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Propylbenzilylcholine mustard is selective for rat heart muscarinic receptors having a low affinity for agonists

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1 Propylbenzilylcholine mustard (PrBCM), an irreversible muscarinic antagonist, inactivated receptors with a low affinity for agonists faster than those with a high affinity in rat heart membranes. This result was obtained using either: (a) a low ionic strength buffer (allowing heterogeneity among antagonist binding sites, (b) the same buffer enriched with GTP, or (c) a high ionic strength buffer (where antagonists showed similar binding characteristics to all receptors).

2 These data suggest either that PrBCM is a 'selective' antagonist which detects conformational differences between low and high affinity receptors, or that the agonist affinity of cardiac muscarinic receptors is determined, in part, by the relative concentrations of receptor and GTP binding protein.

Introduction

In contrast to the binding of most antagonists to muscarinic receptors, agonists appear to recognize at least two classes of binding sites – or states – in all tissues studied (for review see Ehlert *et al.*, 1981). This observation has led us to ask two questions: (a) are these 'binding sites' interconvertible or do they reflect the existence of different receptor proteins? (2) Which of these sites is responsible for the biological effects of muscarinic agonists?

Birdsall *et al.* (1978) were able to demonstrate the existence of three classes of muscarinic receptors in a crude preparation of synaptosomes from rat cerebral cortex. In this system, the two major populations of agonist binding sites did not interconvert during the binding experiment and showed the same affinity for antagonists. Quantitative correlation between agonist affinity constants for the low affinity site in the synaptosome preparation and agonist activities in smooth muscle, after elimination of spare receptors, suggested that low affinity agonist binding sites were responsible for the biological effects of muscarinic agonists.

It is increasingly evident that cardiac and brain muscarinic receptors present different binding characteristics (see for example Hammer *et al.*, 1980; Gibson *et al.*, 1983; Vickroy *et al.*, 1984), although the evidence for the origin of these differences is not yet conclusive (Venter, 1983; Schreiber & Sokolovsky, 1984). Since phenoxybenzamine (an irreversible antagonist often used to detect the existence of muscarinic or spare α-adrenoceptors) is able to block calcium channels (Gengo *et al.*, 1984), we believe that propylbenzilylcholine mustard (PrBCM) (Young *et al.*, 1972) will be used increasingly to detect muscarinic spare receptors. We therefore decided to verify whether PrBCM behaves the same way in rat heart as in rat brain (Birdsall *et al.*, 1978), i.e. like a non-selective antagonist. In fact, PrBCM proved to block more effectively the muscarinic receptors with a lower affinity for agonists.

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Methods

Preparation of rat heart membranes

Rat cardiac membranes were prepared according to the method of Snyder & Drummond (1978) with a few modifications. Wistar albino rats, fed *at libitum* on standard chow, were decapitated. The hearts were quickly removed, rinsed at room temperature with isotonic NaCl solution and stored in liquid N_2. All subsequent operations were performed at 4°C. Each heart was homogenized with a glass-Teflon homogenizer in 5 ml of 20 mM Tris-HCl buffer (pH 7.5) enriched with 5 mM $MgCl_2$. After a 2 fold dilution in the same buffer, the homogenate was filtered through two layers of medical gauze and centrifuged at 520 *g* for 10 min. The membrane pellet was washed once with 10 ml of homogenization buffer. The resulting pellet

was resuspended in 5 ml of 20 mM Tris-HCl buffer (pH 7.5) containing 0.25 M sucrose and 5 mM $MgCl_2$. An equal volume of 20 mM Tris-HCl buffer (pH 7.5) enriched with 0.25 M sucrose and 2.5 M KCl was added dropwise. The final mixture was left for 1 h in an ice bath, under continuous stirring, then centrifuged at 37 000 *g* for 10 min. The resulting pellet was washed three times in 10 ml of 20 mM Tris-HCl buffer (pH 7.5) containing 0.25 M sucrose. The membranes were finally resuspended in the last buffer at a final concentration of approximately 6 mg of protein ml^{-1} and stored in liquid N_2. Protein concentrations were determined by the method of Lowry *et al.* (1951).

