British Journal of Pharmacology (1998) 123, 1639–1644

http://www.stockton-press.co.uk/bjp

Pharmacological characterization of muscarinic receptors in the uterus of oestrogen-primed and pregnant rats

[2]Melinda Munns & [1]Jocelyn N. Pennefather

Department of Pharmacology, Monash University, Clayton, Victoria 3168, Australia

1 Radioligand binding and contractility studies were undertaken to determine the subtype/s of muscarinic receptors present in uteri of oestrogen-treated and late pregnant rats.

2 Competition binding studies with uterine membrane preparations and [^{3}H]-QNB (quinuclidinyl benzilate) provided negative log dissociation constants (pK_i) for each antagonist as follows; oestrogen-treated – atropine (7.98) $\geqslant$ himbacine (7.83) $>$ methoctramine (7.52) $\geqslant$ hexahydrosiladiphenidol (HHSiD; 7.32) $\geqslant$ 5,11-dihydro-11-[[[2-[2-[(dipropylamino)methyl]-1piperidinyl]ethyl]amino]-carbonyl]-6H-pyrido-[2,3-b][1,4]-benzodiazepin-6-one (AF-DX 384; 7.10) $>$ 11-[[2-[(diethylamino)methyl]-1-piperidinyl]-acetyl]5,11-dihydro-6H-pyridol]2,3,-b][1,4]benzodiazepin-6-one (AF-DX 116, 6.77) $>$ pirenzepine (6.17); late pregnant – atropine (8.05) $\geqslant$ methoctramine (7.95) $\geqslant$ himbacine (7.71) $\geqslant$ HHSiD (7.52) $\geqslant$ AF-DX 384 (7.34) $>$ AF-DX 116 (6.72) $>$ pirenzepine (6.18).

3 The potency of carbachol in causing uterine contraction was similar in preparations from pregnant and non-pregnant animals (pD_2 = 5.57 and 5.46, respectively). Each muscarinic antagonist caused parallel, rightward shifts of carbachol concentration-response curves. The pA_2 estimates were: oestrogen-treated – atropine (9.42) $>$ himbacine (8.73) $\geqslant$ HHSiD (8.68) $\geqslant$ methoctramine (8.49) $\geqslant$ AF-DX 384 (7.91) $\geqslant$ AF-DX 116 (7.36) $\geqslant$ pirenzepine (7.26); late pregnant – atropine (9.48) $>$ himbacine (8.37) $\geqslant$ HHSiD (8.22) $\geqslant$ methoctramine (8.01) $\geqslant$ AF-DX 116 (7.73) $\geqslant$ AF-DX 384 (7.44) $\geqslant$ pirenzepine (6.92).

4 The relative pK_i estimates for antagonists obtained in membrane preparations from oestrogen-treated rats suggest the presence of muscarinic M_2 subtypes. In functional studies pA_2 values indicated the additional presence of muscarinic M_3 receptor or, possibly an atypical receptor subtype. The similarity between pK_i and pA_2 estimates obtained in uteri from oestrogen-treated and pregnant animals, respectively, indicates that pregnancy does not affect myometrial muscarinic receptors in the rat.

Keywords: Myometrium; uterine contractions; pregnancy; muscarinic receptors; carbachol; [^{3}H]-QNB; M_2 muscarinic receptors; M_3-muscarinic receptors; oestrogen

Introduction

Investigations of the subtype/s of muscarinic receptors responsible for mediating contractions of uterine smooth muscle in various species have led to contradictory results. Leiber *et al*. (1990) studied the effects of specific agonists and antagonists on carbachol-induced contractions of guinea-pig uterine tissue and measured accumulation of inositol phosphates and cyclic AMP inhibition, and concluded that in the myometrium of the guinea-pig the contractile event is triggered by the M_3 receptor subtype and may be modulated by the M_2 receptor subtype. However, other studies with the guinea-pig uterus have indicated that the M_2, M_3 or M_4 receptors are actively involved in mediating uterine contraction (Eglen *et al*., 1989; 1991; Dörje *et al*., 1990a,b; Doods *et al*., 1993). More recent studies by the latter group indicate that the muscarinic receptor present in the guinea-pig uterus may be a novel subtype (Boxall *et al*., 1997). In the rabbit uterus, studies with receptor antibodies indicate the presence of M_2 muscarinic receptors (Dörje *et al*., 1991a), while earlier functional studies indicate the presence of M_3 and M_2 muscarinic receptors (Crankshaw, 1984).

By measuring inhibition of carbachol-induced accumulation of inositol monophosphate in the presence of selective muscarinic antagonists, and from binding studies, Varol *et al*. (1989) concluded that both M_2 and M_3 receptors exist in the immature rat uterus, but that only the latter are responsible for mediating contractions, although these were not measured. Binding studies conducted by Pennefather *et al*. (1994) indicated the presence of a homogeneous population of M_2 binding sites in the mature rat uterus, again studies of uterine contractions were not conducted. A major aim of the present study was to examine the potencies of a range of subtype-selective muscarinic receptor antagonists on contractions induced by a muscarinic receptor agonist in the uterus of this species.

