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The human astrocytoma cell line 1321 N1 contains M_2-glandular type muscarinic receptors linked to phosphoinositide turnover

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1 Muscarinic receptors present in the human astrocytoma cell line 1321 N1 were characterized in radioligand binding studies and in functional studies of carbachol-stimulated phosphatidylinositol (PI) turnover.

2 In radioligand binding studies the muscarinic receptor in intact cells could be labelled using [^{3}H]-N-methylscopolamine ([^{3}H]-NMS) but not by [^{3}H]-pirenzepine. In the intact cells these receptors displayed low pirenzepine affinity (pKi = 6.83) indicating that they were not of the M_1 subtype. Furthermore, the 1321 N1 muscarinic receptors displayed low affinity for the two M_2-cardiac selective ligands methoctramine (pKi = 5.82) and AF-DX 116 (pKi = 6.29). This pharmacology was consistent with the 1321 N1 cells containing a single population of muscarinic receptors that displayed a similar pharmacology to the M_2-receptor present in exocrine gland tissue.

3 The M_2-gland nature of the receptors was further indicated in the functional studies where antagonist affinities were determined from their ability to antagonize carbachol-stimulated PI turnover in 1321 N1 cells. pA_2 values for pirenzepine (7.31), methoctramine (6.10) and AF-DX 116 (6.52) were similar to those determined in the binding studies.

4 From these studies we conclude that 1321 N1 astrocytoma cells contain an M_2-gland muscarinic receptor which mediates muscarinic receptor-mediated stimulation of PI turnover in these cells.

Introduction

Muscarinic receptors mediate a diverse range of physiological actions including regulation of cardiac, exocrine gland, smooth muscle and CNS functions. Pharmacological studies have provided evidence that these events are not mediated through just one type of muscarinic receptor, rather they are mediated by at least two muscarinic receptor subtypes (Hammer *et al.*, 1980; Hammer & Giachetti, 1982). In addition to this heterogeneity of the muscarinic receptor it has also become clear that the subtypes of the muscarinic receptor can interact with multiple effector systems such as the adenylate cyclase and inositol phospholipid second messenger systems (Harden *et al.*, 1986) and may also interact with ion channels (Yatani *et al.*, 1987).

A question which has remained unresolved in recent years concerns whether a single receptor subtype couples to a single effector system or whether each muscarinic receptor subtype can interact with multiple effectors.

Hammer & Giachetti (1982) initially proposed that the M_1 and M_2 receptors identified in binding studies, and differentiated on the basis of their affinity for pirenzepine, interacted with discrete effector systems. In their scheme, the M_1 receptor was coupled positively to phosphatidyl inositol (PI) turnover and the M_2 receptor was coupled negatively to adenylate cyclase. Harden *et al.* (1986) have favoured such a system and have even proposed that muscarinic receptors be classified on the basis of their effector system (Hughes & Harden 1986; Hepler *et al.*, 1987).

One major problem with these studies has been the finding that in chick heart a muscarinic receptor with high pirenzepine affinity and therefore an M_1 receptor under the classification scheme of Hammer & Giachetti (1982) is coupled to adenylate cyclase (Brown *et al.*, 1985) while in the human astrocytoma

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cell line 1321 N1 a muscarinic receptor with low affinity for pirenzepine and therefore an M_2 receptor by definition (Hammer & Giachetti, 1982) is coupled to PI turnover (Evans *et al.*, 1984; Brown *et al.*, 1985). On the basis of these pharmacological studies it would appear that the M_1 and M_2 muscarinic receptors can couple to either the PI pathway or to the adenylate cyclase system.

In recent years it has become evident that muscarinic receptors can no longer be classified as belonging to only two subtypes as originally proposed by Hammer & Giachetti (1982). Indeed, considerable evidence from radioligand binding studies has accumulated to indicate that at least three muscarinic receptor subtypes exist (Waelbrook *et al.*, 1986; De Jonge *et al.*, 1986). These have been termed M_1, M_2 and M_3 (De Jonge *et al.*, 1986) and correspond, respectively, to the M_1, M_2-cardiac and the M_2-glandular receptors. The M_1 receptor displays high pirenzepine affinity while M_2-cardiac and M_2-gland receptors display low affinity for pirenzepine. The M_2-cardiac and M_2-gland receptors can be differentiated on the basis of their affinity for AF-DX 116 (Hammer *et al.*, 1986), methoctramine (Michel & Whiting, 1988) and himbacine (Delmendo *et al.*, 1988).

