Effects of temperature and ethanol on agonist and antagonist binding to rat heart muscarinic receptors in the absence and presence of GTP

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The effect of temperature on the binding of four agonists and three antagonists to rat heart muscarinic receptors was studied in the absence and presence of GTP. The binding of agonists to two states (or classes) of receptors, in the absence of GTP, led to enthalpy and entropy changes that decreased sharply above 25 °C, suggesting that agonists induced 'isomerization' reactions (large conformational changes and/or receptor-effector association). Both temperature increase and ethanol decreased hydrophobic interactions, thereby hindering binding and/or agonist-induced 'isomerization' reactions. Addition of GTP to the incubation medium also appeared to reverse (or prevent) 'isomerization' reactions. For agonist binding to the low-affinity state, in the presence of GTP, and for antagonist binding, the thermodynamic parameters observed could be readily explained by simple receptor-ligand associations; large entropy increases and small enthalpy increases, provoked by hydrophobic and ionic interactions, were partly neutralized by entropy and enthalpy decreases, due to hydrogen bonds and van der Waals interactions. The muscarinic antagonists used (atropine, *n*-methylscopolamine and dextimide), being more hydrophobic molecules than the agonists tested (carbamylcholine, oxotremorine and pilocarpine), induced larger entropy changes or more negative enthalpy changes.

INTRODUCTION

At least two classes of muscarinic receptors have been described in rat heart membranes (Waelbroeck *et al.*, 1982 and references cited therein): the receptors with highest affinity for the agonists [possibly identical with the brain 'SH' (super-high) receptors] have a 10–100-fold higher affinity than the other class of receptors ['H' (high) receptors] (Birdsall & Hulme, 1976; Birdsall *et al.*, 1978). Both classes of receptors may be converted to a low-affinity state by addition of GTP (Waelbroeck *et al.*, 1982). It is conceivable that the association of the receptors to a guanine nucleotide binding regulatory protein, N_i , in the absence of GTP, is necessary to allow formation of high-affinity agonist-receptor complexes (Kurose & Ui, 1983; Harden *et al.*, 1983; Birdsall *et al.*, 1984).

While agonists and antagonists are thought to occupy the same 'binding site' on the receptors, agonists alone lead to activation of the receptors and information transfer. This difference can often be examined by the thermodynamic analysis of agonist and antagonist binding. Indeed, agonist binding to muscarinic receptors (Galron *et al.*, 1981) appears to induce lower enthalpy and entropy changes than does antagonist binding.

Major conformational changes or protein-protein association are known to provoke characteristic thermodynamic behaviour: for example, reactions of protein

association (Ross & Subramanian, 1981) and protein denaturation (Kauzmann, 1959; Tanford, 1969, 1970) may lead to large heat capacity changes (variations of the enthalpy change with temperature) and curvilinear van't Hoff plots. We therefore decided to study the effect of temperature on the binding of three muscarinic antagonists and four agonists to rat heart muscarinic receptors, in the absence and presence of GTP, in a relatively wide temperature range (5–40 °C).

MATERIALS AND METHODS

Chemicals

[³H]NMS (sp. radioactivity 54 Ci/mmol) and [³H]Oxo-M (sp. radioactivity 84 Ci/mmol) were obtained from New England Nuclear (Dreieich, Germany). Oxotremorine sesquifumarate was obtained from Aldrich Chemical Co. (Beerse, Belgium), atropine from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and carbamylcholine from Federa (Brussels, Belgium). GTP was purchased from Boehringer (Mannheim, Germany). Dextimide was kindly given by Janssens Pharmaceutica (Beerse, Belgium).

Methods

Preparation of rat cardiac membranes. Rat cardiac membranes were prepared according to the method of

Abbreviations used: [³H]NMS, [*N*-methyl-³H]methylscopolamine chloride; [³H]Oxo-M, [*methyl*-³H]oxotremorine M acetate; L-QNB, L-quinuclidinyl benzilate; EC₅₀, concentration of drug necessary to inhibit 50% of tracer binding; K_D , equilibrium dissociation constant; N_i , guanine nucleotide binding regulatory protein responsible for inhibition of adenylate cyclase.

