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The effects of agonists on the components of the cardiac muscarinic receptor

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1 The binding of eleven agonists to muscarinic receptors in the rat heart has been measured in competition with [^{3}H]-N-methylscopolamine.

2 Full analysis of binding required the resolution of three components (SH, H and L).

3 The proportion of the H component was independent of agonist structure. The proportion of the SH component ranged from 2–36% of the total and was dependent on the agonist. The proportion of the L component varied in a complementary way from 59–22% of the total.

4 The ratios of the affinities of ten of the agonists to the three components of the receptors were constant; the weakest binding agonist, choline, had lower ratios of affinity.

5 When saturated with guanylylimidodiphosphate (GppNHp) the SH receptor population was no longer detectable and the H receptor population was reduced by 63%: about 85% of the receptors was in the L population.

6 The affinity constants of agonists for the H and L forms of the receptors were not changed by GppNHp.

7 The results are interpreted in terms of the effects of accessory proteins on the proportions of binding and non-binding conformations of the receptor.

Introduction

It is now very well established that the binding of agonists to muscarinic receptors in broken cell preparations from various tissues are not governed by a simple mass-action relationship. The complexities have been attributed to two possible causes. Either the receptor is partitioned between several substates so that the overall binding is due to the superposition of binding to these separate states (see for instance Birdsall *et al.*, 1978) or, alternatively, the receptor is involved in complex ternary complexes with other proteins which give rise to the low slope of the binding curve (see for example Ehlert, 1985). It is also known that the detailed character of the binding can be modified by the ionic composition of the medium and by cyclic nucleotides (Berrie *et al.*, 1979; Rosenberg *et al.*, 1980; McKinney *et al.*, 1984). A preliminary examination of the dependence of these properties on the nature of the agonist has been performed on the receptors in the cerebral cortex (Birdsall *et al.*, 1980b) for receptors that are predominantly M_1 in character.

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The purpose of the present study was to examine this aspect of agonist structure-activity relationships in the heart where the receptors belong to the M_2 group. After this study was submitted the very interesting study by Wong *et al.* (1986) on receptors in the hamster heart appeared.

Methods

The membrane preparations were from the whole heart of rats as described by Burgen (1986). All determinations were made in a buffer consisting of 100 mM NaCl, 2 mM $MgCl_2$ and 20 mM Tris HCl adjusted to pH 7.5. In the case of the cholinesterase-sensitive agonists acetylcholine and butyrylcholine, 10 μM neostigmine was present throughout. Incubations at 25°C lasted for 30 min.

The binding curves were analysed by a computer program giving least squares fitting to a sum of up to three superimposed binding sites. The concentration of receptor in each subgroup was expressed as a percentage of the total and hence there were two

independent variables for the concentration of sites, but three independent variables for binding constants. In those instances where no significant concentration of a third binding site was found, the computation was repeated with a reduced number of variables. Data from the individual experiments were analysed independently. Average values and their standard errors were then calculated to give the values presented in Tables 1–3.

Calculations for a system in which the receptor was associated with a second subunit, which modified its affinity, were carried out using a procedure essentially the same as that described by Lee *et al.* (1986). Like these authors we did not find that this method of analysis gave results that stood up to tests of internal consistency and they will not be used in the analysis presented.

No correction was made for the occupancy of the receptor by [^{3}H]-N-methylscopolamine ([^{3}H]-NMS). Since the concentration of NMS was 0.2 nM and the affinity constant for NMS was $2.5 \times 10^9\,M^{-1}$ the agonist binding constants need to be multiplied by 1.5 to obtain the intrinsic values.

Preliminary experiments showed that the concentration of GppNHp required to generate half the full effect on the binding curve was approximately 10 nM.

The concentration used in most of the experiments listed in Table 3 was 100 μM. In a few instances checks were also made at 1 mM but these did not produce a greater effect so we are confident that the GppNHp effects are maximal.

Methylfurmethide was kindly supplied by Dr E.W. Gill. Guanylylimidodiphosphate (GppNHp) was obtained from Boehringer.

