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$[^3H]$ VERAPAMIL BINDING SITES IN SKELETAL MUSCLE TRANSVERSE TUBULE MEMBRANES

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SUMMARY : $[^3H]$verapamil binding to muscle tubule membrane has the following properties. K_D = 27 ± 5 nM and maximum binding capacity B_{max} = 50 ± 5 pmol/mg of protein. A 1 = 1 stoichiometry of binding was found for the ratio of $[^3H]$verapamil versus $[^3H]$ nitrendipine binding sites. The dissociation constant found at equilibrium is near that determined from the ratio of the rate constants for association (k_1) and dissociation (k_{-1}). Antiarrhythmic drugs like D600, diltiazem and bepridil are competitive inhibitors of $[^3H]$verapamil binding with K_D values between 40 and 200 nM. Dihydropyridine analogs are apparent non competitive inhibitors of $[^3H]$verapamil binding with half-maximum inhibition values ($K_{0.5}$) between 1 and 5 nM.

INTRODUCTION : The most potent calcium channel inhibitors have an important pharmacological and clinical interest. Molecules with these properties include the dihydropyridine derivatives such as nitrendipine or nimodipine and compounds like verapamil, D600, diltiazem and bepridil (reviewed in 1 and 2). Receptors of $[^3H]$nitrendipine or $[^3H]$nimodipine have recently been indentified in nerve, cardiac, skeletal and smooth muscle membranes (reviewed in 2).

Electrophysiological experiments have shown that voltage-dependent calcium channels in frog skeletal muscle are primarily localized in the transverse tubular membranes (T-tubules). Purified skeletal muscle T-tubule membranes have indeed been found to be richer in $[^3H]$nitrendipine binding sites than surface muscle membranes (4, 5) and a confirmation of this result was obtained with $[^3H]$nimodipine and d-cis-$[^3H]$diltiazem (6-8). Pure T-tubule membranes from rabbit skeletal muscle have at least 50-100 times more $[^3H]$nitrendipine binding sites than other known tissues (4-5). Electrophysiological experiments on heart and muscle have shown that verapamil is a blocker of voltage-dependent Ca^{2+} channel with properties differing from those of nitrendipine and analogs (9, 10).

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In the present work [^{3}H]verapamil has been used to characterize the biochemical and pharmacological properties of its specific binding site on purified rabbit skeletal muscle T-tubule membranes.

MATERIALS AND METHODS : T-tubule membranes were isolated from rabbit skeletal muscle as already described (11) and stored as in (5). Protein concentration was determined by the method of Hartree using bovine serum albumin as a standard. [^{3}H]nitrendipine binding experiments were carried out as previously described (5). Binding experiments with [^{3}H]verapamil were done as described for [^{3}H]nitrendipine (5). In the routine assay, samples of membranes were incubated at 10°C in 1 ml of a solution containing 50 mM Tris-Cl buffer, pH 7.5 and the required concentration of [^{3}H]verapamil. The final concentration of membranes was 0.06–0.07 mg of protein/ml. In all binding experiments, [^{3}H]verapamil was added last. In standard equilibrium binding experiments, incubation lasted 70–80 min. Incubations were stopped by rapid filtration of 400 µl samples of the incubation mixture through polyethylene imine treated whatman GF/C glass fiber filters, under reduced pressure. The filters were immediately washed twice with 8 ml of a cold solution containing 200 mM choline chloride and 20 mM Tris-Cl at pH 7.5. Experiments in duplicate were systematically carried out. For measurements of nonspecific binding, unlabelled verapamil was present in the medium at a final concentration of 10 µM. Binding of [^{3}H]verapamil to the filters alone was shown to be non-negligible and was displacable in the presence of an excess of verapamil (10 µM). Therefore, for each measurement in a given assay, a control was carried out with the filters without membranes under the same conditions as in experiments with T-tubule membranes and this control was substracted from total binding. The filters used in the experiments were pretreated during 10 min with 0.05 % polyethylene imine in order to minimise [^{3}H]verapamil binding to filters. Inhibition of [^{3}H]verapamil binding by different drugs was measured under equilibrium conditions as described above, in the presence of 0.9–1.0 nM [^{3}H]verapamil. N-methyl-[^{3}H]verapamil at 75 Ci/mmol and 5-methyl-[^{3}H]nitrendipine at 85 Ci/mmol were obtained from NEN. All other compounds used were obtained as described in (5).

