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EFFECTS OF DIHYDROPYRIDINE CALCIUM CHANNEL BLOCKING DRUGS ON RAT BRAIN MUSCARINIC AND α-ADRENERGIC RECEPTORS

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Abstract—The dihydropyridine (DHP) Ca^{2+} channel blocking drugs nicardipine, nitrendipine, nimodipine, felodipine, nifedipine and nisoldipine were examined for activity in inhibiting specific (−)-[^{3}H]QNB and [^{3}H]WB4101 binding to the muscarinic and α-adrenergic receptors, respectively, of rat brain. Muscarinic receptor binding was affected most by nicardipine, with felodipine having less activity; the other DHP drugs were essentially inactive at 3×10^{-5} M. The (+)-stereoisomer of nicardipine ($K_I = 4.07 \times 10^{-7}$ M) was 27 times more potent than the (−)-isomer in inhibiting [^{3}H]QNB binding, and this inhibition was found to be competitive. This inhibitory effect of nicardipine was not mediated via interaction with the high-affinity DHP binding site assumed to be associated with a Ca^{2+} channel. (+)-Nicardipine inhibited the binding of [^{3}H]nitrendipine to this DHP binding site of brain, with a K_I of 9.01×10^{-11} M, and was 10 times more potent than the (−)-isomer. Thus, the muscarinic receptor was 4200 times less sensitive to (+)-nicardipine than was this DHP binding site. Nicardipine was also the most potent DHP drug inhibiting [^{3}H]WB4104 binding to the α-adrenergic receptor, although the other drugs were also somewhat active, in the rank order sequence listed above. This effect of nicardipine on the adrenergic receptor was also stereoselective, with (+)-nicardipine ($K_I = 3.46 \times 10^{-7}$ M) being about 3 times more potent than the (−)-isomer, in producing competitive inhibition of radioligand binding. These data suggest that the effects on brain receptors occur as a result of direct, stereospecific effects of DHP drugs on these receptors and are not due to Ca^{2+} channel blocking activity of these drugs.

The Ca^{2+} entry blocking drugs verapamil and its methoxy analog, D600, are known to inhibit the binding of labeled agonists or antagonists to muscarinic, α-adrenergic and opiate receptors assayed variously in brain, liver, heart and NG 108–15 neuroblastoma-glioma cells [1–4]. These effects on different types of receptors in brain have been suggested to occur as a result of a common non-specific action such as perturbation of membrane fluidity which then might lead to impairment of receptor-binding function [1]. Since binding of the radioantagonist [^{3}H]QNB to the muscarinic receptor of the guineapig ileum is inhibited equally by racemic D600 and by its stereoisomers whereas the Ca^{2+} entry blocking function of D600 is stereoselective [5], it appears that the effects of D600 on the guinea pig ileal muscarinic receptor, and perhaps on receptors generally, are not related to the effects of D600 on Ca^{2+} entry.

The dihydropyridine (DHP) Ca^{2+} entry blocking drugs such as nifedipine and its analogs bind with high affinity to various tissues [6–10]. These drugs appear to act primarily on channels controlling Ca^{2+} entry in smooth muscle and in that manner are more specific than D600, so that the DHP drugs might be useful in exploring further any association between Ca^{2+} channel blocking and receptor functions. This report describes the inhibitory effects of a number of DHP drugs on radioligand binding to muscarinic and α-adrenergic receptors of rat brain, shows that the highly asymmetrical DHP drug nicardipine was the most active of these drugs studied, and demonstrates that the (+)- and (−)-stereoisomers of nicardipine differed in inhibitory potency. The results of this study suggest that interaction of the dihydropyridine channel blockers with the brain high-affinity DHP binding site (and possibly, therefore, with brain Ca^{2+} channels) does not lead to perturbation of receptor radioligand binding, but that the effects on brain receptors occur as a direct result of DHP drug interaction with these receptors.

