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Influence of monovalent cations on the binding of a charged and an uncharged ('carbo'-)muscarinic antagonist to muscarinic receptors

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1 The effect of the buffer concentration on binding of [^{3}H]-N-methylscopolamine to muscarinic receptors M_2 was tested in rat heart. Tracer binding was of low affinity in a 20 mM imidazole buffer (pK_D 8.3), inhibited by an increase from 10 to 100 mM of the sodium phosphate buffer concentration (pK_D 9.92 to 9.22), slightly inhibited by an increase of the Tris/HCl buffer concentration from 20 to 100 mM (pK_D 9.70 to 9.47) and unaffected by an increase of the histidine/HCl buffer concentration from 20 to 100 mM (pK_D 9.90 to 9.82). We chose the last buffer to analyse the effect of ions on antagonists binding to cardiac M_2 receptors and to transiently expressed wild-type and (Y533→F) mutant m3 muscarinic receptors in COS-7 cells.

2 Equilibrium [^{3}H]-N-methylscopolamine binding to cardiac M_2 receptors was inhibited, apparently competitively, by monovalent salts (LiCl ≥ NaCl ≥ KCl). In contrast, binding of the uncharged 3,3-dimethylbutan-1-ol ester of diphenylglycolic acid (BS-6181) was facilitated by addition of monovalent salts (LiCl ≥ NaCl ≥ KCl) to the binding buffer. This cation binding pattern is consistent with interaction with a large, negative field strength binding site, such as, for instance, a carboxylic acid.

3 In the presence of 100 mM NaCl, [^{3}H]-N-methylscopolamine had a similar affinity for the wild-type m3 receptor (pK_D 9.85) and for a (Y533→F) mutant m3 receptor (pK_D 9.68). However, in the absence of added salts, the tracer had a significantly lower affinity for the mutated (pK_D 10.19) as compared to the wild-type (pK_D 10.70) m3 receptor. BS-6181 had a significantly lower affinity for the (Y533→F) mutant m3 muscarinic receptor, as compared to the wild-type m3 receptor, both in the absence (pK_D 6.19-6.72) in the presence (pK_D 6.48-7.40) of 100 mM NaCl. The effects of NaCl on binding of the uncharged ester and of [^{3}H]-N-methylscopolamine to the m3 receptor were decreased by the mutation.

4 Taken together, these results support the hypothesis that monovalent cations from the buffer may interact with the cation binding site of the receptors (an aspartate residue in the third transmembrane helix of muscarinic receptors). Buffer cations may inhibit competitively the binding of (charged) muscarinic ligands having a tertiary amine or ammonium group, while facilitating the receptor recognition by uncharged, isosteric 'carbo-analogues'. Mutation of the (Y533→F) of the m3 receptor decreased the affinity of the receptor for positive charges, including the sodium ion.

Keywords: Muscarinic receptors; quaternary nitrogen; uncharged muscarinic antagonist; coulombic interaction; affinity; BS-6181

Introduction

Five distinct muscarinic acetylcholine receptors (m1 to m5) have been identified by molecular biological techniques (Bonner, 1989; Levine & Birdsall, 1989). The binding properties of the m1 to m4 proteins, expressed in eucaryotic cells, are similar to those of the corresponding pharmacological muscarinic receptor subtypes found in rat brain and other tissues (M_1 in brain and human neuroblastoma NB-OK-1 cells, M_2 in rat heart, M_3 in rat pancreas and M_4 in rat brain) (see review by Levine & Birdsall, 1989).

The natural ligand for the muscarinic receptor is a quaternary ammonium derivative, acetylcholine. It is generally assumed that formation of an ionic bond between the cationic head of muscarinic agonists or antagonists (protonated amine or quaternary ammonium) and an anionic (aspartate) group from the receptor is essential for high affinity binding. Two methods have been used to test this hypothesis: (1) Investigation of the pharmacological properties of isosteric uncharged drugs (containing quaternary carbon instead of quaternary ammonium residues) (Burgen, 1965; Banister & Whittaker, 1951; Barlow & Tubby, 1974; Barlow *et al.*, 1992) or of isosteric 'uncharged' receptors (replacement of the charged aspartate residue of the muscarinic binding site located in the third transmembrane domain by an uncharged asparagine residue) (Fraser *et al.*, 1989; Hulme, 1995; Spencer *et al.*, 1995). (2) Measurement of the drugs' affinities at very high or very low pH, thereby neutralising the charge of the amine or of the aspartate residue (Barlow & Winter, 1981; Barlow & Chan, 1982; Asselin *et al.*, 1983; Birdsall *et al.*, 1989; Ehlert & Delen, 1990).