Results

As noted in the Methods section the analysis of results used here is based on the superposition of independent binding sites, each of which depends on a simple mass action relationship. An important decision is therefore the number of components that are necessary and sufficient for the analysis. It has previously been shown that this can be decided on statistical grounds in terms of goodness of fit (Birdsall *et al.*, 1980a; Munson *et al.*, 1984). Evaluation of the binding of carbachol to the receptors in the present case has shown that residuals are smaller (rms 1.1%) and no larger than expected from experimental error when three components were used but were considerably larger when only two components were used (rms 3.0%). As seen in Table 1 the errors in most of the parameters are also smaller when three components are used in the analysis despite the influence of the increased degrees of freedom which would tend to increase the error estimates. In the central nervous system three components were also indicated. Is there a limit to the number of components that should be postulated? There is no theoretical limit, but the practical limits are in resolution and also what is sufficient to provide a self consistent scheme.

The importance of the method of analysis is very clearly illustrated in Table 1, where it can be seen that varying the number of components produces large changes in the magnitude of the calculated affinity constants. The relevance of this will become clearer later when we consider the effects of GppHNp on the receptor.

In Table 2 we show the results of application of three component analysis to the binding of 11 agonists. It can be seen that the high binding component f_H is essentially the same for all agonists at 42.2% ± 3.84 (s.d.). While the fraction of the superhigh component f_{SH} might be regarded as not too dissimilar for the first six agonists in the list, there is a marked fall away seen in the latter five agonists. The agonists have been arranged as a declining series in f_{SH}–it is not a declining series in f_H and hence is a generally increasing series in f_L. The strong correlation between f_{SH} and f_L and the lack of correlation between f_{SH} and f_H are evident in Figure 1. The last two agonists in Table 2 are very weak agonists and there is indeed some correlation between f_{SH} and the binding constant K_{SH} but it is not very strong and is heavily influenced by choline and piperidine (Figure 2).

Analysis of the relationship between the binding constants for the subpopulations reveals the interest-

Table 1 Carbachol binding

Population of binding components	f_{SH}	f_H	f_L
Two component analysis	—	42.6 ± 10.1	57.4 ± 10.1
Three component analysis	28.5 ± 6.1	45.8 ± 2.6	25.7 ± 4.4
Affinity constants (log)	pK_{SH}	pK_H	pK_L
Two component analysis	—	7.31 ± 0.20	5.20 ± 0.07
Thee component analysis	7.71 ± 0.19	5.98 ± 0.12	4.61 ± 0.09

Table 2 Population of receptor components

Agonist	f_{SH}	f_H	f_L
Acetylcholine (4)	35.8 ± 1.6	42.5 ± 7.1	21.7 ± 5.7
(±)-Carbamyl β-methylcholine (5)	31.0 ± 5.3	38.0 ± 1.1	31.0 ± 5.0
Furmethide (3)	29.6 ± 7.2	35.3 ± 2.1	35.1 ± 3.3
Carbachol (6)	28.5 ± 6.1	45.8 ± 7.1	25.7 ± 4.4
Methylfurmethide (3)	28.2 ± 2.3	47.3 ± 2.7	24.5 ± 4.9
Arecoline (3)	27.2 ± 5.0	47.4 ± 4.4	25.4 ± 4.5
Oxotremorine (4)	21.5 ± 5.4	43.2 ± 2.2	35.3 ± 5.6
Pentyl TMA (3)	16.3 ± 3.0	40.4 ± 0.9	43.3 ± 3.6
Pilocarpine (4)	13.4 ± 7.6	43.5 ± 2.0	43.1 ± 7.2
Choline (3)	12.3 ± 4.2	41.6 ± 6.8	46.1 ± 3.5
Piperidine (3)	2.4	39.1 ± 4.8	58.5 ± 4.4

The number of experiments is shown in parentheses. Pentyl TMA = pentyltrimethylammonium.

