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DILTIAZEM ENHANCEMENT OF [^{3}H]NITRENDIPINE BINDING TO CALCIUM CHANNEL ASSOCIATED DRUG RECEPTOR SITES IN RAT BRAIN SYNAPTOSOMES

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The binding of the dihydropyridine calcium channel antagonist [^{3}H]nitrendipine to whole rat brain synaptosomes was studied. Binding was specific, saturable, and of high affinity (K_d = 170 pM). The calcium channel antagonist diltiazem enhanced [^{3}H]nitrendipine binding in synaptosomes in concentrations of 1 and 10 μM. Equilibrium saturation analysis demonstrated that this effect was mediated by a decrease in the dissociation constant, due to a 3-fold reduction in the rate of ligand-receptor complex dissociation. It is concluded that diltiazem allosterically modulates the calcium channel drug receptor labeled by [^{3}H]nitrendipine in this preparation.

INTRODUCTION

The importance of calcium in the functioning of biologic systems has long been realised. Calcium entry into various cell types via the slow calcium channel is inhibited by a heterogeneous group of structurally diverse drugs that collectively have been termed 'calcium antagonists' (1). The structural heterogeneity among the different calcium antagonists is consistent with the differential pharmacological and electrophysiological effects of these drugs (2,3). Among the clinically utilized classes of calcium antagonists are the phenylalkylamines verapamil and methoxyverapamil (D-600), the benzothiazepine derivative diltiazem, and a number of dihydropyridine derivatives which include nitrendipine, nifedipine and nimodipine. Recently, [^{3}H]nitrendipine (4-10), [^{3}H]nimodipine (11) and [^{3}H]nifedipine (12) have been introduced as specific, high affinity probes for a dihydro-

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pyridine drug receptor site associated with the slow calcium channel. The inhibitory potency order of various dihydropyridine derivatives and multivalent cations corraborates the pharmacological significance of the [^{3}H]nitrendipine labeled drug receptor site (4-10). In contrast to the dihydropyridines, verapamil and D-600 inhibit [^{3}H]nitrendipine binding by a mechanism of negative heterotropic cooperativity through an allosteric modulatory site (6,7). Since the pharmacological and electrophysiological effects of diltiazem differ from those of both the phenylalkylamines verapamil and D-600, and the dihydropyridine derivatives (2,3), it was of interest to delineate the molecular interaction between diltiazem and the slow calcium channel associated drug receptor site labeled by [^{3}H]nitrendipine.

METHODS

Male Sprague-Dawley rats (200-250 g) were killed by decapitation and the brain from each animal was removed rapidly and placed on a petri dish on ice. The whole brain was then placed in a beaker of ice-cold sucrose (0.32 M) to remove surface vessels and excess blood and homogenized in 19 volumes of 0.32 M sucrose, using a teflon-in-glass homogenizer (10 full strokes at 850 rpm). The homogenate was centrifuged at 900 x g for 10 minutes to remove remaining blood, intact cells, and nuclei (P_1). The supernatant was centrifuged at 11,500 x g for 30 minutes to form a mitochondrial-synaptosomal pellet (P_2). This pellet (P_2) was resuspended in Krebs phosphate buffer (119 mM NaCl, 4.8 mM KCl, 2.1 mM $MgSO_4$, 1.3 mM $CaCl_2$, 20.3 mM Na_2HPO_4, 3.2 mM HCl, 10 mM D-glucose, pH 7.4). Resuspension of the P_2 fraction was performed with 9 volumes of Krebs-phosphate buffer (final tissue concentration 10 mg/ml buffer) by a teflon-in-glass homogenizer (4 full strokes at 400 rpm).

