled nifedipine to give a final concentration of 1.0 μM, or by the addition of a 100-fold excess of binding buffer containing no dihydropyridine.

RESULTS

Membrane isolation and purification. Table 1 shows the distribution of five membrane-associated enzyme activities and of relative β-adrenergic receptor content, and of enhancement of activities relative to those present in the initial homogenate. The results are from three preparations of pooled nonpregnant human myometrium each containing 400–600 g of tissue. (Na^+,K^+)-ATPase, 5′-nucleotidase, and [^{3}H]DHA binding, considered to be reliable markers for plasmalemma [18, 25–27], were highest in the lowest buoyant density sucrose gradient fraction S_1 and enriched 27-, 15- and 35-fold, respectively, over the homogenate. In contrast, succinic dehydrogenase, a marker for inner mitochondrial membrane [20], was concentrated in the heavier fractions, as was the activity of NADPH–cytochrome *c* reductase, a marker for endoplasmic reticulum [26, 27].

Characterization of [^{3}H]*PN*200-110 *binding.* Since the specific binding of [^{3}H]PN200-110 was found to be directly proportional to membrane protein concentration up to 250 μg/ml, binding studies were routinely done with 50 μg protein in 0.50 ml reaction volume. [^{3}H]PN200-110 binding in preparation fractions correlated closely with plasmalemma markers and was greatest (nearly 600 fmoles/mg protein) in S_1, that fraction most enriched in plasmalemma (Fig. 1). Since the relative binding shown in Fig. 1 was obtained with subsaturating concentrations of ligand in order to minimize nonspecific binding, and since the difference in binding between fractions could be due either to organelle-specific receptor sites of differing affinities or to various concentrations of the same receptor, saturation isotherms were obtained for each fraction. As shown in Fig. 2, the equilibrium dissociation constants were nearly identical (0.14, 0.16 and 0.21 nM) for mitochondrial (F_3), crude microsomal (F_4), and plasmalemma (S_1) fractions, respectively, whereas the maximal binding capacities were, respectively, 108, 161, and 405 fmoles/mg protein. Similarly, results for fractions S_2, S_3 and S_4 showed nearly identical dissociation constants and decreasing binding capacities (data not shown). Furthermore, only a single class of binding sites was identified in each fraction. Since fraction S_1 had the highest concentration of binding sites, it was used for all further studies.

Saturation isotherms were employed to study [^{3}H]PN200-110 binding at 0–4°, 22°, and 37°. Specific binding at 0–4° after 60 min of reaction was found to be only 30–50 fmoles/mg protein. Reaction times were extended to 4 hr and then to overnight without a significant increase in specific binding. This low binding capacity, less than 10% of that obtained at 22°, precluded further examination at this temperature. Although the maximal binding capacity varied

Table 1. Comparison of the subcellular distribution of membrane markers from human myometrial membrane fractions

Fraction	(Na^+,K^+)-ATPase (μmoles/mg protein/hr)	5′-Nucleotidase (μmoles/mg protein/hr)	NADPH–cytochrome *c* reductase (μmoles/mg protein/hr)	[^{3}H]DHA binding (fmoles/mg protein)	Succinic dehydrogenase (mg iodonitrotetrazolium formazan/ mg protein/hr)
Homogenate, F_1	0.99 ± 0.24 (1)*	4.04 ± 1.06 (1)	0.24 ± 0.04 (1)	9.20 ± 1.03 (1)	0.31 ± 0.11 (1)
100,000 *g* pellet, F_2	2.75 ± 0.60 (2.8)	35.15 ± 8.15 (8.7)	0.70 ± 0.06 (2.9)	65.12 ± 11.11 (7.1)	0.80 ± 0.19 (2.6)
17,000 *g* pellet, F_3	2.50 ± 0.93 (2.5)	36.03 ± 8.48 (8.9)	0.49 ± 0.06 (2.0)	106.10 ± 7.04 (11.5)	2.54 ± 0.41 (8.2)
100,000 *g* KCl pellet, F_4	10.80 ± 1.41 (10.9)	44.40 ± 9.0 (11.0)	0.75 ± 0.05 (3.1)	192.42 ± 33.34 (20.9)	1.15 ± 0.20 (3.7)
Sucrose gradient:					
S_1, 0.25/0.7 M Sucrose	26.91 ± 3.77 (27.2)	64.41 ± 8.21 (15.4)	0.40 ± 0.07 (1.7)	323.88 ± 84.83 (35.2)	0.35 ± 1.10 (1.1)
S_2, 0.7/0.9 M Sucrose	9.78 ± 1.10 (9.9)	42.08 ± 7.54 (10.4)	1.12 ± 0.14 (4.7)	177.10 ± 27.71 (19.3)	1.34 ± 0.30 (4.3)
S_3, 0.9/1.5 M Sucrose	5.94 ± 2.20 (6.0)	31.35 ± 9.29 (7.8)	1.26 ± 0.08 (5.2)	116.23 ± 15.08 (12.6)	2.95 ± 0.67 (9.5)
S_4, Pellet	4.11 ± 1.82 (4.2)	19.30 ± 5.70 (4.8)	1.32 ± 0.12 (5.5)	84.92 ± 8.91 (9.2)	1.34 ± 0.26 (4.3)

