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Characterization of the muscarinic receptor in isolated uterus of sham operated and ovariectomized rats

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1 The pharmacological characteristics of muscarinic receptors in rat isolated uterus were studied in ovariectomized (ov.) and sham operated (sh.) animals.

2 Competition radioligand binding studies, using uterine membranes and [^{3}H]-NMS, were undertaken with several muscarinic receptor antagonists. Most of the antagonists indicated a one-site fit with apparent affinity estimates (pK_i) unchanged by ovariectomy. The selective M_2 antagonist, tripitramine revealed high (representing 33 ± 8 and $38\pm2\%$) and low (67 ± 8 and $62\pm2\%$) affinity binding sites in both sh. and ov. rat uterus, respectively. These sites likely represented muscarinic M_2 and M_3 receptors and the proportions were not significantly different in the two conditions.

3 Carbachol induced concentration-dependent contractions which were surmountably antagonized by several muscarinic receptor antagonists (pK_B, sh.; ov.): zamifenacin (9.19; 9.18), p-F-HHSiD (8.50; 9.06), tripitramine (7.23; 7.54), himbacine (7.21; 7.41), methoctramine (6.79; 7.49), pirenzepine (6.48; 7.21), AF DX 116 (6.26; 6.61), MTx 3 (<7.00; <7.00) and PD 102807 (<7.00; <7.00).

4 The apparent affinity values obtained in functional studies using the uteri from both sh. and ov. animals correlated most closely with values reported at human recombinant muscarinic M_3 receptors. This suggests that the muscarinic M_3 receptor mediates contraction under both conditions.

5 Radioligand binding experiments indicate the presence of M_2 receptors, in addition to M_3 receptors, which probably explains the discrepancies between functional and binding affinities. These data further suggest that the pharmacological profile and proportions of the two populations of muscarinic receptors are unaffected by ovariectomy.

Keywords: Muscarinic receptors; muscarinic M_3 receptor; uterus; smooth muscle contraction; ovariectomy

Abbreviations: AF DX 116, [11-((((dimethylamino)-methyl)-1-piperidinyl)acetyl)-5,11-dihydro-6H-pyrido(2,3-b)(1,4)benzodiazepine-6one]; MTx 3, Mamba toxin 3; [^{3}H]-NMS, [^{3}H]-*N*-methyl scopolamine; Ov., ovariectomized; PD 102807, (3,6a,11,14-Tetrahydro-9-methoxy-2-methyl-12H-isoquino[1,2-b]pyrrolo [3,2-f][1,3]benzoxazine-1-carboxylic acid ethyl ester); p-F-HHSiD, para fluoro hexahydrosiladifenidol hydrochloride; Sh., sham operated

Introduction

In smooth muscle from several peripheral tissues, muscarinic receptors are operationally and genomically divided into five subtypes, M_1, M_2, M_3, M_4 and M_5. Despite evidence of an m_5 receptor gene product (Bonner *et al*., 1987; 1988), no isolated tissue response has yet been shown to be mediated by this subtype (Caufield & Birdsall, 1998). In many smooth muscle tissues, mixed populations of muscarinic receptors are present, with both M_2 and M_3 receptors involved in the contractile function. Nonetheless, the precise function remains speculative (see Eglen *et al*., 1997, for review). Moreover, the nature of the muscarinic receptor subtype mediating myometrial contraction, from various species, has yet to be definitively classified.

In guinea-pig uterus, evidence for the presence of muscarinic M_2 (Eglen *et al*., 1989; 1992; Doods *et al*., 1993), M_3 (Leiber *et al*., 1990), M_4 (Dörje *et al*., 1991) and an atypical muscarinic receptor (Boxall *et al*., 1998) has been presented. The rabbit uterus has not been extensively studied but M_2, M_3 and M_4 receptors have been identified using immunoprecipitation techniques (Crankshaw, 1984; Dörje *et al*., 1991). In rat myometrium, muscarinic M_2 and M_3 receptors have been identified (Varol *et al*., 1989; Pennefather *et al*., 1994), and the subtype mediating contraction has been suggested to be atypical (Munns & Pennefather, 1998). By contrast, radioligand binding studies in both guinea-pig (Boxall *et al*., 1998) and rat (Munns & Pennefather, 1998) uterine membranes reveal the presence of only muscarinic M_2 receptors. The muscarinic receptor mediating contraction of human myometrial tissue is not known.

The objective of the present study was to pharmacologically characterize the muscarinic receptor subtype in rat isolated myometrium from both sham operated and ovariectomized rats. Hormonal changes, notably in ovarian steroid levels, modify smooth muscle contractility and the density of the population of muscarinic receptors in the uterus (Fernandez *et al*., 1995; Matucci *et al*., 1996). However, it is unknown if these steroids influence the proportion and nature of the muscarinic receptor subtypes expressed. Therefore, several selective ligands, including several only recently identified ligand, such as tripitramine (M_2-selective), PD 102807 (M_4-selective) and the mamba snake toxin 3 (MTx 3, M_1/M_4-selective) have been used to examine the effects of ovariectomy on muscarinic receptor pharmacology and expression. Apparent affinity estimates determined using tissue bath contractile studies were compared with affinity estimates previously determined at muscarinic receptors stably expressed in CHO cells (Doods *et*

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al., 1993; Eglen *et al*., 1997; Hegde *et al*., 1997) and with apparent antagonist affinity estimates from competition radioligand binding studies using uterine membranes.