With these recent findings in mind we have undertaken studies to reevaluate the receptor identity of the muscarinic receptors found in the human astrocytoma cell line 1321 N1. The data obtained indicate that the muscarinic receptor subtype present in these cells is of the M_2-gland subtype.

Methods

Cell culture

Human astrocytoma cells (1321 N1) were provided by Dr J.H. Brown, San Diego, U.S.A. Cells were cultured at 37°C in a humidified atmosphere (5% CO_2) as a monolayer culture using a low glucose DMEM/5% foetal calf serum medium. Cells were seeded at a density of 4×10^4 cells ml^{-1} and grown until confluent (usually 5–7 days). After 3 days the medium was replaced with fresh medium. Cells were harvested by incubating with trypsin (0.025%) for 4 min at 37°C. When cells were grown in 150 cm^2 flasks the yield of cells was in the order of 20–30 $\times 10^6$ cells per flask which provided enough cells for between 10–30 assay tubes. In some studies cells were harvested with a cell scrapper. In these studies cell viability was only 65% compared to >95% when using trypsin. In two experiments using the non-trypsin treated cells the binding parameters for pirenzepine, methoctramine and AF-DX 116 were not significantly different from those obtained with cells that were treated with trypsin.

Membrane preparations

Membrane preparation was performed as described previously (Michel & Whiting, 1987; Kunysz *et al.*, 1988). Briefly EDTA washed (Cheung *et al.*, 1982) cerebrocortical, cardiac and submaxillary gland membranes were prepared from 200–300 g male Sprague-Dawley rats. For some experiments 1321 N1 cells, harvested as described above, were also used. Tissues were homogenized in 50 mM Tris, 5 mM Na_2EDTA buffer (pH 7.4 at 4°C) with a polytron P10 tissue disrupter (setting 10; 2 × 10 s bursts). The homogenate was centrifuged at 48,000 *g* for 15 min. The pellet obtained was washed, by resuspension and centrifugation, once in homogenizing buffer and twice in 50 mM Tris, 0.5 mM EDTA buffer (pH 7.4 at 4°C). Membranes were stored under liquid nitrogen until required.

Binding assays

Binding assays were conducted as described previously (Michel & Whiting, 1987) with minor modifications. In all studies a Tris-Krebs assay buffer of the following composition was used (mM): NaCl 144, KCl 4.7, KH_2PO_4 1.7, $CaCl_2:(H_2O)_6$ 2.5, $MgCl_2$ 1.1, glucose 10, Tris 10, pH 7.4 at 37°C. Assays were conducted at 37°C in a final volume of 1 ml at pH 7.4. In all studies atropine (1 μM) was used to define non-specific radioligand binding (NSB). In competition studies the radioligand [^{3}H]-N-methylscopolamine ([^{3}H]-NMS) was present at a fixed concentration of 0.1 nM while in saturation studies the concentration of [^{3}H]-NMS was varied between 0.02 and 4 nM. In competition experiments with [^{3}H]-pirenzepine, a fixed concentration of 0.5 nM was employed while in saturation studies the concentration of [^{3}H]-pirenzepine was varied between 0.05 and 80 nM.

Incubations were for 2 h at 37°C and were terminated by vacuum filtration over Whatman GF/B glass fibre filters using a Brandel 48 well cell harvester. After filtration the filters were washed with 15 ml of room temperature water in those studies using [^{3}H]-NMS and with ice cold water in studies where [^{3}H]-pirenzepine was used. The filters were pretreated with 0.1% polyethyleneimine 18 h before use in order to reduce filter binding of the radioligands. Radioactivity retained on the filters was determined by liquid scintillation counting.