RESULTS AND DISCUSSION : [^{3}H]verapamil binding to T-tubule membranes. Fig. 1 A illustrates a typical equilibrium binding experiment of [^{3}H]verapamil to rabbit T-tubules membranes. The linearity of the Scatchard plot shown in Fig. 1A (inset) shows specific binding to a single class of sites and gives values of 53 pmol/mg of protein for the maximum number of [^{3}H]verapamil sites (B_{max}) and 27 nM for the equilibrium dissociation constant (K_D) of the verapamil-receptor complex. Specific binding of [^{3}H]verapamil varied linearly with increasing concentrations of pure T-tubule membranes in the range of 30 – 90 µg of membrane proteins/ml (not shown). Measurements of [^{3}H]nitrendipine binding were carried out in parallel and we confirmed our previous results (6, 7) giving a B_{max} = 55 ± 6 pmol/mg protein and a K_D value of 1.8 ± 0.3 nM. Thus a 1:1 stoichiometry exists between [^{3}H]verapamil and [^{3}H]nitrendipine binding sites on T-tubule membranes.

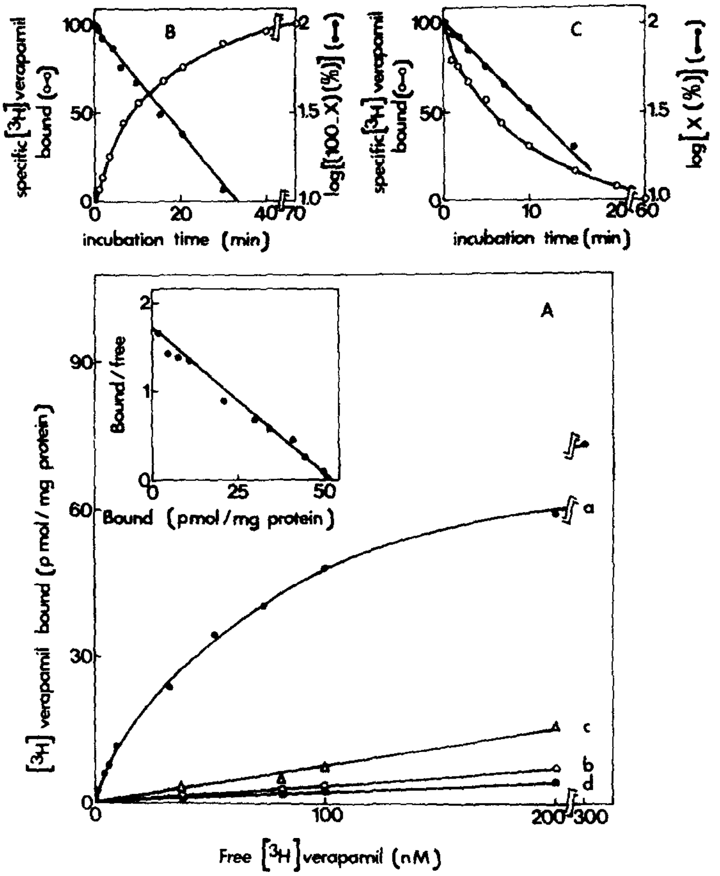

Fig. 1. Equilibrium binding of [^{3}H]verapamil to T-tubule membranes and kinetics of association and dissociation. Equilibrium binding was measured using[^{3}H]verapamil prepared at 11 Ci/mmol and 0.07 mg/ml of membranes (pH 7.5 and 10°C). A, Binding to T-tubule membranes in the absence (a, ●) or presence (b, ○) of 10 μM unlabelled verapamil and comparative binding to GF/C filters, alone, without membranes, in the absence (c, △) or presence (d, ■) of 10 μM unlabelled verapamil. The specific binding component is calculated as (a-b) - (c-d). A, inset, Scatchard plot for the specific [^{3}H]verapamil binding component. Note that GF/B filters gave 5 times more binding to filters than GF/C filters and are to be avoided in experiments of that kind. B (○) Direct association kinetics corresponding to specific [^{3}H]verapamil binding. Concentration of [^{3}H]verapamil : 0.8 nM (75 Ci/mmol) and concentration of T-tubule membrane : 0.070 mg of protein/ml, pH 7.5, 10°C. Non-specific binding was measured as usual in the presence of 10 μM verapamil, and substracted from total binding. (●) pseudo-first order representation of the data. X, extent (in %) of the specific [^{3}H]verapamil association to T-tubule membranes. C, After equilibrium has been reached (60-70 min) and checked to be stable for more than 2 h, the dissociation of the complex was triggered by a 20-fold dilution in a 50 mM Tris-Cl buffer at pH 7.5. (○) time course of [^{3}H]verapamil significally bound to its receptor. (●) first-order representation of the data.

