Binding properties of nine 4-diphenyl-acetoxy-N-methyl-piperidine (4-DAMP) analogues to M₁, M₂, M₃ and putative M₄ muscarinic receptor subtypes

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1 We compared the binding properties of 4-diphenyl-acetoxy-N-methyl-piperidine methiodide (4-DAMP) and nine analogues of this compound on muscarinic receptors of human neuroblastoma NB-OK1 cells (M₁ subtype), rat heart (M₂ subtype), rat pancreas (M₃ subtype) and to the putative M₄ subtype in striatum.

2 The requirements for high affinity binding were somewhat different for the four receptor subtypes. In general, the requirements of M₃ receptors were more stringent than for M₁, M₂ or putative M₄ receptors.

3 The abilities of the compounds to discriminate muscarinic receptor subtypes were not correlated with their affinities at any subtype.

4 The temperature-dependence of binding of 4-DAMP analogues to M₂ receptors varied with the drug structure. In particular, the increased affinity of the α -methyl derivative of 4-DAMP could be ascribed to van der Waals interactions.

5 The affinities of most 4-DAMP analogues for M₂ and M₃ receptors were similar to their pharmacological potencies on atrial and ileum preparations, respectively.

6 At concentrations above 1 μ M, all 4-DAMP analogues as well as atropine, reduced the [³H]-N-methyl scopolamine ([³H]-NMS) dissociation rate from cardiac muscarinic receptors, with no obvious structure-activity relationship.

Keywords: 4-Diphenyl-acetoxy-N-methyl-piperidine (4-DAMP) analogues; atropine; pirenzepine; AF-DX 116; muscarinic receptor subtypes; structure-activity relationship; binding/functional correlations

Introduction

Three muscarinic receptor subtypes have recently been described, based on binding and pharmacological studies *in vitro* and *in vivo* (see Birdsall *et al.*, 1989) and there are indications of the existence of a fourth (Lazareno *et al.*, 1990; Waelbroeck *et al.*, 1990; Dörje *et al.*, 1991). M₁ receptors in the nervous system are characterized by high affinity for pirenzepine (Hammer *et al.*, 1980; Brown *et al.*, 1980). The other muscarinic receptors (with a low affinity for pirenzepine), have been subdivided using the cardioselective muscarinic antagonists methoctramine (Giraldo *et al.*, 1988) and AF-DX 116 [11-((2-[diethylamino)-methyl]-1-piperidinyl)acetyl)-5,11-dihydro-6H-pyrido (2,3b)(1,4)benzodiazepin-6-one] (Hammer *et al.*, 1986; Giachetti *et al.*, 1986). Cardiac M₂ receptors from mammalian heart show high affinity for methoctramine and AF-DX 116 and low affinity for pirenzepine. By contrast, secretory gland and smooth muscle M₃ receptors have a low affinity for methoctramine, AF-DX 116, and pirenzepine (Batink *et al.*, 1987; Doods *et al.*, 1987; Birdsall *et al.*, 1980; Waelbroeck *et al.*, 1987; Lambrecht *et al.*, 1987). About 40% of the muscarinic binding sites in striatum, like the functional receptors in uterus smooth muscle, have a high affinity for methoctramine and a low affinity for AF-DX 116 and for pirenzepine (Waelbroeck *et al.*, 1990; Dörje *et al.*, 1991). Since their binding/pharmacological properties are different from the previously identified M₁, M₂ and M₃ receptors, and very similar to cloned M₄ receptors, we shall hereafter call these binding sites 'putative M₄ sites'.

We previously demonstrated that these four muscarinic binding sites can be studied using [³H]-N-methyl scopol-

amine ([³H]-NMS) binding to, human neuroblastoma NB-OK 1 cells (M₁), rat heart (M₂), rat pancreas (M₃), and rat striatum (putative M₄ sites having slow [³H]-NMS dissociation rate) respectively (Waelbroeck *et al.*, 1987; 1988a,b; 1990).

4-Diphenyl-acetoxy-N-methyl-piperidine (4-DAMP) (Barlow *et al.*, 1976) was one of the first compounds able to discriminate two receptor subclasses sharing low affinity for pirenzepine (the M₂ and M₃ receptors). It also has high affinity for muscarinic receptors (of the order of 10⁹ to 10⁸ M⁻¹ depending on the subtype studied) (Barlow *et al.*, 1976; Waelbroeck *et al.*, 1987; Doods *et al.*, 1987), suggesting that its discriminatory capacity relies on good fit to high affinity receptors rather than on steric hindrance in low affinity receptors. We have compared the binding properties of nine 4-DAMP analogues to the 4 muscarinic receptor subtypes, in order to define further the structural requirements of the binding sites.

