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VERAPAMIL INTERACTION WITH THE MUSCARINIC RECEPTOR: STEREOSELECTIVITY AT TWO SITES*†

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Abstract—Verapamil, in addition to blocking calcium channels, exhibits such "non-specific" effects on myocardium as inhibition of sodium and potassium conductances and modifications of muscarinic receptor–ligand interactions. To characterize further the effects of verapamil on the cardiac muscarinic receptor, we examined the abilities of the enantiomers of verapamil to modify the binding of the muscarinic antagonist [^{3}H]quinuclidinyl benzilate ([^{3}H]QNB) to purified canine sarcolemmal vesicles. Membranes were incubated with [^{3}H]QNB and various concentrations of racemic, (+)-, or (−)-verapamil (25 or 37°, pH 7.4), and reactions were terminated by rapid filtration. (−)-Verapamil (K_i of 5.3 ± 0.2 μM) was twice as potent an inhibitor of equilibrium binding as (+)-verapamil (K_i of 11.4 ± 0.6 μM), and this effect resulted from the ability of each enantiomer to slow [^{3}H]QNB–receptor association. This degree of stereoselectivity, albeit at nanomolar concentrations, was similar to that observed for each enantiomer to compete for the specific phenylalkylamine site in this preparation. Verapamil also inhibited [^{3}H]QNB–receptor dissociation, but this effect required high concentrations and demonstrated stereoselectivity opposite to that observed for association. These findings support the view that verapamil interacts with two distinct sites, possibly within membrane lipid, each with a different affinity and preference for (+)- and (−)-verapamil, to modify the muscarinic receptor.

Verapamil and other structurally related phenylalkylamine calcium channel blocking agents, in addition to inhibiting transmembrane calcium fluxes through the slow inward channel, exert a number of other effects on membrane function *in vitro*. Verapamil decreases sodium and potassium conductances in nerve and muscle [1–3], alters neurotransmitter reuptake in rat forebrain synaptosomes [4], and inhibits specific radioligand binding to muscarinic, alpha-adrenergic and opiate receptors in membrane preparations from a number of different tissues [5–9]. These diverse and apparently unrelated effects of verapamil have led to the hypothesis that phenylalkylamines alter membrane function through non-specific interactions with membranes [6]. More recent reports, however, suggest that verapamil may alter ligand binding in a more specific manner, possibly by interacting with each receptor or sites closely related to the receptor [7, 9]. If verapamil acts as a specific muscarinic receptor ligand, its interaction with the receptor should, like that of other specific ligands, demonstrate stereoselectivity [10, 11].

The phenylalkylamine receptor in heart and skeletal muscle has been reported to demonstrate stereoselectivity for a number of ligands [12–14]. A preliminary report demonstrated that racemic verapamil had a greater ability than the (+) enantiomer to inhibit muscarinic antagonist binding to crude rat myocardial membranes [15]; but inhibition of muscarinic antagonist binding to guinea-pig ileal or rat vas deferens smooth muscle by D-600 did not demonstrate stereoselectivity [16]. To assess the degree to which the interaction of verapamil with cardiac muscarinic receptors exhibits stereoselectivity, the abilities of verapamil enantiomers to modify muscarinic antagonist binding to a purified canine sarcolemmal preparation were examined.

MATERIALS AND METHODS

Membrane preparation. Purified sarcolemmal vesicles were prepared from canine ventricles according to the method of Jones *et al.* [17]. Detailed characterization of this preparation has been reported previously [18]. Sarcolemmal vesicles were stored at −80° in 0.25 M sucrose, 20 mM HEPES†† (pH 7.8). Protein concentrations were determined as described

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†† Abbreviations: HEPES, N-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; [^{3}H]QNB, tritiated quinuclidinyl benzilate; D-888, desmethoxyverapamil; and NMS, N-methylscopolamine.

by Lowry *et al.* [19] with bovine serum albumin as standard.