MATERIALS AND METHODS

Muscarinic receptor binding activity of rat brain striatal preparations was determined with the (−)-isomer of [^{3}H]QNB, and α-adrenergic receptor binding activity of rat brain cortex preparations was determined with [^{3}H]WB4101, as described previously [1]; specific binding was defined as that binding which was decreased by the presence of 10^{-6} M atropine or 10^{-4} M norepinephrine, respectively, in the incubation mixtures. All assays were run in triplicate, and repeated at least three times. Inhibitions of radioligand binding were calculated from comparisons of experiments run with/without the DHP drug being investigated. Because of the limited aqueous solubility of the DHP drugs, they were made up as stock solutions of 3×10^{-3} M in ethanol; all incubation systems contained a final concentration of 1% (v/v) ethanol, which itself did not affect radio-

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ligand binding. [^{3}H]Nitrendipine binding to rat brain cortex was determined as described previously [9], with the inclusion of 10^{-6} M nifedipine to define the specific binding. K_I values of the radioligands and the DHP drugs were calculated from experimentally determined IC_{50} values as described earlier [1]. Relative lipid solubilities of the DHP drugs (R_m values) were determined as described by Rodenkirchen *et al.* [11]. The drugs were dissolved in methanol and spotted on reverse-phase No. 52021 thin-layer chromatography plates (Analtech, DE) and developed in methanol:water (1:1). R_f values were measured and then R_m values were calculated from the relationship $R_m = \log [(1/R_f) - 1]$. Higher R_m values indicate drugs to be more lipophilic than drugs of lower R_m values. Protein was assayed by the method of Lowry et al. [12] using bovine serum albumin as standard. Radioligands were purchased from the New England Nuclear Corp. Dihydropyridine drugs were contributed by the following companies: Yamamouchi Chemical Co., Japan [(+)- and (−)-nicardipine]; A. B. Hassle, Sweden (felodipine); Miles Laboratories (nimodipine, nitrendipine, nisoldipine); Syntex Laboratories (racemic nicardipine). Nifedipine was synthesized in this laboratory.

RESULTS

The structures of the various DHP drugs used in this study are shown in Fig. 1. It can be seen that the greatest degree of asymmetry on the dihydropyridine ring (substitutions R_1 and R_2) is present in nicardipine. These drugs were examined for their ability to inhibit the specific binding of [^{3}H]QNB to the rat brain muscarinic receptor, each being used in the racemic form at a final concentration of 3×10^{-5} M, which is the highest concentration attainable in the incubation mixture used here. It can be seen from Table 1 that at this concentration nicardipine almost completely inhibited the binding of [^{3}H]QNB, with felodipine being the second most active drug. The other DHP drugs tested had little activity on the binding of this radioligand by the brain muscarinic receptor and were not examined further because of their limiting solubilities in the aqueous assay systems used. Also shown in Table 1 are the R_m values, calculated from the R_f values determined on reverse-phase thin-layer chromatography plates as described in Methods, which reflect the relative lipid solubilities of these DHP drugs; in this comparison nicardipine is seen to be the most lipid soluble drug.

Table 1. Inhibition of the binding of [^{3}H]QNB to the rat brain muscarinic receptor produced by various DHP drugs*

Drug	% Inhibition of [^{3}H]QNB binding	R_m
Nicardipine	96 ± 1.7	1.47
Felodipine	44 ± 6.9	1.00
Nifedipine	6.2 ± 3.6	0.26
Nitrendipine	5.1 ± 3.3	0.60
Nisoldipine	4.9 ± 2.5	0.85
Nimodipine	1.3 ± 0.8	0.71

* Specific [^{3}H]QNB binding to rat brain striatal membranes was determined as described in Methods. Racemic forms of the DHP drugs were tested at a final concentration of 3×10^{-5} M (the highest obtainable in these conditions), and the inhibitions were calculated. Values show the means ± S.E. of at least three replicate experiments, each performed in triplicate. R_m values were calculated from the equation $R_m = \log [(1/R_f) - 1]$ as described in Methods, and reflect the relative lipid solubilities of these drugs, with nicardipine having the greatest lipid solubility.

Since studies with D600 had shown that inhibition of radioligand binding to the guinea pig ileum muscarinic receptor is not stereoselective whereas the Ca^{2+} channel blocking activity of D600 on the same tissue is stereoselective [5], it was of interest to test the inhibitory effects of the stereoisomers of nicardipine on [^{3}H]QNB binding to the rat brain muscarinic receptor. Figure 2 shows probit plots of these inhibitions which indicate that the (+)-stereoisomer of nicardipine ($K_I = 4.07 \times 10^{-7}$ M) was 27 times more potent than the (−)-stereoisomer ($K_I = 1.12 \times 10^{-5}$ M) in inhibiting radioligand binding to this receptor. The mechanism underlying this inhibition of [^{3}H]QNB binding by (+)-nicardipine is shown in the Scatchard plot of Fig. 3. It can be seen that the apparent dissociation constant of the radioligand–receptor interaction was dependent on