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Snyder & Drummond (1978) with a few modifications (Waelbroeck *et al.*, 1982). Proteins were determined by the method of Lowry *et al.* (1951).

Scatchard analysis of [³H]NMS and [³H]Oxo-M binding. Cardiac membranes (0.1 mg of protein/ml) were incubated in 1.2 ml of 50 mM-sodium phosphate buffer (pH 7.4) enriched with 2 mM-MgCl₂, 1% bovine serum albumin, increasing concentrations of [³H]NMS or [³H]Oxo-M (0.2–4.0 nM). Incubation for 90 min at 5 and 10 °C, 45 min at 15 and 20 °C, or 10 min at 25, 30, 33, 35, 37 and 40 °C allowed complete equilibration of binding (results not shown). The incubation was terminated by a filtration method, as previously described (Waelbroeck *et al.*, 1982). Non-specific binding was defined as tracer binding in the presence of 1 μM-atropine, and was subtracted from all other determinations, yielding specific [³H]-NMS binding. The results were analysed according to Scatchard (1949).

[³H]NMS competition curves. Cardiac membranes (0.1 mg of protein/ml) were incubated as indicated above in the presence of 0.5 nM-[³H]NMS and various concentrations of agonist or antagonist. Specific binding was measured as indicated above.

The Hill coefficient (*h*) and EC₅₀ values for agonists and antagonists were estimated by linear regression analysis. The Hill coefficients of agonist (but not antagonist) competition curves were lower than 1.0 in the absence of GTP, suggesting receptor heterogeneity. We analysed the competition curves obtained at different tracer concentrations (0.2–4.0 nM-[³H]NMS) according to a two-classes-of-sites model (Minneman *et al.*, 1979). These competition curves were indeed compatible with the existence of two classes of receptors, having different affinities for the agonists but the same affinity for the antagonists (including [³H]NMS) (see Richardson & Howlett, 1983). We therefore felt justified to calculate the *K_D* values of all unlabelled drugs according to Cheng & Prusoff (1973).

Measurement of the dissociation rate constants (*k_{off}*). Membrane protein (0.1 mg/ml) was incubated in 1.2 ml of 50 mM-sodium phosphate buffer (pH 7.4) enriched with 2 mM-MgCl₂, 1% bovine serum albumin, 0.2 nM-[³H]NMS or 0.4 nM-[³H]Oxo-M and in the presence or absence of 1 μM-atropine for 1 h at 10 °C and 19 °C, 15 min at 25 °C, and 10 min at 30 and 33 °C. Atropine (12 μl of 100 μM) was then added to each tube to prevent association. Specific binding was measured using the filtration procedure indicated above, at the appropriate time intervals (5 min at 10 °C, 1 min at 19 and 25 °C, and 30 s at 30 and 33 °C). The dissociation rate constants were obtained in two experiments, by linear regression analysis of plots of ln(*B*) as a function of time (where *B* represents specific binding), using at least 10 determinations in triplicate (correlation coefficients of the regression were greater than 0.9 in each case).

Interpretation of the effects of temperature on *K_D* and EC₅₀ values. The equilibrium dissociation constant *K_D* is equal to:

$$K_D = e^{-\frac{\Delta G_D}{RT}} = e^{\frac{\Delta G}{RT}} \quad (1)$$

(where ΔG_D and ΔG are the free energy changes corresponding to the ligand–receptor dissociation and association reactions, respectively, *R* is the gas constant and *T* the absolute temperature).

We may therefore write:

$$\ln(K_D) = \frac{\Delta G}{RT} \quad (2)$$

$$\ln(K_D) = \left(\frac{\Delta H}{R}\right) \frac{1}{T} - \frac{\Delta S}{R} \quad (3)$$

Where ΔH and ΔS are the standard enthalpy and entropy changes for the drug–receptor binding reaction.