There is evidence to suggest that steroid hormones regulate the content, turnover and release of neurotransmitters in the nerves of the uterus (Bell, 1972). Histochemical studies have indicated that the number of acetylcholine-esterase positive nerves supplying the uterus decreases during pregnancy, possibly to protect the uterus from autonomic excitation (Wikland *et al*., 1984; Garfield, 1986). Functional studies have also indicated a loss of cholinergic nerves in the uterus of pregnant rats (Sato *et al*., 1989). Despite early findings that in the human uterus, a marked reduction in sensitivity of the myometrium to acetylcholine occurs (Nakanishi & Wood, 1971), there have been few, if any studies of myometrial muscarinic receptors during pregnancy.

In the present investigation we have used selective muscarinic antagonists, in both functional and radioligand binding studies, to identify the subtypes of muscarinic receptors present in the uterus of the rat. Non-pregnant rats were pretreated with oestrogen for these experiments so that all

[1] Author for correspondence.
[2]Present address: Department of Perinatal Medicine, Royal Women's Hospital, Grattan St., Carlton, Victoria, 3052, Australia.

were in an equivalent hormonal state. The effects of pregnancy on muscarinic receptors were determined by repeating the above experiments in uterine preparations from late pregnant (days 17–20) rats. A preliminary account of some of the results of this study was presented to the British Pharmacological Society (Pennefather & Munns, 1995).

Methods

Prior ethical approval for this study was obtained from the Monash University Standing Committee on Ethics in Animal Experimentation.

Animals and treatments

Mature female virgin Sprague-Dawley rats housed at 22°C with a photo-period of 12 h light and 12 h dark were used in these experiments. The rats used were either oestrogen-treated (210–275 g); oestrodiol cypionate in peanut oil was administered (0.1 ml kg^{-1}) subcutaneously at a dose of 200 μg kg^{-1} 24 h before experimentation; or primigravid (300–350 g) at days 17–20 of the 21 day gestation period. Tissue from implantation sites was not used.

Rats, including foetuses in pregnant animals, were killed by a blow to the head followed by cervical dislocation. The abdomen was opened and both uterine horns were removed carefully and separated from fat deposits and mesenteric attachments. Vaginal smears were taken from non-pregnant animals to confirm cornification of the vaginal epithelium following oestrogen treatment. These were stained with Giemsa stain which was made by dissolving Giemsa in citric acid/phosphate buffer (pH 4.0). This buffer contained 0.1 M of citric acid, 0.2 M disodium hydrogen peroxide phosphate in 25% methanol. Slides were dried and left overnight in the stain before being rinsed with methanol.

Radioligand binding experiments

The methodology was identical to that described by Pennefather *et al.* (1994). To prepare membranes for radioligand binding experiments, both uterine horns were removed, placed on ice and weighed. The tissue was then cut finely ready for homogenization. A Na_2HPO_4 buffer (pH 7.4) was used in these experiments for drug dilution, homogenization and washing filters. The solution comprised Na_2HPO_4 7.95 g l^{-1} with pH adjusted by addition of 1 M HCl. Each non-pregnant rat provided enough tissue for one binding experiment (i.e. a competition study with one antagonist or one saturation analysis). For pregnant animals, a similar amount of tissue per experiment was weighed, chopped and in most cases kept overnight, as the homogenate, on ice at 4°C. Pilot studies confirmed that the membrane muscarinic receptor population is preserved under these storage conditions. The chilled tissue preparation was homogenized in 10 ml of buffer with a Kinematica Homogenizer. Homogenization comprised two 20 s bursts at 1/2 setting with a 2 min resting period, during which the homogenate was kept on ice.

By use of a SS34 rotor centrifuge, the homogenate was spun for 12 min at 3000 r.p.m. at 0°C. The supernatant was decanted and used in the experiment except for a small aliquot reserved for protein content determination as described by Schacterle and Pollack (1973). The average protein recovery was approximately 30 μg g^{-1} of tissue for oestrogen-treated rats and 25 μg g^{-1} of tissue for pregnant rats.

Competition binding studies

Triplicate samples of labelled ligand [^{3}H]-QNB 47 Ci $mmol^{-1}$ (200 pM), antagonist (0.5 nM–5 μM for atropine, 2 nM–50 μM for other antagonists) and buffer (made to 0.8 ml for total sample) were preincubated for 10 min at 37°C. Membrane homogenate (200 μl) was added to each tube (total volume 1.0 ml) and again incubated for a further 60 min at 37°C. Each sample was then filtered at 4°C with a Brandell 24 cell Harvester containing glass fibre filters (Type GF/B) that had been soaked overnight in 0.1% polyethyleneimmine and 10 μM atropine. Filters, in a suspension of Ecolite scintillant (3 ml), were counted in a Hewlett Packard Liquid Scintillation Counter (Minaxi Tri-Carb 4000 Series) with an efficiency of 55%. Non-specific binding was determined from the binding obtained under the same conditions with atropine 10 μM present.

Saturation studies

The equilibrium dissociation constant (K_D) for [^{3}H]-QNB was determined by separate saturation experiments. With membranes from three oestrogen-treated rats, saturation curves were constructed by use of seven concentrations of the labelled ligand (50 pM–5000 pM). A similar procedure was undertaken as for competition binding studies, with homogenization, incubation, centrifugation, filtration and counting steps remaining the same. Triplicate samples for each concentration of labelled ligand were counted in the presence and absence of atropine (10 μM) to determine non-specific binding.

