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The effect of ionic strength on cardiac muscarinic receptors

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1 The binding of N-methylscopolamine (NMS) and carbachol to muscarinic receptors in the rat heart has been measured as a function of ionic strength (μ).

2 The binding of NMS was reduced by 3.69 fold for a 10 fold increase in ionic strength.

3 The binding of carbachol was affected in two ways. Firstly, the proportions of the subsites were changed. Above $\mu = 0.5$ M, the superhigh (SH) subsite was converted into the low (L) subsite and above $\mu = 0.8$ M, the high (H) subsite was also converted into the L subsite. Therefore, at high ionic strength, no agonist-determined subsites can be detected. In addition, increase in ionic strength reduced the binding of carbachol to all subsites and to a much greater extent than for NMS.

Introduction

It is generally to be expected that where the interaction of a ligand with a receptor involves a charged group, that the strength of binding will be reduced by an increased concentration of inorganic electrolytes in the reaction medium. This may be due to direct competition for the charged complementary group in the binding site by the inorganic ion or through the general charge shielding effect usually known as the Debye-Hückel effect. The degree to which binding is modified will depend on the energetic contribution of the charge-charge interaction to the total binding energy. The magnitude of the reduction in binding produced by raised electrolyte concentration will therefore provide an indication of the importance of the charged group to binding.

It is also clear that electrolyte concentrations may affect the binding protein through changes in conformation or in the degree to which component proteins in the receptor are mutually associated.

There have been several previous reports on the effects of monovalent inorganic cations on muscarinic receptors in both heart and brain (Birdsall *et al.*, 1979a, 1980a; Rosenberger *et al.*, 1980; Burgen *et al.*, 1981; Hulme *et al.*, 1981). These have suggested that there are both specific and non-specific effects of cations in reducing the binding of both agonists and antagonists. They have also shown that the shape of the agonist binding curve is steepened by high salt concentrations.

In the present study the effects of raising the concentration of sodium chloride on the binding characteristics of an antagonist N-methylscopolamine (NMS) and an agonist, carbachol, on the receptor in the rat heart have been examined quantitatively.

Methods

Membrane receptor preparations were made as follows. Hearts were removed from decapitated Wistar rats (200–250 g) and rinsed in cold 100 mM NaCl. A heart was added to 10 ml buffer (Tris-HCl 20 mM pH 7.5, $MgCl_2$ 5 mM, mercaptoethanol 2 mM) at 10°C homogenized in a Polytron, followed by 10 strokes in a PTFE-glass homogeniser, and filtered through two layers of gauze. The filtrate was centrifuged at 37,000 *g* for 10 min at 5°C. The pellet was resuspended in the same buffer and recentrifuged. The second pellet was suspended in 5 ml of a new buffer (Tris-HCl 20 mM pH 7.5, $MgCl_2$ 5 mM, sucrose 250 mM) at 5°C to which was added slowly with stirring 5 ml of 2.5 M KCl. This was again stirred for a further 1 h at 5°C. It was then centrifuged at 37,000 *g* for 10 min. The pellet was resuspended in Tris-HCl 20 mM, sucrose 250 mM, mercaptoethanol 2 mM and recentrifuged. This was repeated and the final pellet was fast frozen and stored at −70°C and used for up to three weeks.

Immediately before starting an assay, the pellet was thawed and made up to 10 ml with the standard buffer (Tris-HCl 20 mM pH 7.5, $MgCl_2$ 2 mM, NaCl 100 mM) and homogenized in the PTFE/glass homogenizer and centrifuged at 20,000 *g* for 10 min. The pellet was

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resuspended in a buffer containing the appropriate amount of NaCl or other univalent electrolyte, recentrifuged, the pellet resuspended in fresh buffer, recentrifuged and then finally suspended in buffer to a concentration of 0.3 mg protein ml^{-1} and homogenized.

Binding of [^{3}H]-NMS was determined in 1 ml polypropylene microcentrifuge tubes in the Tris HCl 20 mM pH 7.5, $MgCl_2$ 2 mM buffer containing the appropriate additional electrolyte. Incubation was carried out at 25°C for 30 min. The reaction was terminated by centrifugation in a microcentrifuge for 3 min. After pouring out the supernatant the pellet was washed three times with 100 mM NaCl and the tubes left to drain for 2 h. To each tube 0.1 ml Soluene 350 (United Technologies) was added and the tubes left overnight to complete solubilisation of the pellet. The tube tips were then cut off and deposited in 6 ml scintillation fluid (Ready SOLV EP, Beckman) and left for at least 6 h at 4°C before counting.