Treatment of rat heart membranes with PrBCM

Propylbenzilylcholine mustard was dissolved in ethanol, and stored at −20°C. Immediately before use, PrBCM was activated according to Young *et al.* (1972) as follows. The stock solution was diluted in 10 mM phosphate buffer (pH 7.5, final PrBCM concentration: 0.6 mM), preincubated for 1 h at room temperature and used within 10 min. Unless otherwise indicated, rat heart membranes (0.5 to 0.7 mg ml^{-1}) were diluted in 20 mM Tris/HCl buffer (pH 7.5) enriched with 0.25 M sucrose, then treated for 5 min at 25°C with or without the stated concentrations of activated PrBCM or 10 μM GTP. Membranes were then centrifuged for 10 min at 37 000 *g*, and the resulting pellet was washed once in the same buffer. The membranes were then resuspended in the same buffer at a final concentration of approximately 6 mg of protein ml^{-1}.

In some experiments (as stated in the legends to figures), rat heart membranes were centrifuged at 37 000 *g* for 10 min and resuspended in 50 mM sodium phosphate buffer (pH 7.4) enriched with 2 mM $MgCl_2$ (final protein concentration: 0.5 to 0.7 mg ml^{-1}) prior to treatment with PrBCM. The membranes were then treated with PrBCM, washed and resuspended in the same phosphate buffer, as indicated above.

Measurement of [^{3}H]-NMS or [^{3}H]-oxo-M binding

Cardiac membranes (0.1 mg protein ml^{-1}) were incubated at 25°C in 1.2 ml of 50 mM sodium phosphate buffer (pH 7.4) enriched with 2 mM $MgCl_2$, 1% bovine serum albumin, and 1.0 nM [^{3}H]-NMS or 1.5 nM [^{3}H]-oxo-M in the presence or absence of the indicated concentrations of unlabelled drug or 10 μM GTP. Incubation for 10 min at 25°C in the phosphate buffer allowed complete equilibration of binding (not shown). The incubation was terminated by filtering the samples on glass-fibre filters (GF/C; Whatman, Maidstone, Kent, U.K.). The filters were rinsed four times with 2 ml of ice-cold 20 mM Tris-HCl buffer (pH 7.5) enriched with 0.25 M sucrose and 1% bovine serum albumin, dried and the radioactivity determined by liquid scintillation counting. The total contact time between the filters and the rinsing buffer was less than 8 s, so that dissociation of [^{3}H]-NMS and [^{3}H]-oxo-M during the filtration procedure was probably negligible (the dissociation rate of both tracers is decreased at low temperatures).

Non-specific binding (mainly due to interactions with the filters) was defined as tracer binding in the presence of 1 μM atropine. It represented less than 1% of total binding, was unaffected by membrane pretreatment, and was subtracted from total binding, yielding specific binding.

[^{3}H]-NMS-agonist competition curves (measurement of tracer binding in the presence of various concentrations of agonist) were analysed using a programme developed by Minneman *et al.* (1979), assuming the existence of two classes of receptor.

For Scatchard analysis of tracer binding, membranes were incubated for 10 min as described above, in the presence of 0.4 to 8.0 nM [^{3}H]-NMS or [^{3}H]-oxo-M and in the presence or absence of 1 μM atropine. Specific binding was measured as indicated above. The results were analysed according to Scatchard (1949).

Effect of albumin on PrBCM activity

Preliminary experiments indicated that PrBCM was inactivated by the bovine serum albumin generally present in incubation buffers. We therefore performed all experiments using PrBCM at low membrane protein concentration (< 1 mg ml^{-1}) and in the absence of albumin.