Antagonists, as well as agonists in the presence of GTP, appeared to recognize all receptors with a single *K_D* value. By contrast, agonist competition curves, in the absence of GTP, were more complex, with Hill coefficient lower than 1.0, suggesting the presence of at least two classes (or states) of binding sites. We therefore analysed the average competition curves (three to ten experiments) at each temperature, in order to calculate the agonist *K_D* values for each receptor separately, as explained above. The two corresponding van't Hoff plots (Fig. 2) were markedly curvilinear so that we calculated the enthalpy and entropy changes at temperatures below and above 25 °C, by using eqn. (3) (see Table 2).

Interpretation of the effects of temperature on the dissociation rate constant *k_{off}*. The Arrhenius plots obtained by plotting ln(*k_{off}*) as a function of 1/*T* were linear for both tracers (see the Results section). The activation energy *E_a* and activation entropy $\Delta S^\ddagger$ were calculated according to Laidler & Peterman (1979), assuming that:

$$k_{off} = \frac{kT}{h} \cdot e^{\frac{\Delta S^\ddagger}{R}} \cdot e^{-\frac{E_a}{RT}} \quad (4)$$

where *k* is the Boltzmann constant, *h* is Planck's constant, $\Delta S^\ddagger$ and *E_a* are the activation entropy and activation energy, respectively. The activation enthalpy can then be calculated as:

$$\Delta H^\ddagger = E_a - RT \quad (5)$$

RESULTS

Controls

We have chosen [³H]NMS instead of [³H]QNB (Waelbroeck *et al.*, 1982) as tracer because of its greater rate of association to and dissociation from these receptors. The total receptor concentrations measured with [³H]NMS or [³H]QNB (Scatchard, 1949) were identical (results not shown). In addition, the proportion of the two agonist binding sites (in the absence of GTP) and the *K_D* values of all unlabelled drugs studied (calculated by competition curve analysis) were identical with [³H]NMS or [³H]QNB. This indicated that both tracers labelled the same receptor populations.

In the 50 mM-phosphate buffer used in the present studies, [³H]NMS and [³H]Oxo-M recognized rapidly equilibrating, kinetically homogeneous, populations of binding sites at all temperatures studied. First-order dissociation rates suggested the existence of a single equilibrium constant $K = 1/K_D$ (Waelbroeck *et al.*, 1982; Asselin *et al.*, 1983). This was confirmed by the analysis of the [³H]NMS association rate at different tracer concentrations (results not shown), and validated the use

Table 1. Hill coefficients and K_D values of antagonist and agonist binding to rat heart muscarinic receptors

All unlabelled ligands were competing for [3 H]NMS binding. When average competition curves were utilized, mean K_D values were without s.d. (see the Methods section); n.d., not determined. 'SH' and 'H' refer to 'super-high' and 'high' receptors; see the Introduction.

Drugs	Hill coefficients (<i>h</i>) (mean ± s.d., <i>n</i> = 5–7, 25 °C)	Dissociation constants (<i>K</i> _D) (mean ± s.d., <i>n</i> = 3–9)		
		5 °C	25 °C	37 °C
Antagonists		(nM)		
Atropine	1.00 ± 0.09	1.2 ± 0.2	2.1 ± 0.4	4.2 ± 0.2
Dextimide	0.97 ± 0.08	0.9 ± 0.3	1.0 ± 0.4	0.8 ± 0.3
[³ H]NMS	n.d.	0.2 ± 0.05	0.6 ± 0.1	1.0 ± 0.2
Agonists in absence of GTP				
‘SH’ receptors				
[³ H]Oxo-M	n.d.	0.6 ± 0.1	1.5 ± 0.4	n.d.
Oxotremorine	(0.55 ± 0.05)	0.3	0.3	3.2
Carbamylcholine	(0.43 ± 0.04)	7.7	26.0	625.0
‘H’ receptors		μM	μM	μM
Oxotremorine	—	0.04	0.05	0.21
Carbamylcholine	—	1.9	3.4	23.1
Agonists in presence of GTP		(μM)		
Oxotremorine	0.91 ± 0.12	0.31 ± 0.09	0.75 ± 0.02	0.94 ± 0.21
Carbamylcholine	0.95 ± 0.07	13.6 ± 3.7	35.5 ± 10.4	70.9 ± 7.9
Pilocarpine	1.00 ± 0.05	1.86 ± 0.49	3.12 ± 0.41	6.15 ± 1.04