For determination of NMS binding, [^{3}H]-NMS (Amersham) was appropriately diluted with ^{1}H-NMS and added to the suspension in concentrations from 5×10^{-11}–1×10^{-8} M. Non-specific binding was estimated by including tubes that also contained 1×10^{-5} M quinuclidinyl benzilate (QNB).

In carbachol competition experiments, the concentration of [^{3}H]-NMS was 2×10^{-10} M except in the experiments at > 0.35 M when it was 4–8×10^{-10} M. All measurements were made in triplicate.

The concentration-binding curves were analysed by a computer programme giving least squares fitting to a sum of up to three independent binding sites. Since the receptor concentration was expressed as a percentage of the total there were only two independent variables for the concentration of sites, but three independent variables for binding constants.

The calculation of the regression lines in Figures 1 and 4 was by least squares. The curves in Figure 3 were drawn by eye.

The concentrations of electrolytes in all cases is expressed as ionic strength (μ) which is $\frac{1}{2}\Sigma c_i z_i^2$ i.e. half the sum of the molar concentration of all the ions multiplied by the square of their valence.

Results

Binding of N-methylscopolamine

The binding of NMS was adequately accounted for by a single binding site in all our experiments. The progressive shift in the curves indicated a lower affinity as the concentration of NaCl was increased.

Figure 1 is a plot of the log affinity constant against the log ionic strength. The relationship appears to be linear and corresponds to a 3.69 ± 0.25 reduction in affinity for a ten fold increase in ionic strength. It is useful to express this in free energy terms.

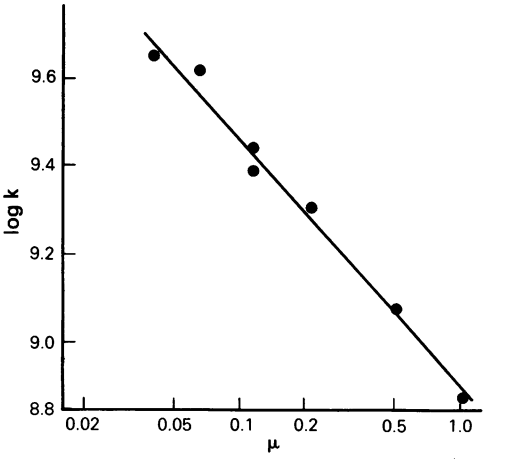

Figure 1 Dependence of N-methylscopolamine (NMS) binding on ionic strength. Ordinate scale: log NMS binding constant. Abscissa scale: ionic strength (log scale).

$$\Delta G = RT \ln K_1/K_2$$

where K_1 is the affinity at some ionic strength and K_2 is the affinity at 10 × the ionic strength. The result is $\Delta G = +0.78 \pm 0.04$ kcal mol^{-1}/10 fold change in ionic strength. It should be noted that the total binding capacity of the receptors was not altered by an increase in ionic strength.

Binding of carbachol

The results with carbachol as expected show more complex changes than found with the antagonist

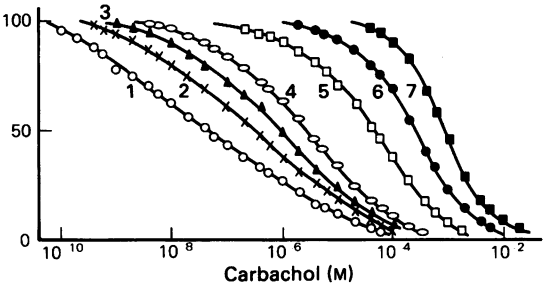

Figure 2 Displacement of [^{3}H]-N-methylscopolamine ([^{3}H]-NMS) by carbachol at different ionic strengths: (1) 0.0405 M: (2) 0.0655 M: (3) 0.1555 M: (4) 0.2655 M: (5) 0.5115 M: (6) 1.0155 M: (7) 1.5155 M. Ordinate scale: % of binding in the absence of carbachol. Abscissa scale: concentration of carbachol (M).