Functional studies

For functional studies with oestrogen-treated rats, each uterine horn was medially transected providing four preparations per animal. The number of preparations obtained from pregnant animals ranged from 8 to 14, depending on the amount of tissue available and the number of binding experiments to be undertaken with the same tissue. The horns were cut in segments of lengths similar to those obtained from non-pregnant animals (approx. 1–1.5 cm).

Each uterine tissue segment was mounted in a 5 or 10 ml organ bath so that longitudinally arranged muscle fibres were vertically oriented. Changes in isometric force were recorded by Grass FT03 transduers coupled to Grass 79B, 79C, 79D, and 7B polygraphs. The tissues were continually bathed and washed in a modified Krebs solution comprising the following substituents ($mmol^{-1}$): NaCl 118, $NaHCO_3$ 25.0, glucose 11.66, KCl 4.7, $CaCl_2.2H_2O$ 2.5, KH_2PO_4 1.18 and $MgSO_4.7H_20$ 0.45. The baths were maintained at a constant temperature of 37°C and bubbled with carbogen (95% O_2, 5% CO_2).

Carbachol log concentration-response curves

Each tissue was allowed to equilibrate for 60 min under 1 *g* force and washed every 15 min before drugs were added. Carbachol concentration-response curves, with a progression ratio of one log unit over 7 orders of magnitude (10^{-9} to 10^{-2} M), were constructed on each tissue by use of the discrete method. An interval of 15 min was allowed between each dose addition, each dose remained in contact with the tissue for 2 min and the tissue was then washed with 2–3 times the bath volume.

Antagonist studies

After the construction of an initial log concentration-response curve to carbachol, three of four tissues from each animal were

equilibrated with an antagonist at 1, 10 or 100 × the initial antagonist concentration for 30 min, then a second carbachol log concentration-response curve was constructed as outlined above. The corresponding concentration of antagonist was re-added between each agonist dose. The remaining tissue was used as a time control in which a second log concentration-response curve was constructed in the absence of antagonist. The peak magnitude (g) for each contraction was measured and the magnitude of any spontaneous contractions immediately before drug addition was substracted from this value.

Drugs and solutions

Carbamylcholine chloride (carbachol) and atropine sulphate were obtained from Sigma. Pirenzepine dihydrochloride 1 H_2O, AF-DX 116 (11-[[2-[(diethylamino) methyl] - 1 - piperidinyl] - acetyl] 5,11- dihydro-6H- pyridol [2,3,-b] [1,4] benzodiazepin -6-one) and AF-DX 384 (5,11-dihydro-11-[[[2-[2-[(dipropylamino) methyl]-1-piperidinyl]ethyl] amino]carbonyl] - 6H - pyrido- [2,3, -b] [1,4]-benzodiazepin-6-one) were gifts from Boehringer Ingelheim. Methoctramine 4 HCl (N,N′-bis[6-[2-methoxybenzyl-amino]hexyl]-1,8-octane-diamine tetrahydrochloride) and HHSiD (hexahydrosiladiphenidol) were obtained from RBI. Himbacine was generously provided by Dr F. Mitchelson and Prof W. Taylor. [^{3}H]-QNB (1-quinuclidinyl[phenyl-4^{3}H]-benzilate) (Specific activity 47 Ci mmol^{-1}) was obtained from Amersham. Oestradiol cypionate was prepared by first dissolving it in 0.1 ml of ethanol and then diluted in peanut oil up to 10 ml; it was protected from light by foil wrapping. Concentrated [^{3}H]-QNB solution was kept wrapped in foil in the freezer and diluted in phosphate buffer to the desired concentration. Both AF-DX 116 and AF-DX 384 were dissolved in 50 μl of HCl before dilution. A minimal amount of ethanol was used to dissolve HHSiD. All other compounds were stored at 4°C and diluted in distilled water for functional studies or Na_2HPO_4 for binding studies.

Data analysis

Data from both competition and saturation radioligand binding experiments were analysed by use of the computer programs EBDA and LIGAND and RADLIG (McPherson, 1985; 1994). These packages provide estimates of Hill coefficients (n_H) and negative log dissociation constants (pK_i), the latter being determined from exact fits to the competition curve. Thus for one-site analysis pK_i is equivalent to the pK_D for each antagonist (McPherson, 1994). Another program in the RADLIG package, i.e. 'ASSAY', was used to allow interpolation of protein content absorbance readings from standard curves.

To determine agonist potencies in functional studies, mean log concentration-response curves were constructed by pooling responses between 15 and 85% of maximum response, and pD_2 values determined. For estimation of antagonist potencies, log concentration-response curves generated in the absence and presence of each antagonist concentration were constructed, and the horizontal shift (log concentration ratio) between each pair of curves was obtained with a computer program 'COMPAR', based on the method outlined in Geigy Scientific Tables (1982; Equation 695). This shift was determined only if the linear portions of the log concentration-response curves were parallel. The log concentration-ratios so obtained were corrected for time-dependent changes and used in Schild regression analysis, in which log concentration-ratio minus one is plotted against antagonist concentration according to the following equation (Arunlakshana & Schild, 1959):

$$\log(CR - 1) = n \log [B] + \log K_{b,}$$

where [B] is the molar concentration of antagonist, n = slope of the linear regression and K_b the dissociation constant of the antagonist-receptor complex. If the linear regression had a slope not significantly different from unity the slope was constrained to unity and the pA_2 determined.

