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The interaction of *para*fluorohexahydrosiladiphenidol at muscarinic receptors *in vitro*

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1 The antagonistic actions of *para*fluorohexahydrosiladiphenidol (pFHHSiD) at muscarinic receptors has been studied in cardiac muscle, smooth muscle and cell culture preparations. In this paper, the classification scheme of Doods *et al.* (1987) is employed. This scheme is based upon differential affinities of muscarinic antagonists. pFHHSiD exhibited high pA_2 values at M_3 receptors mediating contractions of guinea-pig ileum and oesophageal muscularis mucosae (7.8 and 8.2 respectively) whereas low values were determined at M_2 receptors mediating negative inotropic responses in guinea-pig atria (6.0). Intermediate pA_2 values were determined at M_1 receptors mediating contractions of the canine femoral and saphenous veins.

2 The pA_2 values of pFHHSiD at receptors mediating endothelial-dependent relaxation of rat aortic rings, rabbit jugular vein and canine femoral artery (7.6–7.9) were similar to those determined on the ileum. However, the pA_2 values of pFHHSiD at receptors mediating contractions of the guinea-pig trachea (7.1), which has been previously shown to possess M_3 receptors, were different from those determined in the ileum.

3 The similarity in pA_2 values of pFHHSiD between the M_3 receptors in guinea-pig ileum and the receptors mediating endothelial-dependent relaxations provide further evidence for the role of M_3 receptors in this vascular response. Taken together, pA_2 values for pFHHSiD range from 7.1 to 8.2, depending upon the M_3 preparation used. The selectivity of the compound therefore for the M_3 versus the M_2 muscarinic receptor ranged from 13 to 163 fold.

4 At muscarinic receptors mediating stimulation of phosphatidylinositol hydrolysis, pFHHSiD paradoxically displayed a high affinity for the M_1 receptor in the SH-SY5Y cell line ($pA_2 = 7.9$) as well as for the M_3 receptor in the human astrocytoma (1321 N1 cell line ($pA_2 = 7.6$). The value at the M_1 receptor in SH-SY5Y cells was greater than was observed at M_1 receptors mediating contractions of both the canine saphenous and femoral veins (7.1).

5 pFHHSiD, therefore, clearly delineated M_3 from M_2 muscarinic receptors, whilst the separation between M_1 and M_3 receptors was variable. The reason for the anomalous affinity estimates in some functional studies remains unclear. These data indicate that the pA_2 values for pFHHSiD appear to be tissue-dependent since the M_3 selectivity varies according to the preparations studied. As a result the utility of pFHHSiD in muscarinic receptor classification is limited.

Introduction

Genomic cloning studies (see Barnard, 1988 for review) have shown that heterogeneity of muscarinic receptors is due to the expression of a number of distinct, but related gene products (Peralta *et al.*, 1987; Bonner *et al.*, 1987). These have been denoted as m_1, m_2, m_3, m_4 and m_5 (Bonner *et al.*, 1987). Each gene product, on the basis of *in situ* mRNA hybridisation studies, exhibits a unique and specific distribution within the CNS (Buckley *et al.*, 1987) although equivalent studies in the periphery have yet to be reported. The nomenclature of these gene products differs between laboratories, i.e. the HM1, HM2, HM4 and HM3 human gene products identified by Peralta *et al.* (1987) correspond to the m_1, m_2, m_3 and m_4 genes identified by Bonner *et al.* (1987). In the present paper the nomenclature of Bonner *et al.* (1987) is used for purposes of clarity.