An anionic site on the receptor indeed appears to be important for binding, since protons (acidic pH) behave as competitive antagonists and prevent drug binding to the receptor (Birdsall *et al.*, 1989; Ehlert & Delen, 1990). Moreover, replacement of Asp^{105} in the m1 receptors or Asp^{103} in the m2

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receptor by an asparagine residue results in an almost complete inhibition of [^{3}H]-quinuclidinyl benzilate binding (Fraser *et al.*, 1989; Hulme, 1995; Spencer *et al.*, 1995), and 100 to 300 fold decrease of the affinity of [^{3}H]-N-methylscopolamine for the muscarinic receptors (Hulme, 1995; Spencer *et al.*, 1995). Unprotonated scopolamine and unprotonated pirenzepine can bind, although weakly to muscarinic receptors (Barlow & Winter, 1981; Barlow & Chan, 1982; Asselin *et al.*, 1983; Birdsall *et al.*, 1989; Ehlert & Delen, 1990). The pharmacological properties of carbon analogues of classical muscarinic agonists and antagonists (Banister & Whittaker, 1951; Barlow & Tubby, 1974; Barlow *et al.*, 1992; Waelbroeck *et al.*, unpublished observation) in fact suggest that the contribution of the ionic bond to binding is highly variable.

In this study, we analysed the binding properties of an uncharged muscarinic antagonist, the 3,3-dimethylbutan-1-ol ester of diphenylglycolic acid (BS-6181, Figure 1), previously studied by Barlow & Tubby (1974) at native muscarinic M_2 receptors present in rat heart and at cloned m3 receptors expressed in COS-7 cells. Our results indicate that the interaction between this compound and the muscarinic receptors is markedly facilitated by the presence of monovalent cations in the incubation buffer.

Methods

Dissolution of 3,3-dimethylbutan-1-ol ester of diphenylglycolic acid (BS-6181)

Ethanol was used as solvent when preparing BS-6181 solutions for binding studies, because dimethylsulphoxide as well as dimethyl formamide inhibited markedly [^{3}H]-NMS binding at low concentrations. The stock solution of BS-6181 (1 mM) was prepared in ethanol, immediately diluted to 100 μM with water, and BS-6181 was used only at concentrations up to 10 μM (i.e. 1% ethanol) for binding studies. The activity of the stock BS-6181 solution (in ethanol) was stable for over one week, then decreased progressively. This was probably due to transesterification of the drug by the solvent. We therefore used only fresh solutions for experiments.

Homogenate preparation

Male Wistar albino rats were killed by decapitation and the heart immediately removed and homogenized as previously described in a 20 mM Tris/HCl buffer (pH = 7.5), enriched with 250 mM sucrose (Waelbroeck *et al.*, 1991).

COS-7 cells were transfected with wild-type or mutated m3 receptors (Wess *et al.*, 1991). Cells were maintained in Dulbecco's modified Eagle's medium, enriched with 10% foetal calf serum. About 30 μg of plasmid DNA was preincubated for 15 min with 120 μl of lipofectin reagent (GIBCO-BRL) in a final volume of 400 μl, then added in 7 ml of Optimem medium (GIBCO) to a 175 cm^2 flask containing COS-7 cells at 80% confluency. The cells were incubated for 12 h before addition of the complete culture medium. Three days after transfection, the cells were scraped with a rubber policeman, harvested by low speed centrifugation, rinsed once in 20 mM sodium-phosphate buffer enriched with 150 mM NaCl and 1 mM EDTA, then homogenized in the homogenization buffer (20 mM Tris/HCl; pH = 7.5; 250 mM sucrose) and stored in liquid nitrogen until use.

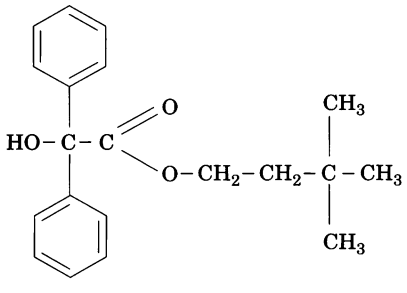

Figure 1 Chemical structure of BS-6181.

Binding studies

All binding studies were performed at 25°C (pH = 7.4) and at equilibrium. [^{3}H]-NMS concentrations were adjusted, to about 2 K_D values. The [^{3}H]-NMS concentrations used in each experiment are indicated in the legends of the Figures and Tables.

Nonspecific [^{3}H]-NMS binding was defined as tracer bound in the presence of 1 μM atropine.

The incubations were stopped by filtration on glass fibre filters (GFC, Whatman, Maidstone, England) soaked in 0.1% polyethyleneimine. The filters were rinsed 4 times with ice cold 50 mM sodium-phosphate buffer (pH = 7.4). The radioactivity bound to the filters was measured by liquid scintillation counting.

Binding of [^{3}H]-NMS and of BS-6181 to cardiac M_2 receptors in different buffers

A few experiments were performed in various buffers, as indicated. For all these experiments, 80 μl of heart homogenates (in the Tris-sucrose buffer) were incubated in a total volume of 1.2 ml of the indicated buffer, in the presence of [^{3}H]-NMS and of the indicated unlabelled drug concentrations.

Effect of salts on the binding of [^{3}H]-NMS and of BS-6181 to rat heart M_2 or to transfected (wild-type and point-mutated) m3 receptors

To study the effect of ions on equilibrium binding of [^{3}H]-NMS and of BS-6181 to cardiac muscarinic M_2 receptors or to transfected (wild-type and mutated) m3 receptors, 80 μl of the homogenate was incubated in a total volume of 1.2 ml of 20 mM histidine-HCl buffer (pH = 7.4), in the absence or presence of the indicated salt concentrations.