Functional and binding data were analysed by 'Quatro-Pro for Windows' from which means, standard deviations, s.e.mean and Student's t values could be obtained. All final results were obtained by averaging estimates obtained from individual experiments and are expressed as mean ± s.e.mean.

One-way analysis of variance (ANOVA) was used when data from more than two groups were analysed. Statistical significance of differences between two means was determined with a Student's unpaired t test. Throughout these studies a value of $P < 0.05$ was considered to be statistically significant.

'Sigma-Plot for Windows' was used to generate linear regression plots with 95% confidence limits and correlation coefficients squared (r^2). 'Sigma Stat' was used to test the null hypothesis that the slope of the regression line was not significantly greater than zero: value of $P < 0.05$ indicates a high probability that the slope was significantly greater than zero, i.e. that one variable could be used to predict the value of the other.

Results

Vaginal smears

Histological examination of vaginal smears from oestrogen-treated rats used in this investigation confirmed cornification of the vaginal epithelium.

Radioligand binding data

From saturation studies conducted in tissues from oestrogen-primed rats the estimate of equilibrium dissociation constant (K_D) for the labelled ligand, [^{3}H]-QNB, was $4.37 \pm 0.84 \times 10^{-11}$ M. Other binding parameters for this ligand on oestrogen-treated uterine preparations were: n_H 1.00 ± 0.045; B_{Max} (mg^{-1} protein) $8.03 \pm 0.52 \times 10^{-13}$ M.

Figure 1 shows the competition binding data for the antagonists with [^{3}H]-QNB as the labelled ligand. pK_D values are listed in Table 1. Although some Hill coefficients differed significantly from one, LIGAND analysis indicated that all curves showed a statistical preference for a one-site rather than a two-site fit. The M_2-selective antagonists, himbacine, methoctramine and HHSiD had high affinities. While the pK_i for HHSiD was significantly greater in tissue from late pregnant rats than in tissue from oestrogen-treated rats, overall there was a high correlation between the two sets of data ($r^2 = 0.92$, $P < 0.05$).

Functional data

To determine whether there was regional variation in sensitivity to carbachol, pD_2 values were compared for each segment of tissue from non-pregnant rats used ($n = 29$); right ovarian half ($pD_2 = 5.80 \pm 1.03$), right cervical half ($pD_2 = 5.38 \pm 0.96$), left ovarian half ($pD_2 = 5.44 \pm 0.93$) and left cervical half ($pD_2 = 5.34 \pm 0.93$). These values did not differ significantly, indicating that the four segments of uterine tissue

can be regarded as homogeneous in response to carbachol. The mean pD_2 values for carbachol measured on estrogen-treated tissues ($pD_2 = 5.46 \pm 0.47$, $n = 29$) and late pregnant tissues ($pD_2 = 5.57 \pm 0.70$, $n = 23$) were similar. Thus antagonist affinities from each of these groups of tissues could be compared with carbachol as the agonist.

Carbachol-induced contractions of the longitudinal muscle layer of the rat uterus were antagonized in a competitive fashion by all of the muscarinic antagonists used. Figure 2 illustrates the mean data for himbacine in preparations from oestrogen-treated rats. The pA_2 values for each of the muscarinic antagonists shown in Table 2 were obtained from Schild plots with slopes constrained to one, since the slope did not differ significantly from unity. Correlation of estimates obtained from oestrogen-treated and late pregnant uterine tissue yielded a correlation coefficient squared (r^2) of 0.84 ($P < 0.05$).

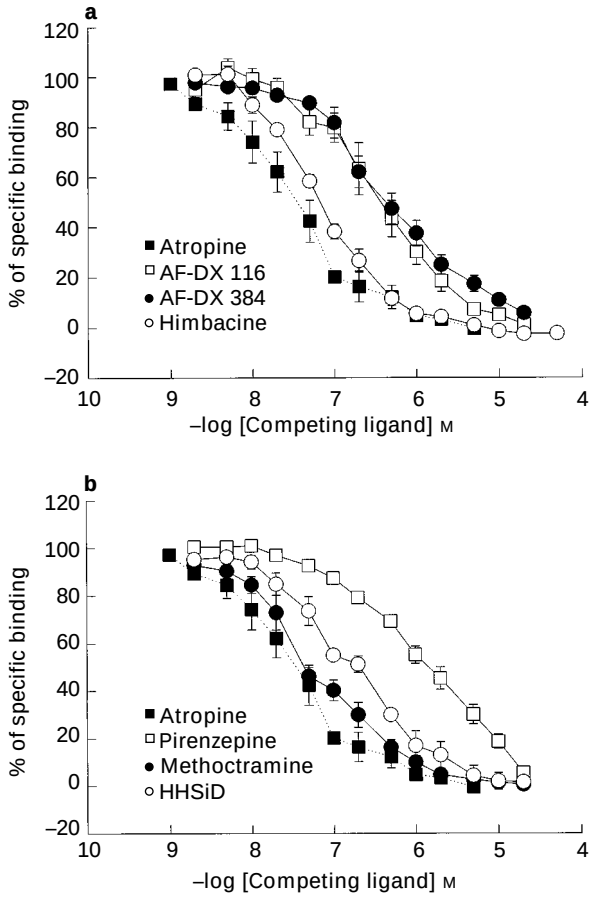

Figure 1 Competition by antagonists with binding of [^{3}H]-QNB to uterine membrane preparations from oestrogen-treated rats. Rat uterine membranes were incubated with 200 pM [^{3}H]-QNB in the absence and presence of unlabelled competing ligands for 30 min at 37°C. (a) Curves for atropine, AF-DX 116, AF-DX 384 and himbacine and (b) those for atropine, pirenzepine, methoctramine and HHSiD. Each point represents the means with the s.e.mean (vertical lines) of four triplicated experiments, except for pirenzepine where $n = 6$.

