Ligand-Binding Properties of the Muscarinic Acetylcholine Receptor in Mouse Neuroblastoma Cells

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1. Muscarinic acetylcholine receptors in a plasma-membrane fraction derived from mouse neuroblastoma clone NIE-115 bind [^3H]quinuclidinyl benzilate according to the Law of Mass Action ($K_{\text{dissociation}}$ 40 pM, h 0.96). 2. Antagonist and agonist binding to the receptor was studied by displacement of [^3H]quinuclidinyl benzilate with non-radioactive ligands. The data show good agreement with similar data obtained on rat brain and ileal smooth muscle [Birdsall & Hulme (1976) *J. Neurochem.* 27, 7–16] indicating that the receptor is very similar in these three tissues.

The binding of agonists and antagonists to muscarinic receptors in membrane fractions from the brain and ileum has been studied extensively (for reviews, see Snyder *et al.*, 1975; Birdsall & Hulme, 1976) and it has been possible to demonstrate significant correlations between the binding properties and pharmacological activities of these muscarinic ligands (Birdsall & Hulme, 1976; Birdsall *et al.*, 1978). However, to study the 'cascade' of events that occurs between the initial interaction of the agonist with the receptor and the production of a physiological response, it is necessary to have a suitable system in which these events may be monitored *in vitro*. Tissue slices and homogenates provide two potential systems but each has its drawbacks. In either case analysis of the 'cascade' is complicated by cellular heterogeneity; in tissue slices there are also problems with diffusion of drugs to the receptor sites, whereas in homogenates it has not yet been possible to demonstrate biochemical events that are linked to agonist-occupancy of the receptor.

Neuroblastoma cells, grown in tissue culture, afford a homogeneous cell system and have many of the properties of nerve cells. Some clones possess muscarinic receptors and have membranes responsive to cholinergic stimuli (Matsuzawa & Nirenberg, 1975). These cells therefore may provide a suitable model 'stimulus-receptor-response' system. In the present paper we describe the binding properties of agonists and antagonists to muscarinic receptors in mouse neuroblastoma cells (clone NIE-115) derived from the C1300 tumour (Amano *et al.*, 1972). The results suggest that the receptors on these cells are

very similar in character to those in the brain and ileal smooth muscle.

Experimental

Materials

[^3H]Quinuclidinyl benzilate (13 Ci/mmol) was from The Radiochemical Centre, Amersham, Bucks., U.K. Non-radioactive quinuclidinyl benzilate was generously given by Dr. R. B. Barlow (Department of Pharmacology, University of Bristol). Acetylcholine chloride, ($\pm$)-acetyl- β -methylcholine chloride, arecoline hydrochloride, ($\pm$)-atropine sulphate, eserine sulphate, (–)-scopolamine hydrochloride and (–)-scopolamine-*N*-methyl bromide were from Sigma (London) Chemical Co., Kingston upon Thames, Surrey, U.K. Carbamoylcholine was from Koch-Light, Colnbrook, Bucks., U.K. and oxotremorine and pilocarpine hydrochloride were from Aldrich Chemical Co., Gillingham, Dorset, U.K. Oxotremorine-M {trimethyl-[4-(2-oxopyrrolidin-1'-yl)-but-2-ynyl]ammonium iodide (m.p. 155–156°C)} was synthesized by a modification of the method described by Bebbington *et al.* (1966). Where racemic compounds were used it was assumed that only one isomer was pharmacologically active [(–)-atropine, (+)-acetyl- β -methylcholine, (–)-quinuclidinyl benzilate].

Chemicals for liquid scintillation counting were from Fisons, Loughborough, Leics., U.K.