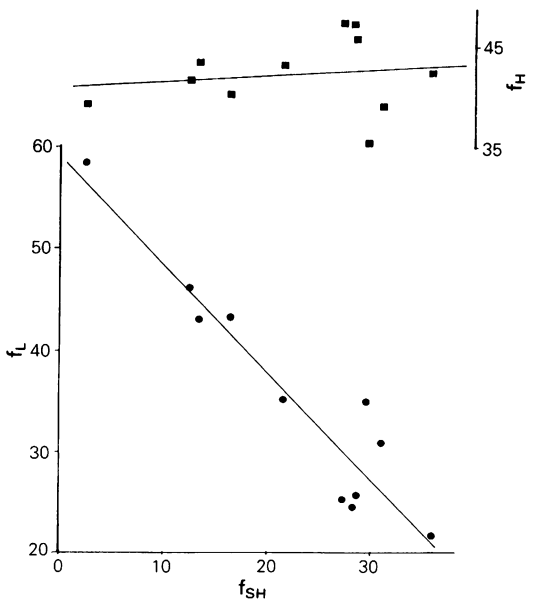

Figure 1 Correlation of the fraction of the SH population (f_{SH}) with the L and H populations (f_L, f_H). The lines are least square fits to the data on the assumption of a linear relationship. The slope of the f_{SH}, f_L regression is −1.06 and that of the f_{SH}, f_H regression + 0.060.

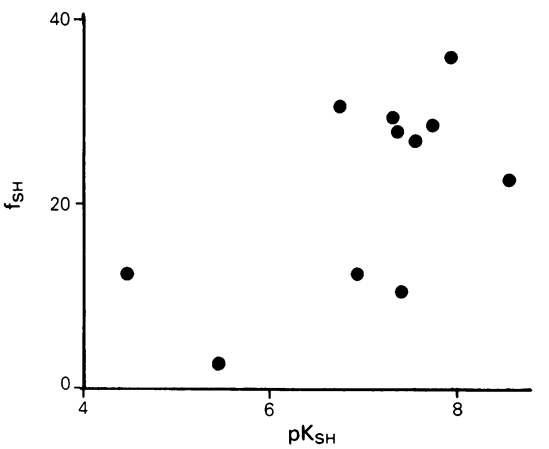

Figure 2 Correlation between the fraction of receptors in the SH form (f_{SH}) and the binding constant for the SH form (K_{SH}).

ing and perhaps surprising finding that, if we exclude choline from consideration, the ratio of the binding constants appear to be themselves constant. The difference between pK_{SH} and pK_L is 2.262 ± 0.208 (s.d.), i.e. the ratio is 419 (s.d. range 259–676) and between pK_H and pK_L 1.408 ± 0.164 (s.d.), i.e. a ratio of 26 (s.d. range 18–37). The values for choline are lower at 59 and 4, respectively, and are statistically different from those of the other agonists.

The effect of a saturating concentration of Gpp-NHp is very clear and is shown in Figure 3 for acetylcholine and pilocarpine. The curves are shifted to the right and the slope is steepened, the magnitude of the effect being greater for acetylcholine than for pilocarpine. The quantitative effect in the whole series is summarised in Table 3. In no case do we find a detectable population of SH receptors, the population of H receptors has been reduced to 15.6% ± 5.3 (s.d.), i.e. to 37% of its previous magnitude and there has been a corresponding increase in the fraction of L receptor which is now about 85% of the total.

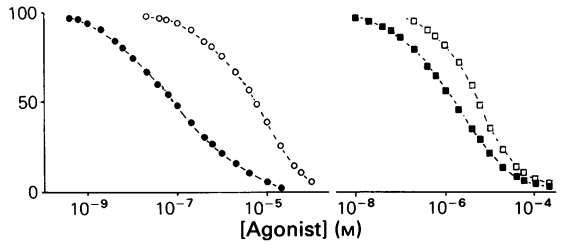

Figure 3 The effect of GppNHp on the binding of acetylcholine and pilocarpine. Ordinate scale: N-methylscopolamine (NMS) bound as % of control. Abscissa scale: concentration of competing agonist. The lines are computer fits to the experimental data. (●) Acetylcholine (+ 10 μM neostigmine): $f_{SH} = 34$; $f_H = 44$; $f_L = 22$. (○) Acetylcholine (+ 10 μM neostigmine) + 10 μM GppNHp: $f_{SH} = 0$; $f_H = 22$: $f_L = 78$. (■) Pilocarpine: $f_{SH} = 6$; $f_H = 47$; $f_L = 47$. (□) Pilocarpine + 10 μM GppNHp: $f_{SH} = 0$; $f_H = 10$; $f_L = 90$.