Discussion

Competition binding and functional studies in uterine preparations from both oestrogen-treated and late pregnant rats have confirmed that this tissue contains muscarinic receptors mediating carbachol-induced contractions. The saturation studies with tissues from oestrogen-primed animals in which [^{3}H]-QNB was used as the labelled ligand indicated the presence of a homogeneous population of binding sites with an equilibrium dissociation constant ($K_D \cong 4 \times 10^{-11}$ M)

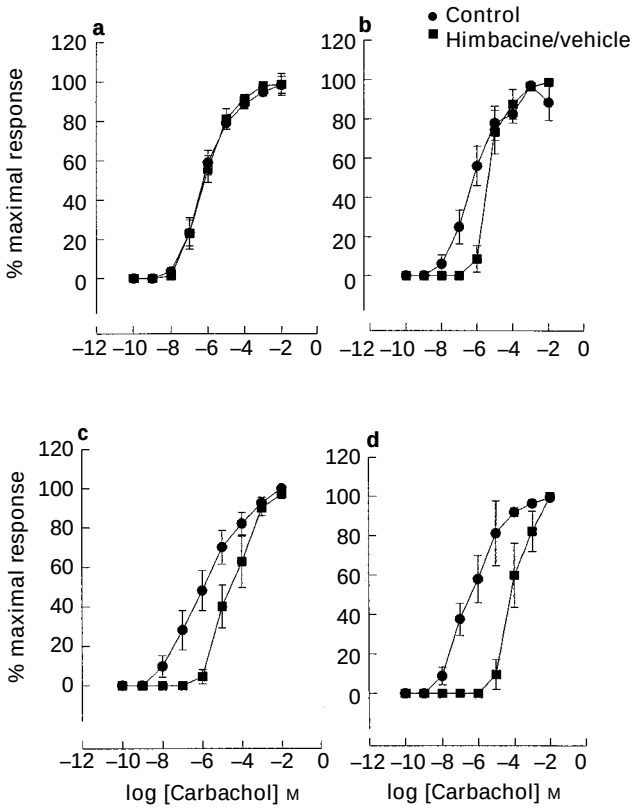

Figure 2 Mean log concentration response curves for carbachol in the absence (control) and presence of vehicle (a) or himbacine 10 nM (b), 100 nM (c) and 1 μM (d) on four segments of uterus from five oestrogen primed rats. The vertical line shows s.e.mean.

Table 1 pK_i and Hill plot slopes (n_H) for antagonist competition with [^{3}H]-QNB binding sites in uterine membranes from oestrogen-treated and late pregnant rats

	Oestrogen-treated		*Late pregnant*	
Antagonist	pK_i	n_H	pK_i	n_H
Atropine	7.98 ± 0.10 ($n = 4$)	0.92 ± 0.04	8.05 ± 0.01 ($n = 4$)	1.04 ± 0.10
Pirenzepine	6.17 ± 0.06 ($n = 6$)	0.91 ± 0.09	6.18 ± 0.06 ($n = 3$)	0.91 ± 0.08
AF-DX 116	6.77 ± 0.01 ($n = 4$)	0.98 ± 0.12	6.72 ± 0.06 ($n = 3$)	0.90 ± 0.03
AF-DX 384	7.10 ± 0.17 ($n = 4$)	0.79 ± 0.03	7.34 ± 0.11 ($n = 3$)	0.76 ± 0.10
Methoctramine	7.52 ± 0.14 ($n = 4$)	0.93 ± 0.08	7.95 ± 0.09 ($n = 3$)	0.77 ± 0.04
HHSiD	7.32 ± 0.01 ($n = 4$)	0.89 ± 0.09	7.52 ± 0.01 ($n = 3$)	1.13 ± 0.04
Himbacine	7.83 ± 0.04 ($n = 4$)	1.02 ± 0.07	7.71 ± 0.09 ($n = 3$)	1.09 ± 0.07

Values are the means ± s.e. of 3–6 experiments performed in triplicate.