Fig. 1. van't Hoff plots of binding of the muscarinic antagonists [3 H]NMS, atropine and dextimide to rat heart membranes

The dissociation constant of [3 H]NMS (●), atropine (■) and dextimide (△) were measured at temperatures between 5 and 40 °C. $\ln(K_D)$ was then plotted as a function of the reciprocal of the absolute temperature. Each point is the average of three to nine determinations. The line was drawn with the ΔH and ΔS values calculated as indicated in the Methods section (see Table 2).

of van't Hoff plots to measure the enthalpy and entropy changes of the reaction (see above).

Preincubation of the membranes and tracer for 2 h at 5 °C and 10 min at 37 °C did not affect the K_D values obtained after a second 10 min incubation at 25 °C (and

vice versa) (results not shown), suggesting that a true equilibrium was reached (Waelbroeck *et al.*, 1979).

Finally, it is known that, when a labelled ligand equilibrates slowly with the receptors, the time necessary to reach equilibrium may be longer in the presence than

Fig. 2. van't Hoff plots of binding of the muscarinic agonist $[^3\text{H}]\text{Oxo-M}$ to rat heart membranes

The dissociation constants of $[^3\text{H}]\text{Oxo-M}$ alone ($\blacktriangle$), oxtremorine ($\bullet$) alone [at super-high (SH) and high (H) binding sites] or in the presence of GTP, and of pilocarpine [in the presence of GTP only ($\triangle$)] were measured at temperatures between 5 and 40 °C. $\ln(K_D)$ was then plotted as a function of the reciprocal of the absolute temperature. Each point is the average of at least three determinations. The lines were drawn using the ΔH and ΔS values calculated as indicated in the Methods section, using the K_D values obtained at 5–25 °C and 30–40 °C (see Table 2).

in the absence of an unlabelled drug (Aranyi, 1980). We therefore verified that equilibrium was indeed reached after a 10 min incubation at 25 °C in the presence of the unlabelled drugs studied here (results not shown).

Binding of most drugs studied here (with the exception of pilocarpine) is independent of pH in the range 6.5–8.5 at 25 °C (Asselin *et al.*, 1983). Since the pH of the phosphate buffer varied very little with temperature, we did not study the effect of pH on muscarinic receptors at all temperatures (Weiland *et al.*, 1979).

Effect of temperature on $[^3\text{H}]\text{NMS}$ and $[^3\text{H}]\text{Oxo-M}$ binding

$[^3\text{H}]\text{NMS}$ Scatchard plots were linear at all temperatures studied (correlation coefficient $r > 0.9$ in all individual experiments). The total number of receptors, measured by linear regression analysis of Scatchard plots (314 ± 40 fmol/mg of protein), did not change with temperature from 5 to 40 °C. In the 50 mM-phosphate buffer used, $[^3\text{H}]\text{NMS}$ binding was not affected by GTP, at any of the temperatures studied (results not shown). The average K_D values of $[^3\text{H}]\text{NMS}$ at 5, 25, and 37 °C are indicated in Table 1; the K_D value increased with increasing temperature. The van't Hoff plots [$\ln(K_D)$ as a function of $1/T$ (eqn. 3)] were linear (Fig. 1) (correlation coefficient $r > 0.9$ for each experiment).

We had previously shown that, under our assay conditions, the muscarinic agonist $[^3\text{H}]\text{Oxo-M}$ recognizes exclusively the 'super-high affinity' state for rat heart muscarinic receptors (Waelbroeck *et al.*, 1982). Scatchard plots obtained with this agonist at temperatures between 5 and 30 °C were linear (correlation coefficient $r > 0.9$ in all experiments) and the number of 'super-high

Table 2. Enthalpy and entropy changes for binding to rat heart muscarinic receptors

Where s.d. values are mentioned, six experiments were considered separately. See Table 1 and the Introduction for definition of 'SH' and 'H'.