Muscarinic receptors have been divided on a pharmacological basis into three major subtypes, which have been denoted as M_1, M_2 and M_3 (Doods *et al.*, 1987; Hammer *et al.*, 1987) and the evidence for each subtype has been reviewed elsewhere (Eglen & Whiting, 1986; Mitchelson, 1988). The M_1 and M_2 subtypes most closely correspond to the m_1 and m_2 gene products (Peralta *et al.*, 1987), whilst the function of the m_3 and m_4 gene products remains unclear. Numa and his group, cited in a review by Barnard (1988), have suggested that the m_3 gene product most closely resembles the M_3 receptor. Buckley *et al.* (1989) have also shown that the radioligand binding profile of the m_3 gene product expressed in CHO-K1 cells was similar to M_3 receptors. Peralta *et al.* (1987) have shown that NG108-15 cells express m_4 receptor mRNA. In addition, Michel *et al.* (1989) have shown that the radioligand binding profile of the receptor present in NG108-15 and PC-12 cells was similar to that shown by Buckley *et al.* (1989) at m_4 receptors expressed in CHO-K1 cells.

M_1 receptors are most sensitive to the antagonists, pirenzepine and *o*-methoxy-silahexocyclium, whilst M_2 receptors are sensitive to methoctramine, AF-DX 116 and himbacine (Michel & Whiting, 1988; Melchiorre *et al.*, 1986; Eglen *et al.*, 1988; see Mitchelson, 1988 for review). Until recently, no antagonist has been available which exhibits a high affinity for the M_3 receptor only, and a low affinity for both M_1 and M_2 receptors. 4 Diphenyl acetoxy-N-methyl piperidine methiodide (4-DAMP; Barlow *et al.*, 1980), whilst differentiating M_3 receptors in smooth muscle from M_2 receptors in the atria, does not clearly distinguish M_1 receptors in the superior cervical ganglion from M_3 receptors in smooth muscle (Brown *et al.*, 1980). Similar conclusions may be drawn with regard to other putative M_3 antagonists such as silabenzhexol or hexahydrosiladiphenidol (see Mitchelson, 1988 for review). However, *para*fluorohexahydrosiladiphenidol (pFHHSiD) has recently been shown (Lambrecht *et al.*, 1988) to be a relatively selective M_3 receptor antagonist since it exhibits pA_2 values of 7.8, 6.7 and 6.1 at M_3, M_1 and M_2 receptors, respectively. The activity of pFHHSiD at expressed gene products has yet to be reported.

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We have undertaken a further analysis of this compound using functional techniques in a range of isolated tissues. In view of the relatively large M_3 vs M_2 receptor selectivity of the compound, in comparison to 4-DAMP, it has been used to characterize further a number of isolated tissues which have previously exhibited anomalous pA_2 values with respect to the current pharmacological classification (Doods et al., 1987; Hammer et al., 1987) i.e. the oesophageal muscularis mucosae, urinary bladder and the vascular endothelium.

A preliminary account of this work has been communicated to the British Pharmacological Society (Eglen et al., 1989).

Methods

The muscarinic receptor subtypes present in the preparations used in this paper are summarised in Table 1.

Isolated tissue studies

Guinea-pigs (male, Dunkin-Hartley, 300–350 g) and rats (male, Sprague-Dawley, 200–250 g) were killed by CO_2 asphyxiation. Guinea-pig proximal ileum, oesophageal muscularis mucosae (OMM) and urinary bladder were isolated and placed in Tyrode solution (pH 7.4, 34°C). Guinea-pig left atria, trachea and rat aortic rings (2 mm) were isolated and placed in Krebs physiological salt solution (pH 7.4, 37°C). Rings (2 mm) of canine saphenous vein, femoral vein and artery, rabbit (male, New Zealand white, 2.5–3 kg) jugular vein were isolated from animals previously anaesthetized with sodium pentobarbitone ($60\,mg\,kg^{-1}$, i.p.) and also placed in Krebs solution (pH 7.4, 37°C). The composition of the Tyrode and Krebs solution is given below.

The detailed methods for each preparation have been described previously i.e. ileum (Clague et al., 1985), OMM (Kamikawa et al., 1985), urinary bladder (Eglen & Whiting, 1987), left atria (Blinks, 1966) and trachea (Eglen & Whiting, 1988). Rings of femoral and saphenous veins, thoracic aorta or jugular vein were prepared according to the method of O'Rourke & VanHoutte (1987). The endothelium of the vascular rings was left intact, as judged by a relaxant response (to $1\,\mu M$ acetylcholine) of a ring precontracted with $0.1\,\mu M$ phenylephrine. Preliminary experiments showed that the canine femoral and saphenous veins exhibited only contractile responses to muscarinic agonists, irrespective of the presence or absence of an intact endothelium. The finding that removal of the endothelium in these tissues did not enhance the contracture to muscarinic agonists is in agreement with the report by Rubanyi & Vanhoutte (1988).