Table 2 pA_2 values and Schild plot slopes for muscarinic antagonists versus carbachol on uterine preparations from oestrogen-treated and late pregnant rats

	Oestrogen-treated rats		*Late pregnant rats*	
Antagonists	*pA_2*	*Slope*	*pA_2*	*Slope*
Atropine	9.42±0.17 ($n=4$)	0.94±0.10	9.48±0.22 ($n=3$)	1.05±0.16
Pirenzepine	7.26±0.29 ($n=4$)	1.24±0.22	6.92±0.28 ($n=3$)	0.79±0.25
AF-DX 116	7.36±0.27 ($n=4$)	0.91±0.20	7.73±0.22 ($n=3$)	0.80±0.11
AF-DX 384	7.91±0.21 ($n=4$)	0.87±0.17	7.44±0.23 ($n=3$)	0.82±0.19
Methoctramine	8.49±0.26 ($n=5$)	0.83±0.19	8.01±0.25 ($n=3$)	0.94±0.18
HHSiD	8.61±0.26 ($n=4$)	0.88±0.26	8.22±0.31 ($n=4$)	0.94±0.13
Himbacine	8.73±0.22 ($n=5$)	0.89±0.14	8.37±0.21 ($n=4$)	0.82±0.18

All results are expressed as mean±s.e., $n=3-5$.

similar to that previously found (Pennefather *et al*., 1994), for uterine membranes from untreated rats.

In previous competition binding studies with [^{3}H]-QNB and uterine preparations from untreated mature rats, Pennefather *et al*. (1994) estimated pK_i values for pirenzepine, AF-DX 116, hexahydrosiladifenidol and himbacine of 6.18, 6.98, 6.88 and 7.84, respectively, and concluded that muscarinic receptor binding sites of the M_2 subtype are present. The corresponding values obtained in the present study were virtually identical, except that for hexahydrosiladifenidol which had a higher affinity ($pK_i=7.32$ and 7.52) in tissues from both oestrogen-primed and pregnant rats. The latter values are marginally within the range found for this antagonist at M_3 receptors (Buckley *et al*., 1989; Lambrecht *et al*., 1989; Lazareno *et al*., 1990; Dörje *et al*., 1991b; Watson *et al*., 1995). Varol *et al*. (1989) suggested previously that muscarinic M_3 receptors are detectable in binding studies with uterine membranes from immature rats. They investigated the displacement of [^{3}H]-QNB binding by 4-diphenylacetoxy-N-methyl-piperidine methobromide and pirenzepine.

M_2 and M_3 receptors are co-expressed in many types of smooth muscle tissue (Eglen *et al*., 1994; 1996), with the proportion of M_2 binding sites exceeding that of M_3 receptor population by 4:1 or more; thus it is conceivable the two receptor subtypes are co-expressed in the rat uterus. Alternatively M_4 or a novel subtype may be present. However, the high binding affinity for methoctramine is not clearly consistent with the presence of either M_3 or M_4 receptors (Lazareno *et al*., 1990; Eglen *et al*., 1994).

Since the muscarinic receptor binding sites in oestrogen-dominated and late pregnant rat uterus displayed a very low affinity for pirenzepine the presence of M_1 and M_4 receptors is unlikely. The pK_i value obtained for himbacine is much higher than that found in binding studies on cloned m5 receptors, suggesting the latter subtype is not present (Dörje *et al*., 1991b). The values we obtained for AF-DX 384 (7.10 and 7.35, in tissues from oestrogen-primed and pregnant animals, respectively) were similar to that obtained in glandular M_3 cells by Doods *et al*. (1993), higher than those these workers obtained with m4 receptors in Chinese Hamster Ovary (CHO) cells (6.8), but lower than that found by this group in the guinea-pig uterus (8.22). The latter tissue has excited considerable controversy as to whether an M_2, M_4 or a novel subtype is present (Eglen *et al*., 1989; Dörje *et al*., 1990a,b; Doods *et al*., 1993; Boxall *et al*., 1997).

In the present competition binding study the majority of the Hill slope coefficients did not differ from one, and one-site fits for all competition curves were statistically better than two site fits in all analyses. Nevertheless, n_H values obtained for AF-DX 384 in both oestrogen-primed and pregnant rats and for AF-DX 116, methoctramine and HHSiD in late pregnant rats were significantly different from one (Table 1). This is consistent with the possibility of a mixed population of receptors, particularly in uterine tissue from late pregnant rats. In contrast, in the functional studies in which carbachol was used as the agonist, Schild regression lines for the antagonists used did not differ significantly from unity, *prima facie* suggesting that the presence of a heterogeneous population of receptor subtypes is not likely. Nevertheless, a Schild plot slope of one does not necessarily exclude the presence of multiple receptor sites.

A notable feature of the binding studies we conducted in rat tissues was the overall high correlation between the respective estimates of pK_i obtained in tissues from oestrogen-primed and pregnant animals. This strongly suggests that pregnancy does not affect the nature of the binding sites present in the uterus in this species. Our functional studies also support the notion that pregnancy does not significantly influence muscarinic receptors in the rat uterus. Firstly, carbachol was equipotent in tissues from oestrogen-primed and pregnant animals, and secondly, no significant differences in the pA_2-estimates we obtained for each antagonist in tissues from both groups of animals were observed, with mean values differing at most by only $\cong 0.5$ log units.

A difference in the results of our functional and binding studies was that the pA_2 values we obtained for the antagonism of carbachol for all antagonists, including atropine, were higher than the corresponding pK_i values obtained in the binding studies. Eglen *et al*. (1996) have recently noted that similar discrepancies, particularly for the M_2 receptor subtype, have been obtained when hypotonic media were used in binding studies. The differences between the functional and binding values may also reflect differences in the tissue used in the two types of experiment. Thus contractile activity was recorded from only the longitudinal layer of the rat uterus, whereas in binding studies both the longitudinal and circular layers and endometrium were homogenized into a single membrane preparation.

