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EFFECTS OF DIHYDROPYRIDINE DERIVATIVES AND ANTICONVULSANT DRUGS ON [^{3}H]NITRENDIPINE BINDING AND CALCIUM AND SODIUM FLUXES IN BRAIN

R. ADRON HARRIS,*† SUSAN B. JONES,‡ PATRICK BRUNO‡ and DAVID B. BYLUND‡

*Denver Veterans Administration Medical Center and Department of Pharmacology, University of Colorado School of Medicine, Denver, CO 80262; and ‡Department of Pharmacology, University of Missouri Health Sciences Center, Columbia, MO 65212, U.S.A.

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Abstract—The binding of [^{3}H]nitrendipine to rat cortical membranes was reduced by phenytoin, phenobarbital, and pentobarbital. The IC_{50} values were 0.09, 0.40, and 0.76 mM respectively. The drugs reduced the apparent binding affinity of [^{3}H]nitrendipine with little effect on the maximum number of binding sites. The inhibitory effects of the drugs were similar in the absence and presence of calcium (4.5 mM). Neither nimodipine (10^{-8} to 10^{-5} M) nor nifedipine (10^{-8} to 10^{-7} M) altered the voltage-dependent uptake of $^{45}Ca^{2+}$ by synaptosomes from rat cortex. Phenytoin inhibited $^{45}Ca^{2+}$ influx, and this inhibition was not altered by nifedipine. Nimodipine and nifedipine (10^{-6} M) produced a small inhibition of the voltage-dependent uptake of $^{24}Na^{+}$ by synaptosomes. Ethanol, phenytoin or pentobarbital reduced $^{24}Na^{+}$ influx, and this action was not altered by nimodipine. Thus, sedative-anticonvulsant drugs reduced the binding of dihydropyridines to brain membranes, but these interactions did not appear to involve either calcium or sodium channels.

Nitrendipine and other dihydropyridine derivatives (such as nimodipine, nifedipine, and nisoldipine) are potent blockers of calcium channels in smooth muscle [1]. These drugs also bind with high affinity to brain membranes [2, 3], but studies to date have not demonstrated pronounced effects of the drugs on neuronal calcium fluxes [4, 5]. There is evidence that dihydropyridines may affect sodium channels more than calcium channels in some tissues, as Douglas and Taraskevich [6] found that 6 μM nifedipine reduces sodium, but not calcium, spikes in pars intermedia cells. In addition, veratridine, an activator of sodium channels, reduces the binding of [^{3}H]nitrendipine to smooth muscle [7]. These reports raise the possibility that dihydropyridines alter brain sodium channels. The drugs do not have pronounced CNS effects, but in mice nimodipine produces ataxia, decreases aggressive behavior, reduces pentylenetetrazole convulsions and potentiates hexobarbital anesthesia [8]. These behavioral effects are also produced by sedative-anticonvulsant drugs such as barbiturates, hydantoins, benzodiazepines, and alcohols. These latter drugs are known to reduce voltage-dependent calcium and sodium fluxes by isolated brain synaptosomes [9, 10], and it is possible that dihydropyridines and sedative-anticonvulsants share a common action on one or both of these channels. This postulate predicts that dihydropyridines would reduce voltage-dependent sodium or calcium fluxes and that sedative-anticonvulsant drugs would alter the binding of dihydropyridines to brain membranes. In the present study, we tested these predictions.

MATERIALS AND METHODS

Binding of [^{3}H]nitrendipine to rat cortical membranes was studied by a slight modification of the method reported by Gould *et al.* [2]. Male Sprague–Dawley rats (Sasco, Inc., Indianapolis, IN) were decapitated, and the cortex was removed and frozen at −20° until use. Initial studies indicated identical binding with fresh and frozen–thawed tissues. However, repeated freezing and thawing reduced binding, and all experiments were performed with tissue that had been frozen and thawed once only. Tissue was homogenized with 50 mM Tris–HCl (pH 7.7) (120 ml/g tissue) using a Tekmar Tissumizer at maximum speed for 20 sec. The membranes were pelleted at 48,000 *g* for 10 min, and the pellet was washed twice and resuspended with the Tris buffer. In some experiments the Tris buffer contained 4.5 mM $CaCl_2$. Aliquots of the membrane suspension containing 0.3 to 0.4 mg protein were incubated in a 1-ml volume with drugs and [^{3}H]-nitrendipine (87 Ci/mmole, New England Nuclear, Boston, MA) for 30 min at room temperature. The mixture was then rapidly filtered through Whatman GF/B filters. The filters were washed with 10 ml of ice-cold Tris–HCl using an automated Brandel filter apparatus. The amount of radioactivity on the filters was determined by liquid scintillation spectrometry.