Values are mean ± SE.
* Ratio of specific activity to that in homogenate.

Fig. 1. Subcellular localization of PN200-110 binding. Specific PN200-110 binding to membrane fractions was measured using 0.6 nM [^{3}H]PN200-110 and 0.050 mg membrane protein per 0.50 ml reaction volume. Equilibration was carried out for 60 min at 37° before rapid filtration. Duplicate estimates of nonspecific binding obtained by the inclusion of 100 nM nifedipine were subtracted from triplicate estimates of total binding for each determination. The mean values of determinations done on three separate preparations are shown. The error bars represent one standard deviation. Fraction designations are given in Materials and Methods. S_1 is the sarcolemma fraction.

2- to 3-fold between preparations, when membranes from the same preparations were used, B_{max} was found to be nearly the same at 22° and 37° (350–450 fmoles/mg), whereas the dissociation constant was actually greater at 22° (0.58 nM) than at 37° (0.19 nM) (Fig. 3).

As expected, [^{3}H]PN200-110 binding was inhibited by the structurally similar dihydropyridine nifedipine (Fig. 4). In this preparation, B_{max} was 280 fmoles/mg. Acting as a competitive inhibitor, addition of nifedipine increased the apparent dissociation constant but had no effect on the B_{max}, whereas diltiazem did not affect the dissociation constant but effectively increased the total number of binding sites.

Diltiazem effects on [^{3}H]*PN200-110 binding.* Figure 5 shows the changes in specific binding as diltiazem concentration was increased from 0 to 200 μM. Note that in the absence of diltiazem specific binding of PN200-110 at 0–4° was less than 10% of binding at 22°. Diltiazem had no apparent effect at

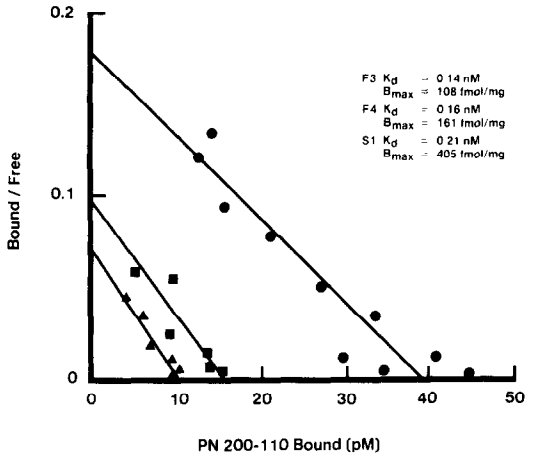

Fig. 2. Characterization of PN200-110 binding to membrane fractions. The dissociation constant, K_d, and the maximal binding capacity, B_{max}, of PN200-110 for mitochondrial (F_3, ▲—▲), purified microsomal (F_4, ■—■), and sarcolemmal (S_1, ●—●) fractions were determined by Scatchard analysis using [^{3}H]PN200-110 concentrations ranging from 0.1 to 10 nM and 0.050 mg protein in 0.50 ml. Nonspecific binding, K_d, and B_{max} were determined using the computer method noted in Materials and Methods.