The smooth muscles and vascular rings were suspended under 1.0 g tension, whilst the left atria was suspended under 0.5 g tension. This latter tissue was electrically paced for the duration of the experiment, using parallel platinum electrodes (5 Hz, threshold voltage + 20%, 5 ms duration) after the method of Blinks (1966).

All preparations were allowed 60 min to equilibrate, during which time the bathing solution was replaced every 15 min. Concentration-response curves were then constructed, with carbachol as the agonist, in either a cumulative (OMM, urinary bladder, atria and vascular tissues; van Rossum 1963) or non-cumulative (ileum; 30 s exposure on a 5 min dose cycle; Clague et al., 1985) manner. Endothelial-dependent relaxations of the femoral artery, jugular vein and aorta were determined in tissues precontracted with phenylephrine ($0.1\,\mu M$) in all experiments except those using methoctramine. The α_1-adrenoceptor antagonist activity of this antagonist (Melchiorre et al., 1986) precluded the use of phenylephrine. U46619 ($0.1\,\mu M$) was used instead to raise the tension in the tissue (Dainty et al., 1988).

All responses were determined as changes in isometric tension (mg), measured with either a Grass FT03 force transducer and displayed on a Grass Polygraph or a Hugo Sachs K30 transducer connected to a Graphtec-Watanabe linearecorder (WR3101).

After construction of the initial concentration-response curve the tissues were allowed 60 min to equilibrate at each antagonist concentration, during which time the bathing solution was replaced every 15 min. A second concentration-response curve was then constructed in the presence of the antagonist. Each tissue was exposed to only one concentration of antagonist.

The concentration-ratios calculated at each of 6 concentrations (0.1, 1, 3, 10, 30 and $100\,\mu M$) of pFHHSiD were determined using EC_{50} (concentration of agonist which elicited 50% of the maximal response) values in the absence and presence of the antagonist. The EC_{50} values were calculated by fitting the data to a non-linear iterative curve fitting procedure (Parker & Waud, 1971) using RS1 software (BBN Software Products Corp., Cambridge, MA, U.S.A.). The dissociation constants of the antagonists were estimated by determination of the pA_2 value, according to the method of Arunlakshana & Schild (1959) by determining the intercept of the resulting straight line with the abscissae and these parameters. In order to obtain an accurate estimation of the dissociation constant, the Schild slopes were determined by linear regression using least squares analysis. When the slopes were not significantly different from unity, they were constrained to unity (Kenakin, 1987).

Phosphatidylinositol hydrolysis studies

Human astrocytoma cells (1321 N1) were cultured and phosphatidylinositol hydrolysis studies were conducted as described previously (Kunysz et al., 1989). Human SH-SY5Y cells were cultured at 37°C in a humidified atmosphere as a monolayer culture in a medium consisting of 45% Hams F12, 45% Dulbecco's modified Eagle's medium (DMEM) and 10% foetal calf serum. Cultures were passaged at a ratio of 1:4 and grown until confluent (usually 5–7 days). After 3 and 5 days of culture the media was replaced with fresh medium. Cells were harvested by incubation with 0.05% EDTA in phosphate buffered saline for 1 min at 37°C.