Methods

Cell growth. Neuroblastoma clone NIE-115 was grown in Dulbecco's Modified Eagle's Medium

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[Smith *et al.*, 1960; no pyruvate, 4.5g of glucose/litre, from Gibco-Biocult, Paisley, Renfrewshire, Scotland, U.K.: lx liquid (catalogue no. 196G) or made up from powder (catalogue no. H21)] containing 10% (v/v) foetal calf serum (Gibco-Biocult), penicillin (100 units/ml) and streptomycin (100 µg/ml) at 37°C in an humidified atmosphere of CO₂/air (1:9) on plastic tissue-culture dishes [Falcon (obtained from Scientific Supplies, London EC1R 5EB, U.K.) or Corning (obtained from Fisons)]. Typically about 5 × 10⁵ cells, obtained by a double treatment with trypsin solution (0.05% in Dulbecco's Modified Eagle's medium without serum) of a monolayer culture, were seeded into 6.0ml of medium in a 55cm² dish, the medium being changed on days 3, 5 and 6 and the cells used on day 7. At this time the cells were nearly confluent and many showed long processes (a 55cm² dish contained 5 × 10⁶–8 × 10⁶ cells and 8–11mg of cell protein).

Preparation of plasma membranes. A crude neuroblastoma plasma-membrane fraction was prepared essentially by the method of Truding *et al.* (1974). Cells were removed mechanically from their plastic dishes with a Teflon 'policeman', collected by centrifugation (8min, 130g) and washed twice with ice-cold sodium phosphate buffer (pH7.4, 20mM, 40ml; the quantities given here are for 16–20 55cm² dishes) containing NaCl (0.15M). The washed cell pellet was resuspended in ZnCl₂ solution (1mM, 40ml) for 7min at room temperature (about 16°C), 6.0ml of sucrose (650g/l) in water added and the cells were collected by centrifugation (200g, 5min). The pellet was resuspended in 19ml of ice-cold sodium phosphate (20mM, pH7.4) for 5min and the cells were homogenized in a Teflon/glass or Teflon/stainless steel homogenizer (clearance 0.1mm, 60 strokes). Sucrose (6.4ml; 650g/l) in sodium phosphate buffer (pH7.4, 20mM) was added and the mixture layered on to 14ml of sucrose (180g/l) in sodium phosphate buffer (pH7.4, 20mM) and centrifuged at 200g for 17min at 4°C. All the sucrose solution except for the cell-debris pellet was collected and centrifuged at 17500g for 30min at 4°C and the resultant pellet resuspended by vortex mixing in 5.3ml of ice-cold bicarbonate buffer [this buffer contained: NaCl (0.11mol/l), KCl (5.3mmol/l), CaCl₂ (1.8mmol/l), MgSO₄ (0.8mmol/l), sodium dihydrogen orthophosphate (0.9mmol/l), glucose (25mmol/l) and NaHCO₃ (44mmol/l) buffered to about pH7.5 with CO₂/O₂ (1:19)]. Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Receptor-binding studies. [3-³H]Quinuclidinyl benzilate binding was assayed by a centrifugation assay similar to that described by Terenius (1974). (±)-[3-³H]Quinuclidinyl benzilate (30pM–3nM) was incubated at 30°C for 1h in quadruplicate with NIE-115 membrane fragments (0.5–1.0mg/ml) in a final

volume of 1ml in the above bicarbonate buffer in capped plastic centrifuge tubes (1.5ml, Sarstedt, Leicester, Leics., U.K.). Preliminary studies showed that under these incubation conditions quinuclidinyl benzilate binding was at equilibrium. Known concentrations of competing drugs were added where appropriate at the beginning of the incubation. The bound radioactivity was determined by rapid centrifugation (10000g, 30s) in a microcentrifuge (Quickfit, supplied by Northern Media Suppliers, Hull, Yorks., U.K.) followed by superficial washing of the pellet in the above bicarbonate buffer. The ends of the tubes were cut off, placed into 10ml of scintillant [Triton X-100/toluene/water, 4:8:1, by vol. with 0.4% 5-(4-biphenyl)-2-(4-*t*-butylphenyl)-1-oxa-3,4-diazole], the pellets dispersed by vortex mixing, and the radioactivity was determined in Packard 2450 or 3255 liquid-scintillation spectrometers operating in the external-standard mode. [3-³H]Quinuclidinyl benzilate-binding inhibited by 10µM(–)-atropine was defined as the specific (receptor-associated) quinuclidinyl benzilate binding, the remainder being non-specifically trapped or bound. In a typical 'direct' binding experiment quadruplicate assays were made at different (–)-[3-³H]quinuclidinyl benzilate concentrations with and without 10µM(–)-atropine so that the specifically bound (–)-[3-³H]quinuclidinyl benzilate could be calculated for each concentration. In a typical displacement experiment quadruplicate assays with a single (–)-[3-³H]quinuclidinyl benzilate concentration were made: (i) with no further additions, to define the sum of the specifically and non-specifically bound (–)-[3-³H]quinuclidinyl benzilate; (ii) with 10µM(–)-atropine, to define the non-specifically bound radioactivity {approx. 50% of total binding at 0.3nM free (–)-[3-³H]quinuclidinyl benzilate}; (iii) with various concentrations of the displacing ligand, to determine the percentage inhibition of the specific (–)-[3-³H]quinuclidinyl benzilate binding. The results were calculated by assuming that only the (–)-isomer of the benzilate bound specifically to the receptor under the conditions used (Meyerhoffer, 1972) and that this isomer represented half the total added radioactivity in an assay. The non-specifically bound (–)-isomer was calculated assuming that the remaining (+)- and (–)-isomers contributed to non-specific binding in proportion to their relative concentrations. The unbound (–)-quinuclidinyl benzilate was then calculated by subtraction.