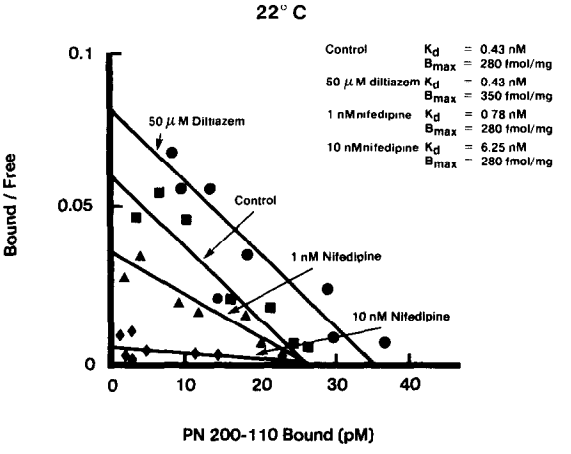

Fig. 4. Effects of nifedipine and diltiazem on PN200-110 binding at 22°. PN200-110 binding was measured in the presence of O (■—■), 1 nM (▲—▲) or 10 nM (◆—◆) nifedipine, or 50 μM diltiazem (●—●). Nonspecific binding, K_d, and B_{max} were determined using the computer method noted in Materials and Methods.

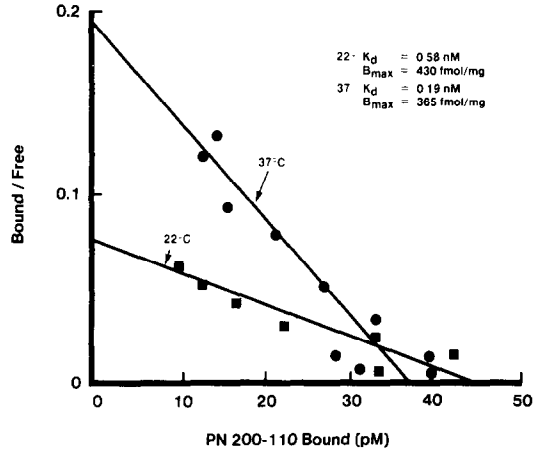

Fig. 3. Temperature dependence of PN200-110 binding. Binding conditions are as noted in Materials and Methods. Equilibration at 22° was carried out for 120 min and at 37° for 60 min. Although B_{max} was unchanged, binding occurred with higher affinity at 37°.

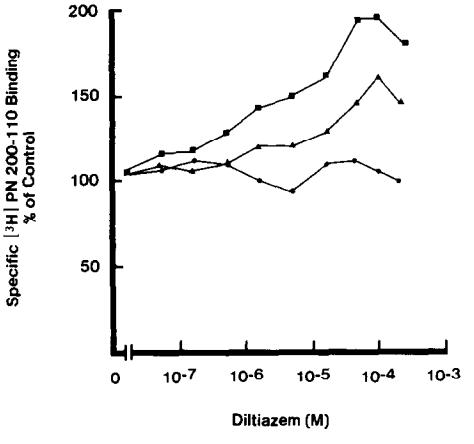

Fig. 5. Temperature dependence of diltiazem stimulation of PN200-110 binding. The effect of diltiazem on PN200-110 binding was measured at 40° (●—●), 22° (▲—▲), and 37° (■—■) using 0.8 nM PN200-110. Nonspecific binding was estimated by the inclusion of 100 nM nifedipine. Specific binding at 4° was only 10% of that at 22°.

0–4°. At 22° and 37° there were significant increases in bound PN200-110 in the presence of increasing concentrations of diltiazem. Stimulation was maximal at 10^{-5} to 10^{-4} M *d-cis*-diltiazem and decreased slightly at higher concentrations.

Increased binding was found to result from an apparent increase in maximal binding capacity rather than a change in dissociation constant. In a representative saturation experiment at 37° total specific binding capacity increased from 390 fmoles/mg protein to 790 fmoles/mg protein (Fig. 6). The dissociation constants were statistically indistinguishable [24]. Similar results were obtained at 22° (Fig. 4). In this determination, B_{max} increased from 280 to 350 fmoles/mg protein.

Kinetic analysis of the effect of diltiazem on [^{3}H]PN200-110 binding at 37° is shown in Fig. 7. The pseudo first order observed association constant k_{obs} was identical in the presence and absence of 50 μM *d-cis*-diltiazem (0.09 min^{-1}), although the amount of [^{3}H]PN200-110 bound at equilibrium was 50% higher in the presence of diltiazem. In the absence of diltiazem, dissociation of [^{3}H]PN200-110 initiated by the addition of 1 μM nifedipine was quantitative and the first order dissociation constant, k_{-1}, was 0.029 min^{-1}. In the presence of 50 μM diltiazem, however, a biphasic dissociation curve was observed.