Materials

[^{3}H]-NMS ([1-(N-methyl^{3}H)]-scopolamine methyl chloride, 80 to 85 Ci mmol^{-1}) was obtained from Amersham (Bucks, England). Atropine sulphate, bovine serum albumin (Fraction V) and polyethyleneimine were from Sigma Chemical Co. (St. Louis, MO, U.S.A.), and glass fibre filters GFC, from Whatman, Maidstone, England. L-Histidine (96–98%) was obtained from Janssens Chemica (Beerse, Belgium). Tissue culture media and lipofectin were obtained from GIBCO Life Technologies (Gent, Belgium). The 3,3-dimethylbutan-1-ol ester of diphenylglycolate, BS-6181, was prepared according to Funcke *et al.* (1959). All the other reagents were of the highest grade available.

Data analysis

All data are presented as means ± s.d. of at least 3 experiments. All saturation and competition curves were analysed by non-linear curve fitting, using an 'all purpose' curve fitting programme, developed by S. Swillens (Brussels University) that accepts user-defined equations. The inhibition constants K_i were calculated with the Cheng & Prusoff (1973) equation, assuming competitive binding to a single receptor subtype. The pK_i and pK_D values were then calculated as $-\log K_i$ and $-\log K_D$. Differences between mean values were tested for statistical significance by F test; $P < 0.05$ was accepted as being significant.

Results

Effect of buffer composition on the [^{3}H]-NMS and BS-6181 binding properties

The binding of [^{3}H]-NMS and BS-6181 to M_2 receptors was analysed in rat heart, in different buffers and at two buffer concentrations. Some of the saturation curves are shown in Figure 2, and the results are summarised in Table 1. As shown in Figure 2, the affinity of [^{3}H]-NMS was significantly higher and the B_{max} values, identical or not (statistically) different in the 20 mM histidine buffer, 50 mM sodium-phosphate buffer or 20 mM imidazole buffer. In the Tris-HCl buffer, the [^{3}H]-NMS saturation curve was probably biphasic (see Hulme *et al.*, 1981), but our experimental data could be fitted assuming a single site, with a slightly lower B_{max} ($80 \pm 5\%$ of the B_{max} found in the phosphate or histidine buffers).

When two buffer concentrations were compared (10→100 mM sodium phosphate, 20→100 mM histidine-HCl or Tris-HCl), we observed no significant change of the tracer's affinity or B_{max} in the histidine buffer, a decreased [^{3}H]-NMS affinity with no change in B_{max} in the sodium phosphate buffer and a slight decrease in [^{3}H]-NMS binding but no change in the apparent B_{max} value in the Tris-HCl buffer.

BS-6181 behaved as a competitive antagonist (decreasing the affinity of the tracer without affecting the receptor number) in rat heart (Figure 3). It had a significantly lower affinity in the histidine-HCl and Tris-HCl buffers as compared to the sodium phosphate buffer (Table 1).

In view of these results, we decided to study the effect of ions on [^{3}H]-NMS and BS-6181 binding, using the 20 mM histidine-HCl buffer as 'baseline'.

Effect of salts on [^{3}H]-NMS and BS-6181 binding

The total [^{3}H]-NMS binding capacity (B_{max}) in rat heart homogenates was not significantly affected in the presence of salts (Figure 2 and results not shown). The affinity of the tracer for cardiac M_2 receptors decreased with increasing ionic strength, as indicated in Figure 4 and Table 2. Competition curves with LiCl, NaCl and KCl gave Hill coefficients equal to 1 (Figure 4). Assuming competitive inhibition of tracer binding, K_i values of respectively, 18 ± 5 mM ($n=6$; LiCl), 22 ± 6 mM ($n=6$; NaCl) or 36 ± 9 mM ($n=6$; KCl) were calculated. BS-6181 enhanced [^{3}H]-NMS binding inhibition by the salts. Binding affinities of BS-6181 increased dose dependently in the presence of the salts, as shown in Table 2.

To analyse these results, we assumed that muscarinic receptors can recognise either [^{3}H]-NMS (R.NMS*) or BS-6181 (R.BS) or a cation (Na^+, Li^+ or K^+) (R.Na) or, simultaneously, BS-6181 and a cation (R.BS.Na):

$$\text{R.BS} \underset{\longleftarrow}{\overset{K_{BS}}{\longrightarrow}} \text{BS} + \text{R} + \text{NMS}^* \underset{\longleftarrow}{\overset{K_D}{\longrightarrow}} \text{R.NMS}^*$$

$$\begin{array}{ccc} + & & + \\ \text{Na}^+ & & \text{Na}^+ \\ K_{Na} \uparrow\downarrow & & \uparrow\downarrow \alpha K_{Na} \end{array}$$

$$\text{R.BS Na} \underset{\longleftarrow}{\longrightarrow} \text{BS} + \text{RNa}$$

$$\alpha K_{BS}$$

The equation describing this model is:

$$B = \frac{\text{R.NMS}^*}{R_o} = \frac{\text{NMS}^*/K_D}{1 + \text{NMS}^*/K_D + \text{Na}/K_{Na} + \text{BS}/K_{BS} + \text{Na.BS}/\alpha K_{Na} K_{BS}}$$

