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Relative affinities of drugs acting at cholinoceptors in displacing agonist and antagonist radioligands: the NMS/Oxo-M ratio as an index of efficacy at cortical muscarinic receptors

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1 Radioligand binding assays using [^{3}H]-N-methylscopolamine (NMS) and [^{3}H]-oxotremorine M (Oxo-M) have been devised to predict the efficacy of test compounds at muscarinic receptors in rat cerebral cortex.

2 Muscarinic antagonists, including non-selective and both M_1- and M_2-selective compounds, displayed similar affinity for both binding assays.

3 Full agonists such as carbachol and muscarine possessed a ratio of potencies against the antagonist versus the agonist ligand (NMS/Oxo-M ratio) of >4000.

4 Compounds which have been shown previously to display partial agonist activity in functional assays e.g. pilocarpine and RS86 had intermediate NMS/Oxo-M ratios of 100–150. A second group of compounds which included oxotremorine had somewhat higher ratios (500–1400).

5 The ratio of affinity constants for the two assays predicted the ability of agonists to stimulate cortical phosphatidyl-inositol turnover.

6 These results suggest that the NMS/Oxo-M ratio may be a useful prediction of efficacy for novel compounds acting at cortical muscarinic receptors.

Introduction

Muscarinic receptor binding assays provide a rapid means of screening for the discovery of novel agents acting at cholinoceptors. It has always been a limitation of these techniques, however, that they provide information about receptor affinity, but not relative efficacy. Several means have been used to overcome this problem. In binding studies using antagonists as the radioligand, displacement curves with muscarinic antagonists have steep Hill coefficients ($N_H = 1.0$) whereas muscarinic agonists yield Hill coefficients significantly less than 1.0 (Birdsall *et al.*, 1978; Hulme *et al.*, 1978; Fisher *et al.*, 1983). Non-linear regression analysis of such curves indicate that muscarinic agonists show a significantly better fit to a two site rather than to a single site model, with high- and low-affinity binding components (Birdsall *et al.*, 1978; Fisher *et al.*, 1983; Evans *et al.*, 1985; McKinney *et al.*, 1985). Similarly the ability of guanine nucleotides to shift agonist displacement curves has been shown also to be related to agonist efficacy (Evans *et al.*, 1985).

All of these methods depend on the availability of detailed dose-response curves and accurate curve fitting procedures, which are very dependent on the assay conditions used and the linkage of receptors to guanine nucleotide binding proteins. Furthermore, whilst it is relatively easy to identify antagonists, partial agonists and full agonists it is often more difficult to distinguish differing degrees of partial agonism without detailed secondary biochemical tests e.g. phosphatidyl-inositol turnover or adenylate cyclase assays (Olianas *et al.*, 1983; Fisher *et al.*, 1984).

In the present study we describe a simple binding assay which predicts the efficacy of muscarinic compounds in rat cerebral cortex, using two well established radioligand binding assays. The muscarinic agonist [^{3}H]-oxotremorine-M (Oxo-M) was used to label the high affinity agonist state of the receptor, whereas [^{3}H]-N-methylscopolamine (NMS) was used

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to label both high affinity and low affinity states of the receptor.

The experimental conditions were chosen to maximise the difference between agonists and antagonists as displacing ligands, and the ratio of affinity constants for the two assays is a useful index of agonist efficacy.

Methods

Brain membrane preparation

Crude synaptosomal-mitochondrial membranes were prepared by homogenizing rat (250–300 g) cerebral cortices in 0.32 M ice-cold sucrose (1:10 w/v) in a motor-driven teflon/glass homogenizer at 500 r.p.m. (10 strokes). The homogenate was centrifuged at 1000 *g* for 15 min and the resulting supernatant centrifuged at 17,000 *g* for 20 min. This yielded the crude synaptosomal mitochondrial pellet (P_2), which was used fresh or stored at − 20°C before use.