Studies on inositol phosphate turnover

In these studies 1321 N1 cells were cultured in 12 well Costar plates. When the cells became confluent,

[^{3}H]-myo-inositol (1 μCi per well) was added to the medium and the cells were incubated for 18 h. After removing the medium the cells were washed once with 1 ml of oxygenated modified Krebs-Bicarbonate buffer of the following composition (mM): NaCl 118, $NaHCO_3$ 25, KCl 4.7, KH_2PO_4 1.2, $CaCl_2$: $(H_2O)_6$ 1.25, $MgCl_2$ 1.1, glucose 11. The cells were then incubated at 37°C for 30 min in 0.90 ml of the modified Krebs-bicarbonate supplemented with LiCl (5 mM) and the antagonist drugs when studied. After this pre-equilibration period, carbachol (100 μl) was added to the cells and allowed to act for a further 30 min at 37°C before terminating the reaction by aspirating the buffer and replacing with 1 ml of chloroform/methanol (1:2, vol:vol). After several minutes, the chloroform/methanol was transferred to a scintillation vial. To effect extraction of the inositol phosphates (IP) the wells were sequentially washed with 1 ml of water, 2 ml of chloroform/methanol (1:2, vol:vol), 1 ml of chloroform and 1 ml of water. The combined washings were shaken for 20 min before determination of total IP by a procedure based upon that of Minneman & Johnson (1984). Briefly, 2 ml of the aqueous layer of the cell washings were applied to Dowex columns (200–400 mesh in the formate form). Following two 10 ml washes with 5 mM myo-inositol, the inositol phosphates were eluted with 2 ml of 1 M ammonium formate in 0.1 N formic acid; 1.8 ml of this eluate was transferred to a scintillation vial containing 10 ml aquasol and counted.

Data analysis

All data were analysed by iterative curve fitting techniques. Saturation binding data were analysed with LIGAND (Munson & Rodbard, 1980) while competition binding data were analysed by a curve fitting programme (Michel & Whiting, 1984) based on the method of Parker & Waud (1971). In the latter case IC_{50} values were converted to K_i values using the Cheng-Prusoff approximation (1973). In the functional studies carbachol concentration-response curves (CRC) were analysed by iterative curve fitting techniques (based on the method of Parker & Waud, 1971) in order to determine agonist ED_{50} values and to determine the slope and maxima of the carbachol CRC. pA_2 values were calculated according to the method of Arunlakshana & Schild (1959). The pA_2 value and the slope of the Arunlakshana & Schild (AS) plot were calculated by linear regression analysis.

Materials

[^{3}H]-NMS (specific activity 72 Ci mmol^{-1}) and [^{3}H]-Pir (specific activity 87 Ci mmol^{-1}) were obtained from Amersham and New England Nuclear, respectively. [^{3}H]-myo-inositol (specific activity 15 Ci mmol^{-1}) was obtained from American Radiolabeled Chemicals Inc. Pirenzepine hydrochloride was obtained from Boehringer Ingelheim. Atropine sulphate was purchased from Sigma Chemical Company as were all chemicals and reagents used. 4-DAMP (4-diphenylacetoxy-N-methylpiperidine methiodide) was obtained from Research Biochemical Inc. AF-DX 116 (11-[[2-(diethylamino)-methyl]-1-piperidinyl]-acetyl-5,11-dihydro-6H-pyrido[2,3-b]-[1,4]benzodiazepine-6-one), CPPS (cyclohexylphenyl(2-piperidinoethyl)silanol), and methoctramine (N,N′-bis[6-[(2-methoxybenzyl)amino]-hexyl]-1,8-octanediamine tetrahydrochloride) were synthesized by J. Berger, Dr R. Clark and D. Repke (IOC, Syntex, Palo Alto). Himbacine was obtained from Dr Taylor, University of Sydney, Australia.

Results

Intact cell binding assays

[^{3}H]-NMS bound to intact 1321 N1 cells in a saturable manner and with high affinity (see below). Measurable levels of binding could be achieved using as little as 0.5×10^6 cells per assay tube, although in routine studies $1–2 \times 10^6$ cells were added to each assay tube.

In contrast to studies with [^{3}H]-NMS, it was not possible to detect any binding of [^{3}H]-pirenzepine to 1321 N1 cells using our filtration assay even when concentrations of 10 nM [^{3}H]-pirenzepine were used.

Binding of [^{3}H]-NMS to 1321 N1 cells was rapid and fully reversible at 37°C (data not shown). In two experiments equilibrium was attained within 30 min and was maintained for up to 4 h when using a concentration of 0.2 nM [^{3}H]-NMS. The half life for dissociation of [^{3}H]-NMS was 10 min in two experiments.