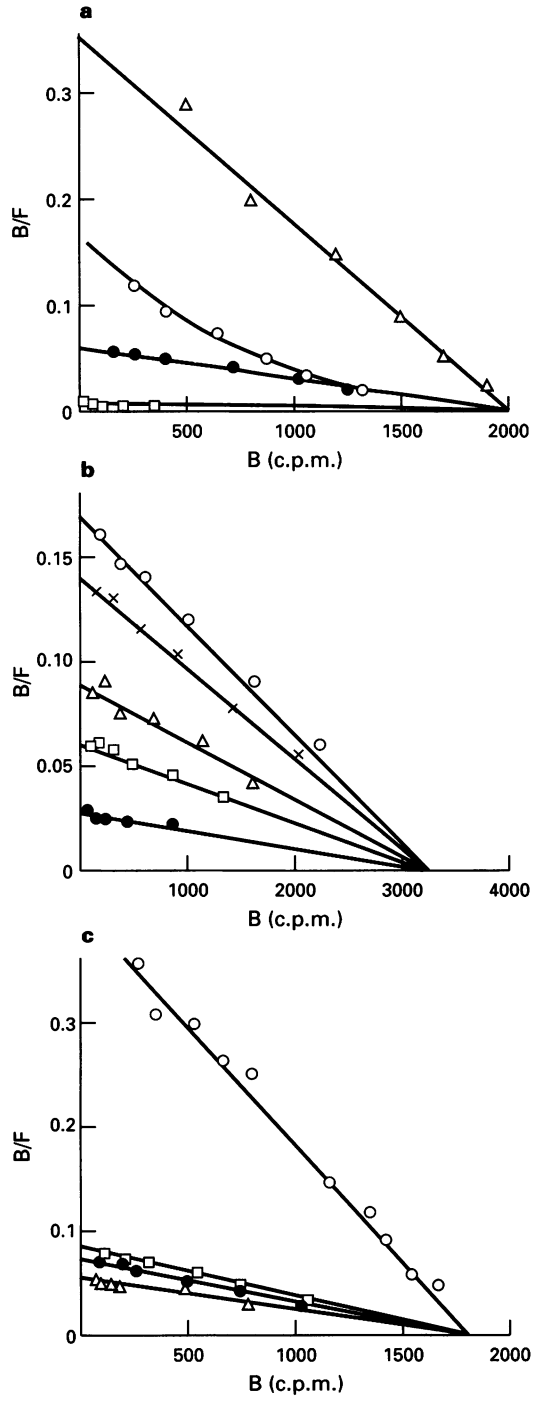

Figure 2 Scatchard representation of [^{3}H]-NMS saturation curves obtained in rat heart in different incubation buffers. (a) [^{3}H]-NMS saturation curves in a 20 mM histidine-HCl (△), 20 mM Tris-HCl (○), 50 mM sodium-phosphate (●) and 20 mM imidazole-HCl (□) buffer, respectively. One representative of three experiments in duplicate. (b) [^{3}H]-NMS saturation curves in a 50 mM sodium-phosphate buffer, in the absence (○) or presence of 0.1 μM (X), 0.2 μM (△), 0.5 μM (□) or 1 μM (●) of BS-6181. Representative of two experiments in duplicate. (c) [^{3}H]-NMS saturation curves in rat heart, in a 20 mM histidine-HCl buffer, in the absence (○) or presence of 100 mM KCl (□), NaCl (●) or LiCl (△). Representative of three experiments in duplicate.

where B represents the fractional receptor occupancy; R_o, the total receptor concentration; and α, an 'allosteric constant' describing the effect of Na^+ on BS-6181 binding and *vice versa* (with all the constants used as equilibrium dissociation constants).

Table 1 pK_D or pK_i values ± s.d. of [^{3}H]-NMS and BS-6181 binding to cardiac M_2 receptors in different buffers

	[^{3}H]-NMS	*BS-6181*
Sodium-phosphate 10 mM	9.92 ± 0.06	7.00 ± 0.10
Sodium-phosphate 100 mM	9.22 ± 0.04*	7.30 ± 0.15*
Imidazole 20 mM	8.3 (7.9–8.7)[a]	5.98 (5.83–6.13)[a]
Histidine 20 mM	9.90 ± 0.10	5.94 ± 0.25†
Histidine 100 mM	9.82 ± 0.06	5.95 ± 0.25†
Tris 20 mM	9.70 ± 0.06	5.70 ± 0.19†
Tris 100 mM	9.47 ± 0.02*	5.72 ± 0.10†

[a]The [^{3}H]-NMS concentrations were varied between 50 and 1700 pM for the saturation curves. The [^{3}H]-NMS concentration-range was too low to approach saturation in the imidazole buffer. We therefore preferred to calculate 95% confidence interval of the [^{3}H]-NMS K_D value in this buffer. The extreme values were then used to calculate the 95% confidence interval of the BS-6181 K_i values. For competition curves, the [^{3}H]-NMS concentration was 800 pM. (Average of 3 to 5 experiments).
*Significantly different from the value obtained with the less concentrated buffer.
†Significantly different from the value obtained in the sodium phosphate buffers.