Drugs	Enthalpy changes (ΔH^0) (kJ·mol ⁻¹) (mean $\pm$ S.D., n = 6)		Entropy changes (ΔS^0) (J·mol ⁻¹ ·K ⁻¹) (mean $\pm$ S.D., n = 6)	
Antagonists	5–40 °C			
Atropine	–27.6 $\pm$ 15.5		+72.4 $\pm$ 52.3	
Dextimide	+3.3 $\pm$ 3.4		$\pm$ 183.3 $\pm$ 22.2	
[³ H]NMS	–38.1 $\pm$ 5.0		+50.6 $\pm$ 31.8	
Agonists in the absence of GTP				
³ SH' receptors	< 25 °C			
[³ H]Oxo-M	–31.8 $\pm$ 3.8		+59.8 $\pm$ 13.4	
	< 25 °C	> 30 °C	< 25 °C	> 30 °C
Oxotremorine	+12.6	–201	+226	–469
Carbamylcholine	–42	–238	+4.2	–649
³ H' receptors				
Oxotremorine	+4.2	–167	+159	–410
Carbamylcholine	–21	–142	+42	–364
Agonists in the presence of GTP	5–40 °C			
Oxotremorine	–39.3 $\pm$ 17.6		–14.6 $\pm$ 58.2	
Carbamylcholine	–40.6 $\pm$ 11.7		–51.8 $\pm$ 38.1	
	5–25 °C			
Pilocarpine	–9.2 $\pm$ 7.9		73.6 $\pm$ 25.1

Fig. 3. Arrhenius plot of $[^3\text{H}]\text{Oxo-M}$ and $[^3\text{H}]\text{NMS}$ dissociation rates

The dissociation rate constants (k_{off}) of $[^3\text{H}]\text{NMS}$ (●) and $[^3\text{H}]\text{Oxo-M}$ (○) from the muscarinic receptors were measured at different temperatures from 10 to 33 °C. $\ln(k_{\text{off}})$ was plotted as a function of the reciprocal of the absolute temperature to allow evaluation of the activation energy and entropy (see the text).

affinity' binding sites (109 ± 18 fmol/mg of protein) remained constant. This number was also not affected irreversibly by preincubation for 10 min at 30 or 37 °C (results not shown). Above 30 °C, $[^3\text{H}]\text{Oxo-M}$ binding was too low to allow an accurate estimate of K_D and of the number of 'super-high affinity' receptors. The K_D values of $[^3\text{H}]\text{Oxo-M}$ binding measured at 5 and 25 °C are indicated in Table 1. The van't Hoff plots (Fig. 2) of $[^3\text{H}]\text{Oxo-M}$ binding were linear in all experiments from 5 to 25 °C (correlation coefficients $r > 0.9$) but the K_D of $[^3\text{H}]\text{Oxo-M}$ at 30 °C was higher than expected for a linear van't Hoff plot (Fig. 2) (see the Discussion).

Effect of temperature on atropine and dextimide binding

The Hill coefficients of atropine and dextimide competition curves (for binding of $[^3\text{H}]\text{NMS}$) were not significantly different from 1.0 (Table 1), indicating that both antagonists recognized all muscarinic receptors with the same affinity. Average K_D values obtained at 5, 25 and 37 °C are indicated in Table 1. In contrast with dextimide (K_D value independent of the temperature), the K_D value of atropine increased with increasing temperature (Table 1 and Fig. 1). The enthalpy and entropy changes of dextimide and atropine binding are shown in Table 2.

Effect of temperature on carbamylcholine, oxotremorine and pilocarpine binding in the presence of GTP

In the presence of GTP, muscarinic agonists recognized all rat heart receptors with the same (low) affinity at all temperatures studied (results not shown; Waelbroeck *et al.*, 1982). The observed K_D values increased with increasing temperature (Table 1).