DRUG	SUBSTITUTIONS AT POSITION			
	R_1	R_2	S_m	S_o
Nicardipine	$-(CH_2)_2.N.CH_3.CH_2.C_6H_6$	$-CH_3$	$-NO_2$	H
Felodipine	$-CH_2.CH_3$	$-CH_3$	$-Cl$	$-Cl$
Nitrendipine	$-CH_3$	$-C_2H_5$	$-NO_2$	H
Nifedipine	$-CH_3$	$-CH_3$	H	$-NO_2$
Nisoldipine	$-CH_3$	$-CH_2.CH(CH_3)_2$	H	$-NO_2$
Nimodipine	$-CH_2.CH_2.OCH_3$	$-CH(CH_3)_2$	$-NO_2$	H

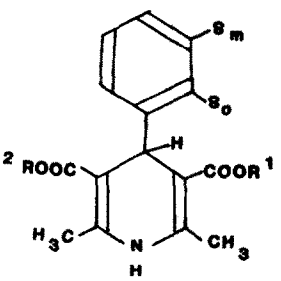

Fig. 1. Structures of the dihydropyridine Ca^{2+} channel blocking drugs used in this study.

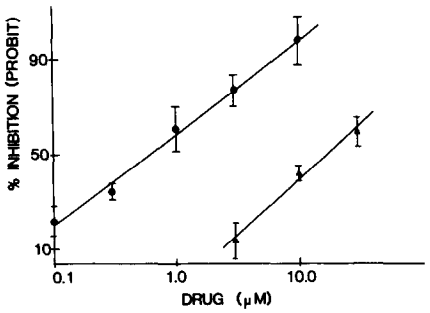

Fig. 2. Probit plot of the inhibition of specific [^{3}H]QNB binding to the rat brain muscarinic receptor produced by the stercoisomers of nicardipinc. [^{3}H]QNB binding was measured in three separate experiments as described in Methods, and either (+)-nicardipine (●) or (−)-nicardipine (▲) was present. IC_{50} concentrations from these plots were 6.1×10^{-7} M and 1.68×10^{-5} M for the (+)- and (−)-stereoisomers, which gave calculated K_I values of 4.07×10^{-7} M and 1.12×10^{-5} M respectively. The ratio of these K_I values is 27.

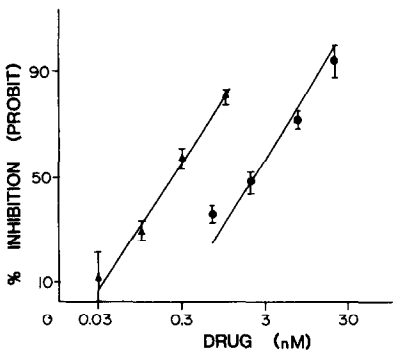

Fig. 4. Probit plot of the inhibition of specific [^{3}H]nitrendipine binding to rat brain membranes produced by the stereoisomers of nicardipine. [^{3}H]nitrendipine binding was measured in three separate experiments as described in Methods and either (+)-nicardipine (▲) or (−)-nicardipine (●) was present. IC_{50} Concentrations from these plots were 2.28×10^{-10} M and 2.26×10^{-9} M for the (+)- and (−)-stereoisomers, which gave calculated K_I values of 9.01×10^{-11} M and 8.93×10^{-10} respectively. The ratio of these K_I values is 10.

the concentration of (+)-nicardipine, whereas the maximum number of binding sites was unaffected, i.e. the inhibition appeared to be competitive.