Preliminary accounts of these findings have been presented previously to the Eighth International Symposium on subtypes of muscarinic receptors (Choppin *et al*., 1999b) and to the British Pharmacological Society (Choppin *et al*., 1999a).

Methods

Tissue isolation

All uteri were isolated from female, Sprague-Dawley rats (200–300 g), previously euthanized by CO_2 asphyxiation. Two groups of animals were used: first, those animals with the ovaries removed and allowed to recover for 4 months prior to experimentation. Second, animals having undergone sham surgical procedures but otherwise treated in a similar manner.

Radioligand binding studies

Uteri were dissected out, chopped with scissors, 40 ml of buffer added and homogenized for 7 s using a polytron at medium speed. The connective tissue was removed from the suspension and the homogenate was centrifuged at 19,500 r.p.m. for 15 min at 4°C. The supernatant was decanted, discarded and the pellet was resuspended in 20 ml buffer. A second homogenization, using the same parameters, was performed. The assays, performed with 500 μg of protein, were conducted with 1.5 nM [^{3}H]-*N*-methyl scopolamine ([^{3}H]-NMS; specific activity 81 Ci mmol^{-1}) in a final volume of 0.25 ml, 50 mM Tris-HCl, 1 mM EDTA (pH 7.4) at 25°C. Nonspecific binding was determined with 1 μM atropine. Saturation experiments were conducted with eight concentrations of radioligand (0.04–5 nM) and most competition displacement curves were generated with six concentrations of antagonists. To define M_2 and M_3 receptor proportions, 24 concentrations of tripitramine, a muscarinic antagonist with a marked degree of selectivity for M_2 over M_3 receptors (Chiarini *et al*., 1995; Roffel *et al*., 1997), were used. Incubations were carried out to equilibrium (60 min at room temperature). Samples were then rapidly filtered over polyethyleneimine-treated GF/B unifilter plates using a Packard Filtermate Harvester and washed with ice-cold 50 mM Tris/HCl, 1 mM EDTA buffer. Scintillation cocktail was added to each filter plate and bound radioligand determined by liquid scintillation spectrophotometry.

In vitro *contractile studies*

The uterine horns, cleared of adhering adipose tissue, were placed in oxygenated Sund's solution (composition, in mM: NaCl 154.0, KCl 5.6, $CaCl_2$ 0.5, $MgCl_2.6H_2O$ 1.0, $NaHCO_3$ 6.0 and dextrose 2.8) containing indomethacin (10 μM), corticosterone (30 μM) and cocaine (30 μM). Four longitudinal strips (approximately 1 cm) were cut from each horn and were mounted in 10 ml organ baths containing Sund's solution, maintained at 32°C and constantly aerated with 95% O_2/5% CO_2 (pH = 7.4). Grass FT03 transducers were used to measure changes in isometric tension and these were displayed on a Grass 7E polygraph. The tissues were maintained at a resting tension of 0.5–1 g during an equilibration period of 60 min. Tension adjustments were made as necessary and the tissues were washed at 15 min intervals.

The tissues were exposed three times to KCl (30 mM) for 5 min to suppress spontaneous contractions and subsequently washed prior to starting the experimental protocol. After washing, tissues were re-equilibrated for 10 min and allowed to regain baseline tension. Cumulative concentration-effect curves to carbachol, a non-selective muscarinic agonist (1 μM–1 mM), were then constructed in each tissue. Thereafter, tissues were equilibrated in either the absence (time control) or presence of antagonist for a 60 min period during which tissues were washed every 10 min. Subsequently, a second concentration-effect curve to carbachol was constructed. Each tissue was exposed to only one concentration of antagonist.

Data analysis

Data from saturation and competition binding studies were analysed using non-linear curve fitting programs (iterative through least sum of squares). The concentration of antagonist displacing 50% of radioligand (IC_{50}) was determined and converted into pK_i (−log of inhibition dissociation constant) by the method of Cheng & Prusoff (1973).

For functional studies, all contractions were recorded as changes in isometric tension from baseline and normalized to the maximum response of the first agonist concentration-effect curve. Agonist concentration-response curves were fitted using a nonlinear iterative fitting program (Origin, Microcal Software, Inc., Northampton, MA, U.S.A.) according to the relationship of Parker & Waud (1971). Agonist potencies and maximum response were expressed as pEC_{50} (−logarithm of the molar concentration of agonist producing 50% of the maximum response) and E_{max}, respectively. Concentration-ratios (CRs) were determined from EC_{50} values in the presence and absence of antagonist. Apparent antagonist affinities (pK_B values) were determined using one concentration of each antagonist, with the equation described by Furchgott (1972; $pK_B = -\log([\text{antagonist}]/CR-1)$).

Pearson correlation coefficients (r) and associated P values were calculated using the method described by Dixon & Massey (1983). The sum of squares of differences in affinity estimates for each plot ($\Sigma\,(y-x)^2$, noted ssq) defines the proximity of the data points to the line of identity ($y = x$). All results are given as means ± standard error of the mean.