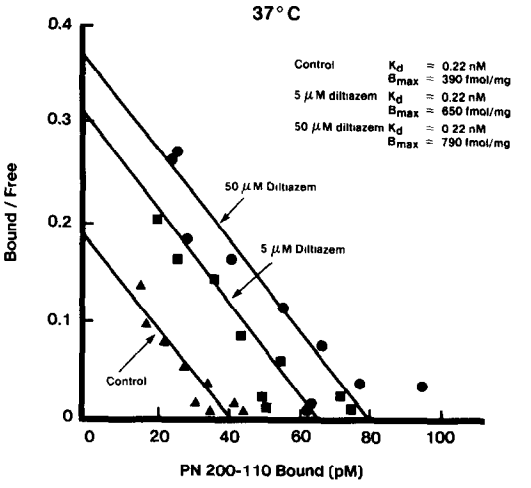

Fig. 6. Effects of diltiazem on PN200-110 binding—equilibrium parameters. Binding was measured in the presence of 0 (▲—▲), 5 (■—■), and 50 (●—●) μM diltiazem. Nonspecific binding, K_d, and B_{max} were determined by the computer method noted in Materials and Methods. In this presentation of the data, K_d was assumed to be constant and thus calculated using all data points. The B_{max} for each diltiazem concentration was then calculated using the same value of K_d. Nearly identical results were obtained if the K_d and B_{max} were calculated separately for each diltiazem concentration.

The slower rate constant, estimated from data obtained between 100 and 500 min was approximately 0.001 min^{-1}. To determine the faster rate constant, the extrapolated slower component was subtracted from the data between 1 and 90 min of dissociation, and the rate constant was calculated from a first order plot of the remainder. The faster dissociation constant in the presence of diltiazem was found to be nearly equal to the dissociation constant noted in the absence of diltiazem (0.029 to 0.035 min^{-1}). Dissociation induced by 100-fold dilution into binding buffer gave similar results and yielded a first order dissociation constant of 0.022 min^{-1}. Since under pseudo first order conditions $k_{obs} = [L_T]\ k_1 + k_{-1}$ [28] where $[L_T]$ is the concentration of total ligand, a value of approximately 0.1 $nM^{-1}\ min^{-1}$ can be calculated from these data, giving a kinetically derived equilibrium dissociation constant, K_d, of 0.3 nM which is in good agreement with that determined by equilibrium binding.

DISCUSSION

In myometrium as in other smooth muscle the major source of the increased cytoplasmic Ca^{2+} necessary for the initiation of contraction appears to be the extracellular space, and the major flux of Ca^{2+} appears to occur through "channels" in the plasmalemma [29]. Nanomolar concentrations of dihydropyridine calcium antagonists impede the inward Ca^{2+} flux [30] and inhibit smooth muscle contraction. The specific, saturable, and reversible dose–response effects of these drugs suggest that they do so by interacting with specific "receptors" [4].

To confirm the site of dihydropyridine interaction with the human myometrial smooth muscle cell, we developed a method of fractionation of cellular membrane which substantially separated those membrane fragments that contained markers characteristic of plasmalemma from those that contained fragments of endoplasmic reticulum or mitochondria.

The dihydropyridine (+)-PN200-110 was found to bind to myometrial membranes in a saturable manner and to be rapidly and completely displaced by the structurally similar dihydropyridine nifedipine. The highest concentration of binding sites was found in the myometrial plasmalemma, confirming previous studies of dihydropyridine binding [31, 32]. In contrast to other reports, we found that binding at 0–4° was minimal despite extended reaction times [13, 16, 25, 33, 34]. Furthermore, both specific and nonspecific binding was found to be greater at 37° than at 24°. Interestingly, this increase did not represent a change in B_{max} but rather higher affinity at higher temperatures.

In contrast to competitive inhibition of binding of PN200-110 by the dihydropyridine nifedipine, the structurally unrelated calcium antagonist diltiazem actually increased PN200-110 binding in a concentration-dependent manner. Diltiazem did not affect either the equilibrium dissociation constant for PN200-110 or the kinetic association constant but did increase B_{max} and produce a biphasic dissociation curve for PN200-110. The rapid component was found to be nearly equal to the dissociation rate in the absence of diltiazem. Moreover, the proportion of the more slowly dissociating component (determined from the intercept of the semilog plot for the slower component) was found to be nearly equal to the increase in binding induced by diltiazem.

A nonlinear first order dissociation curve could imply heterogenous binding sites, cooperative site–site interactions or a ligand-induced conformational change in the receptor [28]. The fact that linear Scatchard plots were observed in the presence of diltiazem supports the third possibility and implies that diltiazem induces a change in the membrane binding site such that a proportion of the dihydropyridine is bound with a 30-fold greater affinity.