Radioligand binding studies. Equilibrium binding of tritiated quinuclidinyl benzilate ([^{3}H]QNB) was examined following incubation of 10 μg of sarcolemmal vesicles with [^{3}H]QNB (15–900 pM) for 90 min at 25° in 50 mM Tris–HCl (pH 7.4), 5 mM NaH_2PO_4, 5 mM $MgCl_2$, in the presence or absence or selected concentrations of racemic, (+)- or (−)-verapamil (0.1 to 200 μM). Reactions were terminated by rapid vacuum filtration through Whatman GF/B glass fiber filters mounted on a Brandel Cell Harvester (Gaithersburg, MD). The filters and membranes were washed three times with 5 ml of 50 mM Tris–HCl (pH 7.4) and added to 4 ml of Biofluor; membrane-associated radioactivity was determined by liquid scintillation spectroscopy (Delta 300, Searle Analytic Inc., Des Plaines, IL). Specific [^{3}H]QNB binding was defined as the difference in bound ligand measured in reactions carried out in the presence and absence of 1 μM atropine sulfate. Non-specific binding was not influenced by verapamil and was less than 15% of total binding at all [^{3}H]QNB concentrations examined. Binding of [^{3}H]-*N*-methylscopolamine ([^{3}H]NMS) (0.001 to 40 nM) to sarcolemmal vesicles was determined using the same conditions as those described for QNB except that reactions were terminated after 60-min incubations.

The time course of QNB binding to sarcolemmal vesicles (45 pM [^{3}H]QNB) was determined by measuring specific QNB binding at selected times after initiation of a binding reaction. Conditions were as described above except that the temperature was 37° (see below).

Dissociation rate constants were calculated from the time course of the decrease in specific [^{3}H]QNB binding (45 pM [^{3}H]QNB) after addition of 1 μM atropine sulfate with or without racemic, (+)-, or (−)-verapamil (0.1 to 500 μM) to a binding reaction that had reached equilibrium. These experiments were performed at 37° to facilitate [^{3}H]QNB dissociation. The half-maximum concentration (IC_{50}) of verapamil to inhibit equilibrium [^{3}H]QNB binding to sarcolemmal vesicles was not different at 25° or 37°.

The stereoselectivity of the sarcolemmal phenylalkylamine binding site was determined by competition experiments in which 0.1 mg/ml sarcolemmal vesicles was incubated at 25° in 50 mM Tris–HCl (pH 7.4) with 1 nM [^{3}H]-(−)-D-888 (83 Ci/mmol) in the presence of 0.001 to 3 μM (+)- or (−)-verapamil or 0.0003 to 1 μM (−)- or (+)-D-888. Bovine serum albumin (0.1%) was included in the reaction mixtures to decrease non-specific binding of [^{3}H]D-888. Binding reactions were terminated after 90 min by filtration through Whatman GF/C filters (presoaked in 0.3% polyethylenimine), and specific D-888 binding was determined as described above. Non-specific binding was determined from the amount of [^{3}H]D-888 bound in the presence of 10 μM unlabeled (−)-D-888 and was approximately 25% of total binding at the K_D (approximately 10 nM).

Materials. All chemicals were of reagent grade and were purchased from the Sigma Chemical Co. (St. Louis, MO). [^{3}H]QNB (33 Ci/mmol), [^{3}H]NMS (85 Ci/mmol) and Biofluor were purchased from New England Nuclear (Boston, MA). [^{3}H]-(−)-D-888 (83 Ci/mmol) and the enantiomers of verapamil and D-888 were gifts from Dr. Rolf Kretzschmar (Knoll AG, Ludwigshafen, FRG).

Data analysis. The maximum number of sarcolemmal QNB and D-888 binding sites (B_{max}) and their apparent affinities for the ligands (K_D) were estimated by linear least squares analysis of Scatchard plots generated from [^{3}H]QNB and [^{3}H]-(−)-D-888 saturation binding isotherms. The IC_{50} values were estimated from indirect Hill plots [20] of verapamil competition experiments. At verapamil concentrations below 100 μM, the affinity of the putative muscarinic binding site for verapamil (K_i) was determined by applying the equation of Cheng and Prusoff [21]. The affinities of the phenylalkylamine site for verapamil and D-888 were determined in a similar manner.

The observed [^{3}H]QNB association rate constant (k_{obs}) was determined by the pseudo-first-order method using the following equation [20]:

$$\ln\left(\frac{[R\text{-QNB}]_e}{[R\text{-QNB}]_e - [R\text{-QNB}]_t}\right) = k_{obs} * t$$

where $[R\text{-QNB}]_e$ and $[R\text{-QNB}]_t$ are the concentrations of specifically bound QNB at equilibrium (e) and each time (t) respectively. The dissociation rate constant (k_{-1}) was determined from the slope of a plot of ln ($[R\text{-QNB}]_t/[R\text{-QNB}]_e$) versus time.