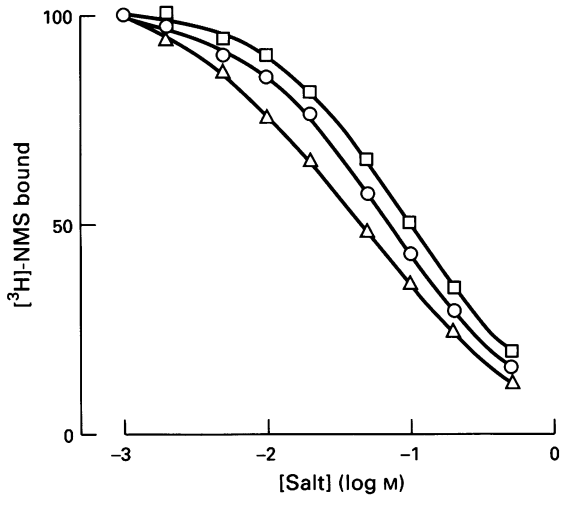

Figure 3 Inhibition of [^{3}H]-NMS (200 pM) binding to rat heart M_2 receptors in the histidine buffer, by LiCl (△), NaCl (○), and KCl (□) (average of 3 experiments in duplicate). The s.e.mean (less than ±3%) corresponds to the size of the symbols.

This equation was used to fit simultaneously the BS-6181 competition curves in the absence or presence of 10 and 100 mM salt, and the salt competition curves, in the absence or presence of 0.5 μM BS-6181. The results of this analysis are shown in Figure 4 for the BS-6181-NaCl interaction. We obtained 'K_{Na}' values of 18, 22 and 36 mM, respectively for LiCl, NaCl and KCl; and identical α-values (0.10 ± 0.05) for the three salts.

Our results therefore suggested that BS-6181 had a 6 to 20 fold higher affinity for the receptor-cation complex than for the muscarinic receptor itself, and that monovalent cations were 6 to 20 fold more easily accommodated in the muscarinic receptor in the presence than in the absence of BS-6181.

Binding of [^{3}H]-NMS and BS-6181 to wild-type and mutant m3 receptors

The affinities of BS-6181 for the wild-type and six point-mutated m3 receptors were investigated. A single preliminary experiment (not shown) suggested that BS-6181 recognised the Y148→F, T231→A, T234→A, Y506→F or Y529→F mutant receptors just like the wild-type m3 receptor. In the absence of NaCl, the BS-6181 pK_i values were between 6.5 and 6.8 for these mutants, as compared to 6.7 for the wild-type receptor. The pK_i increased to values between 7.1 and 7.6 (7.4 for the wild-type receptor) when NaCl was included in the incubation

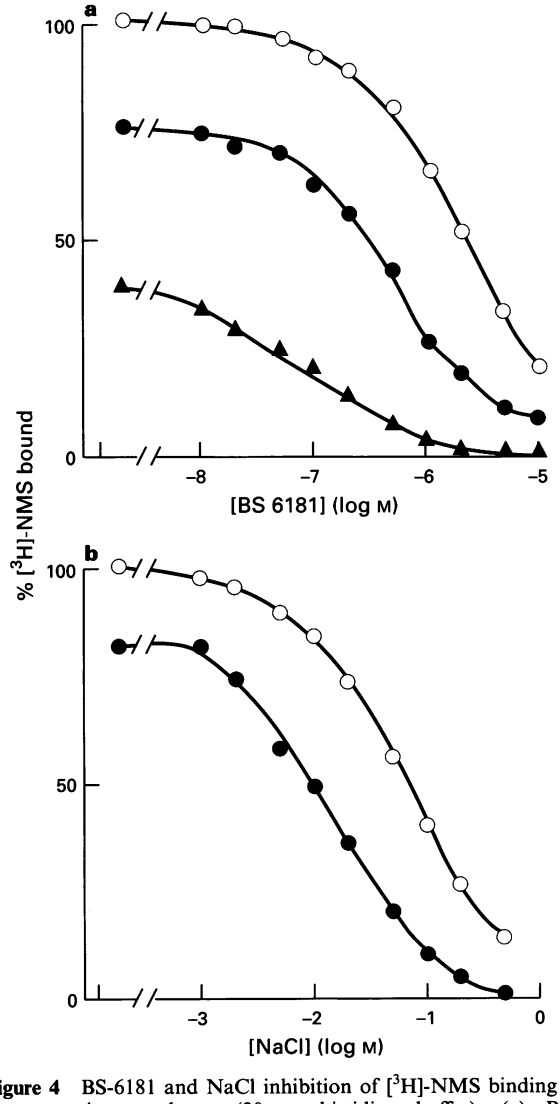

Figure 4 BS-6181 and NaCl inhibition of [^{3}H]-NMS binding to M_2 receptors in rat heart (20 mM histidine buffer). (a) BS-6181 competition curves were measured in the histidine buffer in the presence of 200 pM [^{3}H]-NMS in the absence (○) or presence of 10 mM (●) or 100 mM (▲) of NaCl. (b) Inhibition of [^{3}H]-NMS (200 pM) binding by NaCl, in the absence (○) or presence (●) of 0.5 μM BS-6181. All the data are expressed as % of control specific [^{3}H]-NMS binding, in the absence of salt and of BS-6181 (average of 3 experiments in duplicate). The s.e.mean (±3%) corresponds to the size of the symbols.