In saturation experiments, six concentrations of [^{3}H]nitrendipine ranging from 0.004 to 2.1 nM were tested. The binding affinity (K_D) and maximum number of binding sites (B_{max}) were calculated from

† Address all correspondence to: Dr. R. A. Harris, Department of Pharmacology (C236), University of Colorado School of Medicine, Denver, CO 80262.

saturation experiments by the method of Rosenthal [11]. Inhibition studies used 0.3 to 0.4 nM [^{3}H]nitrendipine. In both saturation and inhibition studies, the non-specific binding was defined as the amount of radioactivity bound in the presence of 100 nM nifedipine (Pfizer Laboratories, New York, NY).

Drugs to be tested were added in a volume of 75 μl/ml of assay volume. Tetracaine HCl and sodium pentobarbital (Sigma Chemical Co., St. Louis, MO) were dissolved in water; phenobarbital (Sigma) was dissolved in water with NaOH, pH 8; diphenylhydantoin (Sigma) was dissolved in dimethyl sulfoxide (DMSO)–water, 1:9. The concentration of DMSO used for the latter drug [0.75% (v/v) in the assay] did not alter the binding of [^{3}H]nitrendipine.

Measurement of synaptosomal sodium uptake. Ficoll gradient centrifugation was used to prepare synaptosomes from rat brain cortex [12]. The uptake of $^{24}Na^+$ was measured by a slight modification of the procedure of Krueger and Blaustein [13]. Synaptosomes were removed from the Ficoll gradients, diluted slowly with low calcium buffer [145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM glucose, 0.02 mM $CaCl_2$ 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES), pH adjusted to 7.5 at 25° with Tris base], and pelleted at 17,000 *g* for 6 min. The pellet was resuspended in incubation buffer (140 mM choline Cl, 5 mM NaCl, 5 mM KCl, 1.4 mM $MgCl_2$, 1.0 mM $CaCl_2$, 2.0 mM NaH_2PO_4, 10 mM glucose, 20 mM Tris base, 20 mM HEPES, pH 7.4, at 25°), and 0.18-ml aliquots (0.7 to 0.9 mg protein) were incubated for 10 min at 30°. Veratridine (Sigma) was then added (0.01 ml, 0.06 mM) followed immediately by addition of 0.01-ml aliquots of drugs. All concentrations of drugs are expressed as the amount of the initial incubation volume. Ten minutes later 0.05 ml of incubation buffer containing about 150 nCi $^{24}Na^+$ and kept at 30° was added while vortexing. Uptake of $^{24}Na^+$ was timed by metronome and stopped 2 sec later by addition of 5 ml of ice-cold incubation solution and rapid filtration (Whatman GF/C). Filters were washed twice with 5 ml of wash buffer, and radioactivity was determined by liquid scintillation counting (Beckman LS9000 or LS6800 with isotope decay correction programs). The veratridine-dependent $^{24}Na^+$ uptake was calculated as the difference between the uptake in the presence of veratridine and uptake in the absence of veratridine. $^{24}NaCl$ was prepared for each experiment by the University of Missouri Research Reactor.

Uptake of $^{45}Ca^{2+}$. The following is a modification of the method of Nachshen and Blaustein [14]. Synaptosomes were prepared as described above for sodium uptake but were suspended in low calcium buffer (described above) rather than the incubation buffer used for sodium uptake. A 0.19-ml aliquot of synaptosomes (0.5 to 0.7 mg protein) was incubated for 10 min at 30°. Drug solutions or the drug vehicle (control) were then added in a volume of 0.01 ml, and incubation was continued for 10 min. Uptake was started by addition of 0.2 ml of buffer (kept at 30°) containing $^{45}Ca^{2+}$ (1.5×10^5 dpm). $^{45}CaCl_2$ was obtained from New England Nuclear (Boston, MA). For resting uptake ($-K^+$) the low calcium buffer (described above) was used, and for depolarized uptake ($+K^+$) potassium was substituted for sodium giving a final potassium concentration of 77 mM. Substitution of choline for sodium indicates that the stimulation of uptake is due to increasing potassium and not to decreasing sodium ([14], R. A. Harris, unpublished results). Uptake was halted by addition of 4 ml of ice-cold low calcium buffer and rapid filtration on Whatman GF/C filters. The filters were quickly washed with two 4-ml portions of ice-cold low calcium buffer and placed in vials for determination of radioactivity by liquid scintillation spectrometry. The uptake period was 3 sec and was timed by a metronome. The depolarization-dependent uptake (ΔK^+) was calculated as the difference between the uptakes in low potassium ($-K^+$) and high potassium ($+K^+$) solutions.