[^{3}H]-N-methylscopolamine binding ([^{3}H]-NMS)

P_2 fractions were homogenized and resuspended at a final dilution of 1:600 (wet wt/v) in ice-cold Krebs HEPES buffer pH 7.4 (composition, mM: NaCl 118, KCl 4.7, $MgSO_4$ 1.2, $NaHCO_3$ 5, KH_2PO_4 1.2, $CaCl_2$ 2.5, glucose 11 and HEPES 20). Binding of [^{3}H]-NMS was determined using 0.01–1.0 nM of ligand and non-specific binding defined with 1 μM atropine. Displacing drugs were added in a volume of 10 μl to give a final assay volume of 1.0 ml. Incubations were initiated by adding 750 μl of membrane solution and were allowed to proceed for 60 min at 30°C. Assays were terminated by filtration using a Brandell Cell Harvester and Whatman GF/B filters. Samples were rinsed with 2 × 10 ml ice cold saline (0.9% NaCl, w/v). Filters were then placed in 10 ml of scintillation fluid (Hydrofluor, National Diagnostics, New Jersey) and radioactivity estimated using ligand scintillation spectrometry.

[^{3}H]-oxotremorine-M binding ([^{3}H]-Oxo-M)

P_2 fractions were washed by resuspending in 10 ml of 20 mM HEPES buffer pH 7.4 and centrifuged at 17,000 *g* for 15 min. The washed membranes were homogenized and resuspended at a final dilution of 1:100 (wet wt/v) in ice cold 20 mM HEPES buffer pH 7.4. Binding of [^{3}H]-Oxo-M was determined using 0.2–5 nM of ligand and non specific binding defined with 2 μM atropine. Displacing compounds were added in a volume of 10 μl to give a final assay volume of 1.0 ml. Incubations were initiated by adding 750 μl of membrane solution and were allowed to proceed for 40 min at 30°C. Assays were terminated by filtration over Whatman GF/C filters presoaked in 0.05% polyethyleneimine using a Brandell Cell Harvester. Samples were washed with 10 ml of ice cold saline and filters placed in 10 ml of scintillation fluid (Hydrofluor, National Diagnostics, New Jersey) and radioactivity estimated by liquid scintillation spectrometry.

Binding parameters were determined by non linear least squares regression analysis using RS1 (BBN Research Systems, Cambridge, Mass.) and a computerised iterative procedure written by Dr A. Richardson, NRC Terlings Park.

Inositol-phospholipid hydrolysis

Tissue slices of rat cerebral cortex (350 × 350 μm) were prepared with a McIlwain Tissue Chopper and were washed three times in Krebs-bicarbonate buffer, followed by a 30 min preincubation in the presence of [^{3}H]-myo-2-inositol, 2 μCi, (Amersham International, TRK.807 13.8 Ci mmol^{-1}) and 10 mM lithium. Tissue slices were subsequently incubated in the presence of muscarinic agonists for 45 min in a volume of 250 μl. The reaction was terminated by addition of 940 μl of chloroform/methanol (1:2) and water-soluble inositol monophosphates were isolated by ion exchange chromatography. The methods have previously been described in detail by Brown and colleagues (1984). For studies with muscarinic antagonists, tissue slices were incubated with antagonists for 30 min before addition of acetylcholine. Acetylcholine was used for these studies because it produces the most efficacious response of all standard muscarinic agonists (Freedman, 1986). This compound was not included in the binding studies because of the necessity of using a cholinesterase inhibitor, which itself interferred with the binding assays. All test compounds were added in a volume of 10 μl.