Phosphatidylinositol hydrolysis was studied in the SH-SY5Y cell line after the method of Kunysz et al. (1989) as follows: the cells were harvested as described above and incubated for 12 h, at a density of 5×10^6 cells ml^{-1}, in a medium consisting of Hams F10 and 0.1% BSA and $1\,\mu Ci\,ml^{-1}$ [^{3}H]-myo-inositol. After washing the cells twice with buffer to remove excess [^{3}H]-myo-inositol, the cells were resuspended in 0.75 ml of oxygenated modified Krebs-Bicarbonate buffer (see below for composition). The antagonist was added ($100\,\mu l$) and, after a 45 min equilibration period, carbachol ($50\,\mu l$) was added to the cells and allowed to equilibrate for 45 min at 37°C. The reaction was terminated by the addition of 3 ml of chloroform/methanol (1:2 vol:vol) followed by the addition of 1 ml of chloroform and 1 ml of water. The mixture was shaken for 20 min prior to determination of total inositol phosphate accumulation using a procedure based upon that of Minneman & Johnson (1984). Briefly, 2 ml of the aqueous layer were applied to Dowex columns (200–400 mesh in the formate form) and 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. Of this eluate 1.8 ml was transferred to a scintillation vial containing 10 ml Aquasol and counted by liquid scintillation spectrophotometry (Packard Tri Carb). The pA_2 values were calculated as described above for the isolated tissue preparations.

Statistics

All significant differences were assessed by the use of Student's t test, with a value of $P < 0.05$ being considered significant. All values are expressed as mean ± s.e.mean, $n = 6$–12.

Physiological salt solutions

The solutions used were as follows: Krebs solution (mM): NaCl 118.4, KCl 4.9, $MgSO_4 \cdot 7H_2O$, 1.2, KH_2PO_4 1.2, glucose 11.1, $NaHCO_3$ 25.0 and $CaCl_2 \cdot 6H_2O$ 2.5; Krebs-Bicarbonate buffer (mM): NaCl 118, $NaHCO_3$ 25, KCl 4.7, KH_2PO_4 1.2, $CaCl_2 \cdot 6H_2O$ 1.3, $MgSO_4$:$(H_2O)_7$ 1.1, glucose 11 and LiCl 10; Tyrode solution (mM): NaCl 136.9, KCl 2.7, $MgCl_2 \cdot 6H_2O$ 1.1, $NaH_2PO_4 \cdot 2H_2O$ 0.4, glucose 3.6, $NaHCO_3$ 11.9 and $CaCl_2 \cdot 6H_2O$ 1.8.

All physiological salt solutions were gassed with 5% CO_2/95% O_2, and maintained at pH 7.4.

Drugs used

[^{3}H]-myo-inositol (specific activity 15 Ci mmol^{-1}) was obtained from American Radiolabeled Chemicals Inc. *Para*-fluorohexahydrosiladiphenidol (pFHHSiD) and methoctramine were synthesized by Dr R. Clark, IOC, Syntex, Palo Alto, California, U.S.A. Pirenzepine was donated by Boots PLC. The remaining drugs were obtained from Sigma Chemical Co. Ltd.

Results

Isolated tissue studies

Carbachol, with the exception of the urinary bladder ($-\log EC_{50} = 6.1 \pm 0.08$), elicited concentration-dependent contractile responses in all smooth muscle preparations with $-\log EC_{50}$ values ranging between 6.8–7.0. These values were in good agreement with previous data from our laboratory (Eglen *et al.*, 1988). Similar $-\log EC_{50}$ values were determined at receptors mediating endothelial-derived relaxing factor (EDRF)-dependent relaxations of the vascular tissue (6.7–7.0).

The muscarinic receptors present in each tissue studied are summarised in Table 1. However, the canine femoral artery and vein have yet to be classified with regard to the muscarinic receptor subtype present. At receptors mediating contractions of the femoral vein, the pA_2 values (with Schild slopes shown in parentheses), for pirenzepine and methoctramine were 7.86 ± 0.16 (0.98 ± 0.11) and 6.07 ± 0.17 (1.17 ± 0.15), respectively. These values are consistent with responses being mediated through an M_1 receptor (Eglen & Whiting, 1986). The pA_2 values at receptors mediating relaxations of the femoral artery for pirenzepine and methoctramine were 6.26 ± 0.11 (1.07 ± 0.10), and 6.05 ± 0.09 (1.01 ± 0.07), respectively. The Schild slopes in these studies were not significantly different from unity. The concentrations of pirenzepine used were 0.03, 0.1, 1 and 3 μM whilst the concentrations of methoctramine used were 1, 3 and 10 μM. Concentrations of methoctramine above 10 μM were not used due to possible allosteric effects (Eglen *et al.*, 1988).