A series of DHP drugs has been shown by Gould *et al.* [6] to differ somewhat in their high affinities for the [^{3}H]nitrendipine binding site in brain which has been postulated to be closely associated with a brain Ca^{2+} channel. In that study felodipine was the most active of the DHP drugs tested, but nicardipine was not examined. Comparisons were therefore made in this laboratory of the affinities of the two stereoisomers of nicardipine for the high-affinity [^{3}H] nitrendipine binding site of rat brain. Figure 4 shows the probit plots, IC_{50} values and K_I constants of these drugs inhibiting [^{3}H]nitrendipine binding, and indicates that (+)-nicardipine with a K_I of 9.01×10^{-11} M was about 10 times as potent as (−)-nicardipine. Racemic felodipine was found (in other experiments not shown here) to have a K_I of 5.91×10^{-10} M at the DHP binding site and was thus less potent than (+)-nicardipine. Comparison of the affinities of (+)-nicardipine interacting with both this high-affinity DHP binding site ($K_I = 9.01 \times 10^{-11}$ M) and with the muscarinic receptor ($K_I = 4.07 \times 10^{-7}$ M) shows that this receptor was approximately 4200 times less sensitive to (+)-nicardipine than was the DHP binding site. Since inhibition of [^{3}H]QNB binding by the brain muscarinic receptor was also markedly stereoselective (Fig. 2) and since nicardipine has a very bulky sidechain at position 3 on the dihydropyridine ring, it seemed possible that the stereoselective effect of (+)-nicardipine on brain muscarinic receptor binding might be due to a secondary steric hindrance of adjacent muscarinic receptor structure occurring subsequent to a primary interaction of nicardipine with the DHP binding site. To test this possibility, the effect of (+)-nicardipine on [^{3}H]QNB binding was also measured in the presence of a concentration of racemic felodipine which would produce, via competitive inhibition, an obvious decrease in the interaction of nicardipine with the DHP binding site-Ca^{2+} channel, but which would produce little direct effect of felodipine on the muscarinic receptor. Table 2 shows that there was no significant change in the inhibition of specific [^{3}H] QNB binding produced by 10^{-6} M (+)-nicardipine when 10^{-5} M racemic felodipine was also present in the reaction mixture. These results strongly suggest that the inhibitory effect of nicardipine on the brain muscarinic receptor is not mediated via occupation of a DHP binding site associated with the Ca^{2+} channel.

Some experiments were performed to measure the abilities of various DHP drugs to inhibit the binding of the antagonist [^{3}H]WB4101 to the rat brain α-receptor. Table 3 lists these inhibitions and shows

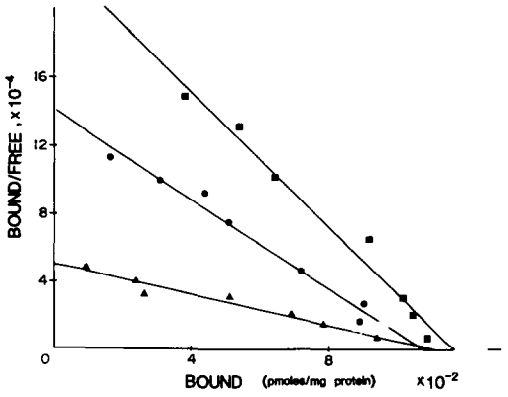

Fig. 3. Scatchard plot analysis of the mechanism of action of (+)-nicardipine inhibiting specific [^{3}H]QNB binding to the rat brain muscarinic receptor. [^{3}H]QNB binding was measured as described in Methods, in the absence (■) of DHP drug and in the presence of 1.5×10^{-7} M (●) and 10^{-6} M (▲) (+)-nicardipine. Intercepts on the X-axis are not statistically different. A representative experiment is shown from the three replicate experiments performed.

Table 2. Effect of addition of felodipine on the inhibition of [^{3}H] QNB binding to the rat brain muscarinic receptor produced by (+)-nicardipine*

Experiment	DHP drug present	% Inhibition
A	(+)-Nicardipine	62
	Felodipine	0
	(+)-Nicardipine + felodipine	66
B	(+)-Nicardipine	63
	Felodipine	8
	(+)-Nicardipine + felodipine	76

* Specific [^{3}H]QNB binding to rat brain membranes was determined as described in Methods. Results of two experiments (A and B) are shown, each run in triplicate. (+)-Nicardipine was present at a 10^{-6} M concentration, and racemic felodipine at 10^{-5} M. The K_I values for (+)-nicardipine and racemic felodipine inhibiting [^{3}H]nitrendipine binding were determined in separate experiments to be 9.01×10^{-11} M and 5.91×10^{-10} M respectively.

that, when the racemic forms of the DHP drugs were tested at concentrations of 3×10^{-5} M, nicardipine again was the most active drug. However, several of the other DHP drugs also exhibited activity against the α-receptor, the rank order of inhibitory activities being nicardipine > nitrendipine > nimodipine > felodipine > nifedipine > nisoldipine. The stereoisomers of nicardipine were then assayed for activity in inhibiting [^{3}H]WB4101 binding by brain membranes, and Fig. 5 shows probit plots of these experiments, and the IC_{50} values, from which the K_I values were calculated. The (+)-stereoisomer of nicardipine ($K_I = 3.46 \times 10^{-7}$ M) was found to be only about 2.8 times more potent than the (−)-nicardipine stereoisomer ($K_I = 9.7 \times 10^{-7}$ M) in inhibiting the brain α-receptor binding, indicating that this receptor was clearly not as differentially affected by the two nicardipine stereoisomers as was the brain muscarinic receptor (Fig. 2). Also, as shown in the Scatchard plot of Fig. 6, the apparent dissociation constant of [^{3}H]WB4101 binding to the α-receptor was dependent on the concentration of (+)-nicardipine added although the number of radioligand binding sites was unaffected, so that nicardipine appears to inhibit this receptor competitively also. Atlas and Adler [4] also have shown that racemic