Compounds used

Indomethacin and KCl were obtained from Sigma Chemical Co (MO, U.S.A.). Corticosterone, carbachol, pirenzepine dihydrochloride, methoctramine hydrochloride and para fluoro hexahydrosiladifenidol (p-F-HHSiD) hydrochloride were obtained from Research Biochemicals Inc. (MA, U.S.A.). Tripitramine, himbacine hydrochloride, AF DX 116 [11-((((dimethylamino)-methyl)-1-piperidinyl)acetyl)-5,11-dihydro-6H-pyrido(2,3-b)(1,4)benzodiazepine-6one], PD 102807 (3,6a,11,14-Tetrahydro-9-methoxy-2-methyl-12H-isoquino[1,2-b]pyrrolo[3,2-f][1,3]benzoxazine-1-carboxylic acid ethyl ester) and zamifenacin fumarate were generously provided by Dr C. Melchiorre (University of Bologna, Italy), Professor W.C. Taylor (University of Sydney, Australia), Boehringer Ingelheim (Germany), Dr R. Schwartz (Parke-Davis Pharmaceutical Research, Ann Arbor, MI, U.S.A.) and Dr Wallis (Pfizer Central Research, Sandwich, Kent, U.K.), respectively. Mamba Toxin 3 (MTx 3) was generously provided by Dr Adem (Karolinska Institute, Sweden).

Results

Proportion of muscarinic receptors in the rat uterus

Radioligand binding studies revealed that the affinity (K_D) and total number of specific binding sites (B_{max}) for [^{3}H]-NMS was 0.12 ± 0.05 nM and 44.7 ± 6.3 fmol mg^{-1} in sham rat uterus ($n=3$) and 0.16 ± 0.02 nM and 67.4 ± 6.2 fmol mg^{-1} in ovariectomized rat uterus ($n=4$). These parameters were not significantly different but the sample size was too small for a definitive conclusion.

Table 1 summarizes the competition binding data (apparent antagonist affinity estimates, pK_i) for several muscarinic antagonists. Although some Hill coefficients differed significantly from unity, the analysis indicated a statistical preference for a one-site rather than a two-site fit. Only tripitramine defined two muscarinic binding sites (Figure 1): a high ($pK_i = 9.46 \pm 0.19$ and 9.12 ± 0.16) and a low ($pK_i = 6.77 \pm 0.11$ and 6.76 ± 0.13) affinity site in sham operated and ovariectomized animals ($n=4$), representing 33 ± 8 and $38 \pm 2\%$, and 67 ± 8 and $62 \pm 2\%$ of the total receptor population, respectively. These proportions, likely representing M_2 and M_3 receptors respectively, were not significantly different in the two tissues.

Characterization of muscarinic receptors mediating contractions of the sham operated rat uterus

Carbachol induced concentration-dependent contractions of the sham operated rat uterus ($pEC_{50} = 5.06 \pm 0.05$; $E_{max} = 1.29 \pm 0.10$ g, $n=56$). Time-control experiments showed that two consecutive concentration-effect curves to this agonist could be constructed in the same tissue without any significant temporal change in the agonist potency and maximum response ($pEC_{50} = 4.93 \pm 0.17$; $E_{max} = 1.11 \pm 0.27$ g and $pEC_{50} = 4.70 \pm 0.26$; $E_{max} = 1.26 \pm 0.32$ g for the first and second agonist curves respectively, $n=7$).

Several antagonists (pirenzepine, methoctramine, zamifenacin, AF DX 116, tripitramine, p-F-HHSiD, himbacine, MTx3 and PD 102807) antagonized carbachol-induced responses and the apparent affinities (pK_B) are summarized in Table 2. Cumulative agonist concentration-response curves were surmountably antagonized by these compounds, with parallel rightward displacements (Figure 2a). The rank order of antagonist affinities (pK_B) was: zamifenacin (9.19 ± 0.16), p-F-HHSiD (8.50 ± 0.08), tripitramine (7.23 ± 0.12), himbacine (7.21 ± 0.18), methoctramine (6.79 ± 0.11), pirenzepine (6.48 ± 0.25), AF DX 116 (6.26 ± 0.12), MTx 3 (<7.00) = PD 102807 (<7.00).

A correlation analysis between the affinities of the antagonists at muscarinic receptors in the sham operated rat uterus and the affinities at human recombinant muscarinic receptors (Figure 3) showed a significant correlation ($r=0.89$, $P=0.003$, ssq = 3.60) at m3. By contrast, poor correlations were observed at m1, m2, m4 and m5 ($r=0.34$, ssq = 10.94; $r=-0.01$, ssq = 15.67; $r=-0.12$, ssq = 11.22; $r=0.61$, ssq = 11.79) respectively.

Characterization of muscarinic receptors mediating contractions of the ovariectomized rat uterus

Carbachol produced concentration-dependent contractions of the ovariectomized rat uterus ($pEC_{50} = 5.15 \pm 0.07$; $E_{max} = 1.22 \pm 0.07$ g, $n=47$). No time-dependent changes in agonist sensitivity were observed during the construction of the second curve ($pEC_{50} = 4.79 \pm 0.24$; $E_{max} = 1.31 \pm 0.30$ g and $pEC_{50} = 4.60 \pm 0.14$; $E_{max} = 1.24 \pm 0.28$ g for the first and second agonist curves, respectively, $n=6$). As before, pharmacological characterization of the muscarinic receptor involved was undertaken by determination of antagonist affinities.