The effect of GppNHp on binding constants is shown in Table 4, where it can be seen that there are essentially no changes in the binding constants for L an insignificant difference -0.014 ± 0.032 (s.e.mean) in pK_L and for pK_H $+0.060 \pm 0.088$ (s.e.mean). Naturally we can say nothing of pK_{SH}.

The importance of using the correct analysis for the receptor components is now evident. If we analyse the system for two components both in the absence and in the presence of GppNHp we would have concluded that pK_H had declined by 1.35 and pK_L by 0.51. This indeed was the conclusion that Walbroeck *et al.* (1982) came to, although in their experiments they were unable to detect an H form of the receptor in the presence of GppNHp.

Discussion

We need first of all to consider what properties of the receptor are consistent with discrete affinity states which are changed in abundance but not in binding characteristics by GppNHp. These properties point very clearly to the operation of a set of G proteins (see Katada *et al.*, 1984) with different details as far as the SH, H and L forms are concerned. In the intact cell the concentration of guanosine triphosphate (GTP) is sufficient to ensure that the shift described here is fully activated and since it is very probable that the agonist promoted inhibition of adenylate cyclase is due to the L form, we presume that the L form itself is coupled to G proteins. Since both SH and H forms are modifed by GppNHp they must also bind G proteins. The most likely situation is that all three bind G_α but differ in the β and γ proteins they bind. The conversion of SH and H forms into L by raised ionic strength (Burgen, 1986) can also be explained on the interaction with auxiliary proteins and a minimalist view would suggest that the same proteins are involved. It is interesting therefore that H to L conversion was distinguishable by greater resistance to ionic strength than the SH to L. The usual assumption would be that the ancillary protein controls the conformation of the receptor. This could be either by influencing the abundance of pre-existing conformations or by generating entirely new conformations. In general we need to think of conformations in pairs corresponding to a ground state conformation in the absence of ligand (perhaps corresponding to the conformation binding antagonists) and the active state selected by the agonist – the first of these could be regarded as a non-binding conformation by agonists. Since in the present situation the same structure-dependence is found for all substates we will assume that the binding conformation is also the same for all three substates. This implies that the only difference between the substates in binding terms is the 'ease'

Table 3 Population of receptor components in the presence of GppNHp

Agonist	f_H	f_L
Acetylcholine (4)	20.7 ± 1.3	79.3 ± 1.3
Carbamyl β-methylcholine (5)	12.6 ± 1.6	87.4 ± 1.6
Furmethide (3)	16.0 ± 4.0	84.0 ± 4.0
Carbachol (6)	24.6 ± 2.7	75.4 ± 2.7
Methylfurmethide (4)	14.6 ± 1.9	86.0 ± 1.9
Arecoline (4)	18.1 ± 6.3	81.9 ± 1.9
Oxotremorine (6)	15.4 ± 2.8	84.6 ± 2.8
Pentyl TMA (3)	5.3	94.7
Pilocarpine (3)	9.7 ± 0.9	90.3 ± 0.9
Choline (3)	14.9 ± 10.0	85.1 ± 10.0
Piperidine (3)	19.8 ± 2.0	80.2 ± 2.0