Chemicals

[N-methyl-^{3}H]-N-2-methylscopolamine ([^{3}H]-NMS: specific radioactivity 54 Ci $mmol^{-1}$) and [methyl-^{3}H]-oxotremorine M acetate ([^{3}H]-oxo-M: specific radioactivity 84 Ci $mmol^{-1}$) were obtained from New England Nuclear Corporation (Dreieich, F.R.G.). Carbamylcholine was obtained from Federa (Brussels, Belgium) and atropine from Sigma Chemical Co (St Louis, MO, U.S.A.). Propylbenzilylcholine mustard (PrBCM) was obtained from Amersham (Bucks, U.K.). Guanosine 5′-triphosphate (GTP) was purchased from Boehringer (Mannheim, F.R.G.).

Results

PrBCM competition curves

To study the interaction of activated PrBCM with rat heart muscarinic receptors, we first performed inhibition curves for [^{3}H]-NMS and [^{3}H]-oxo-M binding, in the 50 mM sodium phosphate buffer previously used

(Waelbroeck *et al.*, 1982), in the absence of bovine serum albumin. As shown in Figure 1, PrBCM inhibited [^{3}H]-NMS and [^{3}H]-oxo-M binding to the same extent, with Hill coefficients of, respectively, 1.12 ± 0.15 and 1.01 ± 0.10 (not significantly different from 1).

Irreversible binding of PrBCM

Dose-dependence As shown in Figure 2, a 5 min preincubation of rat heart membranes in the presence of PrBCM, followed by extensive washing, induced a dose-dependent decrease of the total muscarinic receptor concentration. The affinity of [^{3}H]-NMS for the remaining receptors (slope of Scatchard plots) was not affected by this pretreatment. The decrease of [^{3}H]-NMS binding after pretreatment was not reversed by a second (30 min) incubation in the absence of PrBCM (not shown).

Time-dependence Preliminary experiments suggested that some muscarinic receptors were inactivated by PrBCM during the centrifugation period. To study the time-dependence of [^{3}H]-NMS receptor inactivation, we therefore used as 'control membranes' membranes to which PrBCM was added immediately before centrifugation.

As shown in Figure 3a, the kinetics of [^{3}H]-NMS

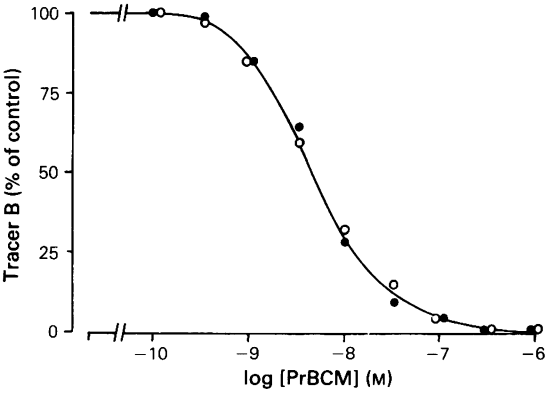

Figure 1 Inhibition of [N-methyl-^{3}H]-N-methylscopolamine ([^{3}H]-NMS) (●) and [methyl-^{3}H]-oxotremorine M acetate ([^{3}H]-oxo-M) (○) binding by propylbenzilylcholine mustard (PrBCM). Rat heart membranes were incubated for 10 min before filtration in the presence of 1.0 nM [^{3}H]-NMS or 1.5 nM [^{3}H]-oxo-M (i.e. concentrations close to their respective K_D values) and the indicated concentrations of activated PrBCM in a 50 mM sodium phosphate buffer (pH 7.4) enriched with 2 mM $MgCl_2$. The results are expressed as percentage of tracer specifically bound (B) in the absence of PrBCM, and are the average of 2 experiments performed in duplicate.