Table 2 pK_D or pK_i values ± s.d. of [^{3}H]-NMS and BS-6181 binding to cardiac M_2 receptors in the absence or presence of LiCl, NaCl, KCl

	[^{3}H]-NMS	*BS-6181*
Control buffer:	9.90 ± 0.10	5.74 ± 0.14
NaCl 10 mM	9.62 ± 0.05*	6.48 ± 0.13*
NaCl 100 mM	9.15 ± 0.08*	6.96 ± 0.13*
LiCl 10 mM	9.56 ± 0.05*†	6.64 ± 0.09*†
LiCl 100 mM	9.10 ± 0.02*†	7.05 ± 0.05*†
KCl 10 mM	9.65 ± 0.03*	6.27 ± 0.14*
KCl 100 mM	9.26 ± 0.05*	6.85 ± 0.06*

The [^{3}H]-NMS concentrations were varied between 50 and 800 pM for the saturation curves. The [^{3}H]-NMS concentration used in BS-6181 competition curves was 200 pM (average of 3 experiments).
*Indicates the values which are significantly different from the values obtained in the control buffer ($P < 0.05$).
†Indicates the values which are significantly different from the values obtained at the same KCl concentration.

buffer. In contrast, the pK_i value of BS-6181 on the Y533→F mutant was 6.2 in the absence of NaCl, but increased only to 6.5 in the presence of NaCl. We therefore decided to investigate further the binding properties of this mutant receptor.

As shown in Table 3, in the absence of added salt, BS-6181 had a 3 fold lower affinity for the Y533→F mutant than for the wild-type receptor (Table 3). In the presence of 100 mM NaCl, this affinity ratio was increased to 8 fold (Table 3). The affinity of [^{3}H]-NMS for the wild-type receptor was decreased 7 fold in the presence of 100 mM NaCl, as compared to only 3 fold for the Y533→F point mutated receptor (Table 3).

Discussion

It is usually assumed that a positive charge in muscarinic ligands is essential for high affinity binding to muscarinic receptors. However, several uncharged 'carbo'-compounds, including BS-6181, have been shown previously to possess considerable affinities (10 to 1000 fold lower than those of the isosteric charged molecules) for muscarinic receptors (Banister & Whittaker, 1951; Barlow & Tubby, 1974; Barlow *et al.*, 1992; Waelbroeck *et al.*, unpublished). It might be argued that these uncharged compounds are highly hydrophobic, and therefore might be able to interact non-specifically with the plasma membrane. However: (1) we recently demonstrated that the 3,3-dimethylbutan-1-ol esters of (**R**)- and (**S**)-cyclohexylphenylglycolic acid and of diphenylglycolic acid (BS-6181) are recognised (stereo) selectively by muscarinic receptors (Waelbroeck *et al.*, unpublished); (2) At least three studies (Burgen, 1965; Banister & Whittaker, 1951; Barlow & Tubby, 1974) demonstrated that the uncharged carboacetylcholine analogue acts as muscarinic agonist on ileum smooth muscle. Taken together, these results suggest that ionic interactions favour the interaction of muscarinic ligands with the receptor but are not absolutely necessary for ligand recognition and activation of the muscarinic receptor.

Birdsall *et al.* (1979) previously observed that increasing the ionic strength of the incubation buffer led to decrease of the affinity of (charged) muscarinic antagonists for the brain muscarinic receptors. Consistent with this study, we found that increasing the buffer concentration generally led to a decrease of the affinity of [^{3}H]-NMS for cardiac M_2 muscarinic receptors. This phenomenon is unlikely to be due simply to a Debye-Hückel effect on ionic interactions (i.e. shielding of receptor and ligand charges by a 'cloud' of counterions). A wide variation of the [^{3}H]-NMS K_D values was indeed observed,

