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Short communication

Opposite effects of alcuronium on agonist and on antagonist binding to muscarinic receptors

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Abstract

Alcuronium is known to retard allosterically the dissociation of [^{3}H]*N*-methylscopolamine from muscarinic M_2 receptors, thereby augmenting the binding of this antagonist. Functionally, alcuronium behaves as a weak antimuscarinic agent and induces in combination with *N*-methylscopolamine an overadditive antimuscarinic action with oxotremorine-M as the agonist. The effect of alcuronium on the binding of [^{3}H]oxotremorine-M was studied in porcine heart homogenates. Agonist binding was concentration dependently inhibited with a $K_i = 0.48 \pm 0.03$ μM (means ± S.D., $n = 3$). Under identical conditions [^{3}H]*N*-methylscopolamine binding was elevated. Alcuronium, 100 μM, which nearly prevented the dissociation of [^{3}H]*N*-methylscopolamine, retarded the rate of dissociation of [^{3}H]oxotremorine-M only by a factor of two. These findings support the notion that the overadditive antimuscarinic action of alcuronium in conjunction with *N*-methylscopolamine is based on a shift by alcuronium of the interplay between agonist and antagonist in favour of the antagonist.

Keywords: Muscarinic receptor; Allosteric modulation; Alcuronium; Agonist; Heart

1. Introduction

Alcuronium is prominent among the various compounds known to modulate muscarinic M_2 receptors allosterically because of two features: alcuronium enhances [3H]*N*-methylscopolamine binding to muscarinic M_2 receptors (Proška and Tuček, 1994), whereas allosteric modulators commonly inhibit antagonist binding. Secondly, among various modulators alcuronium was found to be the most potent allosteric inhibitor of [^{3}H]*N*-methylscopolamine dissociation from muscarinic M_2 receptors (Tränkle et al., 1994), which further recommends alcuronium as a tool to study allosteric actions on muscarinic receptors. Alcuronium also inhibits [^{3}H]*N*-methylscopolamine association to muscarinic M_2 receptors, but since the effect on [^{3}H]*N*-methylscopolamine dissociation is more pronounced, the equilibrium binding of [^{3}H]*N*-methylscopolamine is elevated.

Functionally, alcuronium behaves as an antagonist at muscarinic receptors (e.g. Nedoma et al., 1985). Recently, it was reported that the antimuscarinic effect induced by a combination of alcuronium and *N*-methylscopolamine in contracting guinea pig atria with oxotremorine-M as the agonist was more pronounced than expected for a combination of competitive antagonists (Maaß et al., 1995). As an explanation for this phenomenon we suggested that alcuronium shifted the interplay between agonist and antagonist at the muscarinic M_2 receptors in favour of the antagonist. This suggestion was based on the hypothesis that alcuronium would diminish, and not elevate, agonist binding. It may be assumed that agonist dissociation is less inhibited by alcuronium than antagonist dissociation.

To test the validity of these assumptions, the effect of alcuronium on the equilibrium binding and the dissociation of the radiolabeled agonist, [^{3}H]oxotremorine-M, from muscarinic M_2 receptors was determined. Specific binding of [^{3}H]oxotremorine-M can hardly be measured in contracting guinea pig atria, probably because the receptors are predominantly in the low-affinity agonist binding state under these conditions. Therefore, the binding experiments were performed at muscarinic M_2 receptors in porcine heart membranes, using [^{3}H]oxotremorine-M and [^{3}H]*N*-methylscopolamine as radioligands.

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2. Materials and methods

2.1. Binding assay

The preparation of porcine heart homogenates has been described (Botero Cid et al., 1994). Protein content was determined according to Lowry et al. (1951). Binding of [^{3}H]oxotremorine-M (1 nM, specific activity 86.3 and 85.8 Ci/mmol) and [^{3}H]*N*-methylscopolamine (0.15 nM, specific activity 80.4 Ci/mmol) was measured in triplicate in a final volume of 1.5 ml with a buffer consisting of 3 mM $MgHPO_4$ and 50 mM TrisHCl, pH 7.3 at 23°C. Under control conditions the incubation period was 1 h in the case of [^{3}H]oxotremorine-M and 2 h in the case of [^{3}H]*N*-methylscopolamine.