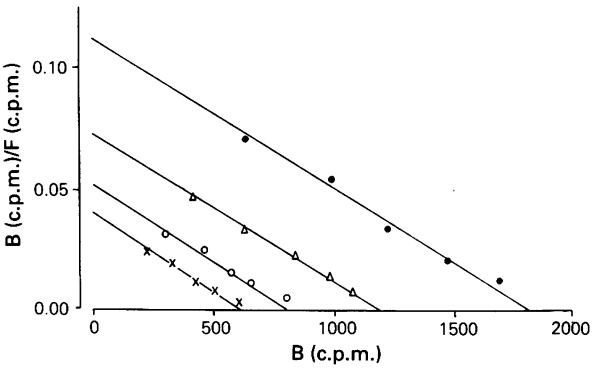

Figure 2 [N-methyl-^{3}H]-N-methylscopolamine ([^{3}H]-NMS) Scatchard plots after propylbenzilylcholine mustard (PrBCM) pretreatment. Rat heart membranes were pretreated with 0 (●), 3 (Δ), 10 (○) or 30 (×) nM PrBCM for 5 min at 25°C, and washed prior to incubation with 0.4 to 8.0 nM [^{3}H]-NMS. The results are presented according to Scatchard (1949). This experiment is representative of 10 others.

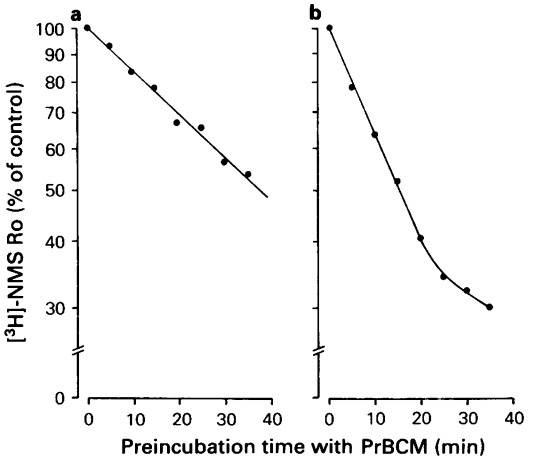

Figure 3 Muscarinic receptor inactivation kinetics. Rat heart membranes were preincubated for 40 min at 25°C, and 3 nM (a) or 10 nM (b) propylbenzilylcholine mustard (PrBCM) was added to each tube at the indicated time intervals before centrifugation. After washing of the membranes, the [N-methyl-^{3}H]-N-methylscopolamine ([^{3}H]-NMS) receptor concentration (Ro) was determined as in Figure 2 by Scatchard plot analysis. The results are expressed as percentage of the [^{3}H]-NMS receptor concentration in membranes centrifuged immediately after PrBCM addition. Each experiment is representative of two.

receptor inactivation – at a low (3 nM) PrBCM concentration – were 'first order' (Young *et al.*, 1972; Burgisser *et al.*, 1982). This suggested that PrBCM itself was stable during preincubation. At a higher (10 nM) PrBCM concentration, however, the rate of receptor inactivation decreased with increasing preincubation periods, indicating that PrBCM might react at different rates with two or more muscarinic receptor subclasses (Young *et al.*, 1972; Burgisser *et al.*, 1982) (Figure 3b).