Association and dissociation kinetics. Typical association kinetics of [^{3}H]verapamil to T-tubule membranes are shown in Fig. 1B. These kinetic studies were carried out with a [^{3}H]verapamil concentration [L_T] of 0.8 nM and a receptor concentration [R_T] of 3.7 nM. The maximal concentration of specifically bound [^{3}H]verapamil $[LR]_E$ which correspond to 100 % in Fig. 1B was only 0.082 nM. Consequently, the kinetic data for association were plotted according to pseudo first-order (12) : $\mathrm{Ln}\ [LR]_E/[LR]_E-[LR] = k_1 \times t \times [L_T][R_T]/[LR]_E$ where [LR]is the concentration of the complex at time t and k_1, the second order rate constant of association. The semi-logarithmic plot in Fig. 1B gives a value of $k_1 = 3.3 \times 10^4\ M^{-1}\ s^{-1}$. Dissociation of [^{3}H]verapamil was obtained by a 20-fold dilution of the preformed complex. Fig. 1C shows that dissociation followed first order kinetics. The value of the rate constant of dissociation was $k_{-1} = 1.6 \times 10^{-3}\ s^{-1}$ corresponding to a half-life of dissociation of 6.9 min at 10°C. The value of the equilibrium constant K_D calculated from kinetic data is K_D = 50 nM, in approximate agreement with the equilibrium binding studies.

The effect of unlabelled verapamil and other drugs active on Ca^{2+} channels on [^{3}H] verapamil binding to T-tubule membranes. Fig. 2A and Table I show the results obtained for a first group of antiarrhythmic drugs including verapamil, D600, diltiazem and bepridil. Increasing concentrations of these different molecules gradually inhibited [^{3}H]verapamil binding to T-tubule membranes. Half-maximum inhibition for unlabelled verapamil is observed at $K_{0.5}$ = 25 nM. Under the experimental conditions (a [^{3}H]verapamil concentration of 0.9 - 1.1 nM which is 30 times lower than the dissociation constant K_D of the verapamil-receptor complex) $K_{0.5}$ represents the equilibrium dissociation constant K_D for verapamil-receptor complex. This value is in good agreement with K_D values found from equilibrium binding (K_D = 27 nM) or from kinetic data (K_D = 50 nM). The K_D values for the association formed by bepridil, D600 and Diltiazem with T-tubule membranes are between 40 and 200 nM (Table I). Fig. 2B and Table I show that molecules of the dihydropyridine family also inhibit [^{3}H]verapamil binding to T-tubule membranes with apparent $K_{0.5}$ concentrations between 1 and 5 nM. Maximum inhibition of [^{3}H]verapamil binding by these drugs reaches 90 %. The molecule BAY K 8644, described as the first dihydropyridine agonist (opener) of the calcium channel (13) also inhibits [^{3}H]verapamil binding with an apparent inhibition constant ($K_{0.5}$) of 30 nM.