All experiments were carried out in duplicate or triplicate and repeated on at least two different preparations. Values are presented as mean ± SEM.

RESULTS

Effect of racemic verapamil on equilibrium [^{3}H]QNB binding. Purified canine sarcolemmal membranes could be characterized as having a single population of high affinity [^{3}H]QNB binding sites with $K_D = 56 \pm 14$ pM and $B_{max} = 8.0 \pm 0.5$ pmol/mg protein (N = 5). Studies with *N*-methylscopolamine (NMS), a muscarinic antagonist with a lower affinity for the receptor ($K_D = 305$ pM, N = 2) yielded a $B_{max} = 9.1$ pmol/mg protein (N = 2).

The major effect of racemic verapamil was to decrease the apparent affinity of the muscarinic receptor for [^{3}H]QNB (Fig. 1). At concentrations up to 40 μM, racemic verapamil did not alter the linear characteristics of the Scatchard plot (Fig. 1B), and Hill plots of the data in Fig. 1 had slopes of approximately 1. The IC_{50} and calculated K_i for the inhibitory effect of racemic verapamil were 13.6 ± 1.6 and 7.3 ± 1.3 μM respectively. Racemic verapamil also inhibited [^{3}H]NMS (100 pM) binding ($IC_{50} = 57.4 \pm 5.0$ μM).

Effects of verapamil enantiomers on equilibrium binding. (−)-Verapamil had a greater inhibitory effect on equilibrium QNB and NMS binding to the cardiac muscarinic receptor (Table 1, Fig. 2). The IC_{50} values for the (−) and (+) enantiomers of verapamil determined from competition studies using 45 pM [^{3}H]QNB as ligand were 9.9 ± 0.4 and 20.5 ± 1.1 μM respectively (Fig. 2). The calculated

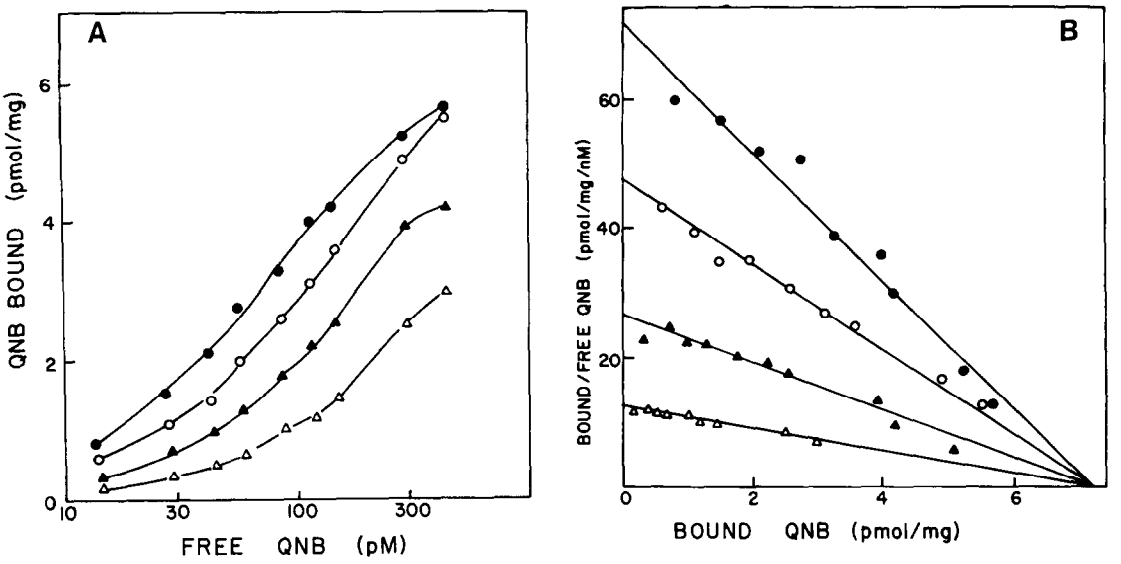

Fig. 1. Effect of racemic verapamil on equilibrium [^{3}H]QNB binding. (A) Binding isotherms. (B) Scatchard plot. Reactions were initiated by addition of 2 μg/ml sarcolemmal vesicles to reaction mixtures containing 0 μM (●), 5 μM (○), 15 μM (▲), or 40 μM (△) verapamil; the mixtures were incubated for 90 min. The values are from a representative experiment.