Protein was measured by the method of Lowry *et al.* [15]. Statistical comparisons were made using Student's *t*-test.

RESULTS

Three of the drugs tested, phenytoin, phenobarbital, and tetracaine, reduced the binding of [^{3}H]-nitrendipine in a concentration-dependent manner (Fig. 1). Complete inhibition of binding was obtained with phenobarbital, but not with phenytoin. Binding of [^{3}H]nitrendipine to brain membranes is reduced by removal of calcium [2], and there is evidence that sedative-anticonvulsant drugs may displace calcium from membranes [16, 17]. Thus, the reduction of [^{3}H]nitrendipine binding may merely reflect the displacement of membrane calcium by the drugs. If this were true, then the inhibitory effects of the drugs would be antagonized by addition of calcium to the buffer. However, this was not the case, as addition of 4.5 mM $CaCl_2$ to the binding assay slightly enhanced the effects of the drugs (Fig. 1).

The inhibition of [^{3}H]nitrendipine binding produced by phenytoin, phenobarbital, and tetracaine

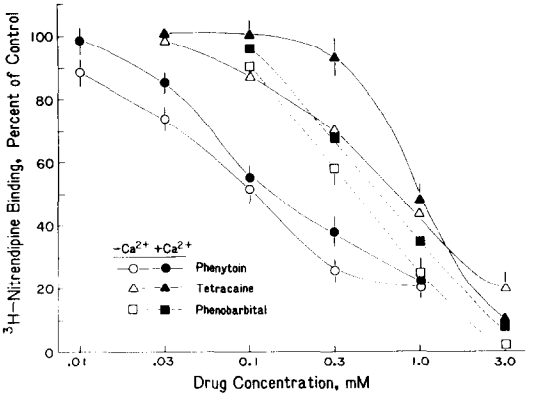

Fig. 1. Effects of phenytoin (circles), tetracaine (triangles), and phenobarbital (squares) on the binding of [^{3}H]nitrendipine to brain membranes. Open symbols represent drug effects in the absence of calcium; filled symbols show drug effects in the presence of 4.5 mM $CaCl_2$. Values are mean ± S.E.M., N = 3–4. Specific binding was 4366 ± 156 cpm/mg protein in the absence of calcium and 5191 ± 154 in the presence of calcium.

Table 1. Saturation analysis of the effects of drugs on the binding of [^{3}H]nitrendipine to brain membranes*

Addition	K_D (nM)	B_{max} (fmoles/mg protein)
Control	0.42 ± 0.02	145 ± 10
Pentobarbital (0.5 mM)	1.37 ± 0.08†	202 ± 11†
Phenytoin (0.2 mM)	2.75 ± 0.60†	186 ± 35
Tetracaine (0.3 mM)	0.98 ± 0.13†	148 ± 14

* Values represent mean ± S.E.M.; N = 5 for all additions except phenytoin, where N = 3.
† Significantly different from control, $P < 0.01$.

Table 2. Effects of anesthetic and anticonvulsant drugs on the binding of [^{3}H]nitrendipine to brain membranes

Drug	IC_{50} (mM)
Phenytoin	0.094 ± 0.007
Phenobarbital	0.400 ± 0.071
Tetracaine	0.475 ± 0.024
Pentobarbital	0.757 ± 0.057
Ethanol	>1000
Inactive at the indicated concentrations	
Valproic acid	(0.1 to 3 mM)
Ethosuximide	(0.1 to 3 mM)
Carbamazepine	(0.03 to 1 mM)
Morphine	(0.01 to 10 μM)
Ketocyclazocine	(0.01 to 10 μM)
Naloxone	(0.01 to 10 μM)
Veratridine	(1 to 30 μM)
Batrachotoxin	(0.1 to 1 μM)
Tetrodotoxin	(0.1 to 3 μM)

could be due either to a change in the affinity of binding (K_D) or the maximal number of binding sites (B_{max}). Saturation experiments demonstrated that the [^{3}H]nitrendipine bound to brain membranes with an affinity of 0.42 nM and a B_{max} of 145 fmoles/mg protein. The affinity is in reasonable agreement with the results of Gould *et al.* [2], who found an affinity of 0.13 nM by saturation, 0.23 nM by kinetic analysis, and 0.55 nM by inhibition. Our B_{max} agrees well with that of Gould *et al.* [2] (13 pmoles/g wet weight), since 8% of the wet weight of the brain is protein [18]. The effect of the drugs was to decrease the affinity of [^{3}H]nitrendipine for the membranes with little change in the number of binding sites (Table 1).