Aliquots (100 µl) of tissue homogenates were incubated with [^{3}H]nitrendipine (New England Nuclear, spec. act. 88.0 Ci/mmole) and other drugs in a total volume of 2 ml containing Krebs phosphate buffer. Incubations were carried out in the dark at 25°C. Membrane bound [^{3}H]nitrendipine was trapped following incubation over Whatman glass fiber filters (GF/B), and the filters rinsed with three 5 ml aliquots of ice cold 0.9% saline. Radioactivity was measured after extraction of the filters in scintillation fluid for at least 4 hours, by liquid scintillation spectroscopy at 46% efficiency. Specific binding was defined as that displaced by 1 µM nifedipine. Protein content of tissue homogenates was determined according to the method of Lowry et al. (13) using bovine serum albumin as the standard.

Experiments were analyzed by conventional graphical techniques and by a program, provided by Susan H. Yamamura, written in Basic for an Apple II microcomputer using least squares linear regression and nonlinear least squares regression by an iterative procedure. Statistical evaluations were performed with a two-tailed student's t-test. Data are given as the mean $\pm$ SEM of n independent experiments.

Drugs were generously supplied by Knoll Pharmaceutical Co. (verapamil) and Warner-Lambert (diltiazem). All other chemicals were obtained from commercial sources.

RESULTS

The dihydropyridine calcium channel antagonist, [^{3}H]nitrendipine binds with high affinity to intact whole rat brain synaptosomes. Binding at 25°C was saturable and consistent with mass action behavior as can be seen from the linear Scatchard plot (K_d = 170 ± 21 pM, B_{max} = 114 ± 8 fmol/mg protein, n=4) (Fig. 1). The dihydropyridine, nifedipine inhibited [^{3}H]nitrendipine binding (50 pM) to rat brain synaptosomes with high affinity, IC_{50} = 112 ± 13 pM. The residual binding which accounted for about 15% of the total was assumed to be nonspecific binding. Sucrose up to concentrations of at least 16 mM was found to have no significant effect on [^{3}H]nitrendipine binding. In addition, the calcium channel antagonist, verapamil, inhibited [^{3}H]nitrendipine binding (50 pM) to rat brain synaptosomes with half maximal inhibition of 178 ± 26 nM. Maximal inhibition was achieved at 10 μM and was 65 ± 3% of the specific [^{3}H]nitrendipine bound.

The structurally dissimiliar calcium channel antagonist, diltiazem, was found to significantly increase [^{3}H]nitrendipine binding in this tissue

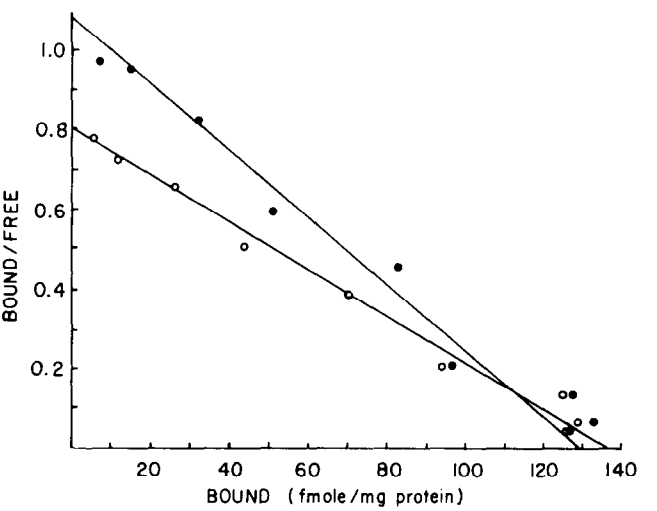
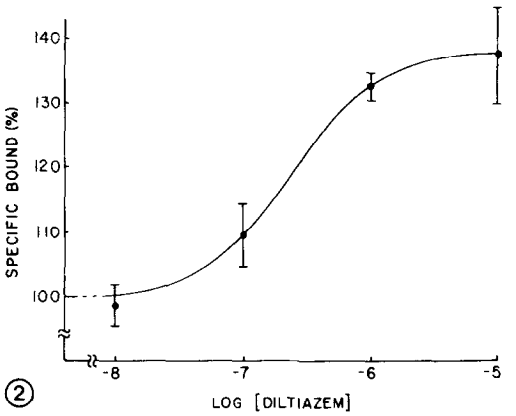