The van't Hoff plots obtained with oxotremorine in the

Fig. 4. van't Hoff plots for carbamylcholine in the absence and presence of GTP and ethanol (1 M)

The EC_{50} of carbamylcholine in the absence (○, ●) and presence (△, ▲) of GTP were measured at different temperatures in the absence (○, △) and presence (●, ▲) of 1 M-ethanol. The van't Hoff plots were drawn with the ΔH and ΔS values calculated as indicated in the Methods section (see Table 2).

Fig. 5. Effects of temperature and ethanol on carbamylcholine binding

Upper panel: carbamylcholine competition curves were measured at 25 °C (○), 30 °C (●) and 40 °C (×). The lines were drawn using the following K_D values for the two classes of receptors:

Temperature	Class 1 (45% of total receptors)	Class 2 (55% of total receptors)
25 °C	13 nM	2 μ M
30 °C	60 nM	4 μ M
40 °C	1100 nM	7 μ M

Lower panel: carbamylcholine competition curves were measured at 25 °C, in the absence (○) or presence of 1 M- (●) or 2 M- (×) ethanol. The lines were drawn using the following K_D values for the two classes of receptors:

Condition	Class 1 (45% of total receptors)	Class 2 (55% of total receptors)
Control	13 nM	2 μ M
Ethanol (1 M)	80 nM	8 μ M
Ethanol (2 M)	600 nM	100 μ M

The proportion of high-affinity receptors was held at 45% for this Figure.

presence of GTP were linear (Fig. 2). The corresponding enthalpy and entropy changes are indicated in Table 2.

Very similar results were obtained when using carbamylcholine instead of oxotremorine (Table 2).

The van't Hoff plot obtained with pilocarpine under the same conditions was not linear (Fig. 2). This was probably due to the low value of the pK_a of pilocarpine (7.05) as compared with the pH of the incubation medium (7.40); indeed, a decrease of the pK_a value with increasing temperature (as generally observed for amines) would lead to a marked apparent increase of the K_D value of pilocarpine binding to the muscarinic receptors because of the decreased proportion of protonated ('bindable')

pilocarpine (Asselin *et al.*, 1983). We therefore believe that the values of enthalpy and entropy changes due to pilocarpine binding to the receptors shown in Table 2 represent minimal values.

Effect of temperature on carbamylcholine, pilocarpine and oxotremorine binding in the absence of GTP

In the absence of GTP, agonists recognize two classes of binding sites in rat heart membranes, with different affinities (Waelbroeck *et al.*, 1982). Oxotremorine and carbamylcholine competition curves for [3 H]NMS binding were compatible with the existence of two, non-interconvertible, classes of binding sites (of constant relative concentrations) at all temperatures studied. The average K_D values (for the two classes of binding sites) obtained at 5, 25 and 37 °C are indicated in Table 1.

The van't Hoff plots obtained by plotting $\ln(K_D)$ as a function of $1/T$ (eqn. 3) were not linear (Fig. 2). The enthalpy and entropy changes, calculated by using the two average K_D values at each temperature (see the Materials and methods section) are indicated in Table 2.

The results obtained with pilocarpine were qualitatively similar to those of carbamylcholine and oxotremorine. We however suspected that the pilocarpine ionization might be temperature-dependent (see above). Furthermore, this analogue being somewhat less selective than the two other agonists (Waelbroeck *et al.*, 1982), pseudo Hill coefficients very close to 1.0 were observed at 37 and 40 °C (0.85 ± 0.06 and 0.92 ± 0.08 , respectively). We therefore did not attempt to calculate the enthalpy and entropy changes of binding of this agonist to both muscarinic receptors.

Effect of temperature on [3 H]NMS and [3 H]Oxo-M dissociation rates (k_{off})

[3 H]NMS and [3 H]Oxo-M dissociation rates were first-order at all temperatures investigated (10–33 °C). A large temperature dependence of k_{off} values was observed, the Arrhenius plot [$\ln(k_{off})$ as a function of $1/T$] being linear (Fig. 3). The average activation enthalpies and entropies of the dissociation reactions (calculated according to eqns. 4 and 5) were, respectively, 86.2 kJ·mol $^{-1}$ and 11.7 J·mol $^{-1}$ ·K $^{-1}$ for [3 H]NMS and 110.5 kJ·mol $^{-1}$ and 87.0 kJ·mol $^{-1}$ ·K $^{-1}$ for [3 H]Oxo-M.

