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EFFECTS OF VERAPAMIL ON THE BINDING PROPERTIES OF RAT HEART MUSCARINIC RECEPTORS : EVIDENCE FOR AN ALLOSTERIC SITE

MAGALI WAELBROECK, PATRICK ROBBERECHT, PHILIPPE DE NEEF and JEAN CHRISTOPHE[1]

Department of Biochemistry and Nutrition, Medical School, Université Libre de Bruxelles, Bld. de Waterloo 115, B-1000 Brussels, Belgium

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The calcium channel antagonist verapamil is known to inhibit competitively antagonist binding to rat heart muscarinic receptors. The present data suggest that this drug recognized two binding sites on the muscarinic receptors: 1) an allosteric site modulating the tracer dissociation rates and 2) the muscarinic drug binding site. The affinity of verapamil for the allosteric site and the efficacy of its effect on muscarinic ligand dissociation rates depended on the ligand studied.

Verapamil is often considered as a potent and selective voltage dependent calcium channel antagonist. Whereas this is indeed true at low concentration (below 1 μM) (1), other inhibitory effects have been demonstrated on sodium or potassium conductances, at higher verapamil concentrations (in the 10 to 100 μM concentration range) (2-5). In the same "non specific" concentration range, verapamil and its derivative D-600 act as competitive antagonists for muscarinic [6,7] and α-adrenergic [7,8] receptors.

Rat heart muscarinic receptors behave as a homogeneous class of receptors, when binding antagonists (in the buffer used in the present study), while they can be distinguished into subclasses with respect to their affinity for agonist molecules (9,10) : the labelled agonist [^{3}H] Oxo-M binds exclusively to rat cardiac muscarinic receptors with the highest affinity (the SH receptors)

[1]Correspondence should be addressed to: Dr. J. CHRISTOPHE, Department of Biochemistry and Nutrition, Medical School, Université Libre de Bruxelles; Bld. of Waterloo, 115 B-1000 Brussels, Belgium.

Abbreviations used :
[^{3}H]Oxo-M, (methyl[^{3}H])oxotremorine M acetate; [^{3}H]NMS, (N-methyl[^{3}H]) scopolamine methyl chloride; SH receptors, cardiac receptors with a high affinity for muscarinic agonists; H receptors, cardiac receptors with a low affinity for muscarinic agonist.

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and not to muscarinic receptors with lower affinity (the H receptors) (10). In the present work, we compared the effects of verapamil on the binding and dissociation rate constants of [^{3}H] NMS and [^{3}H] Oxo-M.

MATERIALS AND METHODS

Rat hearts kept frozen until use (11), were thawed then homogenized (5 %, w/v homogenate) at 4°C in a buffer consisting of 20 mM Tris-HCl (pH 7.4), 2 mM dithiothreitol, and 5 mM $MgCl_2$. After filtration through two layers of medical gauze, the homogenate was centrifuged at 520 x g for 10 min. Heart membranes were collected from this crude particulate extract as previously described (11).

To study binding equilibrium, heart membrane proteins (90-150 µg) were incubated for 10 min at 25°C with 1.0 nM [^{3}H] NMS (54 Ci/mmole) or 1.5 nM [^{3}H] Oxo-M (84 Ci/mmole) (from New England Nuclear Corporation, Dreieich, F.R.G.), in 1.2 ml of 50 mM sodium phosphate buffer (pH 7.4) enriched with 2 mM $MgCl_2$ and 1 % bovine serum albumin, and in the absence or presence of 1 µM atropine (to determine non specific binding) or 0.1 to 1000 µM verapamil. Membrane-bound radioactivity was separated from free radioactivity by filtration through glass-fiber filters GF/C (Whatman, Maidstone, U.K.) and washed three times with ice-cold buffer, as previously detailed (10).

For measuring dissociation rates, 1 µM atropine was added simultaneously with 0 to 1 mM verapamil, after a 10 min preincubation of the membranes and tracer. Specific tracer binding was measured as above, at the indicated time intervals after atropine and verapamil addition.

RESULTS AND DISCUSSION

Verapamil competition for [^{3}H] NMS and [^{3}H] Oxo-M binding sites is shown on Fig. 1. The concentrations of verapamil necessary to decrease [^{3}H] NMS and [^{3}H] Oxo-M binding by 50 % were not significantly different. The K_i values, calculated according to Cheng and Prusoff [12], were 3.5 $\pm$ 1.0 µM and 2.5 $\pm$ 0.8 µM (n = 3 experiments in duplicate) for, respectively, [^{3}H] NMS and [^{3}H] Oxo-M binding inhibition, suggesting that verapamil recognized all