The synthesis and the pharmacological properties, in ileum (M₃ receptors) and atria (M₂ receptors), of the nine 4-DAMP analogues studied have been described by Barlow & Shepherd (1985, 1986).

Methods

NB-OK 1 cells: Culture and homogenates preparation

NB-OK 1 human neuroblastoma cells (a generous gift from Dr N. Yanaihara, Shizuoka, Japan) were maintained in RPMI 1640 medium enriched with 100 unit ml⁻¹ penicillin and

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Figure 1 Structure of 4-diphenyl-acetoxy-N-methyl-piperidine (4-DAMP) and of analogues 1 to 9: 4-diphenylacetoxy-N-methyl piperidine *etho* bromide (1), 4-diphenylpropionyl-N-methyl piperidine methobromide (homo-4-DAMP) (2), 4-(α -methyl-4)-DAMP methobromide (3), 4-(di-*p*-tolyl)acetoxy-N-methyl piperidine methobromide (4), pentamethylene-*bis*-4-(diphenyl-acetoxy)-N-methylpiperidinium bromide (5), 4-(fluoren-9-carboxy)-N-methyl piperidine methobromide (6), 4-(xanthen-9-carboxy)-N-methyl piperidine methobromide (7), 4-(dibenzo cycloheptane-carboxy)-N-methylpiperidine methobromide (ethane-bridged 4-DAMP) (8), 4-(di-*p*-chlorophenyl)acetoxy-N-methylpiperidine methobromide (9).

100 $\mu\text{g ml}^{-1}$ streptomycin, and 10% foetal calf serum at 37°C, as previously described (Waelbroeck *et al.*, 1988a). For [³H]-NMS binding studies, cells were harvested in a 20 mM sodium phosphate buffer enriched with 1 mM EDTA and 150 mM NaCl (pH 7.4); centrifuged at 500 *g* for 5 min, then resuspended, and homogenized in 20 mM Tris-HCl buffer enriched with 5 mM MgCl₂ (pH 7.5) and stored in liquid nitrogen until use. Samples (80 μl), containing 160 to 200 μg protein per tube, were used. Protein concentration was determined according to Lowry *et al.* (1951).

Cardiac homogenate preparation

Male Wistar albino rats (200–250 g) were killed by decapitation, the heart removed and rinsed in isotonic (150 mM) NaCl. The homogenate was prepared as previously described (Waelbroeck *et al.*, 1990) and was used either fresh or stored in liquid nitrogen until use; 80 μl of this homogenate (400 to 500 μg protein per tube) were used.

Pancreas homogenate preparation

The pancreases were removed, minced with scissors and homogenized as previously described, in a buffer containing 500 kallikrein inhibitor units ml^{-1} Trasylol, 0.2 mg ml^{-1} baci-

tracin and 1% bovine serum albumin (Waelbroeck *et al.*, 1987). Samples (1 ml), 800 to 1000 μg pancreas protein per tube, were used.

Striatum homogenate preparation

The striatum was removed, homogenized as previously described (Waelbroeck *et al.*, 1990) and stored in liquid nitrogen until use.

[³H]-N-methyl scopolamine binding experiments

[³H]-NMS binding was measured at 25°C in a total volume of 1.2 ml, in the following incubation buffer: 50 mM sodium phosphate (pH 7.4), 2 mM MgCl₂ and 1% (w/v) bovine serum albumin. For binding studies to pancreas receptors, we also added Trasylol and bacitracin (see above) to inhibit further proteolytic activity.

The [³H]-NMS concentration used (0.25 nM for binding studies to NB-OK 1 and pancreas homogenates, 1.0 nM for binding studies to cardiac homogenates) corresponded to approximately twice the *K_D* values in the systems used. Receptor concentrations in the assays were 50 to 200 pM. Non-specific binding was defined as [³H]-NMS binding in the presence of 1 μM atropine in the three systems.

