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BINDING OF AGONISTS AND ANTAGONISTS TO MUSCARINIC RECEPTORS

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The binding of one irreversible and two reversible radioactive antagonists to muscarinic receptors in synaptosome preparations of rat cerebral cortex has been studied. The ligands all bind to the same receptor pool and directly and competitively yield self-consistent binding constants closely similar to those obtained by pharmacological methods on intact smooth muscle. The binding process for antagonists seems to be a simple mass action-determined process with a Hill slope of 1.0. The quantitative correlations strongly support the view that the receptor studied by ligand binding corresponds to the receptor studied by pharmacological methods.

Inhibition of antagonist binding by most agonists shows a reduced Hill slope which also applies to direct binding studies of [^{3}H] acetylcholine. Mechanisms that might account for the behavior of agonists are discussed but do not conclusively point to any single mechanism.

INTRODUCTION

The identification of pharmacological receptors in a subcellular preparation depends on measuring the binding properties of characteristic ligands. It is necessary that the ligands that are known to react with the receptor in intact tissue should also react with the subcellular preparation and that the ligands not relevant to the receptor system should not so interact. In the case of acetylcholine receptors, the two major classes of receptor, nicotinic and muscarinic, are well separated in their properties in intact tissues and this distinction is fully maintained in subcellular preparations of muscarinic receptors.

However, our understanding of the molecular events associated with receptor-ligand interaction is at present inadequate to make exact predictions of the relationship between binding of ligands and the resultant physiological events. Such predictions clearly depend on presumptions about the mechanism of receptor action that are at present speculative and need validating by experiment. The experiments reported here were designed to examine quantitative aspects of binding of antagonists and agonists to the muscarinic receptor.

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Abbreviations: PrBCM: N-2′-chloroethyl-N-propyl-2-aminoethylbenzilate; PrBCh: NN-Dimethyl-N-propyl-2-aminoethylbenzilate; Atr: atropine; ACh: acetylcholine.

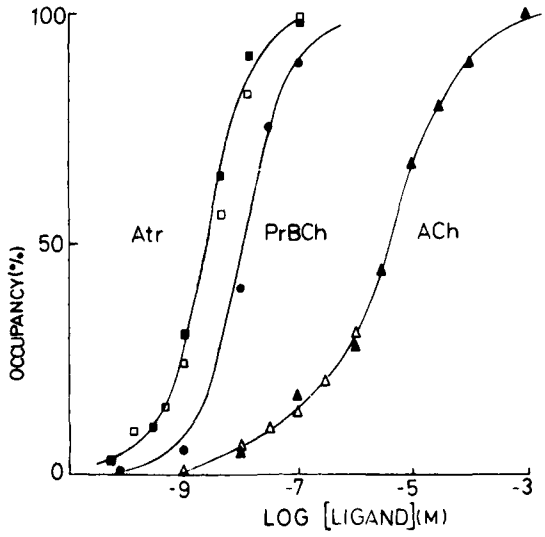

Fig. 1. Binding of Atr (squares), PrBCh (circles) and ACh (triangles) to the muscarinic receptor of the rat cortex. ■, ^{3}H-Atr binding; □, ^{3}H-PrBCh-Atr competition; ●, ^{3}H-PrBCh binding; △, ^{3}H-ACh binding; ▲, ^{3}H-PrBCh-ACh competition. No correction is applied for depletion of the free ligand concentration as a result of binding to the receptor.

METHODS

[^{3}H] Atropine (0.4 Ci/mmole) and [^{3}H-$\overset{+}{N}Me_3$] acetylcholine (10 Ci/mmole) were obtained from the Radiochemical Centre, Amersham. Tritiated propyl benzilylcholine [(NN-dimethyl-N-{2′3′-$^3H_2$} propyl-2-aminoethyl benzilate) bromide] (40 Ci/nmole) was synthesized by reduction of allylbenzilylcholine with tritium gas. Tritium-labeled propylbenzilylcholine mustard (0.6 Ci/mmole) was purified by thin-layer chromatography. All radioactive compounds were > 95% pure by chromatography in several solvent systems.

The two methods used for the study of the binding of radioactive ligands were a filtration method (1) or the rapid centrifugation of the membrane fragments using a Microfuge (2).

RESULTS

Binding of Radioactive Antagonists

We have previously reported the irreversible binding of the alkylating antagonist [^{3}H]PrBCM to the receptor (1). The atropine-sensitive part of the binding follows simple bimolecular reaction kinetics. The reaction can be inhibited by the simultaneous presence of a reversible antagonist and, from the change in reaction rate, the binding curve for the reversible agent can be determined. Affinities so determined agree well with values determined by pharmacological antagonism on intact tissues. The affinities of [^{3}H]-atropine and [^{3}H]PrBCh have also been determined by direct binding, making allowance for nonspecific (or low affinity) binding and utilizing both self-saturation and cross-saturation experiments with the nonradioactive ligand. Both types of saturation experiment give identical affinity constants (Fig. 1).

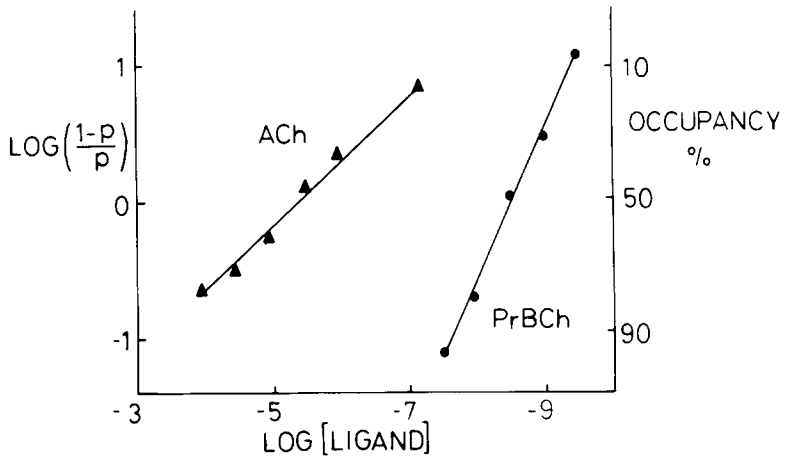

Fig. 2. Hill plots of PrBCh (●) and ACh (▲) binding curves. The slope for PrBCh is 1.0 and the slope for ACh is 0.5.