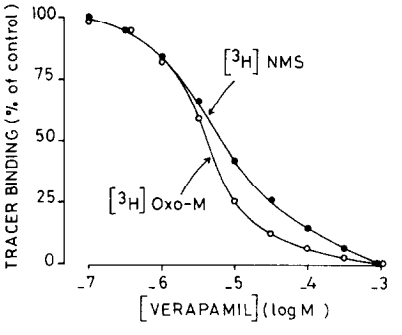


Fig. 1. Inhibitory effects of verapamil on [^{3}H] NMS (•) and [^{3}H] Oxo-M (o) binding at equilibrium. Data were expressed as percent of control values in the absence of verapamil. Mean of 3 experiments performed in duplicate.

cardiac muscarinic binding sites (SH and H receptors) with the same affinity. A comparison of competition curves obtained at different [^{3}H] NMS concentrations (data not shown) confirmed that verapamil increased the apparent K_D value for [^{3}H] NMS without affecting the total receptor concentration, as already observed by Karliner et al. (7). Slight but significant deviations from the law of mass action as applied to competition curves in Fig. 1 (n_H = 0.79 ± 0.05 and 0.86 ± 0.07 (n = 3 experiments in duplicate) for [^{3}H] NMS and [^{3}H] Oxo-M competition curves, respectively) were, however, observed. This phenomenom was further investigated. Indeed, we know that the dissociation rates of muscarinic antagonists are modulated allosterically by the neuromuscular blocking agents gallamine (13,14), pancuronium (14) and d-tubocurarine [unpublished results] so that apparent receptor heterogeneity (reflecting in fact "negative cooperativity" interactions) is induced (14). To test whether verapamil provoked similar effects, we compared the dissociation rate constants of [^{3}H] NMS and [^{3}H] Oxo-M, in the absence and presence of verapamil.

As shown in Fig. 2, dissociation kinetics of [^{3}H] NMS and [^{3}H] Oxo-M were first order with the buffer system utilized. Addition of verapamil (simultaneously with atropine) reduced the dissociation rate of [^{3}H] NMS markedly, whereas that of [^{3}H] Oxo-M was reduced slightly (but still significantly). The relative rate constants of tracer dissociation, measured 3 min after addition of 1 μM atropine and 0 to 1 mM verapamil, are shown in Fig. 3.

A decrease of the tracer dissociation rate constant will lead to a decrease of the tracers equilibrium dissociation constant, i.e. to an increase of the tracer affinity constant ($K_D = k_{off}/k_{on} = 1/K_a$). This effect will partially compensate the competitive inhibition of [^{3}H] NMS and [^{3}H] oxo-M binding by verapamil, and distort the competition curves.

To estimate the extent of the "distortion" of the verapamil competition curves, due to its effects on the tracer dissociation rates, we calculated

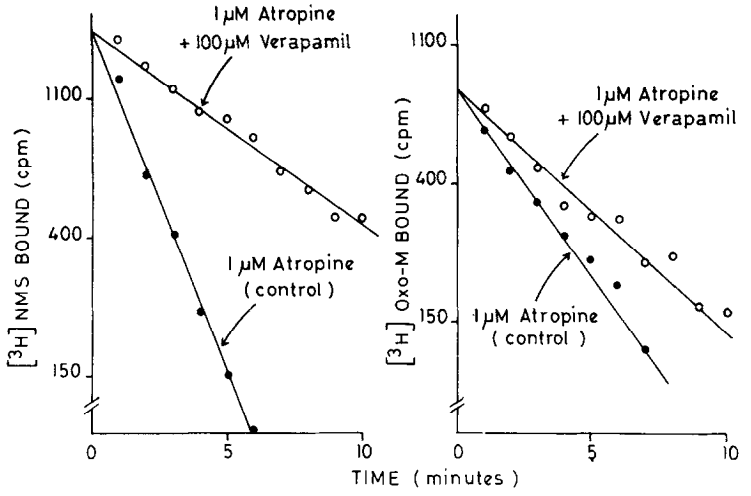


Fig. 2. [^{3}H] NMS and [^{3}H] Oxo-M dissociation induced by addition of 1 μM atropine alone (●) or by simultaneous addition of 1 μM atropine and 100 μM verapamil (o). Tracer binding, measured at the indicated time intervals, was plotted on a semilogarithmic scale. Control k_{off} values (measured after addition of atropine alone) were 0.38 ± 0.05 min^{-1} and 0.24 ± 0.04 min^{-1} for [^{3}H] NMS and [^{3}H] Oxo-M, respectively (n = 3 experiments in duplicate). This experiment is representative of 3 experiments performed in duplicate.

the tracer apparent association rate constants k_{on} at each verapamil concentration, assuming that :

$$B = \frac{L}{K_D + L}$$

where B is the fraction of receptors occupied by the tracer, L is the tracer's concentration,

and $$K_D = k_{off}/k_{on}$$

is the tracer's equilibrium dissociation constant. The values of k_{on} obtained by this procedure, using the experimental data shown in Figs. 1 and 3, are shown in Fig. 3.

The pseudo Hill coefficients of "k_{on} inhibition curves" by verapamil were equal to 0.98 and 1.02 for [^{3}H] NMS and [^{3}H] Oxo-M, respectively (Fig. 3).