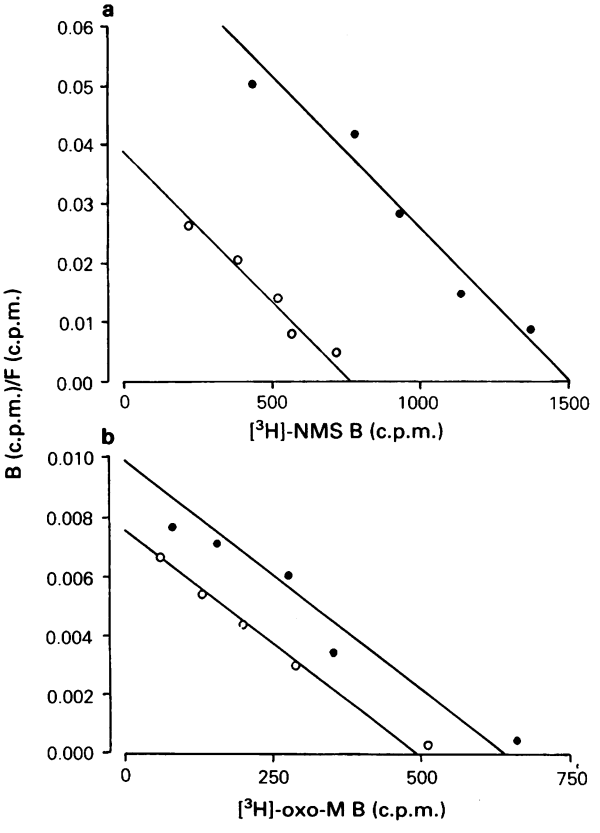

Figure 4 Comparison of the effects of propylbenzilylcholine mustard (PrBCM) pretreatment on [N-methyl-^{3}H]-N-methylscopolamine ([^{3}H]-NMS) (a) and [methyl-^{3}H]-oxotremorine M acetate ([^{3}H]-oxo-M) (b) binding. Rat heart membranes were pretreated with 0 (●) or 10 (○) nM PrBCM then washed. Specific 0.4 to 8.0 nM [^{3}H]-NMS and 0.1 to 4.0 nM [^{3}H]-oxo-M binding to rat heart membranes was then analysed according to Scatchard (1949). The protein concentrations were identical in control and PrBCM-treated membranes (0.09 and 0.15 mg of membrane protein ml^{-1}, respectively, for [^{3}H]-NMS and [^{3}H]-oxo-M binding). This experiment is representative of 7 others.

Effect of PrBCM on agonist binding

[^{3}H]-oxo-M binding By contrast with [^{3}H]-NMS, which recognized all rat heart muscarinic receptors with the same affinity in the 50 mM phosphate buffer (Asselin *et al.*, 1983), [^{3}H]-oxo-M selectively labelled the receptor subclass with a higher affinity for agonists (Waelbroeck *et al.*, 1982). As shown in Figure 4, [^{3}H]-oxo-M receptors were markedly less affected than [^{3}H]-NMS receptors by a pretreatment with PrBCM. The results of seven similar experiments, at various PrBCM concentrations, are summarized in Figure 5. Up to 50% of [^{3}H]-NMS receptors could be inactivated by PrBCM before the [^{3}H]-oxo-M receptor concentration decreased by more than 10% (Figure 5).

Carbamylcholine competition curves. The preferential inactivation of low affinity receptors by PrBCM was also evident in [^{3}H]-NMS -agonist competition curves. Indeed, as shown in Figure 6, membrane pretreatment with PrBCM induced a dose-dependent leftward shift of carbamylcholine competition curves, indicating an increase in the average affinity of the remaining muscarinic receptors for the agonist.

A computer analysis of competition curves confir-

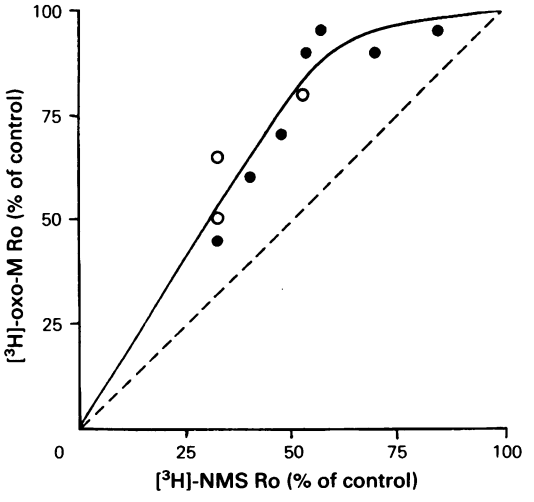

Figure 5 Comparison of the effects of propylbenzilylcholine mustard (PrBCM) pretreatment on the number (Ro) of [^{3}H]-oxo-M and [^{3}H]-NMS receptors. Rat heart membranes were pretreated with PrBCM (1 to 10 nM) in either Tris-HCl buffer (●) (4 experiments) or sodium phosphate buffer (○) (3 experiments), then washed and assayed for muscarinic receptors as indicated in Figure 4. All results are expressed as percentage of control binding to membranes pretreated in the absence of PrBCM (5 determinations performed in duplicate at different tracer concentrations). For abbreviations see legend to Figure 4.