Figure 1 Scatchard analysis of [^{3}H]nitrendipine binding with (•) and without (o) 1 μM diltiazem using rat brain synaptosomes. Free concentrations of [^{3}H]nitrendipine varied from 10 to 3000 pM. Shown in this figure is a representative experiment of four separate determinations, each done in triplicate. In this experiment a receptor density (B_{max}) of 128 and 136 fmol/mg protein and an affinity (K_d) of 119 and 170 pM were obtained in the presence and absence of diltiazem, respectively.



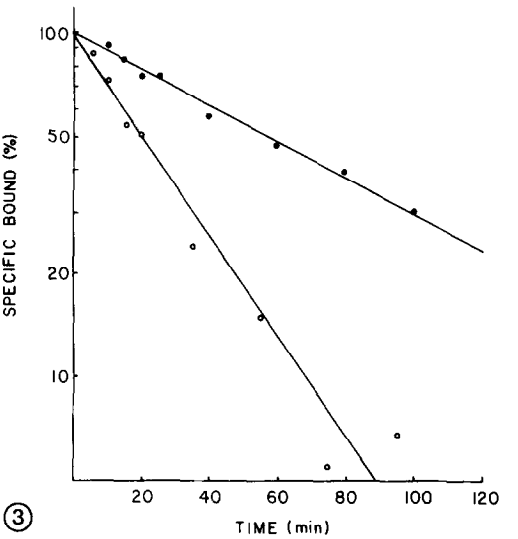


Figure 2 Dose response curve of diltiazem enhancement of 50 pM [^{3}H]nitrendipine binding in rat brain synaptosomes. An EC_{50} value of 0.2 μM was obtained with a maximal enhancement of 38% at 10 μM diltiazem. The data represent the mean binding values ± SEM of three independent experiments, each done in triplicate.

Figure 3 Time course of dissociation of [^{3}H]nitrendipine from rat brain synaptosomes, with (•) and without (o) 1 μM diltiazem. Dissociation was initiated at equilibrium (90 min at 25°C) by addition of 1 μM nifedipine. Shown in this figure is a representative experiment of three separate determinations, each done in triplicate. The dissociation rate constant (k_{-1}) for this experiment was 0.032 min^{-1} in the absence of diltiazem and 0.012 min^{-1} in the presence of 1 μM diltiazem.

preparation. The enhancement of [^{3}H]nitrendipine binding was significant at concentrations 1 and 10 μM ($p < 0.05$ and $p < 0.025$, respectively). Maximal enhancement was determined to be 138% of control with an EC_{50} value of 0.2 μM (Fig. 2).

The enhancement by diltiazem (1 μM) was found to be due to an increase in [^{3}H]nitrendipine binding affinity (K_d = 121 ± 16 pM, n=4, compared to control K_d = 170 ± 21 pM, n=4, paired data $p < 0.02$) with no significant change in the receptor density (B_{max} = 111 ± 6 fmol/mg protein, n=4, compared to control B_{max} = 114 ± 8 fmol/mg protein, n=4). A representative experiment is shown in Figure 1.

We investigated the mechanism of the enhancement of [^{3}H]nitrendipine (50 pM) binding by diltiazem (1 μM) using kinetic analysis. We found a highly significant decrease in the dissociation rate constant in the presence of 1 μM diltiazem (k_{-1}= 0.0116± 0.0002 min^{-1}, n=3) as compared to controls (k_{-1}=

0.0355 ± 0.0022 min^{-1}, n=3, p<0.001) (Fig. 3). A smaller insignificant change was found in the respective association rate constants (k_{+1} = 4.82± 2.07x $10^{-4}pM^{-1}$ min^{-1} in the presence of 1 μM diltiazem and k_{+1}= 6.45± 1.09x $10^{-4}pM^{-1}$ min^{-1}, in controls, n=3). The ratio k_{-1}/k_{+1} gives kinetically determined dissociation constants (K_d) for [^{3}H]nitrendipine of 18 pM in the presence of diltiazem (1 μM) and 74 pM in the absence of diltiazem.