We next tested other anticonvulsant and anesthetic drugs to determine if the reduction of [^{3}H]nitrendipine binding produced by phenytoin and phenobarbital was a general attribute of either of these drug classes. Pentobarbital inhibited binding of [^{3}H]-nitrendipine, but was less potent than phenobarbital (Table 2). Ethanol was a weak inhibitor; a concentration of 1000 mM reduced binding by only 30–40%. Several anticonvulsant drugs were without effect on [^{3}H]nitrendipine binding, as were opiate agonists and antagonists. Toxins that activate (veratridine, batrachotoxin) or block (tetrodotoxin) neuronal sodium channels also failed to alter binding (Table 2). At the concentrations tested, each of these toxins alters brain synaptosomal sodium fluxes [19].

The sedative-anticonvulsant drugs that inhibit [^{3}H]nitrendipine binding to brain membranes also inhibit voltage-dependent calcium and sodium channels of brain synaptosomes [9]. This raised the possibility that sedative-anticonvulsant drugs act through dihydropyridine receptors, as suggested for benzodiazepines [20]. If this were true, then the dihydropyridines might either mimic these drugs to reduce calcium and sodium fluxes or antagonize the inhibition produced by sedative-anticonvulsant drugs. However, dihydropyridines produced only a slight reduction of the voltage-dependent uptake of $^{45}Ca^{2+}$ or $^{24}Na^+$, and they did not alter the inhibitory effects of phenytoin, ethanol, or pentobarbital on these fluxes (Tables 3 and 4). Synaptosomal calcium influx

Table 3. Effects of dihydropyridines alone and in combination with phenytoin on voltage-dependent uptake of $^{45}Ca^{2+}$ by cortical synaptosomes

	$^{45}Ca^{2+}$ uptake* (nmoles/mg protein)	% Change
Vehicle†	0.35 ± 0.01	
Nimodipine, 0.01 μM	0.35 ± 0.01	0
0.1 μM	0.37 ± 0.02	6
1 μM	0.35 ± 0.01	0
10 μM	0.32 ± 0.02	−9
Nifedipine, 0.01 μM	0.36 ± 0.01	3
0.1 μM	0.35 ± 0.01	0
Vehicle + phenytoin, 0.4 mM	0.26 ± 0.03‡	−26
Phenytoin + nifedipine, 0.01 μM	0.23 ± 0.01‡	−34
Phenytoin + nifedipine, 0.1 μM	0.22 ± 0.01‡	−37

* Depolarization-dependent uptake of $^{45}Ca^{2+}$ by synaptosomes from rat cortex. Values are mean ± S.E.M., N = 3–6. Uptake time was 3 sec.
† Dimethyl sulfoxide (0.25%, v/v) was the vehicle for the dihydropyridines.
‡ Significantly different from vehicle alone, $P < 0.05$.

Table 4. Effects of dihydropyridines alone and in combination with other drugs on voltage-dependent uptake of $^{24}Na^+$ by cortical synaptosomes

	$^{24}Na^+$ uptake* (nmoles/mg protein)	% Change
Vehicle†	2.10 ± 0.11	
Nitrendipine, 1 μM	1.91 ± 0.09	−9
Nifedipine, 1 μM	1.73 ± 0.10‡	−18
Nimodipine, 1 μM	1.81 ± 0.05‡	−14
Nimodipine, 0.1 μM	1.88 ± 0.19	−10
Vehicle† + ethanol, 400 mM	0.79 ± 0.22‡	−62
Ethanol + nimodipine, 0.1 μM	0.68 ± 0.10‡	−68
Vehicle† + phenytoin, 0.1 mM	1.36 ± 0.16‡	−35
Phenytoin + nimodipine, 0.1 μM	1.44 ± 0.18‡	−31
Vehicle† + pentobarbital, 0.8 mM	1.51 ± 0.31‡	−28
Pentobarbital + nimodipine, 0.1 μM	1.11 ± 0.16‡	−47

* Veratridine-dependent uptake of $^{24}Na^+$ by synaptosomes from rat cortex. Values are mean ± S.E.M., N = 3–6. Uptake time was 2 sec.