pFHHSiD (1 nM–10 μM) did not elicit responses itself in any of the preparations. Parallel dextral shifts, which were not accompanied by any diminution in the maxima of the concentration-response curves to carbachol were observed in all preparations studied, with the exception of the urinary bladder. In the latter tissue, the concentration-response curves were shifted dextrally in a non-parallel fashion and the maxima were also depressed by pFHHSiD in a concentration-dependent manner. The pA_2 values and slopes of the Schild regressions are shown in Table 2. The slopes were not significantly different from unity in the majority of tissues with the exception of those determined from experiments in the urinary bladder and relaxations of the femoral artery. As a result, the Schild slopes were not constrained to unity in these tissues.

The pA_2 values for pFHHSiD at muscarinic receptors mediating relaxations of the canine femoral artery, rat aorta and jugular vein were not significantly ($P > 0.05$) different either from each other or from pA_2 values determined at receptors mediating contractions of the guinea-pig ileum. However, the pA_2 value for pFHHSiD in the OMM was significantly ($P < 0.05$) greater than those obtained in the tissues listed. Conversely, the pA_2 values at receptors mediating contractions of the canine femoral and saphenous veins, guinea-pig trachea, urinary bladder and negative inotropic responses were significantly ($P < 0.05$) lower than those in the ileum. The lowest pA_2 value was obtained at receptors in the left atria.

When the pK_B values (obtained after imposing the unity constraint on the Schild plot) were considered, good agreement was observed between the pK_B values for receptors present in the ileum, OMM, aorta and jugular vein. The pK_B values at M_1 receptors in the femoral and saphenous veins (7.22 and 6.87) were lower than those observed at M_3 receptors, whilst the lowest pK_B value was that observed at atrial M_2 receptors. The pK_B value at receptors mediating contractions of the trachea (7.08) was lower than those observed at M_3 receptors mediating ileal contractions.

In terms of selectivity, therefore, pFHHSiD was 70 fold more selective for M_3 receptors mediating ileal contractions than for M_2 receptors mediating negative inotropic responses, and 6 fold more selective than for M_1 receptors mediating contractions of the saphenous vein. However, when all the functional M_3 pA_2 values for the isolated tissues are considered (in which the pA_2 values range from 7.1 (trachea) to

Table 1 Muscarinic receptor subtypes mediating responses in various bioassays

Bioassay	*Muscarinic receptor subtype*	*Reference*
Canine saphenous vein	M_1	O'Rourke & VanHoutte (1987)
Guinea-pig left atria	M_2	Clague *et al.* (1985)
Guinea-pig ileum	M_3	Clague *et al.* (1985)
Guinea-pig OMM	M_3	Eglen & Whiting (1988)
Guinea-pig trachea	M_3	Eglen & Whiting (1988)
Guinea-pig urinary bladder	M_3	Eglen & Whiting (1987)
Rat aorta	M_3	Eglen *et al.* (1988)
Rabbit jugular vein	?	
Canine femoral vein	?	Rubanyi & Vanhoutte (1987)
Canine femoral artery	?	Rubanyi & Vanhoutte (1987)
Human astrocytoma (1321 N1) cells	M_3	Kunsyz *et al.* (1989)
Human SH-SY5Y cells	M_1	Sera *et al.* (1988)

OMM – Oesophageal muscularis mucosae.
? – Muscarinic receptor subtype present remains to be classified.
The classification scheme employed in this table is after that described by Doods *et al.* (1987). This scheme is based upon differential antagonist affinities, estimated in pharmacological studies.