Saturation studies

Equilibrium saturation binding of [^{3}H]-NMS indicated that this ligand bound to an apparently homogeneous population of sites in the 1321 N1 cells. The K_d value was 0.21 ± 0.04 nM while the B_{max} was 23 fmol mg^{-1} protein (Table 1). The K_d for [^{3}H]-NMS in the 1321 N1 cells was marginally lower than in the submaxillary gland and cardiac membranes. In Table 1 saturation data obtained using rat membrane preparations are shown. As can be seen, [^{3}H]-NMS identified apparently homogeneous populations of sites in cardiac and submaxillary gland membranes while [^{3}H]-pirenzepine appeared to

Table 1 Binding parameters for muscarinic radioligands in the intact 1321 N1 cells and in rat membrane preparations

Preparation	K_d (nM)	B_{max} (fmol mg^{-1} protein)
1321 N1 cells	0.14 ± 0.014	23 ± 2
Cardiac membranes	0.47 ± 0.031	495 ± 21
Submaxillary gland membranes	0.26 ± 0.029	340 ± 48

In this table the radioligand used was [^{3}H]-N-methylscopolamine ([^{3}H]-NMS). In rat cerebral cortex membranes [^{3}H]-pirenzepine displayed a K_d of 14.8 ± 1.4 nM and a B_{max} of 1690 ± 230 fmol mg^{-1} protein.

Values shown are the best fit parameters obtained after fitting specific and total binding data either to models describing the binding of the radioligand to one or more specific binding sites or to a model which assumed binding in a cooperative manner. In addition, specific binding data were fitted to a model which assumed the presence of single or multiple populations of radioligand binding sites together with a non-specific component of binding. In all cases the data could only be described in terms of an interaction of the radioligand with homogeneous populations of sites in the respective preparations. Data are those obtained from fitting specific binding data. In each preparation the data are from 4–7 experiments. Values are the mean ± standard error of the mean.

identify a single population of muscarinic receptor binding sites in cerebrocortical membranes.

Competition studies

The affinity estimates for a series of compounds in inhibiting [^{3}H]-NMS binding in the intact 1321 N1 cells are shown in Table 2. In this Table, affinity estimates of the compounds for the three subtypes of muscarinic receptor identified in rat membranes are also presented for comparative purposes. In the membrane preparations the M_1 receptor of cerebral cortex was labelled with [^{3}H]-pirenzepine while the M_2 receptors present in cardiac and submaxillary gland membranes were labelled with [^{3}H]-NMS.

The pharmacology of the three subtypes of muscarinic receptor identified in the membrane preparations can be seen in Table 2. Methoctramine and himbacine were 100 and 10 fold, respectively, selective for the M_2-cardiac over the M_2-gland muscarinic receptor. Pirenzepine was nearly 100 fold more potent at the M_1 than at the M_2-cardiac receptor. The pharmacological differences between the M_1 and M_2-gland receptors were not as dramatic. Pirenzepine and methoctramine were both approximately 10 fold more potent at M_1 than at M_2-gland muscarinic receptors.

In the 1321 N1 cells, all of the compounds studied produced mass action displacement isotherms with Hill coefficients close to unity (Table 2). Given that these compounds displayed up to a 100 fold selectivity between muscarinic receptor subtypes in direct binding studies (Table 2) this would indicate that only one population of muscarinic receptors was being labelled by [^{3}H]-NMS in the 1321 N1 cells.

The pharmacological profile of the muscarinic receptors in the 1321 N1 astrocytoma cells was clearly dissimilar to that of the M_1 and M_2-cardiac muscarinic receptors depicted in Table 2 but showed striking similarities to the M_2-gland receptor. Thus, both methoctramine and pirenzepine, which could differentiate M_1 and M_2-cardiac muscarinic receptors, displayed approximately 10 fold lower potency for muscarinic receptors of 1321 N1 cells than for M_1 muscarinic receptors in rat cerebral cortex. These data indicated that the muscarinic receptors in the 1321 N1 cells were unlikely to represent M_1 muscarinic receptors. Furthermore, the 1321 N1 muscarinic receptors were also dissimilar to the M_2-muscarinic receptor of cardiac membranes since both AF-DX 116 and himbacine were some 5 fold, and methoctramine some 100 fold less potent at the 1321 N1 muscarinic receptor than at the M_2-cardiac muscarinic receptor. In marked contrast, affinity estimates of the ligands at the M_2-gland muscarinic receptor and at the 1321 N1 muscarinic receptor were almost identical. In membrane preparations of the 1321 N1 cells the pKi values obtained for AF-DX 116 (6.22 ± 0.08; $n = 3$), atropine (8.92 ± 0.06; $n = 3$), himbacine (6.72 ± 0.09; $n = 3$), methoctramine (5.82 ± 0.11; $n = 3$) and pirenzepine (6.81 ± 0.05; $n = 3$) were not significantly different from those obtained in the intact cells.