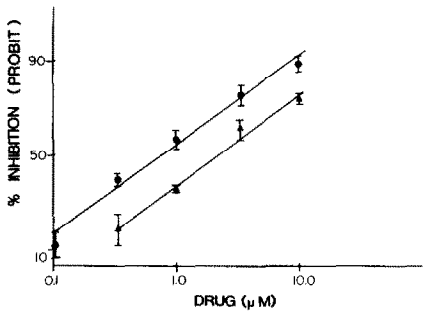

Fig. 5. Probit plots of the inhibition of specific [^{3}H]WB4101 binding to the rat brain α-adrenergic receptor. [^{3}H]WB4101 binding was measured in three separate experiments as described in Methods, and either (+)-nicardipine (●) or (−)-nicardipine (▲) was present. IC_{50} Concentrations from these plots were 7.5×10^{-7} M and 2.1×10^{-6} M for the (+)- and (−)-stereoisomers, which gave calculated K_I values of 3.46×10^{-7} M and 9.7×10^{-7} M respectively. The ratio of these K_I values is 2.8.

Table 3. Inhibitions of the binding of [^{3}H]WB4101 to the rat brain α-adrenergic receptor produced by various DHP drugs*

Drug	% Inhibition of [^{3}H]WB4101 binding
Nicardipine	90.5 ± 6
Nitrendipine	45 ± 4
Nimodipine	32 ± 6.1
Felodipine	27 ± 7.6
Nifedipine	21 ± 8.1
Nisoldipine	14 ± 11

* Specific [^{3}H]WB4101 binding to rat brain membranes was determined as described in Methods. Racemic forms of the DHP drugs were tested at 3×10^{-5} M (the highest obtainable in these conditions), and the inhibitions of radioligand binding were calculated. Values show the means ± S.E. of at least three replicate experiments, each performed in triplicate.

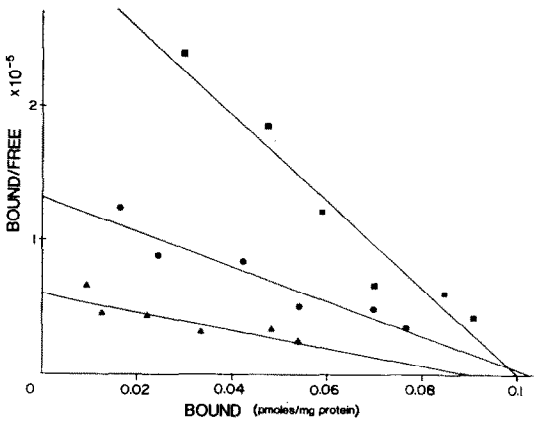

Fig. 6. Scatchard plot analysis of the mechanism of action of (+)-nicardipine inhibiting specific [^{3}H]WB4101 binding to the rat brain α-adrenergic receptor. [^{3}H]WB4101 binding was measured as described in Methods, in the absence (■) of DHP drug and in the presence of 2×10^{-7} M (●) and 1.5×10^{-6} M (▲) (+)-nicardipine. Intercepts on the X-axis are not statistically different. A representative experiment is shown from the three replicate experiments performed.

nicardipine (YC-93) inhibits the binding of [^{3}H] WB4101 to rat brain membranes, with a dissociation constant of 0.93×10^{-6} M.

DISCUSSION

Earlier work from this laboratory had shown that the Ca^{2+} entry blocking drug D600 additionally caused inhibition of radioligand binding to rat brain muscarinic, α-adrenergic and opiate receptors and therefore suggested caution in interpreting effects of D600 on biological systems as being solely due to Ca^{2+} channel effects [1]. A relationship between effects on receptors and on Ca^{2+} channels was then suggested to be possibly due to some non-specific action on membrane structure. Also, a direct relationship between effects on receptors and on Ca^{2+} channels was suggested to be unlikely because the Ca^{2+} blocking activity of D600 was stereoselective whereas the effects of D600 on muscarinic receptors occurred equally well with the two stereoisomers [5]. The availability of several dihydropyridine Ca^{2+} channel blocking drugs having greater selectivity than D600 permitted us to re-examine the question as to whether blockade of radioligand binding by selected brain receptors occurred as a result of effects at brain DHP binding sites-Ca^{2+} channels. The results indicate that, although all the DHP drugs tested can bind with high affinity to the DHP binding site-Ca^{2+} channel, nicardipine was by far the most potent of these drugs in inhibiting brain muscarinic receptors. It would thus appear that interaction with the DHP binding site by these DHP drugs does not generally lead to receptor inhibition.