Effect of ethanol on [3 H]NMS and carbamylcholine binding

We analysed [3 H]NMS and carbamylcholine binding in the presence of 1 M-ethanol, at 5, 15, 25 and 30 °C. The van't Hoff plots of [3 H]NMS binding (not shown) and carbamylcholine binding in the presence of GTP (Fig. 4) were linear both in the absence and in the presence of ethanol. The enthalpy changes decreased from -38.1 ± 4.6 to -46.0 ± 5.4 kJ·mol $^{-1}$ ([3 H]NMS) and -40.2 ± 11.7 to -45.2 ± 14.2 kJ·mol $^{-1}$ (carbamylcholine + GTP) when 1 M-ethanol was added to the incubation buffer (mean values $\pm$ S.D., $n = 4-6$). The corresponding entropy changes also decreased from 50.6 ± 31.8 to 38.5 ± 8.4 kJ·mol $^{-1}$ ·K $^{-1}$ ([3 H]NMS) and from -46.0 ± 71.1 to -71.1 ± 54.4 kJ·mol $^{-1}$ ·K $^{-1}$ (carbamylcholine + GTP) in the presence of ethanol.

In the absence of GTP and at 25 °C, ethanol used at a 1 M concentration induced a slight straightening of the carbamylcholine competition curve then, at higher (2 M) concentration, a parallel shift of the competition curve (Fig. 5, lower panel). This was similar to the effect of a

temperature increase (Fig. 5, upper panel). The inhibition by ethanol of carbamylcholine binding was markedly increased at high temperature (25 and 30 °C) as compared with 15 or 5 °C (Fig. 4).

DISCUSSION

Antagonist binding to rat heart muscarinic receptors

Antagonist binding was systematically entropy-driven (positive ΔS values). It was also enthalpy-driven (negative ΔH values) in the case of atropine and [^3H]NMS but not for dextimide (Table 2). This was in agreement with the results of Barlow *et al.* (1979), on rat brain and ileum and those of Martin *et al.* (1984) on rat heart. Entropy and (small) enthalpy increases, due to 'dehydration' of binding molecules, are expected from both hydrophobic and ionic interactions (Ross & Subramaniam, 1981; Asselin *et al.*, 1983). We believe therefore that other forces, including hydrogen bond formation, dipole-induced dipole interactions, and London dispersion forces (Kauzmann, 1959; Tanford, 1969, 1970; Ross & Subramanian, 1981) were responsible for the large enthalpy decrease (Table 2) and selectivity of fitting of atropine and *N*-methylscopolamine with muscarinic receptors while hydrophobicity played a comparatively greater role in dextimide (this work) and quinuclidinyl benzilate (Martin *et al.*, 1984) binding. If this is true, a comparison of the temperature dependence of a series of related, well characterized, muscarinic antagonists might allow further insight in muscarinic binding site topology (Lien *et al.*, 1976; Barlow *et al.*, 1979).

Agonist binding in the presence or absence of GTP

Agonist binding, in the presence of GTP, induced smaller entropy and/or less negative enthalpy changes than antagonist binding (Table 2). This was not surprising, as hydrophobic interactions were probably far less extensive for agonist (as compared with antagonist) binding to the receptors (Stephenson, 1956; Asselin *et al.*, 1983).

In the absence of GTP, the van't Hoff plots obtained with the four agonists studied (Fig. 2, and results not shown) were not linear. Analysis of the competition curves suggests that the binding enthalpy and entropy changes to both classes of muscarinic receptors decreased markedly above 25 °C.