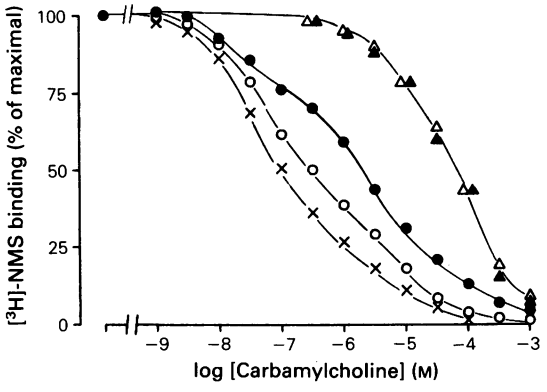

Figure 6 Comparison of [N-methyl-^{3}H]-N-methylscopolamine ([^{3}H]-NMS) vs carbamylcholine competition curves in rat heart membranes pretreated with no (●,▲), 3 nM (○) or 30 nM (×,△) propylbenzilylcholine mustard (PrBCM) and washed. The membranes were then incubated in the absence (●,○,×) or presence (▲,△) of 100 μMGTP. The remaining [^{3}H]-NMS receptor concentrations were, respectively, 63 and 35% of control after pretreatment with 3 and 30 nM PrBCM (not shown). In the absence of GTP, the high affinity agonist receptor represented, respectively, 24, 44 and 58% of [^{3}H]-NMS binding after pretreatment with no, 3 or 30 nM PrBCM (that is: 116 and 85% of the high affinity agonist receptors were still present, after treatment with 3 or 30 nM PrBCM). This experiment is representative of 3 experiments performed in duplicate.

med that receptors with a high affinity for agonists decreased in number only at a high PrBCM concentration. In good agreement with the results shown in Figure 5, up to 50% of the [^{3}H]-NMS labelled receptors could be inactivated, without inducing a significant decrease of the high-affinity agonist receptor concentration (see legend of Figure 6).

Reagents modifying sulphydryl residues such as dithiobisnitrobenzoate and N-ethyl maleimide prevent GTP inhibition of agonist binding (Uchida *et al.*, 1985; Harden *et al.*, 1982). We therefore measured the effect of GTP on agonist binding to PrBCM-treated membranes.

In the presence of 100 μM GTP, carbamylcholine inhibited [^{3}H]-NMS binding with a single, low affinity (IC_{50} = 80 μM; n_H = 1.0). The competition curves obtained using control and PrBCM-treated membranes were able to be superimposed (Figure 6).

Influence of buffer composition on PrBCM binding

In low ionic strength buffers (such as the Tris-HCl buffer used in most of our PrBCM pretreatment experiments), muscarinic antagonists recognized two classes of rat heart muscarinic receptors with very different affinities (Hulme *et al.*, 1981; Burgisser *et al.*, 1982), the 'high affinity antagonist receptors' having probably a low affinity for agonists (Burgisser *et al.*, 1982). This apparent heterogeneity was abolished by GTP addition to the buffer or by an increase in ionic strength (Hulme *et al.*, 1981). To test the hypothesis that low ionic strength could be responsible for the observed PrBCM selectivity, we studied agonist binding, after pretreatment with PrBCM, under two conditions where no antagonist receptor subclasses can be distinguished at equilibrium or by kinetic experiments:

(a) The two 'classes' of rat heart muscarinic receptors are converted to a single high antagonist/low agonist affinity state by adding GTP or guanosine 5'-O-(2-3-imido)-triphosphate (p(NH)ppG) (Hulme *et al.*, 1981; Burgisser *et al.*, 1982). We previously demonstrated that the inhibitory effect of p(NH)ppG on agonist binding to muscarinic receptors is persistent (Waelbroeck *et al.*, 1981) while that of GTP is fully reversible upon washing. This is why we studied the effect of GTP on irreversible PrBCM binding and on subsequent [^{3}H]-NMS and carbamylcholine binding properties (Figure 7). Preincubation with 10 nM PrBCM alone led to a 47% decrease in receptor concentration (not shown) and to a marked (6 fold) decrease of the EC_{50} value for carbamylcholine (Figure 7). In the presence of GTP, PrBCM was