Concentration-effect curves to carbachol were surmountably antagonized by pirenzepine, methoctramine, zamifenacin,

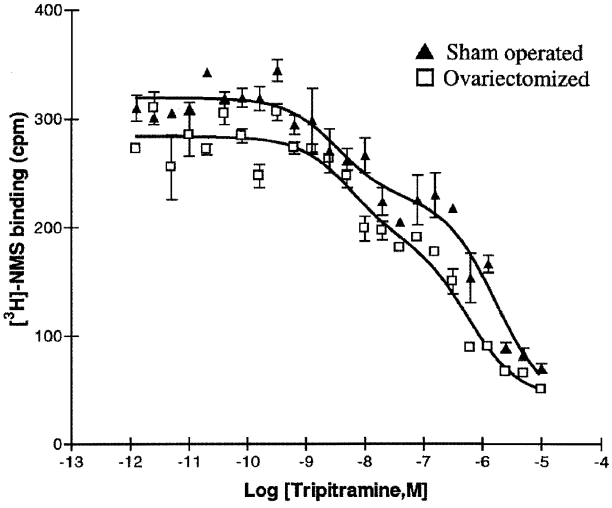

Figure 1 Competition by tripitramine with [^{3}H]-NMS to uterine membranes from sham operated (closed triangle) or ovariectomized (open square) rats. The values shown are means ± s.e.mean, $n=2-4$.

Table 1 Binding affinities (pK_i values) and Hill slopes (n_H) of muscarinic antagonists in sham operated and ovariectomized rat uterus and in oestrogen-treated and late pregnant rat uterus

	Sham operated rat uterus		*Ovariectomized rat uterus*		*Oestrogen-treated rat uterus*		*Late pregnant rat uterus*	
Antagonist	pK_i	n_H	pK_i	n_H	pK_i	n_H	pK_i	n_H
Pirenzepine	6.39 ± 0.06	1.34 ± 0.35	6.41 ± 0.09	0.89 ± 0.05	6.17 ± 0.06	0.91 ± 0.09	6.18 ± 0.06	0.91 ± 0.08
Methoctramine	6.88 ± 0.14	0.86 ± 0.34	7.39 ± 0.16	1.17 ± 0.37	7.52 ± 0.14	0.93 ± 0.08	7.95 ± 0.09	0.77 ± 0.04
Zamifenacin	8.22 ± 0.04	0.65 ± 0.03*	7.88 ± 0.02	1.04 ± 0.07				
AF DX 116	5.68 ± 0.21	0.63 ± 0.14*	5.94 ± 0.23	0.85 ± 0.11	6.77 ± 0.01	0.98 ± 0.12	6.72 ± 0.06	0.90 ± 0.03
Tripitramine	7.26 ± 0.15	†	7.80 ± 0.48	†				
p-F-HHSiD	7.25 ± 0.07	0.66 ± 0.07*	7.23 ± 0.07	0.88 ± 0.05				
Himbacine	7.53 ± 0.23	0.87 ± 0.11	7.49 ± 0.15	0.93 ± 0.40	7.83 ± 0.04	1.02 ± 0.07	7.71 ± 0.09	1.09 ± 0.07

Values shown are means ± s.e.mean, $n=3-6$. *Hill slopes significantly different from unity. †Displacement curve resolved with two components ($pK_{iH} = 9.46 \pm 0.19$ and 9.12 ± 0.16, $pK_{iL} = 6.77 \pm 0.11$ and 6.76 ± 0.13 in sham operated and ovariectomized rat uterus, respectively. Affinity values in oestrogen-treated and late pregnant rat uterus were taken from Munns & Pennefather (1998).

Table 2 Binding affinities (pK_i values) of muscarinic antagonists at human recombinant muscarinic receptors (m1–m5), affinity values (pK_B) in sham operated and ovariectomized rat uterus, and pA_2 values in oestrogen-treated and late pregnant rat uterus

Antagonist	*M1* (pK_i)	*M2* (pK_i)	*M3* (pK_i)	*M4* (pK_i)	*M5* (pK_i)	*Sham operated rat uterus* (pK_B)	*Ovariectomized rat uterus* (pK_B)	*Oestrogen-treated rat uterus* (pA_2)	*Late pregnant rat uterus* (pA_2)
Pirenzepine	8.04	6.28	6.80	6.98	6.90	6.48±0.25	7.21±0.29	7.26±0.29	6.92±0.28
Methoctramine	6.55	7.56	6.11	6.85	6.43	6.79±0.11	7.49±0.18*	8.49±0.26	8.01±0.25
Zamifenacin	7.50	7.13	7.90	6.67	7.35	9.19±0.16	9.18±0.24		
AF DX 116	6.27	7.09	5.68	6.39		6.26±0.12	6.61±0.21	7.36±0.27	7.73±0.22
Tripitramine	8.40	9.38	7.09	7.80	7.31	7.23±0.12	7.54±0.10		
p-F-HHSiD	7.33	6.56	7.51	7.24	6.73	8.50±0.08	9.06±0.13*		
Himbacine	6.62	7.93	6.90	7.44	6.11	7.21±0.18	7.41±0.31	8.73±0.22	8.37±0.21
MTx 3	6.02	<5.40	<5.40	8.25	5.68	<7.00	<7.00		
PD 102807	5.60	5.90	6.80	7.50	5.70	<7.00	<7.00		

Values shown are means±s.e.mean, $n=3-7$. *Values in columns 7 and 8 are significantly different ($P<0.01$). Binding data were taken from Doods *et al.* (1993), Eglen *et al.* (1997) and Hegde *et al.* (1997). Affinity values were taken from Munns & Pennefather (1998).

