A unitary mechanism of calcium antagonist drug action
(dihydropyridine/nifedipine/verapamil/neuroleptic/diltiazem)

KENNETH M. M. Murphy, ROBERT J. Gould, BRIAN L. Largent, and SOLOMON H. SNYDER*

Departments of Neuroscience, Pharmacology and Experimental Therapeutics, and Psychiatry and Behavioral Sciences, Johns Hopkins University School of Medicine, 725 North Wolfe Street, Baltimore, Maryland 21205

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ABSTRACT [3H]Nitrendipine binding to drug receptor sites associated with calcium channels is allosterically regulated by a diverse group of calcium channel antagonists. Verapamil, D-600 (methoxyverapamil), tiapamil, lidocfazine, flunarizine, cinnarizine, and prenylamine all reduce [3H]nitrendipine binding affinity. By contrast, diltiazem, a benzothiazepine calcium channel antagonist, enhances [3H]nitrendipine binding. All these drug effects involve a single site allosterically linked to the [3H]nitrendipine binding site. Inhibition of [3H]nitrendipine binding by prenylamine, lidoflazine, or tiapamil is reversed by D-600 and diltiazem, which alone respectively slightly reduce or enhance [3H]nitrendipine binding. Diltiazem reverses the inhibition of [3H]nitrendipine binding by D-600. Our prediction that drugs allosterically regulating [3H]nitrendipine binding should be calcium antagonists is confirmed by calcium antagonism in guinea pig ileum observed with the antihistamine dimethindene, the neuroleptics thioridazine and mesoridazine, and the anticholinergic biperiden.

Several distinct classes of organic calcium antagonist drugs are important therapeutic agents in cardiovascular and numerous other medical conditions (1, 2). At least four classes of structures are distinguished: the dihydropyridines exemplified by nifedipine (1–3), phenylalkylamines exemplified by verapamil (1–3), benzothiazepines such as diltiazem (1, 2, 4), and diphenylalkylamines such as prenylamine and lidoflazine (1, 2, 5). Receptors for the drugs are labeled with [3H]nitrendipine (6–10), or [3H]nimodipine (10) and regulated in a physiological fashion by calcium (7). The apparent competitive inhibition of [3H]nitrendipine binding by diphenylalkylamines has implied effects at the same site as the dihydropyridines (11). Verapamil and diltiazem respectively inhibit and stimulate [3H]nitrendipine binding allosterically, suggesting actions at different sites (9, 12). Here we show that the phenylalkylamine, diphenylalkylamine, and benzothiazepine drugs, in pharmacologically relevant concentrations, interact at a single drug recognition site allosterically linked to the dihydropyridine receptor. We predict and confirm by bioassay previously unknown calcium antagonist activity for several drugs.

MATERIALS AND METHODS

Drugs were obtained from the following sources: nifedipine, Pfizer; nimodipine, Miles; prenylamine, Hoechst-Roussel Pharmaceuticals (Somerville, NJ); D-600 (gallopamil, methoxyverapamil) and verapamil, Knoll Pharmaceutical (Whippany, NJ); diltiazem, Marion Laboratories (Kansas City, MO) and Tanabe (Osaka, Japan); desmethyl-cis-diltiazem and trans-diltiazem, Tanabe; lidoflazine, flunarizine, and cinnarizine, Janssen Pharmaceutical (Beerse, Belgium); and bepridil, McNeil Pharmaceutical (Spring House, PA).

Binding assays in guinea pig brain membranes, filtration, and liquid scintillation counting were carried out as described (7). All experiments, performed in triplicate, were replicated at least three times with similar results.

Guinea pig ileum longitudinal muscles were prepared for recording as described by Rosenberger et al. (13) and incubated in a modified Tyrode's buffer (14) at 37°C with continuous aeration with 95% O2/5% CO2. Ileum longitudinal muscles were incubated in this buffer for 30 min before Ca2+-dependent contractions were recorded as described by Jim et al. (15).