K_i values were 5.3 ± 0.2 μM for (−)-verapamil and 11.4 ± 0.6 μM for (+)-verapamil. (−)-Verapamil had a greater inhibitory effect on NMS binding than did (+)-verapamil (Table 1).

The K_D for QNB increased from 52 pM in the absence of phenylalkylamines to 570 and 990 pM in the presence of 40 μM (+)- and (−)-verapamil respectively (Fig. 3). Hill plots of the data in Fig. 3 were linear with slopes of approximately 1. Neither enantiomer significantly altered B_{max}.

Effects of verapamil and its enantiomers on QNB association and dissociation. Racemic verapamil slowed QNB–receptor association in a concentration-dependent manner (Fig. 4). The k_{obs} was decreased from 5.5 ± 2.1 (N = 4) to $2.3 \times 10^{-4} \cdot sec^{-1}$ (N = 2; data from Fig. 4) by 30 μM verapamil, and its IC_{50} for this effect was 10.5 ± 1.5 μM. (−)-Verapamil had a greater inhibitory effect on QNB association than (+)-verapamil [IC_{50} = 7.9 ± 2.3 and 15.5 ± 2.1 μM respectively (Figs. 5A and 6)].

The time course of [^{3}H]QNB dissociation after addition of 1 μM atropine consisted of two phases: a small rapid phase followed by a large slow phase (Figs. 5B and 6). Neither verapamil enantiomer affected the initial, rapid phase of dissociation, which was not examined further. However, both enantiomers inhibited the slow phase; this effect required high verapamil concentrations (>50 μM) and demonstrated stereoselectivity opposite to that observed for inhibition of [^{3}H]QNB–receptor association (Figs. 5 and 6). The estimated dissociation rate constant for the slow phase of the reaction was $5.0 \pm 0.5 \times 10^{-5} \cdot sec^{-1}$ and, in the presence of 150 μM (+)-, racemic and (−)-verapamil, this rate

Table 1. Inhibition of muscarinic antagonist binding to sarcolemmal vesicles by verapamil enantiomers

	% Control binding	
Ligand	(+)-Verpamil	(−)-Verapamil
QNB (45 pM)	53.8 ± 3.0 (10)	38.5 ± 3.8* (7)
NMS (100 nM)	69.1 ± 6.1 (12)	57.7 ± 2.6* (12)

Conditions were as described in Materials and Methods. The concentrations of verapamil isomers were 10 and 50 μM, respectively, for the QNB and NMS experiments. Control values for QNB and NMS were 3.2 ± 0.5 pmol/mg (N = 4) and 1.48 ± 0.3 pmol/mg (N = 7) respectively. Values are mean ± SEM, with the number of experiments given in parentheses.

* Significantly different from (+) isomer, $P < 0.05$.

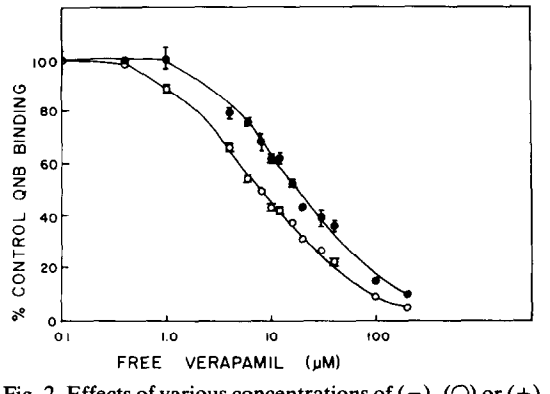

Fig. 2. Effects of various concentrations of (−)- (○) or (+)- (●) verapamil on [^{3}H]QNB (45 pM) equilibrium binding. Reaction conditions were as described in Fig. 1. The values are mean ± SEM for N ≥ 7. Control specific QNB binding = 1.45 ± 0.05 pmol/mg (N = 6).