Table 2 pA_2 values and Schild plot slopes for pFHHSiD at muscarinic receptors in various isolated preparations

Species	*Preparation*	*pA_2*	*Schild slope*
Canine	Femoral vein	7.11 ± 0.11 (7.22)	1.08 ± 0.07
Canine	Femoral artery	7.90 ± 0.14	0.75 ± 0.04*
Canine	Saphenous vein	7.08 ± 0.07 (6.87)	0.92 ± 0.08
Guinea-pig	Left atria	6.01 ± 0.05 (6.17)	1.02 ± 0.10
Guinea-pig	Ileum	7.85 ± 0.08 (7.87)	1.10 ± 0.12
Guinea-pig	OMM	8.22 ± 0.03 (8.06)	0.97 ± 0.04
Guinea-pig	Trachea	7.13 ± 0.11 (7.08)	0.94 ± 0.10
Guinea-pig	Bladder	7.59 ± 0.10	0.74 ± 0.08*
Rat	Aorta	7.67 ± 0.10 (7.71)	1.02 ± 0.16
Rabbit	Jugular vein	7.89 ± 0.13 (7.66)	0.86 ± 0.11
Human	Astrocytoma (1321 N1) cells	7.64 ± 0.10 (7.73)	1.13 ± 0.06
Human	SH-SY5Y cells	7.90 ± 0.10 (7.83)	0.96 ± 0.04

OMM – Oesophageal muscularis mucosae.
Values are mean ± s.e.mean, $n = 6$–10.
* Significantly ($P < 0.05$) different from unity. The values in parentheses are those calculated after imposing the unity constraint (Kenakin, 1987).

8.2 (OMM)) the selectivity for M_3 versus M_2 receptors ranged from 13 to 163 fold, respectively.

Cell culture studies

In both the astrocytoma (1321 NI) and SH-SY5Y cells, pFHHSiD (at concentrations of 0.1, 0.3, 1 and 3 μM) produced dextral shifts in the carbachol concentration-response curve with no suppression of the maximal response. The pA_2 value in 1321 NI cells was similar to those values observed at M_3 receptors in the isolated smooth muscle tissues (Table 2). The pA_2 values for pirenzepine and methoctramine at muscarinic receptors in this cell line have been previously reported (Kunysz *et al.*, 1989) to be 7.3 and 6.0, respectively.

In SH-SY5Y cells, the pA_2 value for pFHHSiD was also similar to values obtained at M_3 receptors in the smooth muscle tissues (Table 2). In order to characterize the receptor mediating this response further, the pA_2 values for pirenzepine and methoctramine were 8.40 ± 0.10 (Schild slope = 1.03 ± 0.05) and 6.80 ± 0.20 (Schild slope = 0.95 ± 0.08), respectively. These values are consistent with responses mediated by stimulation of an M_1 receptor (Eglen & Whiting, 1986).

Discussion

In the present study, we have characterized the action of pFHHSiD, using responses at muscarinic receptors in isolated tissues and cell cultures. The functional data were consistent with data reported by Lambrecht *et al.* (1988). These authors showed that pFHHSiD exhibited a relatively high pA_2 value for M_3 muscarinic receptors mediating ileal smooth muscle contractions and discriminated M_2 receptors (atria) from M_1 receptors (vas deferens). However, other data obtained in the present study indicate that it may also exhibit dissociation constants (as estimated by the pA_2 value) that depend on the M_3 preparation studied.

The pA_2 values at atrial M_2 and ileal M_3 receptors were quantitatively very similar to those obtained by Lambrecht *et al.* (1988). The pA_2 value at M_1 receptors in the canine saphenous vein (7.1) was also similar to that reported by Lambrecht *et al.* (1988) at M_1 receptors in the rabbit vas deferens (6.9). A similar value was found at receptors mediating contractions of the canine femoral vein suggesting that M_1 receptors also mediate contractions in this tissue. In agreement with this postulate, both a high pA_2 value for pirenzepine (7.9), and low value for methoctramine (6.1) were also derived. Previous reports have shown that pA_2 values for pirenzepine at M_1 receptors generally range from 8.0–8.4 (see Eglen & Whiting, 1986; Mitchelson, 1988 for reviews) whereas the low value for methoctramine was indicative of a lack of M_2 receptor function.