RESULTS

All dihydropyridine calcium antagonists evaluated inhibit [3H]nitrendipine binding in a competitive fashion (6–10). Verapamil inhibits [3H]nitrendipine binding (6–10), but Yamamura and associates showed that this effect is not strictly competitive, because verapamil cannot completely inhibit [3H]nitrendipine binding, the extent of inhibition of binding by verapamil decreases at increasing [3H]nitrendipine concentrations, and verapamil accelerates the dissociation of [3H]nitrendipine from receptor sites (9). Because diphenylalkylamines inhibit [3H]nitrendipine binding completely, it was suggested that these drugs act at the same receptor sites as dihydropyridines (11). In our studies, the diphenylalkylamines prenylamine, flunarizine, and lidoflazine and the phenylalkylamine tiapamil maximally inhibit [3H]nitrendipine binding (Fig. 1). However, maximal inhibition of [3H]nitrendipine binding by these drugs declines with increasing [3H]nitrendipine concentration between 0.1 and 3 nM (data not shown), suggesting a similarity in the action of these agents and verapamil. We have confirmed this possibility in dissociation experiments (Fig. 2). As previously reported (9), verapamil speeds the dissociation of [3H]nitrendipine (data not shown), whereas the dihydropyridine nimodipine has no effect on the dissociation rate. Like verapamil, the diphenylalkylamines lidoflazine and prenylamine and the verapamil analog tiapamil accelerate [3H]nitrendipine dissociation. Cinnarizine and flunarizine similarly increase the dissociation rate.

Having established that the diphenylalkylamines influence [3H]nitrendipine binding allosterically, we investigated whether the diphenylalkylamines and phenylalkylamines act at the same site. If so, then increasing concentrations of D-600 (which maximally reduces [3H]nitrendipine binding only 20–30%) (Fig. 3) should block the ability of a diphenylalkylamine to inhibit [3H]nitrendipine binding, with a rightward shift in concentration–response curves. Indeed, increasing concentrations of D-600 do produce rightward shifts in the concentration–response curves for inhibition of [3H]nitrendipine binding by the diphenylalkylamines prenylamine, flunarizine, and lidoflazine (Fig. 1 a–c). Tiapamil, a phenylalkylamine closely similar in structure to verapamil and D-600 (16), produces the same maximal inhibition of [3H]nitrendipine binding elicited by the dih-
librium with 100 nM nimodipine.

The rightward shift differs for the drugs evaluated, being greatest with tiapamil and least with flunarizine. By contrast with these results, the concentration–response curve for inhibition of \( [\text{H}]\)nitrendipine binding by nifedipine is not progressively shifted in the presence of increasing concentrations of D-600 (data not shown).

Because D-600 maximally reduces \( [\text{H}]\)nitrendipine binding less than the other diphenylalkylamines and phenylalkylamines and assuming that the drugs act at the same site, D-600 should overcome inhibition of \( [\text{H}]\)nitrendipine binding exerted by these other drugs. Accordingly, we examined the ability of D-600 to influence \( [\text{H}]\)nitrendipine binding to membranes simultaneously incubated with prenylamine, tiapamil, flunarizine, or lidoflazine (Fig. 3). In the absence of other drugs, D-600 maximally reduces \( [\text{H}]\)nitrendipine binding only about 25% with an EC\(_{50}\) (50% effective concentration) of about 10 nM. By contrast, in the presence of concentrations of prenylamine, tiapamil, flunarizine, or lidoflazine that give nearly maximal inhibition of \( [\text{H}]\)nitrendipine binding, D-600 actually stimulates \( [\text{H}]\)nitrendipine binding (Fig. 3). The percentage stimulation of \( [\text{H}]\)nitrendipine binding by D-600 diminishes with lower concentrations of the other drugs. \( [\text{H}]\)Nitrendipine binding is restored by D-600 to the level of binding that occurs with maximally inhibitory concentrations of D-600 alone. As expected for drugs competing for the same site, D-600 is somewhat less potent at enhancing \( [\text{H}]\)nitrendipine binding in the presence of increasing concentrations of the other inhibitors (Fig. 3).

Whereas diphenylalkylamines and phenylalkylamines inhibit \( [\text{H}]\)nitrendipine binding, diltiazem stimulates binding when evaluated in the absence of other drugs (Fig. 4a) (12, 17). It has been suggested that diltiazem therefore acts at a distinct site, specific for benzothiazepines, and different from the site at which the phenylalkylamines and diphenylalkylamines act (12, 17). If this were the case, no direct interactions should occur between the benzothiazepines and the phenylalkylamines or diphenylalkylamines. To evaluate possible interactions, we explored the effect of diltiazem on \( [\text{H}]\)nitrendipine binding in the presence of D-600, prenylamine, and tiapamil (Fig. 4a). Diltiazem strikingly reverses the inhibition of \( [\text{H}]\)nitrendipine binding produced by tiapamil, D-600, and prenylamine. Moreover, its potency in reversing such inhibition is decreased in the