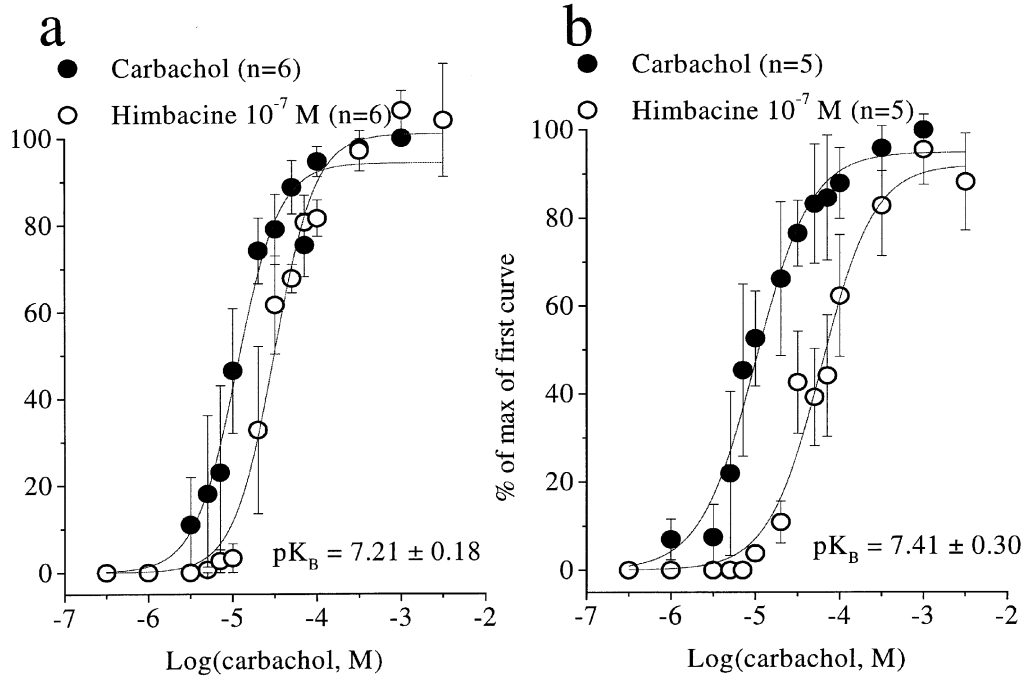

Figure 2 Effects of himbacine on the cumulative concentration-response curves of carbachol (a) on the sham operated rat uterus and (b) on the ovariectomized rat uterus. Contractile effects were expressed as percentages of the maximum response of the control curve. The values shown are means±s.e.mean, $n=5-6$ animals.

AF DX 116, tripitramine, p-F-HHSiD, himbacine, MTx3 and PD 102807 (pK_B are summarized in Table 2). As an example, the effect of himbacine on the cumulative concentration-response curve to carbachol on the ovariectomized rat uterus is shown in Figure 2b.

The most significant correlation between the affinities of antagonists at muscarinic receptor in ovariectomized rat uterus and the affinities at human recombinant receptors was obtained at m3 receptors ($r=0.86$, $P=0.006$, ssq=7.54). In contrast, the correlation was less favourable at the other subtypes ($r=0.39$, ssq=13.38; $r=-0.06$, ssq=17.18; $r=-0.11$, ssq=11.20; $r=0.65$, ssq=18.58 at m1, m2, m4 and m5, respectively (Figure 4)).

Comparison between functional data in the sham operated and ovariectomized rat uterus

When the affinities of antagonists (pA_2; Table 2) in the sham operated rat uterus were compared with the affinities in the ovariectomized rat uterus, a highly significant correlation ($r=0.97$, $P<0.0001$) was obtained, close to the line of identity (ssq=0.97; Figure 5).

Discussion

Previous studies have attempted to characterize the pharmacological nature of muscarinic receptors mediating contraction of guinea-pig isolated uterus (Eglen *et al.*, 1989; 1992; Leiber *et al.*, 1990; Dörje *et al.*, 1991; Doods *et al.*, 1993; Boxall *et al.*, 1998). The most recent study suggests that the contraction is mediated by an atypical muscarinic receptors. Radioligand binding studies using membranes of rat isolated myometrium indicate that muscarinic M_2 receptors form a homogenous population (Pennefather *et al.*, 1994). Operational studies characterizing the muscarinic receptor mediating inositide phospholipid hydrolysis (Varol *et al.*, 1989) or contraction (Munns & Pennefather, 1998) suggest the presence of