Materials

Compounds and reagents for these studies were obtained from the following sources: pirenzepine (The Boots Company PLC), AFDX-116 (11-[[2-(diethyl-amino) methyl] - 1 - piperidinyl]acetyl] - 5, 11- dihydro - 6H - pyrido [2,3-b] [1,4]benzodiazepine-6-one, Boehringer Ingelheim) RS86 (2-ethyl 8 methyl-2,8 diazaspiro-[4,5]decan-1,3-dion hydrobromide, Sandoz Ltd.), McN-A-343 ([4-*m*-chlorophenylcarbamoyloxy)-but-2-ynyl] trimethylammonium chloride, (McNeil Pharmaceuticals) oxotremorine-M (Dr Nigel Birdsall, MRC, Mill Hill) and AF-30 (2-methyl-spiro-(1,3-dioxolane-4,3-)-quinuclidine, Dr J. Saunders, MSD Terlings Park). Radioligands were purchased through New England Nuclear ([^{3}H]-N-methylscopolamine, NET-636, 85–90 Ci mmol^{-1}; [^{3}H]-oxotremorine-M, NET-671, 84.9 Ci mmol^{-1}).

Proteins were assayed by the method of Lowry *et al.* (1951) with bovine serum albumin as the standard.

Results

[^{3}H]-N-methylscopolamine binding to rat cerebral cortex membranes

The non-selective muscarinic antagonist [^{3}H]-NMS bound to rat cerebral cortex membranes in a saturable concentration-dependent manner. Scatchard analysis produced a dissociation constant K_D of 0.14 ± 0.02 nM and a maximum binding capacity of 1400 ± 340 fmol mg^{-1} protein ($n = 5$) in good agreement with previously published results (Hammer *et al.*, 1980). In the presence of 20 mM HEPES Krebs buffer the displacement of 0.1 nM [^{3}H]-NMS by various drugs acting on cholinoceptors showed appropriate pharmacology for a muscarinic receptor (see Table 1). The most potent displacers were atropine and N-methylscopolamine which had affinity constants in the low nanomolar range. Both compounds displayed Hill coefficients not significantly different from 1.0.

In rat cerebral cortex the displacement curves for pirenzepine yielded a corrected IC_{50} value of 72 nM and a Hill coefficient of 0.69 which was significantly different from 1.0 ($P < 0.01$). Upon detailed curve fitting, the data showed a significantly better fit for a two site model compared with a one site model (data not presented). The results are similar to those previously reported by Hammer and colleagues, (1980) and have been used to suggest the presence of both M_1- and M_2- receptors in rat cerebral cortex. In contrast to pirenzepine, the structurally similar molecule AFDX-116, which is claimed to be a cardioselective M_2- muscarinic antagonist (Hammer *et al.*, 1986; Giachetti *et al.*, 1986), was 8 fold weaker. This supports the idea that the majority of the cortical muscarinic receptors are of the M_1- subtype.

Muscarinic agonists were weaker than antagonists in displacing [^{3}H]-NMS, with oxotremorine being the most potent. Muscarine and carbachol were both very weak by comparison with the antagonists. Agonists displayed very flat displacement curves with Hill coefficients $\simeq 0.5$, as reported previously with a variety of antagonist ligands (Birdsall *et al.*, 1978). Partial muscarinic agonists such as pilocarpine, arecoline and the Sandoz muscarinic agonist RS86 (Palacios *et al.*, 1986) were somewhat more potent, and their displacement curves typically had steeper slopes (Hill coefficients approaching 1.0). These data are in good agreement with previously published data (Birdsall *et al.*, 1978; Hammer *et al.*, 1980).

These results suggest that both low and high affinity states of the muscarinic receptor are labelled under these conditions, whilst the relatively low affinity of carbachol and muscarine suggest a predominence of the L states.