In general the pA_2 values we obtained, with the exception of that for hexahydrosiladifenidol, are consistent with the presence of a predominant population of M_2 receptors in the uteri from both oestrogen-primed and pregnant rats. The pA_2 values for pirenzepine (7.26 and 6.92) were approximately ten times lower than those at M_1 and M_4 receptors (Caulfield, 1993) excluding action at these subtypes. Those for AF-DX 116 (7.36 and 7.37) are too high for M_1 and M_3 receptor subtypes, but are broadly compatible with those at M_2 and M_4 (Caulfield, 1993). Those for AF-DX 384 (7.91 and 7.44) are very similar to those found for guinea-pig atria (M_2) by Doods *et al*. (1993).

Methoctramine pA_2 values (8.49 and 7.52) are too high to be compatible with the antagonism at M_1 or M_3 receptors and in particular in tissues from pregnant animals estimates of affinity seem closest to those determined functionally at M_2 or M_4 receptors (Eglen & Watson, 1996). A similar situation

exists for himbacine (8.73 and 8.37). Hexahydrosiladifenidol exhibited high affinity for uterine receptors in functional experiments ($pA_2 = 8.61$ and 8.22), exceeding by ten fold that obtained for guinea-pig uterus and atria by Doods *et al.* (1993), but similar to or higher than that found for the M_3 receptor subtype present in guinea-pig ileum (Boselli & Grana, 1995; Dietrich & Kilbinger, 1995).

Taken together, the estimates of affinity from functional studies, with the exception of that for hexahydrosiladifenidol, are compatible with the presence of a predominant population of M_2 muscarinic receptors in the uterus of the rat. However, the possibility that there is also a population of M_3 receptors present does merit further investigation, in particular when receptor antagonists such as zamifenacin are used (Eglen & Watson, 1996). Zamifenacin apparently disciminates between M_3 receptors in different smooth muscles.

Another possibility that merits further investigation is that a novel muscarinic receptor subtype is present in this tissue, as has recently been proposed for the guinea-pig uterus (Boxall *et al.*, 1997). The underlying reason for the relatively low binding and functional affinities of the uterine receptors for the three tricyclic antagonists used in this investigation compared to those of methoctramine, himbacine and hexahydrosiladifenidol might also be worthy studying.

References

ARUNLAKSHANA, O. & SCHILD, H.O. (1959). Some quantitative uses of drug antagonists. *Br. J. Pharmacol. Chemother.,* **14,** 48–58.

BELL, C. (1972). Autonomic nervous control of reproduction: circulatory and other factors. *Pharmacol. Rev.,* **21,** 657–710.

BOXALL, D.K., FORD, A.P.D.W., CHALLIS, R.A.J., NAHORSKI, S.R. & EGLEN, R.M. (1997). Atypical muscarinic cholinoceptor (mAChR) mediating carbachol-induced contraction of the guinea-pig uterus. *Br. J. Pharmacol.,* **120,** 62P.

BUCKLEY, N.J., BONNER, T.I., BUCKLEY, C.M. & BRANN, M.R. (1989). Antagonist binding properties of five cloned muscarinic receptors expressed in CHO-K1 cells. *Mol. Pharmacol.,* **35,** 469–476.

BOSELLI, C. & GRANA, E. (1995). Model of action of methoctramine, AF-DX-116 and hexahydrosiladifenidol in guinea-pig left atrium and ileum: comparison of Schild and resultant analysis. *J. Auton. Pharmacol.,* **15,** 115–127.

CAULFIELD, M.P. (1993). Muscarinic receptors – characterization, coupling and function. *Pharmacol. Ther.,* **58,** 319–379.

CRANKSHAW, D.J. (1984). Muscarinic cholinoceptors in the rabbit's myometrium: A study of the relationship between binding and response. *Eur. J. Pharmacol.,* **101,** 1–10.

DIETRICH, C. & KILBINGER, H. (1995). Prejunctional M_1 and postjunctional M_3 muscarinic receptors in the circular muscle of the guinea-pig ileum. *Naunyn-Schmiedeberg's Arch. Pharmacol.,* **351,** 237–243.

DOODS, H.N., WILLIM, K.D., BODDEKE, H.W.G.M. & ENTZEROTH, M. (1993). Characterization of muscarinic receptors in guinea-pig uterus. *Eur. J. Pharmacol.,* **250,** 223–262.

DÖRJE, F., FRIEBE, T., TACKE, R., MUTSCLER, E. & LAMBRECHT, G. (1990a). Novel pharmacological profile of muscarinic receptors mediating contraction of the guinea-pig uterus. *Naunyn-Schmiedeberg's Arch. Pharmacol.,* **342,** 284–289.

DÖRJE, F., FRIEBE, T., TACKE, R., MUTSCHLER, E. & LAMBRECHT, G. (1990b). Pharmacological characterisation of muscarinic receptors mediating contractions of guinea-pig uterus. *Naunyn-Schmiedeberg's Arch. Pharmacol.,* **341** (Suppl.), R79.

DÖRJE, F., LEVEY, A.I. & BRANN, M.R. (1991a). Immunological detection of muscarinic receptor subtype proteins (m1-m5) in rabbit peripheral tissues. *Mol. Pharmacol.,* **40,** 459–462.