The possibility was investigated that the displacing ligands were displacing some non-specifically bound [3-³H]quinuclidinyl benzilate [as defined by 10µM(–)-atropine]. In experiments where the unbound (–)-quinuclidinyl benzilate concentration was about 0.2nM, 10µM(–)-atropine displaced 49.6% of the total bound radioactivity, 10µM-carbamoylcholine displaced 30.5% of the total bound radioactivity and

10 μ M-(−)-atropine together with 10 μ M-carbamoylcholine displaced 46.4% of the total bound radioactivity. Since the combination of carbamoylcholine (at concentrations where if used alone it displaces a significant amount of bound radioactivity) with atropine gave a similar displacement to atropine alone, it is unlikely that the test ligands are displacing the non-specifically bound radioactivity.

For direct binding studies, plots of [specifically bound (−)-quinuclidinyl benzilate] $^{-1}$ versus [unbound (−)-quinuclidinyl benzilate] $^{-1}$ were used to determine dissociation constants. In addition, Hill plots of log(fractional receptor occupancy/1−fractional receptor occupancy) versus log(unbound (−)-quinuclidinyl benzilate) were constructed. Where other ligands were used to displace [3 H]quinuclidinyl benzilate double-reciprocal and Hill plots were used to determine ligand-binding parameters where possible.

Results

Direct binding of (−)-[3 H]quinuclidinyl benzilate to neuroblastoma cell membranes

Initial ligand-binding studies using *N*-[3 H]methyl-(−)-scopolamine (E. C. Hulme & P. G. Strange, unpublished work) indicated that there indeed was a muscarinic acetylcholine receptor present on NIE-115 neuroblastoma cells, but in rather low amounts. It was therefore decided to use the antagonist [3 H]-quinuclidinyl benzilate for routine work, because it was available at very high specific radioactivity (13 Ci/mmol). (+)-Quinuclidinyl benzilate is at least 20 times less active pharmacologically than the (−)-isomer (Meyerhoffer, 1972) and in all experiments it is assumed that (+)-[3 H]quinuclidinyl benzilate does not bind significantly to the receptor. This is supported by experiments on rat brain synaptosomes (P. G. Strange & P. R. Golds, unpublished work). Synaptosomes incubated with (±)-[3 H]quinuclidinyl benzilate such that the receptor concentration was greater than the concentration of the (−)-isomer (receptor concentration, 1 nM approx.; (−)-[3 H]-quinuclidinyl benzilate added, 0.47 nM) bound specifically only about half the total available (±)-[3 H]quinuclidinyl benzilate (after subtraction of non-specifically bound radioactivity). This presumably reflects binding of nearly all the (−)-isomer (a small proportion remaining unbound), the (+)-isomer showing no significant specific binding under these conditions. A similar experiment is not feasible with neuroblastoma membranes, because of the lower receptor number, but the conclusions are likely to apply to both tissues.

In initial experiments on whole cells, no saturation of [3 H]quinuclidinyl benzilate binding could be obtained. This may have been due to uptake of

Fig. 1. Specific [3 H]quinuclidinyl benzilate binding to an NIE-115 membrane fraction

Various concentrations of (−)-[3 H]quinuclidinyl benzilate were incubated with an NIE-115 membrane fraction as described in the Materials and Methods section. The dissociation constant (36 pM in this experiment) was determined from a double-reciprocal plot and the curve is a mass-action binding curve drawn using this value.