Table 1 Displacement studies for muscarinic compounds against [^{3}H]-N-methylscopolamine binding to rat cerebral cortex membranes

Compound	K_{app} (μM)		N_H
N-methylscopolamine	0.00028	(0.00015–0.00074)	0.94 ± 0.04
Atropine	0.0010	(0.00092–0.0012)	1.00 ± 0.08
Pirenzepine	0.072	(0.064–0.087)	0.69 ± 0.05
AFDX-116	0.55	(0.51–0.60)	0.87 ± 0.05
Oxotremorine	0.83	(0.59–1.2)	0.79 ± 0.04
Oxotremorine-M	2.2	(2.0–2.4)	0.53 ± 0.02
Pilocarpine	4.0	(2.6–5.3)	0.93 ± 0.03
Methylfurmethide	4.2	(3.1–6.0)	0.72 ± 0.04
AF-30	4.9	(4.0–6.0)	0.90 ± 0.04
RS86	5.0	(4.3–5.4)	0.91 ± 0.05
Arecoline	6.2	(5.1–9.0)	0.76 ± 0.04
McN-A-343	6.9	(6.1–7.4)	0.87 ± 0.04
Muscarine	19	(19)	0.64
Carbachol	24	(23–26)	0.60 ± 0.05
Carbamyl-β-methyl choline	84	(84)	0.79 ± 0.03

Results are expressed as an apparent affinity constant (K_{app}) which has been corrected for ligand occupancy by use of the Cheng-Prusoff equation (1973). Each value is a geometric mean of at least three determinations performed on separate occasions. Each curve is typically 6–10 concentrations performed in triplicate. Numbers in parentheses indicate the range for each value. Inhibition studies were performed with 0.1 nM [^{3}H]-N-methylscopolamine.
N_H: Hill coefficient.

[^{3}H]-oxotremorine-M binding to rat cerebral cortex membrane

Since high ionic strength has been shown to reduce the SH and H affinity components of muscarinic agonist binding, (Hulme *et al.*, 1983; Watson *et al.*, 1986), experiments were performed in a low ionic strength buffer, 20 mM HEPES pH 7.4. Membrane preparations were also washed to reduce possible contamination by endogenous acetylcholine. The potent muscarinic agonist [^{3}H]-oxotremorine-M (Oxo-M) bound saturably to a high affinity binding site in the concentration range 0.1–5.0 nM. At higher concentrations a second component was evident. With the low ionic strength buffer conditions, Scatchard analysis indicated a dissociation constant K_D of 0.68 ± 0.12 nM and a maximum binding capacity of 520 ± 160 fmol mg^{-1} protein ($n = 4$). Although this appears to be a relatively high proportion of the total sites labelled by [^{3}H]-NMS, it should be noted that the different ionic conditions used in the two assays make direct comparison difficult.

The pharmacology of this site is summarised in Table 2. Muscarinic antagonists such as atropine showed high (nanomolar) affinity and again displayed Mass Action curves with Hill coefficients not significantly different from 1.0. In contrast another classical antagonist, N-methylscopolamine displayed Hill coefficients significantly greater than 1.0. This result may be due to the problems associated with depletion of a high affinity compound in a tissue with a large binding capacity. Pirenzepine and AFDX-116 displaced [^{3}H]-Oxo-M binding dose-dependently with pirenzepine showing 5 fold greater affinity. Pirenzepine showed a similar flat displacement curve ($N_H = 0.66$) to that seen with [^{3}H]-NMS, indicating that under these conditions [^{3}H]-Oxo-M was once again labelling both M_1- and M_2- receptors.

The major difference between the [^{3}H]-NMS binding assay and the [^{3}H]-Oxo-M assay was the relative high affinity of muscarinic agonists in displacing [^{3}H]-Oxo-M. The two most potent displacers of binding were oxotremorine and oxotremorine-M, both of which had nanomolar affinities. Agonists such as carbachol and muscarine also showed very high affinity for [^{3}H]-Oxo-M binding, showing an ability to recognise the super high affinity state of the receptor. In contrast to [^{3}H]-NMS binding, the Hill coefficients for these compounds were much closer to 1.0. Somewhat weaker in this assay were compounds which have been previously shown to be partial muscarinic agonists, including pilocarpine, McN-A-343 the selective ganglion agonist (Roszkowski, 1961) and RS86. Generally the Hill coefficients for these muscarinic agonists were also higher than seen for [^{3}H]-NMS binding.