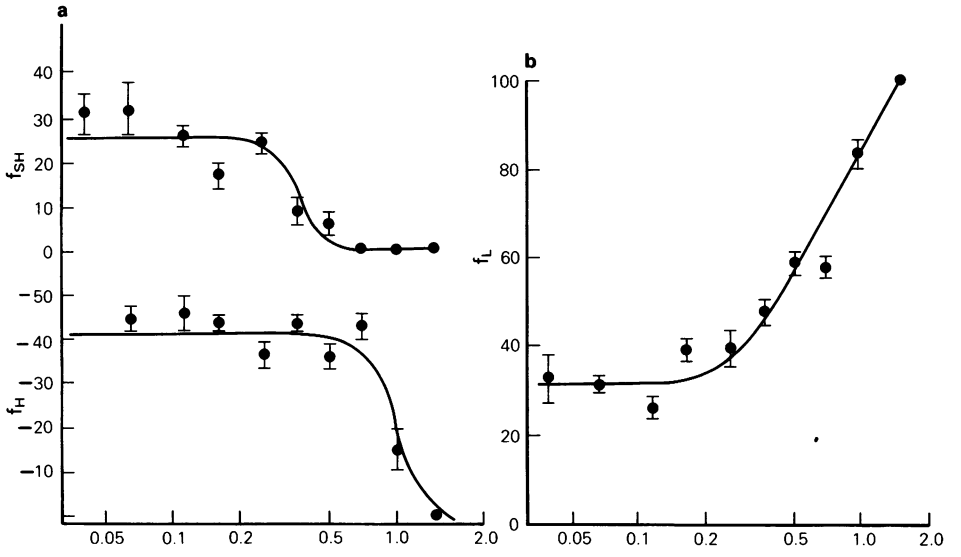

Figure 3 Population of subsites of receptor as a function of ionic strength: (a) fractions of superhigh (SH) and high (H) subsites; (b) fraction of low (L) subsites. Abscissa scale: ionic strength (log scale).

(Figure 2). There is a large shift of the curves to lower concentrations as the ionic strength is raised. The IC_{50} (concentration at which binding of NMS is reduced to 50%) increases from 4.4×10^{-8} M at $\mu = 0.0405$ to 8.3×10^{-4} M at $\mu = 1.515$, a decrease of almost 20,000 fold in the mean binding constant. However, it will be noted that this is accompanied by a marked increase in the steepness of the curves.

Analysis of the curves as described in the methods section shows that at ionic strengths up to $\mu = 0.5$ requires the inclusion of three subsites. Analysis in terms of only two subsites leads to systematic deviations of the calculated curves from the experimental data (Birdsall *et al.*, 1980a,b). Above $\mu = 1.515$ only a single component could be resolved.

In Figure 3, the proportions of superhigh (SH), high (H), and low (L) subsites are shown. It appears that up to $\mu = 0.25$ the proportions of SH, H and L subsites are essentially unchanged, but above $\mu = 0.25$ the proportion of the SH subsite declines and by $\mu = 0.60$ has essentially disappeared. There is no change in the proportion of the H subsite in this ionic strength range and the decrease in SH subsite is compensated by an increase in the proportion of the L subsite.

When the ionic strength exceeds 0.8 the population of the H subsite also starts to decline and when $\mu = 1.515$ the H subsite is no longer detected and the binding of carbachol is then uniformly to the L subsite.

The dependence of the affinity constants for SH, H and L subsites on ionic strength is shown in Figure 4. As with antagonist binding there is a monotonic decrease in binding constant with increase in ionic strength. The slopes of the curves are rather similar and are shown in Table 1.

While the figures for the slopes are suggestive that there is a trend towards a steeper slope for SH than H and for L subsites, none of these differences is statistically significant. However, the slopes of all

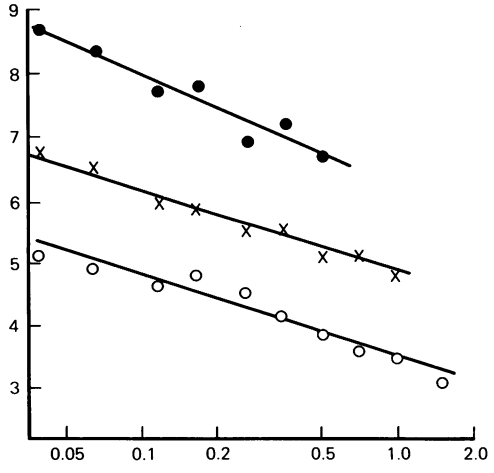

Figure 4 Binding of carbachol to receptor subsites as a function of ionic strength: superhigh (●); high (×) and low (○). Ordinate scale: log binding constant. Abscissa scale: ionic strength (log scale).

Table 1 Dependence of carbachol binding on ionic strength

Subsite	*Affinity at μ_I* / *Affinity at $10 \times \mu_I$*	*Free energy difference $10 \times \mu_I$, compared μ_I, kcal mol^{-1}*
Superhigh	58.9 ± 32.9	+ 2.38 ± 0.28
High	28.1 ± 7.0	+ 1.98 ± 0.13
Low	18.6 ± 7.2	+ 1.74 ± 0.19

three subsites are highly significantly greater than the slope of the NMS curve.