DÖRJE, F., WESS, J., LAMBRECHT, G., TACKE, R., MUTSCHLER, E. & BRANN, M.R. (1991b). Antagonist binding profiles of five cloned human muscarinic receptor subtypes. *J. Pharmacol. Exp. Ther.,* **256,** 727–733.

EGLEN, R.M., HEDGE, S.S. & WATSON, N. (1996). Muscarinic receptor subtypes and smooth muscle function. *Pharmacol. Rev.,* **48,** 531–565.

EGLEN, R.M., MICHEL, A.D., LEVINE, W.B. & WHITING, R.L. (1991). Analysis of muscarinic receptors in guinea-pig oestrogen-dominated uterus, in vitro. *Br. J. Pharmacol.,* **104,** 44p.

EGLEN, R.M., MICHEL, A.D. & WHITING, R.L. (1989). Characterisation of the muscarinic receptor subtype mediating contractions of the guinea-pig uterus. *Br. J. Pharmacol.,* **96,** 497–499.

EGLEN, R.M., REDDY, H., WATSON, N. & CHALLISS, R.A.J. (1994). Muscarinic acetylcholine receptor subtypes in smooth muscle. *Trends Pharmacol. Sci.,* **151,** 114–119.

EGLEN, R.M. & WATSON, N. (1996). Selective muscarinic receptor agonists and antagonists. *Pharmacol. Toxicol.,* **78,** 59–68.

GARFIELD, R.E. (1986). Structural studies of innervation on non-pregnant rat uterus. *Am. J. Physiol.,* **251,** C42–C54.

GEIGY SCIENTIFIC TABLES. (1982). *Introduction to Statistics, Statistical Tables, Mathematical Formulae,* Vol 2, 8th edn. ed Lertner, C. p. 214. Basle, Switzerland: Ciba-Geigy Ltd.

LAMBRECHT, G., FEIFEL, R., MOSER, U., WAGNER-RÖDER, M., CHOO, L.K., CAMUS, J., TASTENOY, M., WAELBROECK, M., STROHMANN, C., TACKE, R., RODRIGUES DE MIRANDA, J.F., CHRISTOPHE, J. & MUTSCHLER, E. (1989). Pharmacology of hexahydro-difenidol, hexahydro-sila-difenidol and related selective muscarinic antagonists. *Trends Pharmacol. Sci.,* **10** (Suppl.), 60–64.

LAZARENZO, S., BUCKLEY, N.J. & ROBERTS, F.F. (1990). Characterisation of muscarinic M_4 binding sites in rabbit lung, chicken heart, and NG 108-15 cells. *Mol. Pharmacol.,* **38,** 805–815.

LEIBER, D., MARC, S. & HARBON, S. (1990). Pharmacological evidence for distinct muscarinic receptor subtypes coupled to the inhibition of adenylate cyclase and to the increased generation of inositol phosphates in the guinea-pig myometrium. *J. Pharmacol. Exp. Ther.,* **252,** 800–809.

MCPHERSON, G.A. (1985). Analysis of radioligand binding experiments – A collection of computer programs for the IBM PC. *J. Pharmacol. Methods,* **14,** 213–228.

MCPHERSON, G.A. (1994). *RADLIG: A collection of programs for the analysis of radioligand binding experiments.* Biosoft, U.K.

NAKANISHI, H. & WOOD, C. (1971). Cholinergic mechanism in the human uterus. *J. Obstet. Gynaecol. Br. Commonwealth,* **78,** 716–723.

PENNEFATHER, J.N., GILLMAN, T.A. & MITCHELSON, F. (1994). Muscarinic receptor binding sites in rat uterus are of the M2 subtype. *Eur. J. Pharmacol.,* **262,** 297–300.

PENNEFATHER, J.N. & MUNNS, M. (1995). Characterization of muscarinic receptor subtype mediating contractions of the uterus of oestrogen-treated and pregnant rats. *Br. J. Pharmacol.,* **115,** 14P.

SATO, S., HAYASHI, R.H. & GARFIELD, R.E. (1989). Mechanical responses of the rat uterus, cervix and bladder to stimulation of hypogastric and pelvic nerves in vivo. *Biol Reprod.,* **40,** 209–219.

SCHACTERLE, G.R. & POLLACK, R.L. (1973). A simplified model for the quantitative assay of small amounts of protein in biological material. *Anal. Biochem.,* **51,** 654.

VAROL, F.G., HADJICONSTANTINOU, M., ZUSPAN, F.P. & HEFF, N.H. (1989). Pharmacological characterisation of the muscarinic receptors mediating phosphoinositide hydrolysis in rat myometrium. *J. Pharmacol. Exp. Ther.,* **249,** 11–15.

WATSON, N., MAGNUSSEN, H. & RABE, K.F. (1995). Pharmacological characterization of the muscarinic receptor subtypes mediating contraction of human peripheral airways. *J. Pharmacol. Exp. Ther.,* **274,** 1293–1297.

WIKLAND, M., DAHLSTROM, A. & HAGLID, K.G. (1984). Structural and functional evidence for the denervation of human myometrium during pregnancy. *Obstet. Gynecol.,* **64,** 503–509.

(Received June 26, 1997
Accepted January 22, 1998)