NMS/Oxo-M ratio

When the ratios of the affinities of the various muscarinic compounds for the two binding assays was

Table 2 Displacement studies for muscarinic compounds against [^{3}H]-oxotremorine-M binding to rat cerebral cortex membranes

Compound	K_{app} (μM)		N_H
N-methylscopolamine	0.00022	(0.00012–0.00065)	1.31 ± 0.12
Atropine	0.00048	(0.00040–0.00064)	0.99 ± 0.07
Oxotremorine-M	0.00052	(0.00035–0.00070)	0.81 ± 0.10
Oxotremorine	0.00096	(0.00072–0.0015)	0.85 ± 0.06
Methylfurmethide	0.0030	(0.0018–0.0038)	0.79 ± 0.08
Muscarine	0.0048	(0.0048)	0.84
Carbachol	0.0058	(0.0048–0.0072)	0.92 ± 0.01
Arecoline	0.011	(0.0098–0.012)	1.07 ± 0.10
Pirenzepine	0.033	(0.025–0.045)	0.66 ± 0.06
AF-30	0.035	(0.033–0.040)	0.99 ± 0.05
RS86	0.040	(0.033–0.046)	0.90 ± 0.05
Pilocarpine	0.040	(0.034–0.043)	0.88 ± 0.07
McN-A-343	0.060	(0.054–0.067)	0.75 ± 0.03
Carbamyl-β-methyl choline	0.097	(0.095–0.099)	0.92 ± 0.00
AFDX-116	0.14	(0.11–0.19)	0.68 ± 0.05

Results are expressed as an apparent affinity constant (K_{app}) which has been corrected for ligand occupancy by use of the Cheng-Prusoff equation (1973). Each value is a geometric mean of at least three determinations performed on separate occasions. Each curve is typically 6–10 concentrations performed in triplicate. Numbers in parentheses indicate the range for each value. Inhibition studies were performed with 3 nM [^{3}H]-oxotremorine-M. N_H: Hill coefficient.

compared (Table 3) it is clear that a relationship with efficacy exists.

Full muscarinic agonists, such as muscarine and carbachol showed in excess of a 4000 fold difference in potency between the two assays, whereas muscarinic antagonists displayed similar affinities in both assays. In the examples examined this appeared to be true both for non-selective antagonists such as atropine and N-methylscopolamine and the selective antagonists such as pirenzepine and AFDX-116.

In contrast to the full agonists carbachol and muscarine, partial agonists exhibited less difference in potency between the two assays, with oxotremorine and methylfurmethide having NMS/Oxo-M ratios of 500–1400 and RS86, pilocarpine and McN-A-343 having ratios of approximately 100.

Relationship between the NMS/Oxo-M ratio and functional efficacy

Phosphatidyl-inositol turnover: rat cerebral cortex Muscarinic agonists have been shown previously to stimulate phosphatidyl-inositol turnover in slices of rat cerebral cortex (Brown *et al.*, 1984; Fisher & Bartus, 1985; Jacobson *et al.*, 1985). Because of the efficiency of coupling of muscarinic receptors in this tissue, compounds with low intrinsic activity behave as partial agonists.

When the NMS/Oxo-M ratio of the compounds was compared with their ability to stimulate inositol-phospholipid (PI) breakdown in rat cerebral cortex (see Table 4) an interesting relationship was evident. The larger the NMS/Oxo-M ratio, the greater the ability of a compound to stimulate [^{3}H]-inositol monophosphate accumulation. Thus muscarine and oxotremorine-M which had ratios of $\simeq$4000 were able to produce a maximal PI response similar to that seen with carbachol. In contrast, arecoline, carbamyl-β-methyl choline and methyl furmethide which had significantly lower ratios (560–1400) were partial agonists producing a maximal response which was between 18–67% of that seen for carbachol (1 mM). A second group of compounds which had lower NMS/Oxo-M ratios of 100–140 e.g. pilocarpine, McN-A-343 and RS86, were very weak partial agonists producing a maximal response of 6–11%. A group of classical muscarinic antagonists, including atropine and N-methylscopolamine, which had NMS/Oxo-M ratios of close to unity were potent antagonists of the acetylcholine (100 μM)-induced PI response in the cortex with IC_{50} values of 16 nM and 3 nM respectively.