A few experiments with oxotremorine gave results qualitatively similar to carbachol but these were not examined in detail. Other univalent electrolytes (LiCl, CsCl, NaCNS and $NaClO_4$) also gave very similar results to those obtained with NaCl.

Discussion

Since in this preparation other univalent electrolytes produced effects similar to those of sodium chloride, it seems that the effects are due to a Debye-Hückel shielding effect rather than to a selective binding of an ion in competition with the ligand. Other reports in the literature have apparently shown effects that are ion-selective (Birdsall *et al.*, 1979a; Rosenberger *et al.*, 1980; Burgen *et al.*, 1981). It may be that ion selectivity depends on the way membranes are prepared and how the binding is measured. This needs further investigation.

The excellent fit of antagonist binding to a simple linear log-log regression on ionic strength (Figure 1) makes it improbable that any phase transition or conformation change occurs over this ionic strength range, at least as regards antagonist binding.

The effects of ionic strength on carbachol binding appears to be due to both a decrease in affinity at all three subsites together with a progressive conversion of the SH and H subsites into the L type.

What is the nature of the difference in constitution of the subsites and the mechanisms by which they can be interconverted? We know that in the heart, the major part of the receptor undergoes a similar conversion to the L type in the presence of certain guanine nucleotides, such as GTP or GppNHp (Berrie *et al.*, 1979; Wei & Sulakhe, 1979; Rosenberger *et al.*, 1980) and further that the solubilized receptor seems to contain a single population of agonist-antagonist binding polypeptides with a molecular weight of about 87K (Birdsall *et al.*, 1979b; Peterson *et al.*, 1984; Haga & Haga, 1985; Shirakawa & Tanaka, 1985). Further, it appears that agonists change the degree of association of these subunits. Current evidence suggests that this system is composed of an agonist binding subunit (receptor), and two other subunits one of which binds guanine nucleotides (G_i or N_i) and a smaller one (β) which does not.

The most likely explanation of our results is therefore that the constitution of the SH, H and L subsites involves different complexes of the receptive subunit with other subunits and that the formation of such complexes alter the conformation of the receptor and hence its binding for ligands.

Note that guanine nucleotides have only a trivial effect on antagonist binding and this leads to the proposition that they only effect the 'excited' conformations of the receptor elicited by agonists. In our experiments also there is no suggestion that the 'ground' state of the receptor (i.e. that detected by NMS) undergoes any change at those ionic strengths (i.e. $\sim\mu = 0.3, 0.8$) at which the population of subsites change. Since we find that the transition in raised ionic strength are SH→L and H→L and not SH→H we must conclude that the modifiers for the SH, and H sites are different. This could be due either to them being of totally different character or due to a combination of modifiers. For instance we could imagine that at the SH site the receptor is complexed with G_i and at the H site with $G_i\beta$ whereas the L site has no coupled subunit. The sequence would then be that raised ionic strength dissociated G_i changing SH into L but the receptors complexed with $G_i\beta$ were more resistant to ionic strength. However, this is merely speculative. It should be noted that when this preparation is maximally affected by GppNHp not all the receptors are converted to the L form; all the SH form disappears but about 15% remains in the H form (Burgen, unpublished). This may represent receptors coupled to K channels which also involve a guanine nucleotide binding protein (Pfaffinger *et al.*, 1985) and probably corresponds to the receptor found in the conducting tissue of the bovine heart (Burgen *et al.*, 1981) which is largely in the H form and whose agonist binding properties are not affected by guanine nucleotides. Our experiments suggest that this receptor form is transformed into the L form by high ionic strength. Our methods are not precise enough to pick out such a subfraction of the H receptor if it were to behave somewhat differently from the guanine nucleotide sensitive fraction. It was noted that the ionic dependence of binding of the antagonist was small suggesting that ionic interaction played only a minor part in the binding of the antagonist and by contrast the ionic dependence of binding the agonist was 2–3 times as great in energetic terms. It is certainly tempting to attribute this to a different exposure of an anionic site in the receptor in the ground and excited states. However, the differences in the structure of the cationic groups in carbachol and NMS could be enough to provide this difference. Nevertheless, it may

be recalled that there is evidence that for antagonists that are tertiary bases the difference in effectiveness between the charged and uncharged forms is not very large, whereas in agonists uncharged forms seem to have very much reduced activity (Burgen, 1965).

I wish to record my appreciation of the excellent technical assistance of Mr C.J. Harbird.

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