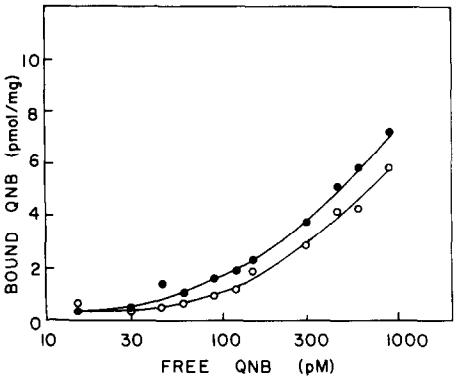

Fig. 3. Effects of 40 μM (−)- (○) or (+)- (●) verapamil on [^{3}H]QNB equilibrium binding isotherm. The values are from a representative experiment.

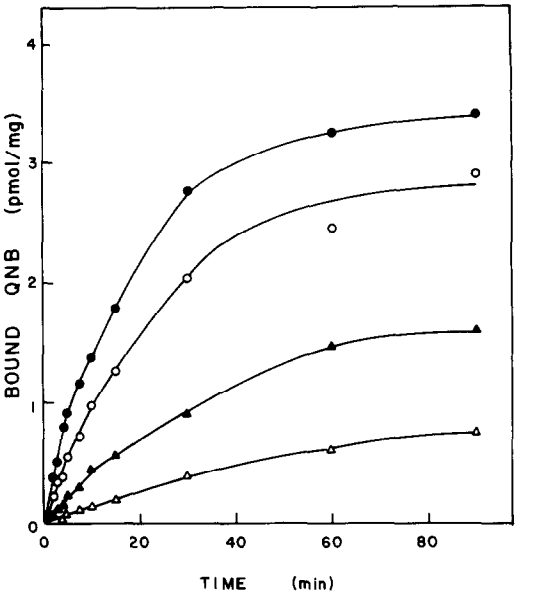

Fig. 4. Effect of racemic verapamil on [^{3}H]QNB–receptor association. Reaction mixtures contained 2.2 μg/ml protein and 45 pM [^{3}H]QNB, at 37° and 0 (●), 5 μM (○), 30 μM (▲), or 100 μM (△) verapamil. The $k_{obs} = 4.2 \times 10^{-4} \cdot sec^{-1}$ for control. The values are from a representative experiment.

constant was 43.3 ± 3.0, 64.0 ± 8.0, and 81.0 ± 8.0% of control respectively (Fig. 6, N ≥ 3).

Stereoselectivity for the phenylalkylamine receptor. Equilibrium binding isotherms for [^{3}H]-(−)-D-888 demonstrated that canine sarcolemma contains a specific and saturable phenylalkylamine binding site with a K_D and a B_{max} of 9.7 ± 0.8 nM and 1.8 ± 0.1 pmol/mg (N = 9) respectively. The (−) enantiomers of verapamil and D-888 were more effective than their respective (+) enantiomers in inhibiting binding of [^{3}H]-(−)-D-888 (Fig. 7). Values of K_i for (−)- and (+)-D-888 were 13.6 and 64.9 nM, respectively, and those for (−)- and (+)-verapamil were 39.6 and 73.7 nM respectively. Although the relative ability of the verapamil enantiomer to inhibit ligand binding to phenylalkylamine and muscarinic receptors in heart was similar, the concentrations required to inhibit [^{3}H]QNB binding were approximately 250-fold greater (K_i = 5.3 and 11.4 μM for (−)- and (+)-verapamil respectively; Fig. 2).

DISCUSSION

The present study is in accord with earlier findings that phenylalkylamine calcium channel blockers