The incubation period was 2 h, when using NB-OK 1 and cardiac homogenates, and 4 h when using pancreas homogenates. In striatum homogenates, [³H]-NMS (at 240 pM) labels approximately 27% *M*₁, 9% *M*₂, 9% *M*₃ and 56% *M*₄ sites (Waelbroeck *et al.*, 1990). We preincubated the homogenates with tracer and unlabelled drug for 2 h at 25°C to monitor [³H]-NMS binding. We then added 1 μM atropine to each sample to prevent reassociation of the tracer, and allowed [³H]-NMS dissociation to proceed for 35 min. After this dissociation period, 85% of the residual [³H]-NMS was bound to *M*₄ sites, 15% to *M*₃ sites.

To terminate the incubation, each sample was diluted with 2 ml of ice-cold 50 mM sodium phosphate buffer (pH 7.4) and filtered on GF/C glass-fibre filters (Whatman), presoaked overnight in 0.05% polyethyleneimine. The filters were rinsed 3 times with the filtration buffer, dried, and the radioactivity (bound tracer) counted by liquid scintillation.

[³H]-N-methyl scopolamine dissociation from cardiac sites

[³H]-NMS was preincubated with cardiac homogenates for 15 min at 25°C. Tracer dissociation was induced either by: (1) addition of 1 μM atropine (control); (2) simultaneous addition of 1 μM atropine and the indicated 4-DAMP analogue concentrations or (3) addition of the indicated atropine concentrations (1 μM to 1 mM), and allowed to proceed for 5 min at 25°C before filtration. The results are expressed as a fraction of the initial tracer binding (before dissociation) on a logarithmic scale.

Temperature-dependence of drug binding to cardiac sites

[³H]-NMS binding to cardiac homogenates was measured as described above, after an incubation of 4 h at 4°C, 2 h at 12°C, 1 h at 25°C and 20 min at 37°C. The tracer concentration used for competition curves was 1.0 nM at all temperatures. [³H]-NMS saturation curves, obtained with tracer concentrations from 0.25 nM to 4.0 nM at each temperature were used to determine the [³H]-NMS *K_D* value and calculate the unlabelled drug *K_i* values as explained below. To calculate the enthalpy and entropy changes associated with drug binding, we plotted the $\ln K_a = -\ln K_i$ values as a function of 1/*T*:

$$\ln K_a = \left[-\frac{\Delta H^\circ}{R} \frac{1}{T} + \frac{\Delta S^\circ}{R} \right]$$

Mathematical analysis

The competition curves were analysed with the competition-assisted curve fitting procedure described by Richardson & Humrich (1984). The K_i values were calculated with the equation of Cheng & Prusoff (1973), using the [^3H]-NMS K_D value in the same tissue.

Compounds

[^3H]-NMS (1-[N-methyl ^3H]-scopolamine methyl chloride) (74 Ci mmol^{-1}) was obtained from Amersham International (Bucks, England). Atropine sulphate was from Sigma Chemical Co. (St Louis, MO, U.S.A.). Tissue culture material and media were obtained from Gibco (Gent, Belgium). All other reagents were of the highest grade available. 4-Diphenyl-acetoxy-N-methyl-piperidine methobromide (4-DAMP) and the nine 4-DAMP analogues were generous gifts from Dr R.B. Barlow. The structure of these analogues is given in Figure 1.

Results

We have previously demonstrated, using selective antagonists, that muscarinic receptor sites from NB-OK 1 cells are 80% M_1 ; from heart, 100% M_2 ; from pancreas, 100% M_3 and that the 'slow-dissociation sites' in striatum are 85% M_4 sites. The competition curves for *bis*-pentamethylene 4-DAMP were steep (n_H 1.5 ± 0.10); all others were compatible with competitive inhibition to a single receptor type (with Hill coefficients varying from 0.95 to 1.05 ± 0.10). We tested the hypothesis that all 4-DAMP analogues and the tracer interacted competitively with the same receptor population by comparing competition curves obtained with 0.3 nM, 1.0 nM

and 3.0 nM [^3H]-NMS, in each of the 4 systems. Since this was apparently true, including for *bis*-pentamethylene 4-DAMP, we used the Cheng-Prusoff equation (Cheng & Prusoff, 1973) to calculate the K_i (inhibition constant) for all antagonists.