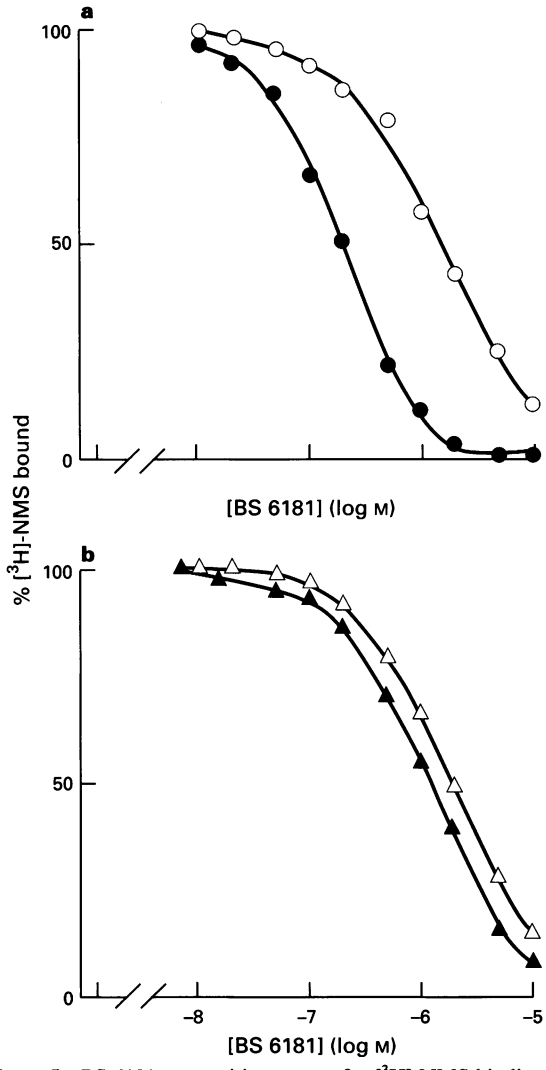

Figure 5 BS-6181 competition curves for [^{3}H]-NMS binding to wild-type m3 receptors and to Tyr533->Phe point-mutated m3 receptors (Y533F receptors) transiently expressed in COS-7 cells (20 mM histidine buffer). The [^{3}H]-NMS concentrations used were 100 pM in the absence of NaCl, and increased to 400 pM in the presence of NaCl, to compensate for the tracer affinity decrease in the presence of salt. The K_i values are summarised in Table 3. (a) BS-6181 competition curves were obtained in the histidine buffer, in the absence (○) or presence (●) of 100 mM NaCl, using cells expressing wild-type m3 receptors. (b) BS-6181 curves were obtained in the histidine buffer, in the absence (△) or presence (▲) of 100 mM NaCl, using cells expressing (Y533F) point-mutated m3 receptors (average of 3 experiments in duplicate). The s.e.mean (±3%) corresponds to the size of the symbols.

depending on the buffer chosen, at comparable ionic strengths. This suggested that the buffer ions *per se* might affect drug binding to the muscarinic receptor, either directly (recognizing the muscarinic binding site) or indirectly. We therefore attempted to find a suitable 'baseline' buffer, to study the effect of ions on BS-6181 and [^{3}H]-NMS binding.

Our results showed that imidazole, and to a lesser extent sodium phosphate, inhibited [^{3}H]-NMS binding to M_2 cardiac receptors (Table 1). Imidazole might be considered as a 'muscarinic antagonist fragment', with a very weak affinity for muscarinic receptors: if the saturation curves in imidazole buffer are compared with saturation curves in other buffers (assuming no change in B_{max}), a K_i value of approximately 0.1 to 0.3 mM can be estimated. In the sodium-phosphate buffer, the [^{3}H]-NMS binding affinity was decreased, and the BS-6181 affinity increased at increasing buffer concentrations. This result suggested that ions from this buffer interacted with the

Table 3 pK_D or pK_i values ± s.d. of [^{3}H]-NMS and of BS-6181 binding to m3 and point-mutated (Tyr 533→Phe, Y533F) m3 receptors, transiently expressed in COS-7 cells, in the absence or presence of 100 mM NaCl

	[^{3}H]-NMS	*BS-6181*
m3 (histidine buffer)	10.70 ± 0.07*	6.72 ± 0.10*
m3 (+100 mM NaCl)	9.85 ± 0.08*	7.40 ± 0.09*
Y533F (histidine buffer)	10.19 ± 0.01*†	6.19 ± 0.08†
Y533F (+100 mM NaCl)	9.68 ± 0.02*	6.48 ± 0.12†

The [^{3}H]-NMS concentrations were varied between 25 and 400 pM for the saturation curves. The [^{3}H]-NMS concentration used in BS-6181 competition curves was 100 pM in the absence of NaCl, 400 pM when NaCl (100 mM) was added to the incubation buffer (average of 3 experiments).
*Indicates the values which are different in the absence and presence of NaCl; †indicates the Y533F mutant receptor values which are significantly different from the wild-type values, obtained in the same conditions (either with, or without NaCl) ($P<0.05$).

receptor and facilitated BS-6181 binding. Tracer and BS-6181 binding were little affected by Tris or histidine at the concentrations tested. Based on these results, both buffers seemed equally suitable 'baseline buffers'. However, in agreement with Hulme *et al.* (1981), our 'Scatchard plots' in the Tris-HCl buffer were compatible with the existence of two populations of binding sites, with high and low affinity for [^{3}H]-NMS. Furthermore, Hosey (1982, 1983) observed that in a Tris-HCl buffer, but not in other buffers, Na^+ facilitated [^{3}H]-quinuclidinyl benzilate binding to chick heart receptors. She therefore suggested that Tris buffer should be avoided in studies of the muscarinic receptor system, since the possibility that Tris interacts with the monovalent cation site on the muscarinic receptors-cyclase system cannot be overlooked (Hosey, 1983). We therefore decided to use the histidine-HCl buffer to investigate the effect of other ions on the binding of [^{3}H]-NMS and of the uncharged antagonist, BS-6181, to cardiac M_2 and transiently expressed m3 receptors.