Functional studies of carbachol-stimulated IP accumulation

In the 1321 N1 cells, carbachol stimulated the accumulation of IP 3–4 fold above basal levels. The ED_{50} for this effect was 8 μM. At the concentrations studied, the muscarinic antagonists indicated in Table 3 were able to inhibit this response in a competitive manner as adjudged by their failure to alter either the slope or maximum of the carbachol CRC and by the demonstration of linear Arunlakshana-Schild (AS) plots for these compounds. The pA_2 values for pirenzepine, methoctramine, AF-DX 116 and himbacine were in close agreement with the pKi values obtained in the radioligand binding studies in the intact cells (Table 2). The functional affinity data are clearly not consistent with the presence of an M_2-cardiac muscarinic receptor in the 1321 N1 cells. For AF-DX 116 and himbacine the pA_2 values were almost identical to their M_1 muscarinic receptor

Table 2 Affinity of competing compounds for radioligand binding sites in 1321 N1 intact cells and in rat membrane preparations

	Membrane preparations			
Ligand	*Cerebral cortex* pKi nH	*Cadiac* pKi nH	*Submaxillary gland* pKi nH	*1321 N1 cells* pKi nH
AF-DX 116	6.55 (0.11) 1.05 (0.06)	7.10 (0.08) 0.92 (0.07)	6.10 (0.11) 1.04 (0.08)	6.29 (0.03) 1.09 (0.08)
Atropine	9.02 (0.09) 0.93 (0.05)	8.67 (0.05) 1.03 (0.10)	8.97 (0.07) 0.93 (0.07)	9.01 (0.05) 0.97 (0.04)
CPPS	8.44 (0.07) 1.02 (0.04)	7.37 (0.08) 1.08 (0.06)	7.73 (0.06) 0.94 (0.07)	7.69 (0.06) 1.04 (0.02)
4-DAMP	8.49 (0.03) 1.06 (0.03)	7.96 (0.03) 0.94 (0.04)	8.68 (0.08) 0.95 (0.04)	8.59 (0.06) 1.00 (0.03)
Himbacine	7.12 (0.08) 0.89 (0.08)	7.72 (0.11) 0.99 (0.03)	6.91 (0.09) 0.92 (0.06)	6.83 (0.11) 1.11 (0.07)
Methoctramine	6.91 (0.07) 1.07 (0.07)	7.86 (0.09) 0.94 (0.08)	6.10 (0.09) 1.07 (0.07)	5.82 (0.06) 0.94 (0.08)
Pirenzepine	7.81 (0.09) 0.98 (0.06)	6.35 (0.02) 0.97 (0.04)	6.81 (0.06) 1.05 (0.07)	6.83 (0.08) 1.04 (0.05)

In all studies the radioligand was [^{3}H]-N-methylscopolamine ([^{3}H]-NMS) except for those using cerebrocortical membranes where the radioligand was [^{3}H]-pirenzepine.

All affinity values are expressed as the negative logarithm of the inhibitory affinity constant (K_i) and are in mol litre^{-1}. The Hill coefficient (nH) values shown were not significantly different ($P > 0.05$) from unity. Parameters shown represent the mean (±s.e.mean) from 4–7 experiments.

AF-DX 116 = 11-[[2-(diethylamino)-methyl]-1-piperidinyl]-acetyl-5,11-dihydro-6H-pyrido[2,3-6][1,4] benzodiazepine-6-one; CPPS = cyclohexylphenyl(2-piperidinoethyl)silanol; 4-DAMP = 4-diphenylacetoxy-N- methylpiperidine methiodide.

affinity and marginally (0.2 to 0.4 log units) different from their M_2-gland muscarinic receptor affinity. For pirenzepine, the pA_2 value was 0.5 log unit different from both the M_1 and M_2-gland muscarinic receptor affinity estimates obtained in binding studies. For methoctramine the pA_2 value was identical to the M_2-gland affinity.