Binding to four muscarinic receptor subtypes at 25°C

The pK_i values for binding of the nine 4-DAMP analogues to the four muscarinic receptor subtypes are given in Table 1, and their selectivities for each pair of sites, in Table 2. The standard deviation of the pK_i values (4 to 6 determinations) was typically 0.10 log units, and always below 0.15 log units. The affinities of the 4-DAMP analogues varied by over 1000

Table 2 Selectivity of the 4-diphenyl-acetoxy-N-methyl-piperidine (4-DAMP) analogues at each pair of sites

Analogue number	M ₁ /M ₂	M ₁ /M ₃	M ₁ /M ₄	M ₂ /M ₃	M ₂ /M ₄	M ₃ /M ₄
4-DAMP	10	1.3	1	-8	-10	-1.3
1	13	1.6	1	-8	-13	-1.6
2	8	3	1.6	-2.5	-5	-2
3	4	2.5	1.3	-1.6	-5	-3
4	1.6	5	5	3	3	1
5	2.5	3	-2.5	1.2	-6	-8
6	3	1.6	2	-2	-1.6	1.3
7	8	3	1.2	-2	-10	-4
8	5	3	1.6	-1.6	-3.2	-2
9	3	6	6	2	2	1

Ratio of the larger K_i value to the smaller: where the first K_i in the comparison is the larger (i.e. the antagonist had a lower affinity for the first site), the ratio is preceded by a minus (-) sign.

Table 1 pK_i values obtained in binding studies for NB-OK 1 (M_1 receptors), heart (M_2 receptors) and pancreas (M_3 receptors) homogenates and to the slowly-dissociating (M_4) sites in striatum^a

Drug	Analogue number	M ₁ NB-OK 1	M ₂ Heart	M ₃ Pancreas	Striatum M ₄ sites
Atropine ^b		9.6	9.0	9.4	9.6
Pirenzepine ^b		8.2	6.6	6.6	7.1
AF-DX 116 ^b		6.3	7.3	5.8	6.3
Himbacine ^b		7.1	8.1	6.5	8.0
Methoctramine ^b		7.3	7.9	6.0	7.6
4-DAMP ^b		9.1	8.1	9.0	9.1
4-Diphenylacetoxy-N-methyl piperidine <i>etho</i> bromide	1	8.9	7.8	8.7	8.9
4-Diphenylpropionyl-N-methyl piperidine methobromide (homo-4-DAMP)	2	6.9	6.0	6.4	6.7
4-(α -methyl-4)-DAMP methobromide	3	9.8	9.2	9.4	9.9
4-(Di- <i>p</i> -tolyl)acetoxy-N-methyl piperidine methobromide	4	8.0	7.8	7.3	7.3
Pentamethylene- <i>bis</i> -4-(diphenyl-acetoxy)-N-methylpiperidinium bromide	5	8.0	7.6	7.5	8.4
4-(Fluoren-9-carboxy)-N-methyl piperidine methobromide	6	8.9	8.4	8.7	8.6
4-(Xanthen-9-carboxy)-N-methyl piperidine methobromide	7	9.9	9.0	9.4	10.0
4-(Dibenzo cycloheptane-carboxy)-N-methylpiperidine methobromide (ethane-bridged 4-DAMP)	8	6.8	6.1	6.3	6.6
4-(Di- <i>p</i> -chlorophenyl)acetoxy-N-methylpiperidine methobromide	9	7.8	7.3	7.0	7.0

^a The standard deviation of the pK_i values was typically ± 0.1 log unit, always below 0.15 log unit. The Hill coefficients varied between 0.95 and 1.05 (standard deviation: ± 0.1) except for compound 5: Hill coefficients of 1.5 ± 0.10 at the four receptors were observed for this compound.
^b Values taken from Waelbroeck *et al.*, 1987, or Waelbroeck *et al.*, 1990.

fold. The values obtained at M_2 and M_3 receptors were in good agreement with their potency in pharmacological assays using, respectively, cardiac atria and ileum (Barlow *et al.*, 1976; Barlow & Shepherd, 1985; 1986). The only exception was *bis*-pentamethylene 4-DAMP, which had a much lower affinity for pancreas M_3 sites than expected from pharmacological studies in the ileum (Barlow & Shepherd, 1986).