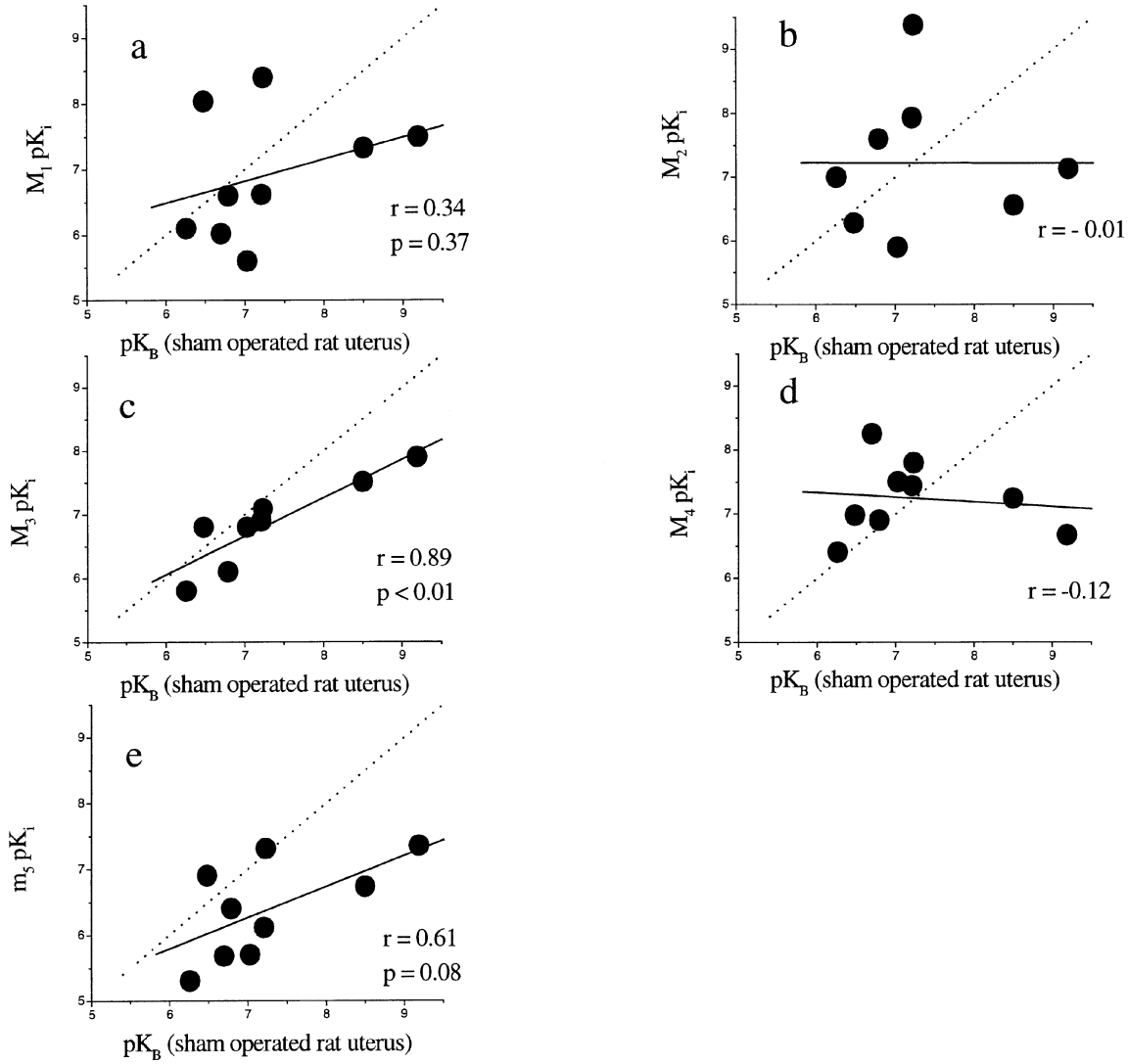

Figure 3 Correlation between the functional affinities (pK_B values) of muscarinic antagonists at muscarinic receptor in sham operated rat uterus and binding affinities (pK_i values) at human recombinant muscarinic receptors (m1–m5; a–e respectively). The binding data were taken from Doods *et al.* (1993), Eglen *et al.* (1997) and Hegde *et al.* (1997). The broken line is the line of identity (x = y) while the solid line is the correlation plot (the inserts give the correlation factors (*r*) and the sum of squares values (ssq)).

muscarinic M_3 or an atypical receptor, respectively. At present, therefore, it may be that the receptor is atypical as has been reported for guinea-pig uterus (Boxall *et al.*, 1998). Alternatively, it may be that the compounds used to characterize the receptor were not sufficiently preferential for one muscarinic receptor subtype.

The present study has examined the pharmacological characteristics of muscarinic receptors in rat isolated myometrium using several novel selective muscarinic antagonists. A secondary aim was to compare the effects of ovariectomy on the pharmacology of the muscarinic receptors present.

Sham operated rat uterus

Competition binding studies in uterine tissue have shown that more than one muscarinic receptor subtype is present in rat myometrium. The apparent affinity (pK_i) values obtained in competition binding experiments with the muscarinic antagonists were intermediate to the affinity expected at either muscarinic M_2 or M_3 receptors (Table 1). Moreover, the most selective compounds (zamifenacin, AF DX 116 and p-F-HHSiD) indicated the presence of M_3 receptors ($pK_i = 8.22$, 5.68 and 7.25, respectively) although a second population cannot be excluded (Hill slopes were significantly different from unity). The displacement curve for tripitramine revealed two components: high ($pK_{iH} = 9.46 \pm 0.19$, representing $33 \pm 8\%$ of the receptors) and low ($pK_{iL} = 6.77 \pm 0.11$, $67 \pm 8\%$) binding sites in sham operated rat tissue. These sites most probably represent muscarinic M_2 and M_3 receptors, respectively and illustrate the utility of this antagonist in defining these two receptor subtypes (Figure 1). Collectively, the most significant correlation between apparent affinity estimates (pK_i) in sham operated rat uterus membranes with those determined at recombinant muscarinic receptors was obtained with the M_3 subtype ($r = 0.84$, ssq = 1.39).