Table 4 Binding characteristics of receptor components

Agonist	pK_{SH} −G	−G	pK_H +G	Δ	−G	pK_L +G	Δ
Acetylcholine	7.89 ± 0.22	6.46 ± 0.15	6.63 ± 0.11	+0.17 ± 0.19	5.20 ± 0.10	5.28 ± 0.22	+0.08 ± 0.24
Carbamyl β-methylcholine	6.75 ± 0.15	5.37 ± 0.18	5.81 ± 0.32	+0.44 ± 0.37	4.16 ± 0.06	4.15 ± 0.10	−0.01 ± 0.12
Furmethide	7.24 ± 0.24	5.93 ± 0.27	5.96 ± 0.30	+0.03 ± 0.40	4.62 ± 0.21	4.50 ± 0.18	−0.12 ± 0.28
Carbachol	7.71 ± 0.09	5.98 ± 0.12	5.96 ± 0.07	−0.02 ± 0.14	4.61 ± 0.09	4.69 ± 0.05	±0.08 ± 0.09
Methylfurmethide	7.35 ± 0.18	5.94 ± 0.12	6.14 ± 0.14	+0.20 ± 0.18	4.87 ± 0.10	4.86 ± 0.02	−0.01 ± 0.10
Arecoline	7.58 ± 0.16	6.05 ± 0.18	6.18 ± 0.10	+0.13 ± 0.21	4.89 ± 0.13	4.94 ± 0.01	+0.05 ± 0.13
Oxotremorine	8.58 ± 0.07	7.20 ± 0.05	7.11 ± 0.19	−0.09 ± 0.20	6.04 ± 0.01	5.86 ± 0.06	−0.18 ± 0.06
Pentyl TMA	6.92 ± 0.05	5.41 ± 0.07	5.62	+0.11	4.12 ± 0.03	4.08 ± 0.04	−0.04 ± 0.05
Pilocarpine	7.39 ± 0.12	6.23 ± 0.04	6.19 ± 0.12	−0.04 ± 0.09	5.09 ± 0.04	5.11 ± 0.05	+0.02 ± 0.06
Choline	4.42 ± 0.06	3.46 ± 0.12	3.80 ± 0.17	+0.34 ± 0.21	2.65 ± 0.08	2.78 ± 0.17	+0.13 ± 0.19
Piperidine	5.42 ± 0.27	3.98 ± 0.07	3.42 ± 0.06	−0.56 ± 0.09	2.91 ± 0.03	2.76 ± 0.04	−0.15 ± 0.07

with which the active conformation can be generated. We can represent the situation thus

$$R_o \overset{\alpha}{\rightleftharpoons} R_1 + D \overset{\beta}{\rightleftharpoons} R_1D$$

in which the constant α controls the abundance of the excited conformation R_1 and hence influences the observed binding of D which will depend on $\alpha\beta/1 + \alpha$. In practice since one must assume that in the absence of an agonist the receptor is predominantly in the ground state then $\alpha < 1$ and hence we can write more simply $K = \alpha.\beta$. Since there is only one active state of the receptor then β will be characteristic of an agonist but the same for all states of the receptor.

Any hypothesis about the receptor has to accommodate three constraints: (1) substates are sensed by agonists but not by antagonists under the conditions considered, (2) the populations of SH and L states are sensitive to the structure of the agonist, (3) the structure-dependence of binding is very similar or identical for all three substates. Our model satisfies the first and third but does not satisfy the second of these criteria.

An alternative model is one in which the agonists combine with the receptor in the ground state but the complex then relaxes into the active conformation – a process usually referred to as conformation induction –

$$R_o + D \overset{\alpha}{\rightleftharpoons} R_oD \overset{\beta}{\rightleftharpoons} R_1D$$

the binding constant is now given by $\alpha(\beta + 1)$ and the difference between subsites depends on discrete values of β, i.e. β_{SH}, β_H, β_L. If R_1 is not identical but shows small variations in detailed conformation with different agonists this might be enough to effect differential binding of the accessory proteins that define the SH and L subtypes. The invariance of f_H implies that the binding of the accessory proteins defining this subtype are not affected in this way.

The relatively small part of the H substate that persists in the presence of GppNHp could simply be the result of damage in preparation but its magnitude is quite constant. It should be recalled that the receptor in the conducting bundle of the beef heart has been found to be unresponsive to GppNHp (Burgen *et al.*, 1981) and was mainly of the H subtype and thus the unresponsive H may be due to the conducting tissue present in the homogenate. The muscarinic receptors associated with the K channel do have another kind of G protein, but it is not known whether this changes the binding parameters of the receptor when G nucleotides are bound (Pfaffinger *et al.*, 1985).

The nearly constant ratio of binding constants found in the heart is quite unlike the findings in the cerebral cortex (Burgen *et al.*, 1980b), nor are the binding constants themselves very close.

Wong *et al.* (1986) have carried out similar

experiments to ours in the hamster heart using a smaller group of agonists. They also found that the fraction of the agonist states depended on the structure of the agonist. However, in their preparations the SH state was not detectable at all for several agonists. This difference may be due to a species difference or, bearing in mind that the balance of substates is easily perturbed by ionic composition, may be due to variation in the details of the binding protocol.

Finally we noted that the results with choline were out of line with those of the other agonists – we cannot at present offer an explanation for this.

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