[3 H]quinuclidinyl benzilate into whole cells or to the presence on whole cells of binding sites with low affinity and high capacity. A crude cell-membrane preparation, however, did show saturation of [3 H]-quinuclidinyl benzilate binding and a representative binding curve is given in Fig. 1. The data conform to the Law of Mass Action indicating a single binding site with a dissociation constant for (−)-[3 H]-quinuclidinyl benzilate of 36 pM; the Hill coefficient, *h*, was 0.96 and the receptor concentration was 57 pmol/g of membrane protein. Although the data fit well to a single binding site, the concentrations of (−)-[3 H]quinuclidinyl benzilate used would not have detected the low-affinity quinuclidinyl benzilate binding sites mentioned by Carson *et al.* (1977). The presence of low-affinity sites cannot therefore be ruled out, but, in the experiments reported below, binding to these sites would not have occurred with the [3 H]quinuclidinyl benzilate concentrations used. The average value from three experiments for the dissociation constant is 40 ± 4.5 pM (mean $\pm$ s.d.). This value is in reasonable agreement with that determined for rat brain synaptosomes (72 pM; P. G. Strange & P. R. Golds, unpublished work).

The concentration of receptors found in separate preparations did not vary markedly; an average value of 44 ± 11 (mean $\pm$ s.d.) pmol of receptor/g of membrane protein was obtained from 27 separate experiments. Also, receptor number did not seem to alter during 20 cell passages or to depend on the exact length of the culture period; cultures were not, for example, grown for several days at confluency as in

the work of Richelson (1977). This receptor concentration corresponds to about 4000 receptors per cell, assuming none are lost during membrane preparation

Fig. 2. Displacement of specific [^3H]quinuclidinyl benzilate binding by ($-$)-atropine ($\circ$) and pilocarpine ($\bullet$). Various concentrations of non-radioactive ligand were used to displace ($-$)-[^3H]quinuclidinyl benzilate (approx. 0.3 nM) binding from NIE-115 membranes as described in the Materials and Methods section. The 0 and 100% inhibition points, though not shown, were determined respectively from the total ($-$)-[^3H]quinuclidinyl benzilate bound specifically and the ($-$)-[^3H]quinuclidinyl benzilate bound in the presence of $10\text{ }\mu\text{M}$ -($-$)-atropine. The curves are mass-action binding curves fitted to the data.

Table 1. Dissociation constants for muscarinic antagonists ($-$)-Quinuclidinyl benzilate data (direct binding) were determined as described in the legend to Fig. 1. All other data were determined by displacement of radioactive antagonist by using non-radioactive antagonist. Neuroblastoma NIE-115 displacement data were obtained by using various concentrations of non-radioactive antagonist to displace [^3H]quinuclidinyl benzilate (approx. 0.3 nM) as described in the Materials and Methods section. The data were corrected for ($-$)-[^3H]quinuclidinyl benzilate-receptor occupancy to give values for dissociation constants.

Antagonist	NIE-115 membrane fraction		Rat brain synaptosomal fraction	
	$-\log K$	h	$-\log K$	h
($-$)-Quinuclidinyl benzilate	10.40*	0.96	10.14*	0.97
($-$)-Atropine	10.09	1.02	9.90†	1.0
($-$)-Scopolamine	9.66	0.96	9.26†*	0.92
N -Methyl-($-$)-scopolamine	9.34	1.10	9.09†	0.98
	9.70	0.90	9.78†*	0.98

* Values determined by direct binding of radioactive ligand.

† Values from E. C. Hulme, N. J. M. Birdsall & A. S. V. Burgen (unpublished work).

and is much lower than in normal rat brain (1 nmol/g of protein).

The low receptor number poses experimental problems because specifically bound radioactivity is low and comparable with the non-specifically bound radioactivity. In addition, the amount of cell protein available from tissue culture in monolayer is limited so that many replicate experiments cannot be made. As a result errors are introduced into the calculation of bound and unbound ($-$)-quinuclidinyl benzilate.