Membranes were separated by rapid filtration of 1 ml incubation medium through glass fibre filters (Schleicher and Schüll, No. 6; Dassel, Germany) followed by two rinses with 5 ml ice-cold incubation buffer ([^{3}H]-oxotremorine-M) or distilled water ([^{3}H]*N*-methylscopolamine). In order to reduce the non-specific binding of [^{3}H]oxotremorine-M the filters were presoaked with 0.25% polyethylenimine solution. In the presence of 1 μM atropine non-specific binding as percentage of the total amounted to: [^{3}H]oxotromorine-M, $20 \pm 5\%$ (means $\pm$ S.D., $n = 12$); [^{3}H]*N*-methylscopolamine, $7 \pm 3\%$ (means $\pm$ S.D., $n = 9$). Membrane-bound radioactivity was determined by liquid scintillation counting.

To assess the effect of alcuronium on equilibrium binding, increasing concentrations of the test compound were applied. The incubation time was 3 h with [^{3}H]-oxotremorine-M as the radioligand. In the case of [^{3}H]*N*-methylscopolamine alcuronium was preincubated with the membranes for 2 h, before the radioligand was added for another 7 h. As suggested by Proška and Tuček (1994) the long incubation time was applied because equilibrium binding of [^{3}H]*N*-methylscopolamine is reached much later in the presence of a highly potent allosteric modulator.

The kinetic assay was performed in a volume of about 20 ml. After a preincubation period of 1 h the dissociation of [^{3}H]oxotremorine-M (2 nM) and [^{3}H]*N*-methylscopolamine (0.15 nM) was made visible by adding 1 μM atropine alone or in combination with the allosteric modulator, alcuronium. Aliquots of 1 ml were taken at the indicated intervals and processed as described above.

2.2. Statistics / data analysis

Non-linear regression analysis was carried out using the Inplot software (GraphPad Version 3.1, San Diego, CA, USA). Curve fitting to competition and inhibition data was based on the general Hill equation. Statistical calculations were done using the Instat software from GraphPad (San Diego, CA, USA).

2.3. Materials

[^{3}H]Oxotremorine-M and [^{3}H]*N*-methylscopolamine were obtained from NEN-Dupont (Homburg, Germany). Alcuronium dichloride was generously provided by Hoffmann-La Roche (Basel, Switzerland). *N*-Methylscopolamine bromide and atropine sulfate were purchased from Sigma (Deisenhofen, Germany), and oxotremorine-M iodide from ICN Biochemicals (Cleveland, OH, USA).

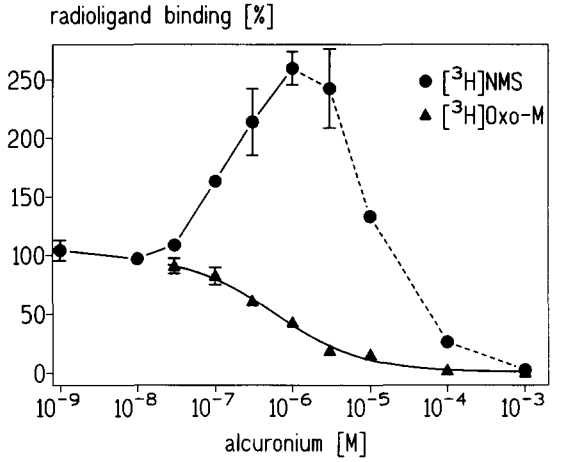

Fig. 1. Effect of alcuronium on the specific binding of [^{3}H]oxotremorine-M (triangles) and [^{3}H]*N*-methylscopolamine (circles) in porcine heart membranes. Points indicate mean values $\pm$ S.D. of triplicate determinations in representative experiments. The control values in the absence of alcuronium were taken as 100%. Error bars are not shown when they do not exceed the symbols. Curve-fitting for [^{3}H]oxotremorine-M data by non-linear regression analysis; point-to-point connection for [^{3}H]*N*-methylscopolamine data, the dashed line indicating that the decline is due to non-equilibrium data (for details see text).

3. Results

The binding of [^{3}H]oxotremorine-M under control conditions was characterized by an equilibrium dissociation constant, $K_d = 3.6 \pm 0.3$ nM and a binding site density of $B_{max} = 38 \pm 5$ fmol/mg protein (means $\pm$ S.D., $n = 4$). The effect of alcuronium on [^{3}H]oxotremorine-M binding in a representative experiment is depicted in Fig. 1. Binding was concentration dependently inhibited. In a total of three experiments the half-maximum inhibitory concentration was $IC_{50} = 0.61 \pm 0.04$ μM (mean $\pm$ S.D., $n = 3$). The slope factor of the curves was $n_H = -0.7 \pm 0.2$ (mean $\pm$ S.D., $n = 3$) and was not significantly different from unity (*t*-test, $P > 0.05$). Accordingly, it is possible to calculate $K_i = 0.48 \pm 0.03$ μM.