It has been assumed that in brain the high-affinity DHP binding site is associated with a Ca^{2+} channel, since such a binding site-Ca^{2+} channel relationship has been demonstrated in guinea pig ileal muscle [8]. However, it is known that the DHP drug nifedipine does not inhibit K^+-stimulated $^{45}Ca^{2+}$ entry into rat brain synaptosomes [13] and it is uncertain whether such DHP drugs block brain Ca^{2+} channels, even though brain contains a high-affinity DHP binding site [6, 7]. It is clear, however, that at high concentrations, effects of the DHP drugs in the brain cannot be assumed to occur solely at Ca^{2+} channels which may be associated with these high-affinity binding sites.

It is not obvious as to why nicardipine was the most active DHP drug of those studied, in interfering with the brain muscarinic receptor. Nicardipine contains a side chain which is possibly protonated at physiological pH, so that perhaps such a protonated structure is partly responsible for receptor interaction. Nicardipine exhibited the greatest asymmetry, having a large hydrophobic group attached to the dihydropyridine ring. This may have some significance since DHP drugs having non-identical ester groups on the heterocyclic ring have been claimed to be more potent than the corresponding dihydropyridines with identical ester groups [14]. Nicardipine was also the most lipid soluble drug of this group, suggesting that distribution of the drug into membrane lipid components may be related to interference with receptor function, although the relative lipid solubility clearly cannot explain the different activities of the nicardipine stereoisomers. Also, the (+)-isomer of nicardipine had the greatest affinity for the DHP binding site, although competition for this binding site produced by the concurrent presence of felodipine (Table 2) did not affect the action of nicardipine on the muscarinic receptor, thus indicating that binding of nicardipine to the DHP site-Ca^{2+} channel is not related to receptor interference.

It is generally accepted that differential biological activity of the stereoisomers of a drug indicates that such activity occurs via interaction with a specific receptor or binding site exhibiting a structure complementary to the active stereoisomer. Other workers have shown stereospecificity of action of DHP drugs in the inhibition of contractility of guinea pig ileal muscle [15], inhibition of the binding of [^{3}H] nimodipine to a skeletal muscle membrane preparation [10] and in effects of nicardipine on vertebral blood flow [16]. Thus, the finding that the (+)-stereoisomer of nicardipine was some 27 times more active than the (−)-isomer in inhibiting [^{3}H]QNB binding to the muscarinic receptor could mean that interference with this receptor results from a direct action of nicardipine at the receptor or at a site affecting receptor activity, rather than a non-specific effect occurring via membrane structure perturbation, unless this perturbation also exhibited stereospecificity. The fact that nicardipine competitively inhibited radioligand binding by the muscarinic receptor could also be taken as evidence for a receptor interaction either at the radioligand binding site or at an allosteric site, although this evidence must be interpreted cautiously since competitive inhibition was also observed earlier in a study of the effects of D600 on these receptors [1]. The fact that effects of nicardipine on the α-adrenergic receptor were also stereospecific and also exhibited competitive inhibition suggests a similar direct interaction mechanism, although the ratio of activity of the nicardipine stereoisomers on this receptor was considerably smaller. However, the finding that both muscarinic and α-adrenergic receptors were affected by nicardipine is puzzling, since the active sites of these receptors are different in that they recognize structurally dissimilar transmitters. Further work is planned to determine if other brain receptors are affected by the DHP drugs.

The results of this study may also have bearing on another problem, which concerns the mechanism by which DHP drugs, known to block Ca^{2+} channels in other tissues, could affect the brain. It seems likely that Ca^{2+} channels of brain are different from those of smooth muscle, since brain Ca^{2+} channels appear not to be blocked by DHP drugs even though brain contains a high-affinity DHP binding site. However, the present study has demonstrated that selected DHP drugs can interact with the brain muscarinic and α-adrenergic receptors. We therefore suggest caution in the interpretation of the effects of DHP drugs in brain, since these drugs at high concentrations will affect neurotransmitter receptors.

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