'Allosteric' interactions with cardiac receptors

To examine whether these analogues interacted with allosteric sites, in addition to binding sites in muscarinic receptors, we measured the effect of atropine, 4-DAMP and 4-DAMP analogues on [^{3} H]-NMS dissociation from cardiac muscarinic receptors (Figure 2). When tested at high concentration, atropine and 4-DAMP decreased the [^{3} H]-NMS dissociation rate with a $-\log EC_{50}$ of 3.7 to 3.5. Analogues 1 to 4 and 7 to 9 had a slightly (3 to 5 fold) greater potency with a $-\log EC_{50}$ value of 4.2 (Figure 2, superimposable curves). Analogues 6 (fluorenyl 4-DAMP) ($-\log EC_{50}$:5.2) and 5 (*bis*-pentamethylene 4-DAMP) ($-\log EC_{50}$:5.7) were the most potent analogues in this respect but, even so, the effects of these analogues on [^{3} H]-NMS dissociation from cardiac receptors were observed at concentrations 100 to 1,000,000 fold above their K_i value.

Thermodependence of 4-DAMP analogues binding to cardiac receptors

We measured the affinity of atropine, 4-DAMP and eight analogues at four temperatures to calculate the enthalpy and entropy changes associated with binding to cardiac M_2 receptors. The van't Hoff plots were linear: ΔH° and ΔS° values were constant.

As shown in Table 3, binding of 4-DAMP and seven 4-DAMP analogues was associated with a negative

Table 3 Changes in enthalpy (ΔH° in kJ mol $^{-1}$) and entropy (ΔS° in J mol $^{-1}$ K $^{-1}$) associated with the binding of 4-diphenyl-acetoxy-N-methyl-piperidine (4-DAMP) or 4-DAMP analogues to cardiac M_2 receptors^a

Analogue number	ΔH°	ΔS°
4-DAMP	-5.987	+136
1	-8.406	+122
2	-11.615	+77 ^d
3	-12.970	+136
4	-15.430 ^b	+98 ^c
6	-9.230	+133
7	-11.113	+136
8	+5.674 ^c	+136
9	-16.707 ^b	+84 ^b

^a We estimated the standard deviation of the enthalpy and entropy changes at ± 5.51 kJ mol $^{-1}$ and ± 18.6 J mol $^{-1}$ K $^{-1}$, respectively.

^b Significantly different ($P > 0.9$) from the corresponding value for 4-DAMP.

^c Significantly different ($P > 0.95$) from the corresponding value for 4-DAMP.

^d Significantly different ($P > 0.98$) from the corresponding value for 4-DAMP.

Figure 2 Effects of atropine, 4-diphenyl-acetoxy-N-methyl-piperidine (4-DAMP) and 4-DAMP analogues on the [^{3} H]-N-methylscopolamine ([^{3} H]-NMS) dissociation rate from cardiac muscarinic receptors. [^{3} H]-NMS dissociation was induced by isotopic dilution, using 1 μ M atropine together with the indicated 4-DAMP or 4-DAMP analogues concentrations, or using the indicated atropine concentrations. Residual [^{3} H]-NMS binding was measured 5 min after the addition of atropine alone (+), or 1 μ M atropine and the indicated concentrations of 4-DAMP (x---x), 4-DAMP analogues 1, 2, 3, 4, 7, 8, or 9 ($\blacklozenge$), analogue 6 ($\blacktriangle$) or analogue 5 ($\triangle$). This experiment was representative of 3 experiments performed in duplicate.

(favourable) enthalpy change and positive (favourable) entropy change. Binding of one 4-DAMP analogue (compound 8), was entropy driven only, the enthalpy change associated with binding being unfavourable. The enthalpy and/or entropy change associated with the binding of four analogues were significantly different from 4-DAMP values: decreased for homo 4-DAMP 2, (di-*p*-tolyl) and (di-*p*-chloro)-4-DAMP (4 and 9) and increased for the ethane bridged 4-DAMP analogue (8).

Discussion

Binding patterns of 4-DAMP analogues to neuronal, cardiac and pancreatic muscarinic receptors

The binding of 4-DAMP and nine analogues was measured in four different systems but under comparable conditions.