Speth *et al.* (1979), studying benzodiazepine receptors, discussed the hypothesis that a phase transition of membrane lipids could be responsible for a 'broken' van't Hoff plot. A sudden increase of k_{off} values upon lipid phase transition might also explain our results. We tested this hypothesis by comparing the effects of temperature on the k_{off} of [^3H]Oxo-M and [^3H]NMS (Fig. 3). The values of the activation enthalpy of dissociation of [^3H]Oxo-M and [^3H]NMS were quite high (110.5 and 86.2 kJ·mol⁻¹, respectively) in line with the hypothesis that breaking short range interactions, such as hydrogen bonds and van der Waals interactions, controlled the dissociation (Ross & Subramanian, 1981). Yet, the Arrhenius plots obtained with both ligands remained linear (Fig. 3), despite the fact that the temperature range explored included the agonist binding 'transition temperature'.

The 'phase transition' hypothesis was, therefore, ruled out. Alternately, the non-linearity of the [^3H]Oxo-M van't

Hoff plot (Fig. 2) could reflect the fact that agonist binding to the receptors, in the absence of GTP, was controlled by a major, agonist-induced, hydrophobically driven, conformational change of the agonist-receptor complex that was not observed during the formation of an antagonist-receptor complex. At low temperature, the enhancement of solvent structure by the dissolution of nonpolar groups is greatest, leading to greater entropy increases for hydrophobically driven reactions. As the temperature increases, the solvent and protein structures are randomized, so that the entropy change decreases, and eventually becomes largely negative. At that stage, the reaction is mostly controlled by enthalpy changes (see for example Waelbroeck *et al.*, 1979; Seelig & Oppenheimer, 1982).

Analysis of agonist binding in the presence of GTP (Fig. 2 and results not shown) indicated that the addition of GTP to the incubation medium reversed (or prevented) this conformational change.

Ethanol prevents the 'isomerization' reaction following agonist binding in the absence of GTP

We had previously suggested (Waelbroeck *et al.*, 1984) that the inhibition of a muscarinic ligand-receptor interaction by alcohol reflects a reduced hydrophobic contribution to the free energy change of the reaction. This conclusion was comforted by the present van't Hoff plots for carbamylcholine and [^3H]NMS in the absence and presence of 1 M-ethanol. Indeed, we observed: (1) small decreases of the changes in binding enthalpy and binding entropy for carbamylcholine and [^3H]NMS, and (2) an increase in the curvilinearity of the carbamylcholine van't Hoff plot in the absence of GTP (Fig. 4). These two effects could be ascribed to a decrease in hydrophobic interactions (Ross & Subramanian, 1981) (see also Fig. 5), due to the increased solubility of hydrophobic structures.

Conclusions

In the absence of GTP, the enthalpy and entropy changes induced by agonist binding to high and super-high affinity muscarinic receptors in rat heart membranes decreased above 25 °C. This might reflect a large (agonist induced) receptor conformational change, and/or receptor-effector interaction (possibly involving N_i , the adenylate cyclase inhibiting GTP binding protein) (Kurose & Ui, 1983).

In the presence of GTP, agonist van't Hoff plots were linear at all temperatures tested. These data suggest that the presence of GTP reversed or prevented the effect of agonist binding on the receptor and effector proteins. Under these conditions, carbamylcholine and oxotremorine binding were enthalpy-driven (with negligible entropy changes) whereas pilocarpine binding was probably entropy-driven (with a negligible enthalpy change) (Table 2). This result suggested that pilocarpine binding to the muscarinic receptor was due mainly to hydrophobic and ionic interactions, with less short range contact (inducing enthalpy and entropy decreases) than carbamylcholine and oxotremorine binding.

Antagonist binding to muscarinic receptors was always entropy-driven, with variable enthalpy changes, and did not lead to major conformational alterations. The ΔH value probably reflected the relative importance of hydrophobic and 'short range' interactions, that is, the quality of drug fitting to the receptor site. If this was true,

tropic muscarinic antagonists (atropine and *N*-methyl-scopolamine) would fit better than dexetimide (this work) or quinuclidinyl benzilate (Harden *et al.*, 1984) to the binding site.

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