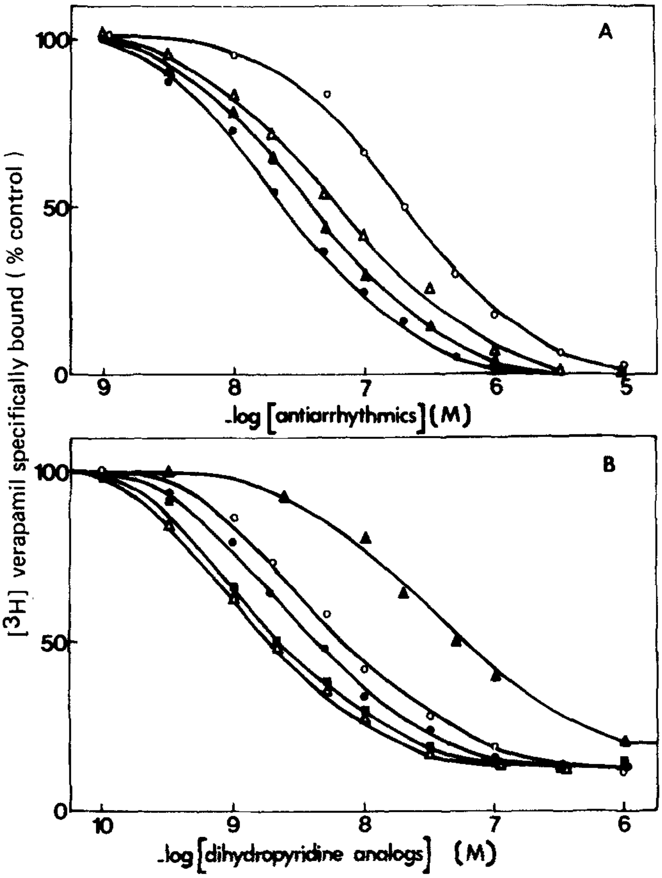

Fig. 2. Effects of different Ca^{2+}-channel blockers on $[^3H]$verapamil binding to T-tubule membranes. A, Inhibition of $[^3H]$verapamil (0.9 nM at 75 Ci/mmol) binding by unlabelled verapamil, D600, diltiazem and bepridil. Binding of $[^3H]$verapamil to T-tubule membranes (0.06 mg/ml) was measured under equilibrium conditions at pH 7.5 and 10°C, in the presence of the indicated concentrations of verapamil (●), bepridil (▲), D600 (△) and diltiazem (○). B, Inhibition of $[^3H]$ verapamil (0.9 nM) binding by nitrendipine, nimodipine, nifedipine, nisoldipine and BAY K 8644 as described in A. Binding was measured in the presence of indicated concentrations of nitrendipine (△), nimodipine (■), nifedipine (●), nisoldipine (○) and BAY K 8644 (▲).

Fig. 3A shows a family of Scatchard plots describing $[^3H]$verapamil binding to T-tubule membranes in the presence of increasing concentrations of nitrendipine. One observes a decrease of the maximal binding capacity (B_{max}) with no change of equilibrium dissociation constant (K_D = 27 nM) when nitrendipine concentrations are increased from zero to 1.7 nM. Nitrendipine behaves as an apparent noncompetitive inhibitor for $[^3H]$verapamil binding to T-tubule membranes. Fig. 3B shows Scatchard plots of $[^3H]$verapamil binding to T-tubule membranes in the presence of increasing concentrations of diltiazem. One ob-