The receptor-selectivities of 4-DAMP analogues (Table 2) were independent of their affinities for any given subtype (Table 1): for example, homo-4-DAMP (2) and the xanthene-derivative (7) had the same $M_4 > M_2$ selectivity as 4-DAMP but, 100 fold lower and 10 fold higher affinities respectively, for these subtypes. The requirements for high affinity binding were somewhat different at the four subtypes. For example: modification of the phenyl rings by *para*-chloro or *para*-methyl substituents (4, 9) decreased 50 or 100 fold the analogue's affinity for M_3 and M_4 receptors, 10 or 16 fold for M_1 receptors and only 1.6 or 5 fold for M_2 receptors. Addition of an α -methyl group (3) increased the receptor affinities 2 to 15 fold depending on the subtype.

The binding properties of the various 4-DAMP analogues gave some indications on binding site conformation. Indeed, on theoretical grounds, when 4-DAMP methobromide binds to muscarinic receptors, its two phenyl rings, instead of rotating freely in the solvent, are presumably blocked by their interaction with the receptor. This must correspond to an entropy decrease, which ought to be compensated to allow high affinity binding. Therefore, the affinity of a 'rigid' analogue should be greater than that of the parent molecule if the two phenyl rings are maintained in the correct position. Calculations by Andrews (1986) indicated that the K_b increase

obtained when preventing two bonds from freely rotating is, on average, of the order of 10 fold: it should be much greater if the groups are maintained in the optimal position. Three of the analogues tested in this work (analogues 6, 7 and 8) can be considered as 'rigid' in the sense that the two phenyl residues are bridged by a covalent bond. The four subtypes of muscarinic receptors showed greater affinities for the xanthene derivative (analogue 7) than for the fluorene (analogue 6) and ethane-bridged (analogue 8) derivatives. This suggests that the two phenyl groups of 4-DAMP were normally accepted in a nearly planar conformation in the muscarinic binding site. The xanthene derivative (analogue 7) had the highest affinity for the three subtypes: either it is closest to the optimal 4-DAMP conformation (in the binding site) or the added oxygen bridge allows favourable interaction(s) with muscarinic receptors.

The affinity increase, when comparing analogue 7 with 4-DAMP, was 10 fold in NB-OK1 (M_1), heart (M_2) and striatum (M_4) and only 3 fold in pancreas, suggesting that position of the two phenyl groups of 4-DAMP in the pancreas (M_3) binding site is different from their position in the three other receptor subtypes.

Thermodependence of drug binding to cardiac M_2 receptors

It has been suggested that hydrophobic association and ionic bond formation are associated with positive enthalpy and entropy changes, whereas hydrogen bond formation and van der Waals interactions are associated with negative enthalpy and entropy changes (Ross & Subramanian, 1981).

In this work we have measured the enthalpy and entropy changes associated with the binding of 4-DAMP and several analogues to cardiac M_2 receptors (Table 3). This subtype was chosen because drug binding to M_2 sites is much faster than binding to M_1 , M_3 or M_4 sites: it is therefore easier to achieve equilibrium at low temperatures (Waelbroeck *et al.*, 1987; 1988a,b). The thermodynamic parameters associated with 4-DAMP binding to M_2 receptors can be explained by good hydrogen bond and van der Waals interactions (negative ΔH°) and ionic bond and hydrophobic interactions (positive ΔS°). The higher affinity of analogues 6 and 7 and the lower affinity of analogue 8 were associated, with more negative and with positive enthalpy changes, respectively. This is compatible with, respectively, more- and less favourable van der Waals interactions between analogues 6, 7 and 8 and the M_2 receptor, as compared to 4-DAMP, and supports the hypothesis that the position of the two phenyl groups are, respectively, closer (6, 7) or further away (8) from the optimal conformation required to recognize the M_2 receptor. We also measured the enthalpy and entropy changes associated with the binding of the N-methyl-(1), homo-(2), α -methyl(3), (di-*p*-tolyl)-(4) and (di-*p*-chloro)-(9) analogues of 4-DAMP to cardiac M_2 receptors.

The affinity, binding enthalpy and entropy changes of 4-DAMP and its N-ethyl analogue (1) were not significantly different. The lower affinity of homo-4-DAMP for cardiac M_2 sites is explained by a significantly smaller entropy change. The results suggest that homo-4-DAMP might induce less favourable ionic or hydrophobic interactions. α -methyl-4-DAMP (analogue 3) induced the same entropy change and a slightly lower enthalpy change than 4-DAMP when binding to M_2 sites: the α -methyl group is probably in close contact with the receptor. Modification of the phenyl rings by a *para*-chloro or *para*-methyl substituent decreased significantly the analogues' affinities for M_2 muscarinic receptors, as well as the enthalpy and entropy changes associated with drug binding. These effects might be explained by differences in the electron distribution of 4-DAMP, analogues 4 and 9, inducing differences in van der Waals interactions between the drugs and the M_2 receptor.