Discussion

The effects of an agonist on a tissue is a result of several interacting phenomenon, only one of which is the receptor affinity. The response produced by an agonist is also related to its intrinsic activity, the number of receptors, and the efficiency of the coupling of the

Table 3 NMS/Oxo-M ratio in rat cerebral cortex

Compound	K_{app} (pM) *[^{3}H]-NMS*	*[^{3}H]-Oxo-M*	*NMS/Oxo-M ratio*
Atropine	0.0010	0.00048	2.1
N-methylscopolamine	0.00028	0.00022	1.3
Pirenzepine	0.072	0.033	2.2
AFDX-116	0.55	0.14	3.9
McN-A-343	6.9	0.060	120
Pilocarpine	4.0	0.040	100
RS86	5.0	0.040	130
AF-30	4.9	0.035	140
Arecoline	6.2	0.011	560
Oxotremorine	0.83	0.00096	860
Carbamyl-β-methyl choline	84	0.097	870
Methylfurmethide	4.2	0.0030	1400
Oxotremorine-M	2.2	0.00052	4200
Carbachol	24	0.0058	4100
Muscarine	19	0.0048	4000

The NMS/Oxo-M ratio is the ratio of the two apparent affinity constants which were derived from Tables 1 and 2.

Table 4 Relationship between NMS/Oxo-M ratio and cortical phosphatidyl-inositol turnover

Compound	NMS/Oxo-M ratio	*Phosphatidyl-inositol turnover rat cerebral cortex* EC_{50} (μM)	% max
N-methylscopolamine	1.3	(IC_{50} 0.0047)	0
Atropine	2.1	(IC_{50} 0.016)	0
Pirenzepine	2.2	(IC_{50} 0.170)	0
AFDX-116	3.9	(IC_{50} 2.3)	0
Pilocarpine	100	5.9	11 ± 1
McN-A-343	120	380	11 ± 1
RS86	130	*	6 ± 1
AF-30	140	12	9 ± 2
Arecoline	560	16	18 ± 2
Oxotremorine	860	*	11
Carbamyl-β-methyl choline	870	230	29 ± 3
Methylfurmethide	1400	27	67 ± 7
Muscarine	4200	36	87 ± 5
Carbachol	4100	170	120 ± 5
Oxotremorine-M	4000	9.8	110 ± 8

EC_{50}: potency of compounds in eliciting breakdown of labelled inositol-phospholipids.
% max: results have been expressed as a % of the maximum response to 1 mM carbachol included in all experiments. The maximum response was equivalent to approximately a ten fold increase over unstimulated controls.
*IC_{50} could not be accurately determined because of low efficacy.
Each experiment value is calculated from between 2–5 independent determinations. Antagonist studies were performed against acetylcholine (100 μM) in the presence of 10 μM physostigmine (see Methods). Curves were fitted to data and analysed by non-linear regression using the Allfit, four parameter logistic curve fitting programme (Delean *et al.*, 1978). The EC_{50} was calculated from the individual maximum response of each compound.

receptors to the stimulus-response mechanism (Kenakin, 1986). The latter effects can be very important since even in the absence of a true receptor selectivity, they can result in compounds showing tissue selectivity (Kenakin, 1986).