† Dimethyl sulfoxide (0.4%, v/v) was the vehicle for the dihydropyridines.

‡ Significantly different from vehicle alone, $P < 0.05$.

consists of a fast and a slow phase [14]. The uptake measured in the present studies (3-sec flux) represents mainly fast phase influx [21, 22]. The lack of effect of dihydropyridines on synaptosomal calcium fluxes is in agreement with other studies of both the fast and slow phases of uptake [4, 23].

DISCUSSION

The two drugs most widely used in the treatment of generalized tonic-clonic seizures and partial seizures, phenytoin and phenobarbital, inhibited the binding of [^{3}H]nitrendipine to brain membranes. Maximal therapeutic serum concentrations of these drugs are about 0.08 and 0.2 mM respectively [24]. The concentrations required to affect [^{3}H]nitrendipine binding are therefore similar to serum concentrations found *in vivo*. It should be noted, however, that phenytoin is 90% bound to serum proteins and phenobarbital is 50% bound [25], indicating that the free concentrations of the drugs in cerebrospinal fluid may be less than was required to affect the calcium antagonist site on brain membranes. Nimodipine, a dihydropyridine compound that is chemically and pharmacologically related to nitrendipine, displays anticonvulsant activity in mice [8]. Thus, it is possible that the dihydropyridine binding site is related to anticonvulsant mechanisms and is one site of action for phenytoin and phenobarbital. The relatively low affinity of the latter drugs for the dihydropyridine site, and the low potency of nimodipine as an anticonvulsant [8], may indicate that this site does not represent the only locus of anticonvulsant drug action. Although phenytoin and phenobarbital produce sedative effects in addition to their anticonvulsant action, it is unlikely that these effects are due to an action on the dihydropyridine site because pentobarbital is a more potent sedative than phenobarbital, yet pentobarbital displays a weaker affinity than phenobarbital for the [^{3}H]-nitrendipine binding site. This observation also rules out membrane disordering as the mechanism responsible for reduction of [^{3}H]nitrendipine binding, because pentobarbital is several times more potent than phenobarbital in perturbing brain membranes [26].

The drugs that were effective in inhibiting binding of [^{3}H]nitrendipine also inhibit voltage-dependent uptake of calcium by isolated brain synaptosomes [9, 10]. However, differences in drug potency make it unlikely that these two effects are related. For example, pentobarbital is more potent that phenobarbital in inhibiting calcium uptake [10, 27], but the opposite is true for [^{3}H]nitrendipine bindings. Likewise, 50–100 mM ethanol reduces synaptosomal calcium uptake [28, 29], yet concentrations of 500–1000 mM were required to significantly reduce [^{3}H]-nitrendipine binding. These data, together with those showing that dihydropyridine compounds do not inhibit synaptosomal calcium uptake [4, 5], suggest that the dihydropyridine binding site in brain is not a part of the voltage-dependent calcium channel.

Bolger *et al.* [7] found that veratridine, a selective activator of voltage-dependent sodium channels, displaces [^{3}H]nitrendipine from binding sites on ileal smooth muscle and suggested that sodium and calcium channels may share similar subunits, including those containing dihydropyridine binding sites. This raised the possibility that in brain these drugs may act on sodium channels rather than calcium channels. However, we found that veratridine, batrachotoxin and tetrodotoxin did not reduce the binding of [^{3}H]-nitrendipine to brain membranes. This suggests a difference between the dihydropyridine sites on brain and ileal membranes. We also studied the veratridine-stimulated uptake of $^{24}Na^+$ by rat cortical synaptosomes and found that nitrendipine, nifedipine and nimodipine were not potent inhibitors of $^{24}Na^+$ fluxes.

In summary, sedative-anticonvulsant drugs reduced the binding of [^{3}H]nitrendipine to brain membranes, and this action may be related to the anticonvulsant activity of dihydropyridines, bar-

biturates, and hydantoins. The reduction of [^{3}H]-nitrendipine binding by sedative-anticonvulsant drugs did not appear to be due to the displacement of membrane calcium, membrane fluidization or effects on voltage-dependent calcium or sodium channels.

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