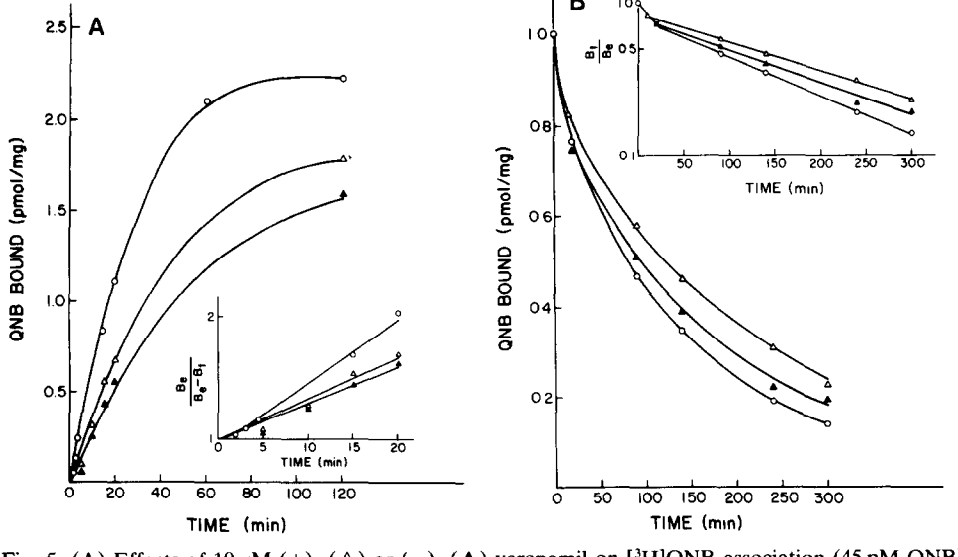

Fig. 5. (A) Effects of 10 μM (+)- (△) or (−)- (▲) verapamil on [^{3}H]QNB association (45 pM QNB) (○) at 37°. Inset: Log plot of $B_e/"B_e - B_t"$ vs t where B_e = ligand bound at equilibrium and B_t = ligand bound at time, t. The k_{obs} for control = $2.4 \times 10^{-4} \cdot sec^{-1}$. Values are from a representative experiment. (B) Effects of 100 μM (+)- (△) or (−)- (▲) verapamil on [^{3}H]QNB (45 pM) dissociation examined by addition of 1 μM unlabeled atropine (○, control), at 37°. Inset: Log plot of B_t/B_e (see panel A) vs time.

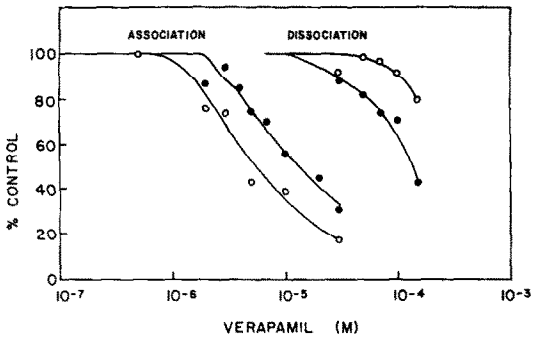

Fig. 6. Relative abilities of (+)-verapamil (●) and (−)-verapamil (○) to decrease the calculated QNB association (k_{obs}) (left) and dissociation (right) rate constants. Data were obtained from a series of experiments similar to that shown in Fig. 5 (N = 5). Control $k_{obs} = 5.5 \pm 2.1 \times 10^{-4} \cdot sec^{-1}$ (N = 4); $k_{-1} = 5.0 \pm 0.5 \times 10^{-5} \cdot sec^{-1}$ (N = 5).

inhibit antagonist binding to cardiac muscarinic receptors. This inhibitory effect (Figs. 1–3) could be characterized as a decrease in the affinity of [^{3}H]QNB with no change in the number of binding sites, i.e. apparent competitive inhibition. Inhibition of equilibrium binding resulted from the ability of verapamil to decrease the rate of [^{3}H]QNB association to the muscarinic receptor, an effect which displays stereoselectivity (Figs. 4 and 6). At concentrations above 10^{-5} M, verapamil inhibited [^{3}H]QNB dissociation [9] (Figs. 5 and 6); while this effect was also stereoselective, the stereoselectivity was opposite to that for [^{3}H]QNB association. Since verapamil inhibited [^{3}H]QNB dissociation only at high concentrations, inhibition of QNB dissociation probably did not modify the competitive-like inhibition of [^{3}H]QNB binding by verapamil at low ligand concentrations (Figs. 1, 4, and 6). These results support the view that phenylalkylamines alter the muscarinic receptor in heart by interacting with two distinct sites [9] having different stereoselectivity and an approximately 25-fold difference in affinity for the phenylalkylamines.