DISCUSSION

High affinity, saturable, and stereospecific binding sites for the dihydropyridine calcium antagonist [^{3}H]nitrendipine have recently been identified in membrane preparations of the brain (6-9), heart (4-10), ileum (5,6), and aortic smooth muscle (10). Evidence has been presented that the sites labeled by [^{3}H]nitrendipine are associated with the slow calcium channel and represent the drug receptor through which the dihydropyridine calcium antagonists exert their pharmacological effects (4-10). The present data indicate that [^{3}H]nitrendipine binds to intact whole rat brain synaptosomes with an affinity, as determined by equilibrium binding studies, similar to that previously reported for a variety of membrane preparations (4-9). Furthermore, binding is potently inhibited by unlabeled nifedipine in agreement with previous observations (6,7). At 25°C ligand receptor association is rapid with a k_{+1} (rate of association) of 4.82x $10^{-4}pM^{-1}$ min^{-1}. Dissociation from equilibrium is monophasic with a half-life of 20 min and a dissociation rate (k_{-1}) of 0.0355 min^{-1}. From these data a kinetically derived dissociation constant (K_d) of 74 pM may be calculated, which is in general agreement with that obtained from equilibrium saturation experiments.

On the basis of pharmacological and electrophysiological characteristics, it has been suggested that the structurally dissimilar organic calcium antagonists affect the slow calcium channel through different modes of action (2,3). The finding that verapamil allosterically (submitted for publication) inhibits [^{3}H]nitrendipine binding to whole rat brain synaptosomes as well as other membrane preparations (6,7), lends further support to this hypothesis.

Furthermore, diltiazem, which differs structurally and pharmacologically from the nitrendipine and verapamil-like agents, appears to affect [^{3}H]nitrendipine receptor sites through a mechanism different from that of verapamil. Thus, diltiazem in concentrations of 1 and 10 μM was found to enhance [^{3}H]nitrendipine binding by 33 and 38%, respectively. Saturation isotherm and subsequent nonlinear analysis indicated that this enhancement is due to a selective increase in the affinity of [^{3}H]nitrendipine for its receptor, caused by a 3 fold decrease in the [^{3}H]nitrendipine-receptor complex rate of dissociation. It may therefore be speculated that the [^{3}H]nitrendipine labeled receptor site is allosterically modulated not only by a phenylalkylamine specific binding site that exerts an allosteric inhibitory effect, but also by a diltiazem-specific binding site that increases the receptor affinity for dihydropyridine analogs. These data differ from previously reported observations (6-9). However, this difference may be attributed to differences in the membrane source and preparation, and the incubation conditions such as temperature and ionic environment.

Ehlert and coworkers (7) have proposed previously that the affinity of [^{3}H]nitrendipine binding may be related to the functional state of the calcium channel. If the interaction between diltiazem and the [^{3}H]nitrendipine labeled drug receptor site demonstrated in the present experiments, is related to the inhibitory effect of diltiazem on calcium entry into the cell, it may be speculated that diltiazem exerts its pharmacological effect by inducing and/or stabilizing the calcium channel in the resting or inactive conformation thereby exposing the drug receptor site accesible to [^{3}H]nitrendipine in its highest affinity state.

In summary, the present data demonstrate that the calcium antagonist diltiazem allosterically modulates the [^{3}H]nitrendipine labeled receptor associated with the slow calcium channel, thereby increasing the receptor affinity of [^{3}H]nitrendipine through a selective effect on the rate of dissociation of the ligand receptor complex. It remains to be established whether

this mechanism represents the molecular mode of action of the inhibitory effect of diltiazem on calcium transport across the cell membrane.

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