Several investigators have noted that diltiazem stimulates binding of dihydropyridines to cellular membranes [5–17] but differ on whether this effect is due to a decrease in the dissociation rate of dihydropyridine [6, 8, 10, 15] or an increase in B_{max} [7, 9, 11, 13, 16]. In neural tissue, the effect seems to be predominantly an effect on the dissociation constant [6, 8, 10, 11, 15] whereas in smooth and cardiac muscle the effect most often observed is an increase in B_{max} [7, 9, 10, 14, 16]. These findings suggest that these calcium antagonists interact with these two different kinds of tissue in fundamentally different manners. Our studies suggest that the apparent increase in dihydropyridine binding to smooth muscle plasmalemma in the presence of diltiazem results from the formation of a fraction of the binding site which binds the dihydropyridine with much higher affinity. Whether this results from the isomerization of the binding proteins before or after PN200-110 binding cannot be determined from the present data. More detailed kinetic analysis as well as identification and characterization of the proteins

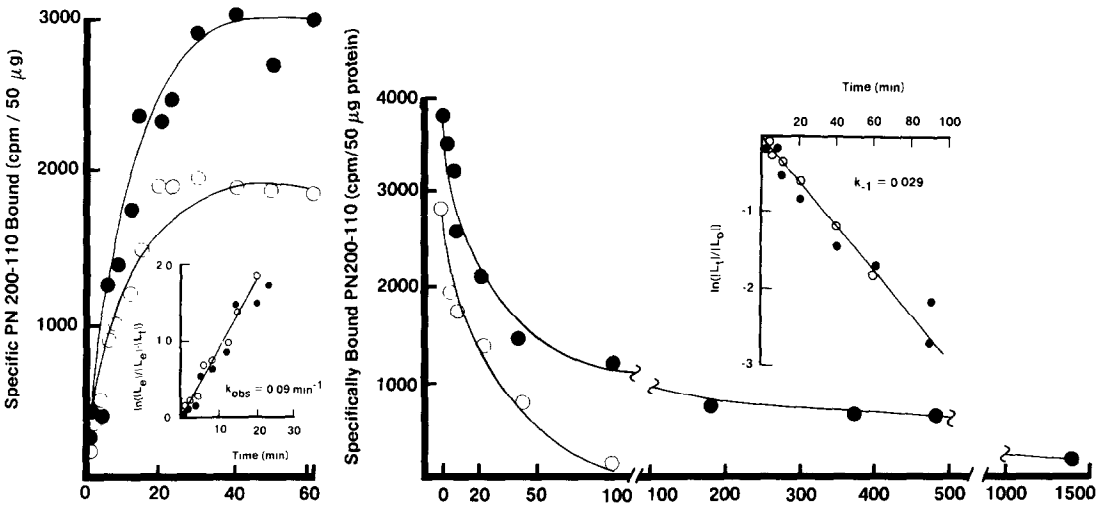

Fig. 7. Effects of diltiazem on PN200-110 binding—kinetic parameters. Kinetic analysis of PN200-110 binding was performed at 37° using 0.6 nM PN200-110. Points represent the average of triplicate determinations. Nonspecific binding was estimated by duplicate determinations in the presence of 100 mM nifedipine. Association and dissociation were measured in the absence (○—○) and presence (●—●) of 50 μM diltiazem. The k_{obs} for association was obtained from a plot of $\ln([L_e]/[L_e]-[L_t])$ versus time, where $[L_e]$ is the concentration of bound PN200-110 at equilibrium and $[L_t]$ that bound at time t. The k_{obs} was found to be the same in the presence and absence of diltiazem. To measure the dissociation rate, binding was allowed to proceed for 60 min after which unlabeled nifedipine was added to a final concentration of 1 μM. The first order dissociation constant, k_{-1}, was estimated from a plot of $\ln([L_t]/[L_0])$ in which $[L_0]$ represents the initial concentration of bound PN200-110. In the presence of diltiazem, a biphasic dissociation curve was noted. The slower apparent rate constant (0.0011 min^{-1}) was determined from the slope of the plot between 100 and 500 min of dissociation. The faster apparent rate constant was calculated by subtracting the slower component from the data between 1 and 90 min of dissociation and determining the slope of the first order plot of the remainder. The faster k_{-1} in the presence of diltiazem (●—●) was found to be nearly equal to the k_{-1} observed in the absence of diltiazem (○—○).

binding PN200-110 in the presence and absence of diltiazem will be needed to answer these questions.

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