These observations disagree with the conclusion of Munns & Pennefather (1998) who reported affinities consistent with the presence of a single M_2 receptor population ($pK_i = 7.52$, 6.77 and 7.83 for methoctramine, AF DX 116 and himbacine, respectively). However, selective antagonists such as zamifenacin and tripitramine (that discriminates muscarinic M_2/M_3 receptors by approximately two orders of magnitude) enabled

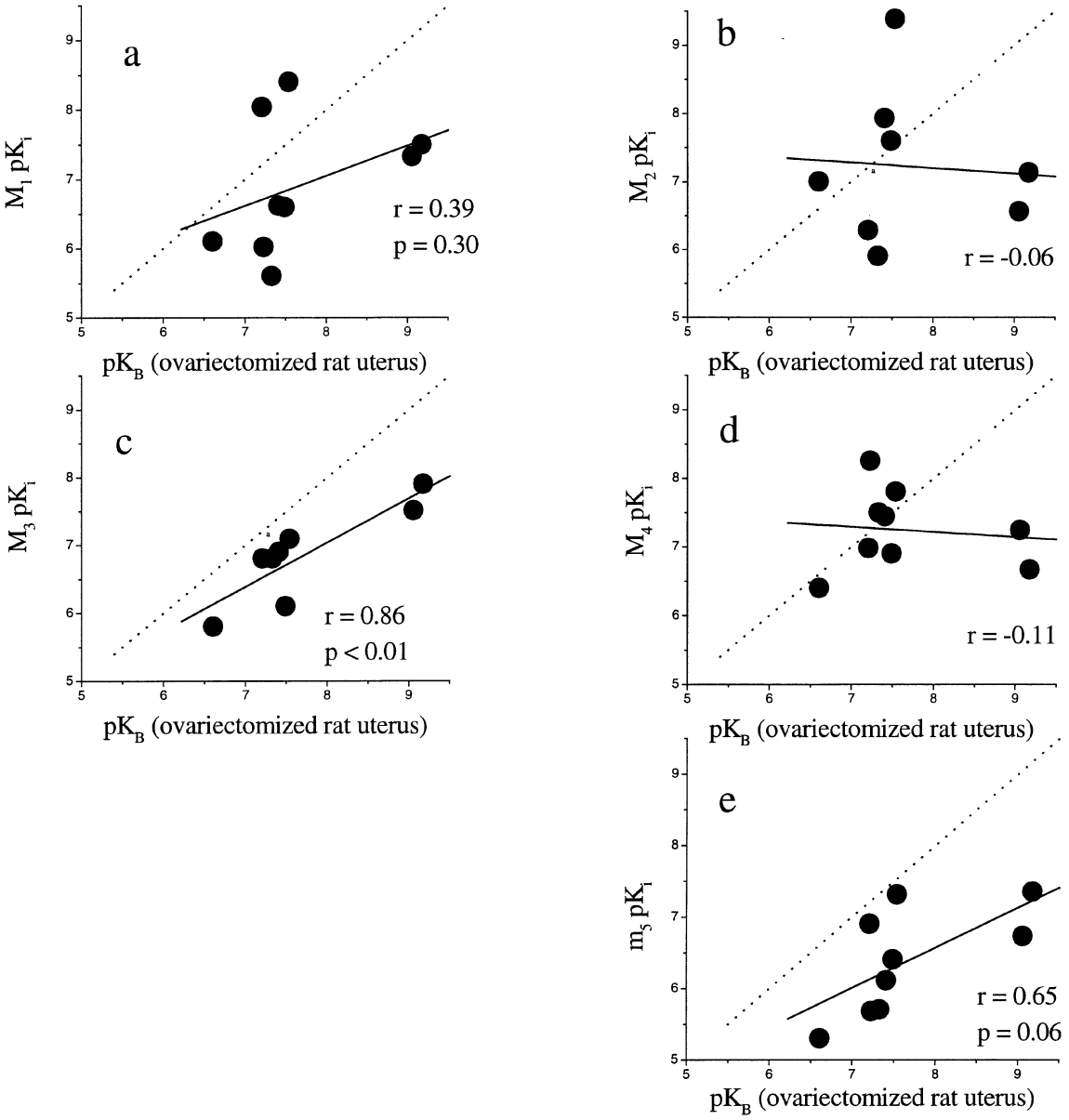

Figure 4 Correlation between the functional affinities (pK_B values) of muscarinic antagonists at muscarinic receptor in ovariectomized rat uterus and binding affinities (pK_i values) at human recombinant muscarinic receptors (m1–m5; a–e respectively). Binding data were taken from Doods *et al.* (1993), Eglen *et al.* (1997) and Hegde *et al.* (1997). The broken line is the line of identity (x = y) while the solid line is the correlation plot (the inserts give the correlation factors (*r*) and the sum of squares values (ssq)).

identification of two receptor populations. In guinea-pig isolated uterus, Boxall *et al.* (1998) were unable to detect a second muscarinic population ($pK_i = 7.6$ and 9.3 for zamifenacin and tripitramine, respectively; Hill slopes were not different from unity). These discrepancies could relate to species differences or the presence of a homogeneous population of atypical muscarinic receptors.