As Levitzki *et al.* (1975) have pointed out, the optimum conditions for direct receptor-binding experiments require a ligand with a dissociation constant comparable with the experimental receptor concentration. Since the receptor concentration here would be about 40 pM (1 mg/ml of protein), [^3H]-atropine (K 0.6 nM) could not easily be used in direct binding studies, whereas [^3H]quinuclidinyl benzilate (K 40 pM) is ideal for this study. Conversely in direct binding studies using [^3H]quinuclidinyl benzilate on normal brain homogenates (receptor concentration about 1 nM at 1 mg of protein/ml) difficulty will be experienced in determining accurately the unbound ligand concentration at 50% receptor occupancy (see, e.g., Yamamura & Snyder, 1974) because of high depletion of the added ($-$)-[^3H]quinuclidinyl benzilate.

Antagonist displacement of ($-$)-[^3H]quinuclidinyl benzilate

Antagonist binding to the receptor was studied by displacement of ($-$)-[^3H]quinuclidinyl benzilate using non-radioactive antagonists. The displacement curve for ($-$)-atropine is shown in Fig. 2. Because of the low receptor number, it was necessary to use a concentration of [^3H]quinuclidinyl benzilate that

Fig. 3. Displacement of specific [^3H]quinuclidinyl benzilate binding by carbamoylcholine

See legend to Fig. 2 for the method. The curve is a least-squares computer fit to the data according to the two-site model of Birdsall & Hulme (1976).

occupied about 90% of the receptor sites to obtain accurately measurable values of the bound radioactivity. The antagonist dissociation constants in Table 1 were obtained by correcting the experimental data for this occupancy assuming simple competitive inhibition. The unbound (–)-[3-³H]quinuclidinyl benzilate concentration changed by about 10% as the bound (–)-[3-³H]quinuclidinyl benzilate was displaced from the receptor by the competing antagonist, so a mean value for the unbound isomer concentration was used for calculation.

The dissociation constants for the neuroblastoma receptor and for the brain receptor (Table 1) agree well. The Hill coefficients for the antagonists were all close to 1 suggesting that, as in the case of brain receptors, antagonists bind to a single class of homogeneous binding sites.

Agonist displacement of (–)-[3-³H]quinuclidinyl benzilate

Agonist binding was also studied by displacement of (–)-[3-³H]quinuclidinyl benzilate using non-

radioactive agonists. The experimental data have been corrected for binding site occupancy by the radioactive ligand (approx. 90%) and Table 4 reports the corrected agonist concentrations that would give 50% receptor occupancy in the absence of other ligands. There is good agreement in most cases between these data and those obtained for rat brain homogenates.

Whereas the data for pilocarpine (Fig. 2) fit well to a mass-action binding curve, those for carbamoylcholine (Fig. 3) give a much flatter curve. These differences have been assessed by measuring the agonist Hill coefficients (Table 2); there is a range of values from weak agonists such as pilocarpine (*h*0.95) to full agonists such as carbamoylcholine (*h*0.36) and there is fair agreement with values obtained for the rat brain receptor. The data for potent agonists (see Fig. 3) show more scatter than for weak agonists and antagonists (see Fig. 2). This is because weak agonist and antagonist displacement curves could be established in a single experiment, whereas two or more experiments were required to provide the greater number of data points for a flat displacement curve

Table 2. *Binding properties of muscarinic agonists*

Data for neuroblastoma-NIE-115 membrane fraction were obtained by displacement of (–)-[3-³H]quinuclidinyl benzilate (approx. 0.3 nM) using non-radioactive agonist as described in the Materials and Methods section. Data for rat brain synaptosomal fractions were from Birdsall & Hulme (1976).

Agonist	NIE-115 membrane fraction		Rat brain synaptosomal fractions	
	–log (Agonist concentration giving 50% binding)	<i>h</i>	–log (Agonist concentration giving 50% binding)	<i>h</i>
Oxotremorine-M	5.91	0.44	5.80	0.25
Carbamoylcholine	4.92	0.36	4.82	0.33
Acetylcholine*	5.69	0.30	5.48	0.42
(+)-Acetyl-β-methylcholine*	6.07	0.38	5.10	0.52
Arecoline	5.53	0.65	5.30	0.75
Oxotremorine	6.01	0.73	6.33	0.80
Pilocarpine	5.32	0.95	5.22	0.95

* Assayed in the presence of 10 μM- eserine.