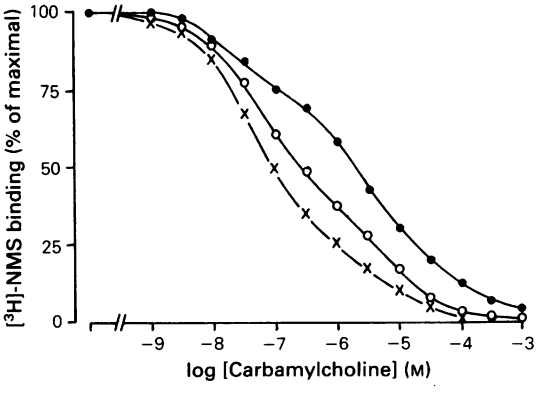

Figure 7 Comparison of [N-methyl-^{3}H]-N-methylscopolamine ([^{3}H]-NMS) vs carbamylcholine competition curves in rat heart membranes pretreated with GTP and propylbenzilylcholine mustard (PrBCM). Heart membranes were preincubated in the absence or presence of 10 μM GTP (●), in the presence of 10 nM PrBCM (○) or in the simultaneous presence of 10 nM PrBCM and 10 μM GTP (×) then washed and assayed for [^{3}H]-NMS binding in the absence or presence of carbamylcholine. The remaining [^{3}H]-NMS receptor concentrations were, respectively, 100, 63 and 16% of control in membranes pretreated with GTP alone (●), PrBCM alone (○), or PrBCM + GTP (×).

markedly more potent, and inactivated 84% of [^{3}H]-NMS receptors (not shown). The average affinity of carbamylcholine for the remaining receptors was further increased as shown by a 12 fold decrease of the EC_{50} value (Figure 7).

(b) [^{3}H]-NMS recognizes a single state of kinetically quivalent receptors in the 50 mM phosphate buffer generally used in our incubations (Gibson *et al.*, 1983 and unpublished results). We therefore investigated agonist binding to rat heart membranes pretreated with 0 to 3 nM PrBCM in this phosphate buffer.

Pretreatment with PrBCM affected [^{3}H]-oxo-M receptors less than [^{3}H]-NMS receptors under both conditions (Figure 5). Carbamylcholine competition curves were also shifted to the left after PrBCM pretreatment in phosphate buffer (not shown).

Discussion

PrBCM behaved in competition studies in a similar manner to classical antagonists. Indeed, after a short incubation period (10 min at 25°C) it inhibited [^{3}H]-NMS and [^{3}H]-oxo-M binding to the same extent, with a Hill coefficient close to 1.0 (Figure 1). This result suggested that PrBCM recognized the high and low agonist affinity receptor subclasses with similar affinity. It also inactivated the receptors rather slowly as compared to its dissociation rate from the receptors in phosphate buffer: indeed Hill coefficients higher than 1.0 would be expected if the muscarinic receptors were rapidly inactivated by PrBCM (Beck & Goren, 1983).

Our analysis of [^{3}H]-NMS and [^{3}H]-oxo-M binding to membranes pretreated with PrBCM and washed confirmed that this antagonist may irreversibly bind to muscarinic receptors (Birdsall *et al.*, 1978; Young *et al.*, 1972). The affinity of the remaining 'unreacted' receptors for [^{3}H]-NMS or [^{3}H]-oxo-M was not affected by the pretreatment (Figure 4). GTP inhibition of carbamylcholine binding to both high and low affinity receptors was unaffected by PrBCM pretreatment (Figure 6), further suggesting that the 'unreacted' receptors were not modified by PrBCM (see, by contrast, the effect of NEM or DTNB on GTP regulation of agonist binding, Harden *et al.*, 1982; Uchida *et al.*, 1985). By contrast, the proportion of receptors with high affinity for agonists was markedly increased following PrBCM treatment, as demonstrated by [^{3}H]-NMS vs carbamylcholine competition curves and by [^{3}H]-NMS/[^{3}H]-oxo-M binding comparison (Figures 4 and 6).