As comparison, the effect of alcuronium on the binding of [^{3}H]*N*-methylscopolamine ($K_d = 0.3 \pm 0.2$ nM and $B_{max} = 138 \pm 41$ fmol/mg protein; means $\pm$ S.D., $n = 4$) was determined under identical conditions. Alcuronium elevated the binding of [^{3}H]*N*-methylscopolamine, as could have been expected in view of the reports of Proška and Tuček (1994) and Jakubík et al. (1995). As pointed out by Proška and Tuček (1994), the declining part of the curve has to be considered as an artefact. In the presence of

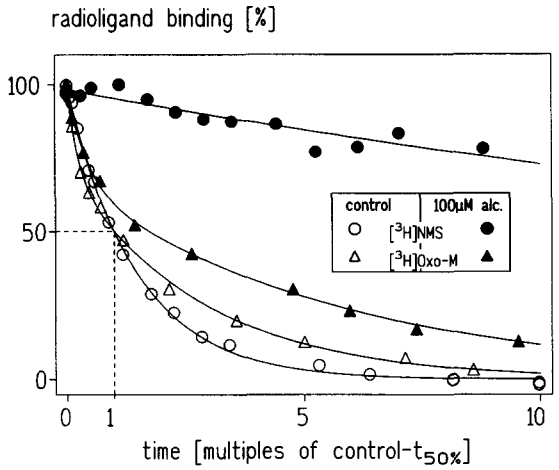

Fig. 2. Effect of 100 μM alcuronium on the dissociation of the agonist, [^{3}H]oxotremorine-M (triangles), and of the antagonist, [^{3}H]*N*-methylscopolamine (circles), in representative experiments; open symbols: control conditions, filled symbols: alcuronium present. Ordinate: radioligand binding as percentage of the starting level. Abscissa: time normalized to the respective control half-life, $t_{50\%}$, i.e. the time at which residual binding amounted to 50% of the starting level. $t_{50\%}$ under control conditions: [^{3}H]oxotremorine-M 4.2 min; [^{3}H]*N*-methylscopolamine 17.0 min. Biexponential curve-fitting in the case of [^{3}H]oxotremorine-M, monoexponential curve-fitting in the case of [^{3}H]*N*-methylscopolamine.

alcuronium the time required to reach the equilibrium binding of [^{3}H]*N*-methylscopolamine is prolonged, thus binding is underestimated in the presence of higher concentrations of alcuronium, even with long incubation periods.

The differential effect of alcuronium on the equilibrium binding of the radioligands was thought to reflect differential allosteric retardation of the radioligand dissociation. The time course of dissociation of [^{3}H]oxotremorine-M under control conditions (Fig. 2) was significantly better described by a biexponential equation than a monoexponential equation (partial *F*-test, $P < 0.05$). The biphasic time course of [^{3}H]oxotremorine-M dissociation from porcine muscarinic M_2 receptors has recently been studied and discussed thoroughly by Hirschberg and Schimerlik (1994). We found, for the fast phase, a capacity of 25 ± 3% of the total [^{3}H]oxotremorine-M binding and a half-life of $t_{1/2} = 0.8 \pm 0.1$ min and for the slow phase a capacity of 75 ± 3% with $t_{1/2} = 8.4 \pm 0.3$ min (means ± S.D., $n = 3$). For our purpose it sufficed to characterize the dissociation of [^{3}H]oxotremorine-M by the time interval after which binding fell to 50% of the starting level. This value amounted to $t_{50\%} = 4.8 \pm 0.5$ min (mean ± S.D., $n = 3$).

In the presence of 100 μM of alcuronium the dissociation of [^{3}H]oxotremorine-M was slightly delayed (Fig. 2). The shape of the dissociation curve was not altered from the control conditions. Biphasic curve-fitting yielded the following data: for the fast phase, the capacity amounted to 30 ± 2%, $t_{1/2}$ was 1.5 ± 0.2 min; for the slow phase the capacity was 70 ± 2% and the $t_{1/2}$ was 20 ± 4 min (means ± S.D., $n = 4$). Thus, the capacities of the two components of [^{3}H]oxotremorine-M dissociation were not affected by alcuronium, whereas the rates of dissociation in these components were reduced by a factor of about two. The $t_{50\%}$ value amounted to 10 ± 2 min (means ± S.D., $n = 4$), being doubled that under control conditions.