Discussion

Previous studies in membrane preparations have not defined the muscarinic receptor subtype present in the 1321 N1 cells. It has been shown that pirenzepine displays similar affinity for both the 1321 N1 receptors and the high affinity pirenzepine binding site (presumably M_1 muscarinic receptor) found in cerebrocortical preparations (Evans *et al.*, 1984). However, in the intact cell the potency of pirenzepine appears to decrease and in both binding and functional studies the muscarinic receptors of 1321 N1 cells display low affinity for pirenzepine (Evans *et al.*, 1984; Brown *et al.*, 1985).

In the present study we used the cardiac M_2 muscarinic receptor subtype selective ligands AF-DX 116 (Hammer *et al.*, 1986), methoctramine (Melchiorre *et al.*, 1987; Michel & Whiting, 1988)

Table 3 pA_2 values for muscarinic antagonists in 1321 N1 cells

Compound	pA_2	*As slope*	*Concentrations tested* (μM)
AF-DX 116	6.52 ± 0.04	1.02 ± 0.04	1, 3, 10, 30
Himbacine	7.03 ± 0.07	1.15 ± 0.08	0.1, 0.3, 1, 3
Methoctramine	6.10 ± 0.06	0.95 ± 0.06	1, 3, 10, 30
Pirenzepine	7.31 ± 0.06	0.93 ± 0.05	0.1, 0.3, 1, 3

The parameters shown represent the mean (±s.e.mean) and were obtained by linear regression analysis of between 8 and 14 data points obtained using the concentrations of antagonist indicated. The AS slopes shown are not significantly different from unity ($P > 0.05$).

and himbacine (Gilani *et al.*, 1986) to characterize the muscarinic receptor of the 1321 N1 cells. The binding results with these ligands confirmed previous findings (Brown *et al.*, 1985) that the receptor does not appear to represent an M_1 receptor and indicated that the receptor was probably of the M_2-gland subtype. Thus, the receptor displayed a low affinity for the M_1 selective ligand pirenzepine (Hammer *et al.*, 1980) and also displayed very low affinity for methoctramine which can also differentiate between M_1 and M_2-cardiac receptors. These findings excluded the possibility that the receptors were of the M_1 subtype.

Methoctramine and himbacine are M_2-cardiac selective ligands that display a 10 and 100 fold, higher affinity at the M_2-cardiac than at the M_2-gland receptor (Delmendo *et al.*, 1989). The data obtained with these ligands indicated that the receptors were not of the M_2-cardiac subtype and therefore were probably of the M_2-gland subtype. This was also suggested by the close similarity of affinity estimates at the M_2-gland receptor present in submaxillary gland membranes and the muscarinic receptor of the intact 1321 N1 cells.

The data obtained in the functional studies provided further evidence that the muscarinic receptors present in the 1321 N1 cells were of the M_2-gland subtype. These studies provided definitive evidence that the receptor was not of the M_2-cardiac subtype since pA_2 values for all four ligands differed 10 fold from that expected of such a receptor.

It was, however, not easy to differentiate between the possibility that the receptor was an M_1 or an M_2-gland receptor. Thus, both himbacine and AF-DX 116 produced pA_2 values consistent with an M_1 pharmacology although the values differed by 0.2 and 0.4 from that expected of an M_2-gland receptor. For pirenzepine the pA_2 value was intermediate between the M_1 and M_2-gland values obtained in membranes although it was at least 10 fold lower than the affinity of pirenzepine for the M_1-receptor in cerebral cortex that is coupled to IP accumulation (Kunysz *et al.*, 1988). The most convincing evidence for the receptor representing an M_2-gland receptor was obtained with methoctramine which displayed an identical pA_2 at the 1321 N1 cells and at the M_2-gland receptor.