![Fig. 1. Inhibition of \( [\text{H}]\)nitrendipine binding by prenylamine, tiapamil, lidoflazine, and flunarizine: Interactions with D-600. Concentration–response curves for the inhibition of \( [\text{H}]\)nitrendipine binding were constructed for prenylamine (a), flunarizine (b), lidoflazine (c), and tiapamil (d) in the absence (o) or the presence of 2.5 nM (c), 25 nM (a), 250 nM (c), 2.5 \( \mu \text{M} \) (a), or 25 \( \mu \text{M} \) (c) D-600. Concentration–response curves are the results of experiments that employed at least six concentrations of each drug assayed in triplicate with 0.25 nM \( [\text{H}]\)nitrendipine. Values are presented as a percent of control \( [\text{H}]\)nitrendipine binding.](image1)

![Fig. 2. Influences of various calcium channel antagonists on dissociation of \( [\text{H}]\)nitrendipine. \( [\text{H}]\)Nitrendipine (0.25 nM) was incubated with brain membranes for 90 min to allow equilibrium association of \( [\text{H}]\)nitrendipine to receptor sites. Dissociations were initiated with 100 nM nifedipine in either the absence (o) or the presence of 2.5 \( \mu \text{M} \) nimodipine (c), 2.5 \( \mu \text{M} \) prenylamine (a), 2.5 \( \mu \text{M} \) tiapamil (d), or 25 \( \mu \text{M} \) lidoflazine (c). Results are presented as a percentage of equilibrium \( [\text{H}]\)nitrendipine binding.](image2)
FIG. 3. D-600 reverses the inhibition of [3H]nitrendipine binding produced by prenylamine, tiapamil, lidoflazine, and flunarizine. Concentration–response curves for the influence of D-600 on [3H]nitrendipine binding to membranes were obtained with at least six concentrations of D-600 and 0.25 nM [3H]nitrendipine. [3H]Nitrendipine binding was determined at each concentration of D-600 in the absence (○), or the presence of 0.07 μM (△), 0.25 μM (△), 0.75 μM (□), 2.5 μM (●), or 25 μM (●) of the four drugs prenylamine (a), tiapamil (b), flunarizine (c), or lidoflazine (d). Values are presented as the percent of control [3H]nitrendipine binding determined in the absence of all inhibiting agents.

The presence of increased concentrations of other drugs. Thus, diltiazem acts at the same site as phenylalkylamines and diphenylalkylamines to enhance receptor affinity for [3H]nitrendipine, whereas the other drugs decrease this affinity.

To ascertain whether this effect of diltiazem is related to the pharmacologic actions of the drug, we compared cis- and trans-diltiazem, because only the cis isomer has pharmacological efficacy. We also evaluated desmethyl-cis-diltiazem, which pharmacologically is about 30–50% as potent as diltiazem (4). Enhancement of [3H]nitrendipine binding in the presence of 2.5 μM tiapamil is elicited by cis- but not trans-diltiazem (Fig. 4b). Desmethyl-cis-diltiazem is only about half as potent as cis-diltiazem. These relative activities are consistent with interactions at the pharmacologically relevant site of action of diltiazem.

Reduction of [3H]nitrendipine binding at high concentrations of cis-diltiazem occurs similarly with trans-diltiazem and desmethyl-cis-diltiazem, indicating no pharmacologic relevance (Fig. 4b).

The phenylalkylamines and diphenylalkylamines possess two widely separated aromatic domains with the amine located centrally, whereas diltiazem possesses only one aromatic domain linked to an alkylamine moiety. We predicted that some drugs with one aromatic domain and an alkylamine moiety might interact with receptors as diltiazem does. We have found that the H1 antihistamines dimethindene and chlorpheniramine, the muscarinic anticholinergic biperiden, the neuroleptics mesoridazine and thioridazine, and the organic calcium antagonist bepridil (18) all display the diltiazem-like action of enhancing [3H]nitrendipine binding (Fig. 5).