The dissociation of [^{3}H]*N*-methylscopolamine under control conditions was monophasic with a half-life of $t_{1/2} = 16.8 \pm 0.5$ min. Alcuronium, 100 μM, induced a pronounced delay of [^{3}H]*N*-methylscopolamine dissociation (Fig. 2). Assuming a monophasic curve, the half-life of dissociation was increased by a factor of 20 in this experiment. On the average, the apparent rate constant of dissociation was reduced by 100 μM alcuronium to 7 ± 2% of the control (means ± S.D., $n = 4$).

4. Discussion

The molecular mode of action of allosteric modulators of muscarinic receptors is being intensively studied (e.g. Tuček and Proška, 1995; Lazareno and Birdsall, 1995). The allosteric compounds show particular features, not only with respect to their mode of interaction with muscarinic receptors, but also with regard to their functional properties. For alkane-bis-ammonium-type modulators as well as gallamine it has been reported that the antimuscarinic effect may saturate at higher concentrations of the compounds (Lüllmann et al., 1969; Clark and Mitchelson, 1976). This phenomenon was, however, not encountered with alcuronium (Maaß et al., 1995). Furthermore, in combination with conventional antimuscarinic compounds such as atropine and *N*-methylscopolamine, allosteric compounds induced non-additive antimuscarinic actions. For instance, gallamine combined with atropine acted as less than additive (Clark and Mitchelson, 1976), whereas alkane-bis-ammonium compounds as well as alcuronium-induced overadditive antimuscarinic effects (Lüllmann et al., 1969; Mitchelson, 1975; Maaß et al., 1995). It is not yet settled how (or even whether) these functional phenomena are related to the uncommon mode of interaction with muscarinic receptors.

Since alcuronium acts allosterically on muscarinic receptors, it is not self-evident that the anti-agonistic effect is based on inhibition of agonist binding – interference with events subsequent to agonist binding may well be imagined. Therefore, it was considered worthwhile to check whether alcuronium inhibits the binding of [^{3}H]-oxotremorine-M in contrast to the binding of [^{3}H]*N*-methylscopolamine, which is known to be elevated. Alcuronium reduced the binding of oxotremorine-M concentration dependently. The slope of the inhibition curve was not significantly different from unity, which corresponds with the functional findings: the antagonistic effect of alcuronium in contracting guinea pig left atria was characterized in the Schild plot by linearity and a slope of unity (Maaß et al., 1995). The $K_i = 0.5$ μM from the binding studies is

of the same order of magnitude as the $pA_2 = 5.7$ from the functional experiments. Thus, the antagonistic action of alcuronium can be explained adequately by its inhibitory effect on agonist binding. Interference with signal transduction needs not be taken into account.

The opposite effect of alcuronium on the binding of [^{3}H]oxotremorine-M and [^{3}H]*N*-methylscopolamine may result from the differential effect on radioligand dissociation. The allosteric inhibition of dissociation is an event promoting ligand binding to the receptor. Due to the marginal effect on [^{3}H]oxotremorine-M dissociation, binding of the agonist is much less favoured by alcuronium than the binding of [^{3}H]*N*-methylscopolamine, the dissociation of which is strongly inhibited. The dependence on ligand type of the inhibitory effect of allosteric modulators on ligand dissociation is a well-established phenomenon (Lee and El-Fakahany, 1988; Waelbroeck et al., 1984, 1988). The divergence is commonly explained by the fact that the effect on dissociation results from the interaction of the allosteric modulator with a receptor already occupied by a ligand. In contrast, the inhibitory effect of allosteric modulators on the association of a ligand results from binding of the alloster to an unliganded receptor. Thus, it does not appear surprising that the inhibition of ligand association by allosteric modulators was found far less dependent on ligand choice. For instance, Waelbroeck et al. (1988) reported that the gallamine concentrations inhibiting ligand association by 50% were identical for [^{3}H]*N*-methylscopolamine and [^{3}H]oxotremorine-M, amounting to 0.35 μM in both cases, whereas the concentrations inhibiting ligand dissociation differed by a factor of 33. Therefore, the divergent effects of alcuronium on the equilibrium binding of [^{3}H]*N*-methylscopolamine and [^{3}H]oxotremorine-M probably arise from the pronounced difference in potency to retard the dissociation of these ligands.

In any case, the fact that alcuronium increases [^{3}H]*N*-methylscopolamine binding while diminishing [^{3}H]-oxotremorine-M binding to muscarinic M_2 receptors offers an explanation for the phenomenon of a more than additive antimuscarinic effect induced by alcuronium in functional experiments with these ligands.

In conclusion, the effects of alcuronium on the binding to muscarinic M_2 receptors of [^{3}H]oxotremorine-M and [^{3}H]*N*-methylscopolamine correspond favourably with the functional interaction of this allosteric modulator with the agonist alone and in combination with the antagonist.

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