Muscarinic receptor subtypes vary in different isolated vascular tissues, depending upon the physiological response, species and anatomical location. The porcine basilar artery and coronary artery exhibit contractile responses which are mediated by M_2 and M_3 receptors, respectively (Van Charldorp *et al.*, 1988), whereas contractile responses in the canine saphenous vein and bovine coronary artery are mediated by M_1 receptors (O'Rourke & Vanhoutte 1987; Duckles, 1988). The canine femoral vein also exhibits a contractile response to muscarinic agonists (Rubanyi & Vanhoutte, 1988) although the receptors have yet to be characterized.

The muscarinic receptor mediating release of EDRF in vascular tissue exhibits a pharmacology similar to the M_3 receptor defined on the basis of differential antagonist affinities. The pA_2 values for pirenzepine, AF-DX 116, 4-DAMP and methoctramine in the rat aorta (Choo *et al.*, 1986; Eglen *et al.*, 1988), rabbit ear artery (Hynes *et al.*, 1986) and bovine coronary artery (Duckles, 1988) were similar to those observed in ileal smooth muscle (Eglen *et al.*, 1988). pFHHSiD, in view of its reported selectivity for M_3 over M_2 and M_1 receptors, may enable a more definitive profile of these tissues to be elucidated.

The pA_2 values at receptors mediating relaxations of the canine femoral artery, rat aorta and rabbit jugular vein were similar to values obtained at ileal M_3 receptors. However, the low Schild slope observed in experiments using the femoral artery, indicate deviation from competitive antagonism. These data suggest that M_3 receptors mediate endothelial-dependent relaxations in a variety of vascular tissues in at least two species, in agreement with previous literature (see Introduction for references). The pA_2 value obtained at receptors in

the astrocytoma (1321 N1) cells was similar to the pA_2 value obtained at receptors in the isolated ileum confirming previous data that M_3 receptors mediate responses in these tissues (Kunysz *et al.*, 1989).

However, anomalous pA_2 values for pFHHSiD were found in some isolated tissues. The pA_2 value was significantly higher in the OMM, a preparation which has been previously shown to possess M_3 receptors (Eglen & Whiting, 1988). Whilst the reason for the higher value is unknown, anomalously higher values have also been reported for pirenzepine (Kamikawa *et al.*, 1985; Eglen & Whiting, 1988). The muscarinic receptor mediating contractions of the oesophageal muscularis mucosae, for example, has been shown (Kamikawa *et al.*, 1985; Eglen *et al.*, 1988) to exhibit pA_2 values for atropine, AF-DX 116, 4-DAMP, gallamine, silabenzhexol and methoctramine similar to those found at ileal and tracheal M_3 receptors. However, dissimilar pA_2 values for pirenzepine were also observed (OMM, 7.4; ileum and trachea, 6.8). The reason for this disparity was unknown, but it was considered not to be due to the presence of M_1 receptors (Eglen & Whiting, 1988).

Conversely, the pA_2 values for pFHHSiD in the trachea were lower than observed in the ileum. The trachea has been previously shown to possess M_3 receptors (Eglen & Whiting, 1988), and the reason for the relatively low value observed in this study is discussed below. The guinea-pig urinary bladder has also been shown (Eglen & Whiting, 1987) to exhibit pA_2 values for pirenzepine and 4-DAMP similar to those at ileal M_3 receptors, although extensive characterization of muscarinic 'receptors' in this tissue has only been undertaken using radioligand binding studies (Nilvebrandt & Sparf, 1988). The relatively low pA_2 value at M_3 receptors in the urinary bladder was accompanied by a Schild slope less than unity, indicating deviation from competitive antagonism. One explanation for the diminution in maxima observed with pFHHSiD may be the low effective receptor reserve for carbachol in this tissue (as judged by the low $-EC_{50}$ value) leading to conditions of hemiequilibrium. Additional experiments are clearly required to elucidate the reason for these relatively 'low' pA_2 values at previously well characterized M_3 receptors.