The binding capacity is also very similar for the three ligands and is in the range 1.3–2.0 nmoles/g protein for P2 pellets prepared from homogenates of rat cerebral cortex. When the homogenate was reacted with PrBCM under conditions in which the receptor would be expected to be fully alkylated, the preparations were devoid of saturable high affinity binding capacity for the reversible ligands, [^{3}H]Atr, and [^{3}H]PrBCh. When the affinity constants of other antagonists were determined by competition, the values obtained were also identical for the different labeled ligands and also consistent with the values obtained from pharmacological experiments. These results show that the antagonists tested are binding with the same receptor pool. In every case where direct or competition binding curves were treated as Hill plots there was no significant deviation from a slope of one (see, for example, Fig. 2). The close correspondence with the pharmacological results provides strong evidence that we are observing binding to the pharmacologically relevant receptor.

Interaction with Agonists

In the presence of muscarinic agonists (e.g., acetylcholine) the reaction of [^{3}H]PrBCM with the receptor is reduced, and so is the binding of [^{3}H]Atr and of [^{3}H]PrBCh (Fig. 1). The specific binding of antagonists can be completely suppressed by agonists. Hill plots of acetylcholine inhibition of [^{3}H]PrBCh binding gave a slope, $n_H = 0.50 \pm 0.05$ (Fig. 2); the same slope was found against the other labeled antagonists. Other full agonists also showed values of $n_H < 1$; pilocarpine, hexyltrimethylammonium, and oxotremorine gave $n_H = 1$.

The binding of acetylcholine to the receptor was measured directly with [^{3}H]-acetylcholine in the presence of cholinesterase inhibitors. Binding is easily measured up to ~30% of the receptor capacity indicated by antagonist binding (Fig. 1); at higher ligand concentrations the low ratio of specific to nonspecific binding makes the estimates of binding imprecise. For this reason it is not possible to state that the specific agonist binding capacity is identical to the specific antagonist binding capacity. However, there are

several indications that this is so. These are: (a) when the acetylcholine binding curve is plotted using the antagonist receptor capacity, the available part of the binding curve is virtually identical in shape to that obtained by competition with antagonists (Fig. 1); the slope of the Hill plot is also identical; (b) when a fraction of the antagonist sites is blocked by PrBCM pretreatment, the acetylcholine binding is reduced proportionately; (c) the displacement of [^{3}H] acetylcholine by a reversible antagonist gives an antagonist binding curve identical to that obtained by the means discussed earlier. The direct binding of acetylcholine thus confirms the results of the competitive experiments.

A comparison of the binding constants of the agonists with their effectiveness in intact tissue offers difficulties since the results can in neither case be analyzed in terms of a simple mass action equation. There are the added difficulties that more than one potency value can be obtained on intact tissue depending on the parameter measured (3) and there is also the problem of spare receptors.

Thus far, all we can say is that the rank orders for potency in smooth muscle and in the receptors do not correlate exactly. However, the lowest Hill coefficients are in general found for the more potent agonists.

DISCUSSION

We are faced in this study with the interesting paradox that while antagonists apparently bind to the receptor in a simple manner, the binding of most agonists does not follow a simple mass action law. Three possible explanations of these findings are being examined. (a) The receptor sites are conformationally coupled. To account for our observations, we would need to postulate either that antagonists do not induce conformational changes or that, if induced, these changes are not coupled; in contrast, agonist-induced changes are coupled in a negatively cooperative manner. It would be expected that if a fraction of the receptors was permanently occupied by alkylation with PrBCM, this should change the properties of the assembly. However, inactivation of up to 90% of the receptors with PrBCM failed to alter the Hill slope of carbachol/[^{3}H]PrBCh competition at the residual sites. This is not compatible with a conformationally coupled assembly of small extent, but does not exclude the possibility of a very large assembly. (b) In intact preparations there is evidence that prolonged exposure to agonists is followed by an inactivation process; this process is time and concentration dependent. It has been suggested that in smooth muscle exposure to high concentration of agonists leads to a decreased affinity for the agonist with an increase in the slope of the dose-response curve, suggesting that the low n_H is due to the presence of a mixed population of native and desensitized receptors (4). In synaptosome preparations, no evidence for this could be found. The centrifugation assay has permitted studies with exposure times to the agonist of 10–900 secs with no evidence of time dependence; pretreatment with high concentrations of agonist also failed to change the response. (c) There are two more classes of muscarinic receptors whose affinities for all antagonists are identical but whose affinities for a given agonist are not identical; this would have the effect of decreasing the slope of the binding curve. This explanation would fit with the pharmacological studies of Burgen and Spero (3) who compared the actions of muscarinic agonists in contraction and cation permeability in smooth muscle. If two receptor classes are present, it would be expected that the Hill slope calculated for values in the middle of the binding range would be less than 1 but that at the extremities of the binding curve these values would approach unity. Analysis of our experiments suggests that the Hill coefficient does increase at least at the low

occupancy end of the curve.

At the present time the reason for the low Hill slopes for agonists thus remains unsettled and requires further experimental investigation. Such evidence as we have available favors explanation (c). It is an intriguing phenomenon that has not been reported with other receptors.

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