We did not attempt to evaluate the enthalpy and entropy changes associated with *bis*-penta-methylene 4-DAMP (5)

binding because competition curves obtained with this analogue gave an anomalous (high) Hill coefficient and because this analogue was the most potent inhibitor of [^{3} H]-NMS dissociation from cardiac receptors (see below).

Allosteric effects of 4-DAMP analogues

To verify whether 4-DAMP and its analogues recognized an allosteric site on the muscarinic receptors in addition to the muscarinic binding site, we measured the effect of these molecules on [^{3} H]-NMS dissociation from cardiac M_2 receptors, a subtype more sensitive in our experience than neuroblastoma M_1 or pancreas M_3 receptors to allosteric effects.

As shown in Figure 1, all analogues tested, like atropine (Birdsall *et al.*, 1987), were capable of decreasing [^{3} H]-NMS dissociation from cardiac membranes provided that very high drug concentrations were used. For [^{3} H]-NMS binding inhibition to the four subtypes, the potency of 4-DAMP analogues varied by over 10^4 fold (from 0.1 nM to 1 μ M) and demonstrated a marked structure-dependence compatible with a specific binding site. By contrast, all analogues showed a very similar (low) potency in decreasing [^{3} H]-NMS dissociation. Fluorenyl-4-DAMP (analogue 6) and *bis*-pentamethylene 4-DAMP (analogue 5) alone were active at 30 to 100 fold lower concentrations than atropine or the other 4-DAMP analogues tested. In fact the drug concentration inducing a half-maximal effect on tracer dissociation seemed to depend mostly on the size of the drug molecule rather than on a particular structure. It has been suggested (Birdsall *et al.*, 1987) that the effect of atropine on the off-rate of [^{3} H]-NMS is due to membrane perturbation rather than to a specific drug-receptor interaction. Indeed, atropine binding is competitive with [^{3} H]-NMS but affected allosterically by gallamine (Birdsall *et al.*, 1987), suggesting that it recognises the muscarinic as opposed to the allosteric binding site.

Since all drugs tested here are amphiphilic molecules with at least one positive charge and a large hydrophobic moiety, we suspect that this 'allosteric' effect might reflect alterations of the lipid environment of the receptor, rather than drug binding to the gallamine (allosteric) site on the receptor protein. At any rate, for all drugs tested in this work, the concentrations necessary to inhibit [^{3} H]-NMS binding were at least 1000 fold lower than the concentrations affecting tracer dissociation, so that this non-specific effect did not affect the competition curves described above.

Comparison with pharmacological data

The 4-DAMP analogues studied in this work all behave as muscarinic antagonists in isolated cardiac atria and in isolated ileum from guinea-pig (Barlow & Shepherd, 1985; 1986). A comparison of the pK_i values in binding studies (this work) and pA_2 values in pharmacological studies (Barlow *et al.*, 1976; Barlow & Shepherd, 1985; 1986) supports the hypothesis that the atrial receptors belong to the M_2 cardiac subtype, whereas the ileum receptors belong to the M_3 pancreatic subtype. The only exception to this rule was *bis*-pentamethylene 4-DAMP, which had a 10 fold lower potency in pancreas binding studies than expected from previous pharmacological data. The significance of this discrepancy is, however, not clear, as: (1) *bis*-pentamethylene 4-DAMP competition curves had a Hill coefficient greater than 1.0 and (2) this compound was the most potent inhibitor of [^{3} H]-NMS dissociation from cardiac receptors; two results which suggest that it might interfere with muscarinic receptors in a 'non classical' manner.

In conclusion, our results confirm that the requirements for high affinity binding to the four muscarinic receptor subtypes are different.

The structural requirements of M_3 receptors were more stringent when compared to M_2 , M_1 or M_4 receptors. As a

result, most of the 4-DAMP analogues studied in this work had a different selectivity pattern from 4-DAMP itself.

At very high concentration, all 4-DAMP analogues modified the [^3H]-NMS dissociation rate from cardiac membranes, their potency correlating with their size, not with a specific

structure, suggesting membrane disturbance rather than binding to a genuine allosteric site.

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