Dimethindene also not only enhances [3H]nitrendipine binding in the absence of other drugs but, like diltiazem, it reverses the inhibition of [3H]nitrendipine binding elicited by D-600, prenylamine, and tiapamil (Fig. 5a). Bepridil, thioridazine, biperiden, chlorpheniramine, and mesoridazine overcome the inhibition caused by 2.5 μM tiapamil, enhancing [3H]nitrendipine binding from 10% to 80% of control (Fig. 5b). Bepridil, thioridazine, and biperiden show biphasic effects like diltiazem, causing an inhibition of [3H]nitrendipine binding at higher concentrations.

To determine whether these effects on binding validly predict pharmacologic calcium channel antagonism, we explored the actions of these drugs on guinea pig ileum contractions (Fig. 6). The Schild plot for dimethindene inhibition of Ca2+-dependent contractions shows a pA2 of 4.95 (Fig. 6), similar to dimethindene's potency in stimulating [3H]nitrendipine binding. Furthermore, 25 μM thioridazine, biperiden, chlorpheniramine, and mesoridazine inhibit Ca2+-dependent contractions of guinea pig ileal muscle by 97%, 95%, 66%, and 51%, re-
respectively. This order of potency corresponds to the drugs' potencies in enhancing [³H]nitrendipine binding (Fig. 5b). The augmentation of [³H]nitrendipine binding by this group of drugs is specific, because the following drugs fail to increase binding at 25 μM: promazine, chlorimipramine, nortriptyline, fluphenazine, clozopine, depropoline, protriptyline, antazoline, spiperone, orphenadrine, promethazine, acetophenazine, pyrilamine, histamine, and atropine.

DISCUSSION

Our present findings clarify actions of calcium antagonist drugs of the known structural classes. Whereas others had suggested that the diphenylalkylamines such as prenylamine act differently than the phenylalkylamines such as verapamil (11), we have demonstrated that both classes of drugs act via the same site to allosterically regulate [³H]nitrendipine binding. It had been suggested that diltiazem acts at a site distinct from the phenylalkylamines and diphenylalkylamines (12, 17), but we find that it acts at the same site. The diphenylalkylamines and phenylalkylamines decrease the affinity of [³H]nitrendipine for receptors, but diltiazem enhances this affinity, as reflected in slowing of the [³H]nitrendipine dissociation rate (12, 17).

These observations suggest a unitary model for regulation of calcium ion channels by drugs (Fig. 7). A dihydropyridine recognition site linked to the calcium channel mediates the pharmacologic actions of this class of calcium channel antagonists. A specific site for multivalent cations influences [³H]nitrendipine binding (7). Divalent cations that mimic calcium physiologically stimulate [³H]nitrendipine binding, whereas cations known to block the physiologic actions of calcium prevent the enhancement of [³H]nitrendipine binding by calcium (7). Phenylalkylamines and diphenylalkylamines act at the same site and in the same fashion, both decreasing [³H]nitrendipine affinity for receptors. These drugs vary in the extent to which they reduce the receptor affinity of [³H]nitrendipine. D-600 and verapamil produce the least effect, reducing [³H]nitrendipine's receptor affinity to 1/2-1/3, whereas tiapamil produces a reduction in affinity to 1/5-1/10 and flunarizine a reduction to 1/25-1/50. Diltiazem and bepridil act at the same site to enhance [³H]nitrendipine affinity.

On the basis of structural similarities, we identified several neuroleptics, antihistamines, and muscarinic anticholinergics that mimic the actions of diltiazem on [³H]nitrendipine binding and have calcium channel antagonist activity in the guinea pig ileum. Thus, effects at a single domain of a bipartite receptor may convey diltiazem-like actions, whereas drugs acting at both domains have verapamil-like profiles. Such binding analyses may help identify calcium antagonists.

Clinical actions of the drugs we have evaluated may relate to their calcium antagonist actions. Of numerous phenothiazines evaluated, thioridazine and mesoridazine were unique in influencing [³H]nitrendipine binding in a diltiazem-like fashion. Blood levels of thioridazine and mesoridazine at therapeutic doses are about 2 μM (19, 20), a concentration at which these drugs display calcium antagonism. Thioridazine and mesori-
dazine elicit more cardiac side effects with Q-T interval prolongation than do other phenothiazines (21–23). Another unique action of thioridazine and mesoridazine is inhibition of ejaculation (23). Because ejaculation involves a massive contraction of the smooth muscle of the vas deferens, the calcium antagonist actions of these drugs may account for such side effects.

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