The pA_2 value for pFHHSiD at receptors in SH-SY5Y cells was approximately 6 fold higher than the pA_2 value obtained at M_1 receptors in the isolated tissue studies but less than the pA_2 values obtained at M_3 receptors. The reason for this disparity is unknown. Sera *et al.* (1989) have proposed, on the basis of antagonist affinities, that M_1 receptors mediate the inositol phospholipid hydrolysis response in the SH-SY5Y cell. The pA_2 values obtained in the present study for pirenzepine and methoctramine are in accord with this hypothesis. However, Lambert & Nahorski (1989) have reported, again using antagonist affinity data, that stimulation of inositol phospholipid hydrolysis is mediated by M_3 receptors in this cell line. The pA_2 value obtained in the present study for pFHHSiD, but not pirenzepine, was indicative of the presence of an M_3 receptor.

It is apparent, therefore, that the pA_2 values for pFHHSiD are critically dependent upon the preparation studied e.g. dissimilar pA_2 values were observed at M_3 receptors in the ileum (7.85) and at M_3 receptors in the trachea (7.08). Similarly different values were observed at M_1 receptors in the femoral vein (7.11) and at M_1 receptors in SH-SY5Y cells (7.90).

Two possible explanations for these data are that either the differences in pA_2 values are a reflection of further muscarinic receptor heterogeneity or that some of the experiments were undertaken under nonequilibrium conditions. We feel that the former is unlikely because (a) the disparities in pA_2 values are too small i.e. less than 10 fold, to use as evidence for heterogeneity (see Eglen & Whiting, 1986 for further discussion) and (b) that these differences between tissues has only been observed in our laboratory with this particular compound. The use of a 60 min preequilibration period and the use of an agonist resistant to degradation or uptake argues against the possibility of the antagonist not being in equilibrium. The trachea has been shown (Roffel *et al.*, 1987) to possess both M_2 and M_3 receptors, and the contribution of both receptors to the response could theoretically result in a low pA_2 value with a unity slope (Kenakin, 1987). However, the ileum also possesses a heterogeneous population of M_2 and M_3 receptors and a higher pA_2 value was observed, consistent with stimulation of M_3 receptors.

The ability of pFHHSiD to exhibit a range of pA_2 values at M_3 receptors raises questions about its suitability for characterization of muscarinic receptors in particular and questions about the use of antagonists to classify receptor subtypes in general. Leff & Martin (1988) have raised similar questions with regard to the use of antagonists to classify receptors for 5-hydroxytryptamine. The affinities of the endogenous ligand, acetylcholine, for each of the putative muscarinic receptor subtypes are very similar (A.D. Michel, unpublished observations), suggesting that the 'receptive site', for the endogenous ligand is similar between subtypes. However, the potential ability of antagonists to bind to accessary sites on the receptor molecule may confer selectivity. In this respect, however, the role of 'tissue specific' binding areas on the membrane is critical. This may lead to a variation in dissociation constants of an antagonist at the same receptor, but located in different tissues. These considerations provide a more parsimonious explanation for the differences in pA_2 values for pFHHSiD at M_3 receptors than further muscarinic receptor heterogeneity. In the majority of bioassays studied in this report, it is unknown which muscarinic receptor subtype is expressed, and where more than one subtype is expressed, which subtype is coupled to a given response. Presumably, when more expression data are available, it may be possible to separate receptor heterogeneity from the role of membrane environment on antagonist affinity.

In summary, the present study has confirmed the ability of pFHHSiD to distinguish between three subtypes of muscarinic receptor in some isolated functional tissues although anomalous pA_2 values were observed at other M_3 receptors. We conclude that pFHHSiD is of limited use in muscarinic receptor classification. Further experiments are required to determine if the disparities observed at M_3 receptors are a reflection of problems of the compound or indicative of further muscarinic receptor heterogeneity. These data emphasize the importance of estimating the putative selectivity of antagonists in a wide range of preparations.

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