The ability of dihydropyridines and phenylalkylamines to inhibit sarcolemmal calcium influx is believed to reflect a specific interaction of these compounds with a membrane protein or proteins related to the calcium selective channel in cardiac and smooth muscle plasma membranes (for review see Ref. 22). The specificity of this interaction is supported by the findings that these agents are effective at nanomolar concentrations and the effects are stereoselective, although the relative effects of the enantiomers of verapamil may vary from 3- to 100-fold in different tissues ([16], see Ref. 23 for review). The present study demonstrates that the interaction of phenylalkylamines with cardiac muscarinic receptors also exhibits a stereoselectivity that is similar in magnitude to that observed for verapamil and D-888 inhibition of phenylalkylamine binding to cardiac sarcolemma (Fig. 7) and skeletal muscle microsomes [24]. Greater stereoselectivity of the phenylalkylamine receptor in heart has also been reported, however [12].

The diverse effects of phenylalkylamines on membrane function that are not attributable to calcium channel blockade [1–9, 23] may reflect a non-specific interaction of these compounds with membranes since these effects occur only at concentrations above 1 μM. Both D-600 and the structurally dissimilar local anesthetic, tetracaine, at similar micromolar concentrations, inhibit ligand binding to muscarinic receptors in brain [6]. The finding that the phenylalkylamine structure can act kinetically as a competitive inhibitor (with similar K_i) of ligand binding to muscarinic (Fig. 1), alpha-adrenergic and opiate receptors [5–9] cannot be explained by the present data. However, the structurally unrelated compound quinidine resembles phenylalkylamines in its ability both to inhibit QNB association to, and also dissociation from, muscarinic receptors in heart [25] and ligand binding to alpha-adrenergic receptors [26]. The finding that a diverse group of plasma membrane receptors are affected similarly by a variety of unrelated molecular structures [9, 27] suggests that the apparent competitive kinetics of the phenylalkylamine effects observed in this study do not reflect binding of these drugs to specific QNB binding sites [27].

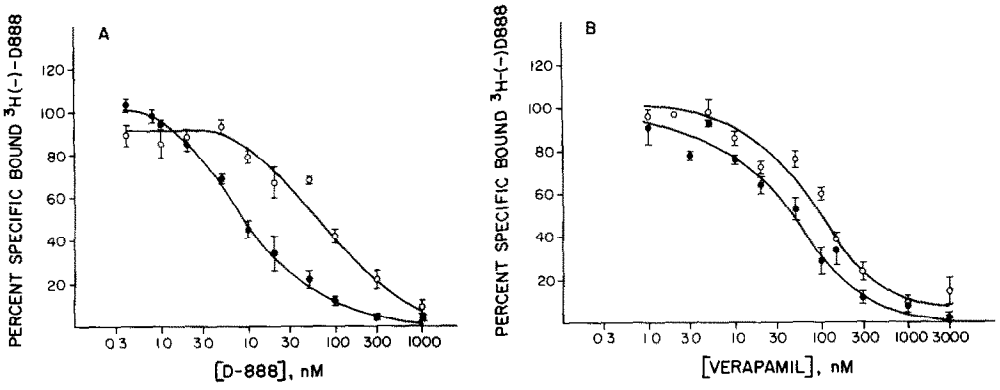

Fig. 7. Competition of (−)- or (+)-D-888 (A), or (−)- or (+)-verapamil (B) with [^{3}H]D-888 (1 nM) for binding to the phenylalkylamine site in canine sarcolemmal vesicles. Each value represents the mean ± SEM (N ≥ 5). Open symbols, (+) isomer; closed symbols, (−) isomer. Control specific (−)-D-888 binding = 0.25 ± 0.01 pmol/mg (N = 18).

The high lipid solubility of the phenylalkylamines suggests that their effects may be mediated by an interaction with hydrophobic membrane domains that are linked functionally to a number of membrane receptors. The importance of the incorporation of calcium channel blocking agents into membrane lipids in their mechanism of action [28] is supported by recent patch clamp studies of single calcium channels [29, 30]. These studies demonstrate that both phenylalkylamines and dihydropyridines can modify calcium channels when added to the aqueous environment outside the patch clamp pipette and so suggest that these drugs can reach a site of action by first entering the membrane bilayer [28, 30]. The stereoselective property of the interaction of phenylalkylamines with the muscarinic receptor reported here could reflect steric constraints of the receptor protein at or near the membrane site where the drug resides [31].

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