The present study has identified a simple method of using the results of previously established binding assays to predict the relative intrinsic activity of various muscarinic compounds. The method is based upon the ability of muscarinic agonists to distinguish between the superhigh (SH), high (H) and low (L) affinity states of the muscarinic receptor, a characteristic which has been known for many years (Birdsall *et al.*, 1978; 1980). This property has been used in several recent studies in which the authors have used detailed non-linear regression analysis to fit dose-response curves for muscarinic agonists to 2 or 3 site models. Fisher and colleagues (1983) distinguished between efficacious and weakly efficacious compounds on phospholipid turnover in brains on the basis of their ability to deviate from mass action binding curves using the radiolabelled antagonist [^{3}H]-quinuclidinyl benzilate. Similar findings were also more recently reported in a murine neuroblastoma clone NIE-115 (McKinney *et al.*, 1985). Ehlert (1985) described the relationship between inhibition of rabbit myocardial adenylate cyclase and the influence of GTP on the binding profiles of muscarinic agonists and showed a significant correlation. Most recently Evans *et al.* (1985) extended these ideas and showed that the relative efficacies of six muscarinic agonists for stimulating phosphatidyl inositol breakdown and calcium mobilisation were significantly correlated with differences in affinities of the low and high affinity states and the extent of the receptor in the H form for each agonist.

The present study appears to do this in a more detailed manner by directly measuring the SH site by labelling it selectively with [^{3}H]-Oxo-M. Some workers under very specific conditions have claimed that [^{3}H]-Oxo-M is selective for M_2- receptors (Potter *et al.*, 1984). However, under the conditions used in this study there are several reasons for thinking that oxotremorine-M labels both M_1- and M_2- receptors. The relative affinities of the selective antagonists pirenzepine and AFDX-116 against [^{3}H]-Oxo-M were similar to those observed against the non-selective antagonist [^{3}H]-NMS. Secondly the low Hill coef-

ficient observed with pirenzepine against both ligands suggests that both M_1- and M_2- receptor types are involved. It is evident from many studies that membrane preparations and ionic conditions are crucial to the extent to which receptors are coupled to guanine nucleotide regulatory proteins (Hulme *et al.*, 1983; Potter *et al.*, 1984). Thus, the findings in this study relate specifically to the conditions that have been used, in particular washes with the chelating agent EGTA have not been used to uncouple muscarinic receptors.

[^{3}H]-N-methylscopolamine has been used extensively to label muscarinic receptors in cerebral cortex and under the conditions used will label the SH, H and L sites as described by Birdsall and colleagues (1978). It is perhaps fortuitous that in the rat cerebral cortex, only a small proportion of the agonist sites are in the SH affinity form under the conditions of the [^{3}H]-NMS binding assay (Birdsall *et al.*, 1980). If this approach were to be used in other tissues, for example the heart, where the opposite is true, then the use of GTP to convert the majority of the sites to L would be required to provide comparable information.

This method has a number of advantages over those previously described for predicting efficacy, namely guanine nucleotide shifts and detailed curve fitting. Firstly the simplicity by which the measurements are made. For example in assessing novel muscarinic agonists, four widely spaced concentrations would enable IC_{50} values to be estimated relatively easily, whereas in contrast, detailed curve fitting procedures would require 20 point displacement curves. Indeed in a previous study, Birdsall and colleagues (1980) determined the ratio of affinity constants using 11 muscarinic agonists by this mechanism. Secondly this procedure can be used in tissues such as the cerebral cortex where GTP shifts cannot easily be demonstrated. Finally it is clear that this assay is very sensitive for agonists of low intrinsic activity e.g. pilocarpine, McN-A-343 and RS86 where ratios of over 100 were demonstrated. These are somewhat higher than values reported previously in studies where detailed curve fitting has been used to measure SH, H and low affinity states e.g. Birdsall *et al.* 1980. The main difference between the two studies is that in this particular protocol we have optimised the binding conditions to produce larger ratios, and therefore have used different conditions for the two assays. These features make the present assay ideal for identifying the degree of intrinsic activity possessed by a range of partial agonists, for example the clear distinction between pilocarpine and arecoline both in terms of NMS/Oxo-M ratio and ability to stimulate phosphatidyl-inositol turnover in rat cerebral cortex slices.