A 'sodium binding site' has been identified in several other G-protein coupled receptors interacting with G_i/G_o (for review, see Tian & Deth, 1993). Na^+ binding to this allosteric site (an aspartate residue in the second transmembrane helix) often facilitates antagonist binding, decreases agonist binding, and impairs the receptor activation (Tian & Deth, 1993).

In contrast, in the present study on cardiac muscarinic M_2 receptors, LiCl, NaCl and KCl inhibited [^{3}H]-NMS binding competitively and favoured the recognition of BS-6181. These results suggest that cations (from the incubation buffer) may interact directly with the muscarinic binding site aspartate residue in the third transmembrane domain. This would indeed prevent formation of an ionic bond between [^{3}H]-NMS and the receptor but facilitate the formation of an electrically neutral ternary complex between the cation, the receptor, and BS-6181.

If this model is correct, the effect of mutations affecting the amino acids which interact with the cationic group of muscarinic ligands on ligand binding might be more reliably detected in low ionic strength buffers. Indeed, if the change of the receptor sequence results in a decreased ionic interaction with cations in general, the decreased affinity for (charged) muscarinic ligands might be compensated by a decreased competition from buffer cations.

To test this hypothesis, we decided to study the binding properties of [^{3}H]-NMS and of BS-6181 to muscarinic receptors with point mutations, in buffers with a high and a low ionic strength.

We did not attempt to investigate directly the interaction of BS-6181 and Na^+ with the binding site aspartate residue (D147 of the m3 receptor), since it has been shown that mutation of this residue to an uncharged aspargine dramatically decreases tracer affinity (Fraser *et al.*, 1989; Hulme, 1995; Spencer *et al.*, 1995). We therefore decided to investigate BS-6181 and Na^+ binding to point-mutated receptors with less drastic modifications of the muscarinic binding site.

We were particularly interested in mutations involving hydroxyl groups close to the binding site aspartate (D147), since we assumed that these groups might contribute to cation binding. We therefore decided to concentrate on several OH-group containing amino-acids (threonine, serine or tyrosine), which are necessary for high affinity agonist binding (Wess *et al.*, 1991; 1992). We hypothesized that one of these hydroxy groups might be close enough to the aspartate (D147) residue to interact with the cation moiety of classical muscarinic antagonists.

In a single preliminary experiment, the [^{3}H]-NMS binding properties ([^{3}H]-NMS affinities and relative B_{max} values) of the wild-type m3 and mutant receptors expressed in COS-7 cells in the presence of 100 mM NaCl were fully comparable to previously published data (Wess *et al.*, 1991; 1992). The wild-type receptor and five of the mutants tested (Y148F, T231A, T234A, Y506F and Y529F) gave almost superimposable BS-6181 competition curves, both in the absence (pK_i values between 6.5 and 6.8) and in the presence of NaCl (pK_i values between 7.1 and 7.6). We therefore did not investigate the binding properties of these mutants any further. The Y533F mutant receptor, in contrast, had a much higher pK_i value for BS-6181 than the wild-type in the presence of NaCl (pK_i values 6.2 without NaCl, 6.5 with NaCl). Subsequent experiments confirmed that the Y533F receptor had a significantly weaker affinity for [^{3}H]-NMS (compared to the wild-type receptor), in the absence of added NaCl (Table 3), and that the affinities of the mutant receptor for BS-6181 and for [^{3}H]-NMS were less affected by the addition of 100 mM NaCl to the incubation buffer (Table 3). This is the result we expected if the mutant receptor has a generally weaker affinity for cations: (1) in the 20 mM histidine buffer, there is almost no inhibition of tracer binding to the mutant receptor by buffer cations and the Y533F mutant receptor had a low affinity for [^{3}H]-NMS (a cation); (2) in the presence of 100 mM NaCl, [^{3}H]-NMS binding to the Y533F m3 receptor was less inhibited, and BS-6181 binding, less favoured by 100 mM NaCl, as if the mutated receptor had a lower affinity for Na^+. The binding of [^{3}H]-NMS and the binding of BS-6181 were affected at comparable salt concentrations. Tyr^{533} of the m3 muscarinic receptor is in fact thought to point towards the muscarinic binding site: if sequences of G-protein coupled receptors and mammalian opsins are compared, this tyrosine residue is aligned with the lysine residue which covalently binds retinal. The mutant receptor had a weaker affinity than the m3 receptor for BS-6181 in the absence of added salt, suggesting that hydrophobic interactions between the drug and the binding site were also decreased. It is possible that the hydroxy group of the tyrosine residue formed hydrogen bond(s) with other residues from the wild-type receptor, so that the Y533F mutation resulted not only in the removal of the 'OH' group, but also in a slight displacement of the aromatic ring.

In conclusion, our results suggest that, in 'physiological buffers' (at rather high ionic strengths), the importance of ionic bonds for binding can be markedly underestimated. This is due to two phenomena: (1) competition of the charged molecule with ions from the buffer, and (2) facilitation of the binding of the uncharged molecule by ion pair formation on the receptor. These effects should be taken into account in the interpretation of the structure-function relationships of charged (amino) and uncharged (carbo) compounds and of wild-type and mutant receptors.

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