We generally used a low ionic strength 20 mM Tris buffer supplemented with 250 mM sucrose to pretreat the membranes with PrBCM. Indeed, the stability of the high-agonist affinity state of muscarinic receptors was enhanced in this buffer (not shown). The inactivation kinetics of cardiac muscarinic receptors by PrBCM in this buffer were not first order (Figure 3), suggesting that PrBCM alkylated two or more receptors subclasses with different overall rate constants (Young *et al.*, 1972; Beck & Goren, 1983). These results were compatible with the hypothesis that PrBCM detects slight conformational differences between the two classes of agonist receptors present in rat heart, and reacts faster with low agonist affinity receptors than with high agonist affinity receptors. In low ionic strength buffers, antagonists (Hulme *et al.*, 1981; Burgisser *et al.*, 1982) and agonists (Burgisser *et al.*, 1982) indeed detect the existence of two receptor types.

This receptor heterogeneity is probably due to an interaction of the receptor with the GTP-binding protein Ni. It can be abolished under two experimental conditions. (1) Guanine nucleotides convert most or all the muscarinic receptors to a state with high affinity for antagonists and low affinity for agonists (Hulme *et al.*, 1981; Burgisser *et al.*, 1982). (2) In the 50 mM phosphate buffer used for binding studies, we were unable to observe any effect of GTP on antagonist binding. [^{3}H]-NMS recognized all cardiac muscarinic receptors with the same association rate, dissociation rate and affinity.

Addition of GTP to the Tris-sucrose buffer increased the [^{3}H]-NMS association rate to our cardiac membranes, without affecting its dissociation rate (not shown).

We assumed that, as with other muscarinic antagonists, PrBCM bound faster or with a greater affinity to the receptor with high antagonist – low agonist affinity in the Tris-sucrose buffer. To test this hypothesis, we pretreated membranes with PrBCM under two conditions where antagonists recognize all muscarinic receptors with the same affinity: (1) in the 50 mM sodium phosphate buffer generally used for binding studies; and (2) in the presence of GTP. As shown in Figures 5 and 7, the PrBCM selectivity was maintained, even under conditions where most or all receptors showed an identical high affinity for antagonists. We have no evidence for a difference in the sensitivity of PrBCM alkylation of high versus low agonist affinity receptors to GTP or the buffer composition. Two hypotheses may account for the PrBCM selectivity:

(1) Agonist binding to both classes of receptors is regulated by Ni, the regulatory GTP binding protein responsible for adenylate cyclase inhibition (Kurose & Ui, 1983). We observed that the receptors with a higher affinity for agonists were more readily converted than the low affinity receptors to a very low affinity state by persistent activation of the membranes by GppNHp (Waelbroeck *et al.*, 1981 and results not shown) or by *Bordetella pertussis* vaccine pretreatment (unpublished observations). These results suggest that the presence of a functional Ni protein is more necessary for the formation of the high than for a low

agonist affinity state, so that the relative concentration of high/low/very low agonist affinity states might depend on the receptor to Ni concentration-ratio. It is possible, therefore, that 'high agonist affinity' and 'low agonist affinity' receptors in rat heart membranes represent different modes of association of the same receptor protein with Ni, the relative concentrations of high/low/very low affinity receptors depending on the relative receptor and Ni concentrations.

(2) On the other hand, it is also possible that, due to conformation or amino acid sequence differences, the receptors with a lower affinity for agonists are more readily inactivated by PrBCM, the distance beteen the aziridinium ion of PrBCM and the reactive nucleophilic residue from the receptor being smaller. Our data do not allow us to exclude either hypothesis.

In summary, these results indicate that PrBCM inactivates the receptors with lower affinity for agonists more efficiently than the receptors with high affinity for agonists. We do not know whether this difference is due to intrinsic differences in receptor conformation or to a modulation of the receptor-GTP binding protein interaction by changes in receptor concentration.

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