Table 3. *Agonist binding data fitted to the two-site model of Birdsall & Hulme (1976)*

For NIE-115 membrane, data from agonist displacement of (–)-[3-³H]quinuclidinyl benzilate (see Table 2) were fitted by a least-squares computer fit to the two-site model of Birdsall & Hulme (1976). Rat brain data are from N. J. M. Birdsall & E. C. Hulme (unpublished work).

Agonist	NIE-115 membrane		Rat brain synaptosomal fraction	
	–log <i>K</i>	Proportion of binding sites	–log <i>K</i>	Proportion of binding sites
Carbamoylcholine	4.3 ± 0.4	60 ± 9	4.1 ± 0.1	68 ± 5
	7.0 ± 0.6	40 ± 9	6.3 ± 0.1	32 ± 5
Oxotremorine-M	5.0 ± 0.4	55 ± 5	5.1 ± 0.1	68 ± 5
	7.6 ± 0.3	45 ± 5	7.6 ± 0.1	32 ± 5

(as given by potent agonists) and the scatter results from differences between separate experiments.

In an alternative method of analysis the data for two agonists has been fitted by computer to the model of Birdsall & Hulme (1976), which assumes two distinct binding sites with different affinities (see curve of Fig. 2 and Table 3). The agreement with similar data for the rat brain receptor is surprisingly good, both in terms of the proportion of binding sites and dissociation constants.

Discussion

These results show good agreement between the ligand-binding properties of the muscarinic receptor in NIE-115 neuroblastoma cells and in rat brain. Similar ligand-binding properties have been demonstrated for muscarinic receptors in smooth muscle strips (Young, 1974; Haigh & Young, 1975; Taylor *et al.*, 1975) and homogenates (Burgen & Hiley, 1974) and we have shown analogous behaviour for a muscarinic receptor in the electric ray *Torpedo marmorata* (M. R. Pickard & P. G. Strange, unpublished work) and frog brain (N. J. M. Birdsall & E. C. Hulme, unpublished work). The receptor is probably the same in all cases, but a definitive proof of identity must await receptor polypeptide isolation and characterization.

The similarity between the neuroblastoma receptor and that in other tissues is important, moreover, because it allows the neuroblastoma system to be used as a model for other muscarinic systems. As a probe of the receptor mechanism, correlations may then be sought between ligand-binding studies and ligand-induced cell responses, e.g. guanosine 3':5'-cyclic monophosphate changes (Matsuzawa & Nirenberg, 1975; Richelson, 1977; Strange *et al.*, 1977) measured in one tissue. Further, the muscarinic receptor in other clones of the C1300 tumour (Amano *et al.*, 1972) and other cell lines derived from it, e.g. neuroblastoma × glioma hybrids (see, e.g., Hamprecht, 1976) is likely to be the same as the one described here in NIE-115 cells.

Specifically, these results show that antagonists bind to a single class of homogeneous binding sites, because their binding data conform to the Law of Mass Action and give h values close to 1 [cf. rat brain (Birdsall & Hulme, 1976)]. The binding data for agonists varies from that for a partial agonist such as pilocarpine which binds in a mass-action fashion to that for a full agonist like carbamoylcholine, which gives a very flat binding curve. Similar data for rat brain have been fitted to a model for the receptor of two distinct agonist binding sites of different affinities (Birdsall & Hulme, 1976). The data for the full agonists carbamoylcholine and oxotremorine-M here also fit quite well to this model, which may

therefore apply to the neuroblastoma receptor too. No functional significance has been given to the two binding sites, although they may be the result of differential receptor-effector coupling (Birdsall *et al.*, 1978). There is some evidence, however, that changing occupancy of the lower affinity site leads to changes in the activity of the enzyme guanylate cyclase (Strange *et al.*, 1977), the resultant guanosine 3':5'-cyclic monophosphate changes leading to a change in the phosphorylation state of a membrane protein (Greengard, 1976). The roles of the higher affinity binding site and possible changes in adenosine 3':5'-cyclic monophosphate are unclear, but it is hoped that studies on the neuroblastoma system can clarify this.

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