This study has clearly demonstrated that McN-A-343 and RS86 are muscarinic agonists with low intrinsic activity. The argument that McN-A-343 is a compound with low efficacy has been recently used by Eglen & Whiting (1986) to outline some of the problems associated with muscarinic receptor classification. Kenakin (1986) has described how such compounds can achieve functional selectivity without true receptor selectivity. In a tissue which possesses an efficiently coupled stimulus-response mechanism such compounds would behave as full agonists, whereas in other tissues, such as cerebral cortex in which the coupling to phosphatidyl-inositol turnover is less efficient or which possess a smaller receptor reserve, the lower efficacy of these compounds may prove insufficient to produce an agonist response.

It is now believed that muscarinic receptors can exist in at least three different forms (Waelbroeck *et al.*, 1986) and it is clear that in a tissue where more than one of these forms exist the relative selectivity of compounds for the high/low affinity states of each receptor may complicate the interpretation of results. Although some workers have suggested that phosphatidyl-inositol turnover is exclusively limited to M_1-receptors, it is more generally accepted that both M_1- and M_2-receptors in the cerebral cortex may be coupled (Lazereno *et al.*, 1985). When the correlation between the NMS/Oxo-M ratio and phosphatidyl-inositol is represented graphically (Figure 1) the relationship can be clearly seen. A threshold value is required for an agonist response and an increased ratio

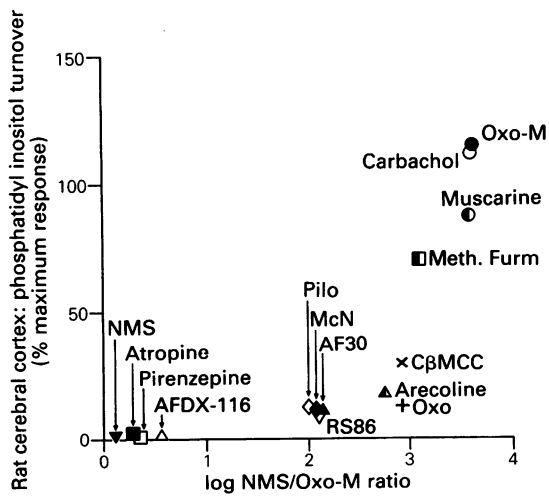

Figure 1 Relationship between the maximum response of cortical phosphatidyl-inositol turnover and the NMS/Oxo-M ratio. Data were derived from Table 4. NMS: N-methylscopolamine; Pilo: pilocarpine; McN: McN-A-343; Oxo: oxotremorine, CβMCC: carbamyl-β-methyl choline chloride, Meth Furm: methylfurmethide; Oxo-M: oxotremorine-M.

produces an increasing maximal effect. Both the threshold for the agonist activity and the ratio required for maximal stimulation will be dependent on the receptor reserve in a particular tissue. It is likely that any exceptions to this may indicate selectivity and to this effect it appears that the results for oxotremorine may be slightly anomalous in comparison to other agonists of similar ratio e.g. methylfurmethide. It is interesting that two groups have suggested the latter compound may exhibit some M_1-selectivity (Newberry 1986; Birdsall & Hulme 1986). However, other selective compounds appear to be less anomalous, for example, the active isomer of AF-30[3(**R**),2'(**S**)] (Saunders *et al.*, 1987) which was claimed by Fisher and colleagues (1976) to be M_1-selective was very weak on the phosphatidyl-inositol response but had a correspondingly low NMS/Oxo-M ratio.

In summary, this method may provide an easy way of predicting the relative efficacy of muscarinic compounds and indeed could be adopted for use in a variety of other neurotransmitter systems where, for example, functional assays have yet to be established.

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