With regard to the difficulty in differentiating between M_1 and M_2-gland receptors, several factors should be borne in mind. Firstly, we have previously noted (Delmendo *et al.*, 1989) that there are no ligands which provide definitive separation of the M_1 and M_2-gland receptors. At best, methoctramine and pirenzepine display a 5–8 fold lower affinity for the M_2-gland than for the M_1 muscarinic receptors while AF-DX 116 and himbacine display low selectivity (2–3 fold) between M_1 and M_2-gland receptors. Secondly for receptor subtypes to be differentiated Furchott (1972) has suggested that at least a 3 fold difference in affinity should be apparent. With this caveat in mind, then it is clear that AF-DX 116 and himbacine would not be expected to differentiate M_1 and M_2-gland receptors. Since the data obtained using the more selective agents methoctramine and pirenzepine were more consistent with the presence of an M_2-gland receptor we feel that it is more likely that the muscarinic receptors of 1321 N1 cells linked to IP accumulation belong to the M_2-gland subtype.

Such a finding, that the muscarinic receptors of the 1321 N1 cells are of the M_2-gland subtype, may explain the difficulty in characterizing this receptor in previous studies since pirenzepine, which has been used exclusively for characterizing this receptor, displays intermediate affinity for the M_2-gland subtype of the muscarinic receptor.

Additional more indirect evidence in favour of the M_2-gland receptor nature of the astrocytoma 1321 N1 muscarinic receptor comes in the recent study of Liang *et al.* (1987). In that study the muscarinic receptor of the 1321 N1 cell line was shown to possess an apparent size of 92,000 Da which differed substantially from that obtained for the M_1 and M_2-cardiac muscarinic receptors. The M_r of the 1321 N1 muscarinic receptor was however similar to that reported for muscarinic receptors in exocrine glands (Hootman *et al.*, 1984) which appear to possess predominantly M_2-gland muscarinic receptors (De Jonge *et al.*, 1986; Delmendo *et al.*, 1989). It is also worth noting that gene sequence studies have identified 4 muscarinic receptor genes one of which has been predicted to exhibit a M_r of 90,000 daltons when glycosylated (Kerlavage *et al.*, 1987). It would be interesting to determine the pharmacology of this receptor, termed the m_3 receptor, when it has been expressed and characterized in order to compare it with the M_2-gland receptor identified in the 1321 N1 cells and in exocrine glands.

Returning to the issue of muscarinic receptor subtypes and their interaction with second messenger systems, the results of the present study would indicate that the M_2-gland receptor present in the 1321 N1 cells can couple to IP turnover. Incidently, if this is correct then the present study represents the first quantitative *in vitro* functional characterization of the M_2-gland receptor. Given the similarities between the M_2-gland muscarinic receptor identified in direct binding studies and the ideal muscarinic receptor identified in functional studies, a comparison of the present functional data on the M_2-gland muscarinic receptor with that obtained in the guinea-pig ileum could enable a direct comparison between these receptors in order to determine if they are separate entities or both belong to the same subtype. Such studies are now in progress.

The demonstration that the M_2-gland receptor subtype couples to PI turnover may indicate that the suggestion by Harden *et al.* (1986) that muscarinic receptors can be characterized by their second messenger system is valid. Thus, there is now ample evidence that M_1 muscarinic receptors couple to PI turnover (Gill & Wolfe, 1985; Lazereno *et al.*, 1985; Fisher & Bartus, 1986; Kunysz *et al.*, 1988). The interaction of the M_2-cardiac muscarinic receptor with the adenylate cyclase system is well established (Flemming *et al.*, 1987) while the present study has indicated that M_2-gland muscarinic receptors can stimulate IP production. It should of course be stressed that a more indirect inference concerning the ability of M_2-gland muscarinic receptors to stimulate PI turnover can be made from the binding classification of exocrine glands as containing M_2-gland muscarinic receptors (De Jonge *et al.*, 1986) and the well-established finding that muscarinic receptor agonists stimulate IP accumulation in exocrine glands (Berrige *et al.*, 1983).

It is still clear that there are receptors with low pirenzepine affinity (presumed to be M_2) which stimulate IP accumulation (Lazereno *et al.*, 1985; Fisher & Bartus, 1986) as well as receptors with high affinity for pirenzepine (presumed to be M_1 receptors) which attenuate adenylate cyclase activity in chick atria (Brown *et al.*, 1985). Perhaps, given the dual problems of classifying receptors using pirenzepine and the demonstration that two muscarinic receptor subtypes can stimulate IP production, further studies are required to reevaluate the pharmacology of the atypical receptors indicated above, in order to clarify the nature of the interaction of muscarinic receptors with effector systems.

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