TABLE I.
Inhibitors of [^{3}H] verapamil binding to T-tubule membranes

Drug	Inhibition constant K_D or $K_{0.5}$ (nM)
Verapamil	25
Bepridil	40
D600	70
Diltiazem	200
Nitrendipine	1.4
Nimodipine	1.6
Nifedipine	3.5
Nisoldipine	5
Agonist BAY K 8644	30

serves an increase of the equilibrium dissociation constant with no change of binding capacity (B_{max} = 50 pmol/mg protein) when diltiazem concentrations are increased from zero (K_D = 29 nM), to 210 nM (apparent K_D = 61 nM). Diltiazem behaves as a competitive inhibitor for [^{3}H]verapamil binding to T-tubule membranes. Fig. 3C shows Scatchard plots of [^{3}H]verapamil binding to T-tubule membranes in the presence of increasing concentrations of bepridil. There is no change of binding capacity (B_{max} = 52 pmol/mg protein) when bepridil concentration is increased from zero (K_D = 28 nM) to 52 nM (apparent K_D = 55 nM). Bepridil also behaves as a competitive inhibitor for [^{3}H]verapamil binding

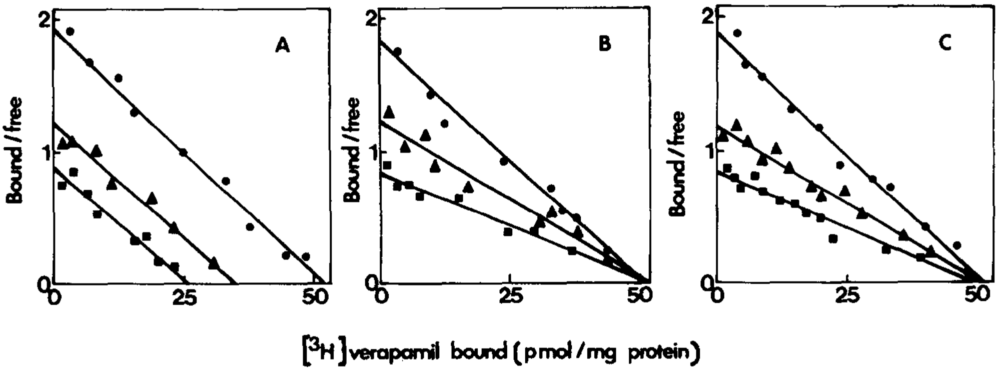

Fig. 3. Equilibrium binding of [^{3}H]verapamil to T-tubule membranes in the absence and in the presence of nitrendipine, diltiazem or bepridil. Binding assays were carried out at pH 7.4 and 10°C with 0.06 mg of membranes/ml.. A, Scatchard plots in the absence (●) and in the presence of 1 nM (▲) or 1.7 nM (■) nitrendipine. B, Scatchard plots in the absence (●) and in the presence of 100 nM (▲) or 210 nM (■) diltiazem. C, Scatchard plots in the absence (●) and in the presence of 24 nM (▲) or 52 nM (■) bepridil.

to T-tubule membranes. The observation that diltiazem and bepridil behave as competitive inhibitors of [^{3}H]verapamil binding is consistant with the proposal that verapamil, diltiazem and bepridil belong to the same family of calcium channel blockers because they all reverse the inhibition of [^{3}H]nitrendipine binding produced by diphenylalkylanine drugs (14). Many other pharmacologically active molecules have been found to have no effect on [^{3}H]verapamil binding to T-tubule membranes. They include classical effectors of the sodium channel like veratridine (100 μM) and tetrodotoxin (10 μM), effectors of different types of K$^+$ channels like tetraethylammonium (10 μM) or apamine (4 μM), tranquizer like diazepam (10 μM), adrenergic blockers (1 μM) like propranolol, yohimbine, prazosin and psychotomimetics (1 μM) like phencyclidine and thienylphencyclidine, antiepileptic like phenobarbital (1 μM) and cholinergic antagonists like atropine and d-tubocurarine (1 μM). Finally it is of interest to remark that affinities found in this work for verapamil (K_d = 25 nM and bepridil (K_d = 40 nM) are similar to EC_{50} values of 30 nM found from electrophysiological experiments for the effects of these molecules on frog skeletal Ca^{2+} channels (10).

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