The results presented in Fig. 3 might be interpreted as follow :
1/ verapamil prevented the binding of [^{3}H] NMS and [^{3}H] Oxo-M to muscarinic receptors with a single K_i value of 2 to 3 μM (Fig. 3 : open circles);
2/ verapamil also interacted with an allosteric site, associated to the muscarinic receptor, thereby decreasing or blocking [^{3}H] Oxo-M and [^{3}H] NMS

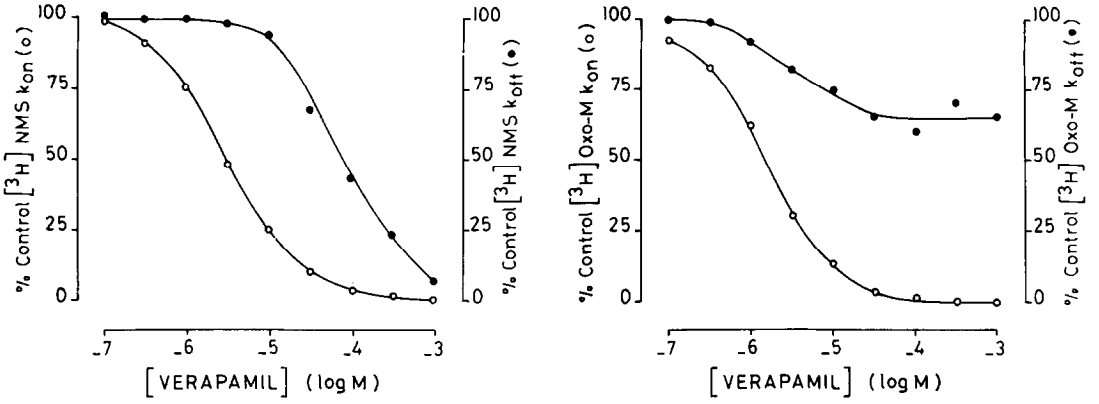
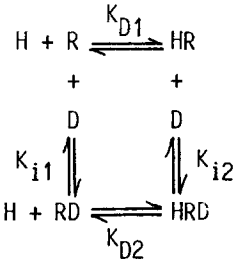


Fig. 3. Decrease, by verapamil, of [^{3}H] NMS (left panel) and [^{3}H] Oxo-M (right panel) dissociation rate constants k_{off} (•) and apparent association rate constants k_{on} (o). The dissociation rate constants (k_{off}) was measured after a 3 min isotopic dilution (in the presence of 1 µM atropine), in the absence or presence of the indicated verapamil concentrations (average of 3 experiments performed in duplicate). The s.d. of the experimental points were below 5 % of the "control value" (n = 3 experiments).

The apparent association rate constants (k_{on}) were calculated as explained in the text, using the verapamil competition curves shown in Fig. 1 and the values of k_{off} determined in parallel experiments (this Fig.). All data were expressed as percent of values obtained in the absence of verapamil ("control").

dissociation from previously occupied receptors, with K_i values of 3 µM and 80 µM, respectively (Fig. 3 : closed circles).

These results cannot be explained by verapamil interaction with an allosteric binding site only, as in the model expanded by Stockton et al. [13]. Indeed, this model assumes that :



, where H is [^{3}H] Oxo-M or [^{3}H] NMS, D an allosteric drug interacting only with a non muscarinic binding site, and R the muscarinic receptor. The affinity of HRD (as compared to H + R + D) should then be independent of the reaction pathway (i.e. association of either H or D to the empty receptor R as the first step), so that : $K_{D1}\ K_{i2} = K_{i1}\ K_{D2}$. Such an equality was for instance

observed by Stockton et al. [13] studying the inhibitory effects of gallamine on heart and brain muscarinic receptors. In the present case : 1/ the inhibition of [^{3}H] Oxo-M binding by verapamil was stable for at least one hour (not shown), implying that the equilibrium K_D constants K_{D1} and K_{D2} for [^{3}H] Oxo-M were markedly different with $K_{D1} << K_{D2}$ (Fig. 1); 2/ in addition, we obtained similar values for K_{i1} (2 to 3 μM) and K_{i2} (3 μM) as derived from k_{on} and k_{off} values for [^{3}H] Oxo-M in Fig. 3. We thus observed $K_{D1}\ K_{i2} << K_{D2}\ K_{i1}$ instead of the equality required above when postulating an exclusive interaction of verapamil with an allosteric site, responsible for decreased [^{3}H] Oxo-M association and dissociation rates.

We conclude that verapamil was able to recognize two binding sites per muscarinic receptor in rat heart membranes : 1/ a verapamil allosteric site (regulating muscarinic drug dissociation rates) with K_i values of 3 μM for [^{3}H] Oxo-M and 80 μM for [^{3}H] NMS (Fig. 3) and 2/ the muscarinic binding site itself with a K_i for verapamil of approximately 3 μM (Fig. 3).

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