Carbachol produced concentration-dependent contractions that were antagonized in a competitive fashion by several muscarinic antagonists. Collectively, these data indicate that muscarinic M_3 receptors mediated the contraction. The low apparent affinity (pK_B) obtained for pirenzepine (6.48) excludes involvement of muscarinic M_1 receptors. The apparent affinity values for methoctramine (6.79), AF DX 116 (6.26) and himbacine (7.21) suggest the presence of either a muscarinic M_4 or M_2 receptor. In contrast, the apparent affinity for tripitramine ($pK_B = 7.23$), was 100 fold lower than its affinity at human recombinant M_2 receptors, thus questioning a role of the latter in the contraction. The relatively low affinity estimates of MTx 3 and PD 102807 (<7.00) argue against activation of a muscarinic M_4 receptor. p-F-HHSiD (Eglen *et al.*, 1990) and zamifenacin (Watson *et al.*, 1995) have been previously suggested to differentiate between muscarinic M_3 receptors in different smooth muscles. In the present study, however, both compounds showed subnanomolar affinity in the rat isolated uterus ($pK_B = 9.19 \pm 0.16$ and 8.50 ± 0.08 for zamifenacin and p-F-HHSiD, respectively) and suggest that the M_3 receptor resembles that seen in tissues such as the rat urinary bladder (Choppin *et al.*, 1997). Collectively, these data support the exclusive role of a muscarinic M_3 receptor in the contraction, even though

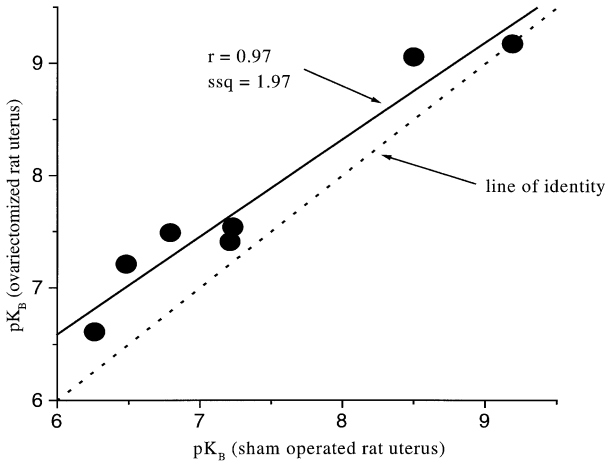

Figure 5 Comparison of the pK_B values of muscarinic receptor antagonists at receptors in sham operated rat uterus and in ovariectomized rat uterus. The broken line is the line of identity (x = y) and the solid line is the correlation plot (the correlation factors (*r*) and the sum of squares values (ssq) are indicated).

both muscarinic M_2 and M_3 receptors are expressed in the tissue.

In support of this conclusion, a significant correlation between affinity estimates with those determined in radioligand binding studies was obtained with human M_3 recombinant muscarinic receptors (apparent affinities (pK_i): pirenzepine 6.80; methoctramine 6.11; zamifenacin 7.90; AF DX 116, 5.80; tripitramine 7.09; p-F-HHSiD 7.51; himbacine 6.90, MTx 3, <5.4 and PD 102807, 6.80; $r=0.89$, ssq = 3.60; Doods *et al*., 1993; Eglen *et al*., 1997; Hegde *et al*., 1997). These data further support the involvement of muscarinic M_3 receptors in the contractile response to carbachol.

Ovariectomized rat uterus

Radioligand binding experiments indicated that the binding profile was similar and that the proportions of sites were not significantly different in tissue from ovariectomized animals in comparison to those having undergone sham surgery. Thus, a population of muscarinic M_2 receptors comprises 38% and a majority of M_3 receptors (62%), as determined by tripitramine binding (high and low affinity binding site, respectively). The corresponding apparent pK_i values of muscarinic antagonists were similar to those obtained in sham operated rat uterus (the correlation parameters between these data were: $r=0.92$; ssq = 0.74), except for methoctramine and zamifenacin (3 fold difference). However, the only antagonist indicating the presence of two binding sites was tripitramine, since none of the other compounds showed a Hill slope different from unity. This is probably attributable to its greater than 250 fold muscarinic M_2/M_3 receptor selectivity.

Carbachol was equipotent in mediating contraction of tissues isolated from sham operated and ovariectomized rats. Using the same series of muscarinic receptor antagonists described above, only pirenzepine and methoctramine demonstrated a 5 fold difference between pK_B values in sham operated and ovariectomized rat uterus (the same difference was observed in binding data) and the pharmacological profile obtained in ovariectomized rat tissue was similar in that the apparent affinity values significantly correlated with the binding affinities at muscarinic M_3 receptors, again reflecting the activation of an M_3 receptor population.

A significant correlation ($r=0.97$, ssq = 1.97) was observed between the apparent affinity estimates in the sham operated and ovariectomized rat tissues. Taken together, these data strongly suggest that ovariectomy did not influence the function of uterine muscarinic receptors. Thus, we were unable to detect any influence of the withdrawal of ovarian steroids on the proportions of muscarinic M_2/M_3 receptors, nature of the subtype mediating contraction or agonist potency.

It is a common finding that muscarinic M_3 receptors mediate smooth muscle contraction despite a predominance of muscarinic M_2 receptors (see Eglen *et al*., 1997 for review). However, radioligand binding studies in rat isolated myometrium (this study) indicated that muscarinic M_2 receptors form only 30–40% of the total population, with M_3 receptors forming the majority. These data contrast to those of Munns & Pennefather (1998) who demonstrated binding to a single population of muscarinic receptors which they determined to be of the M_2 subtype. Similar findings were reported by Boxall *et al*. (1998). The reason for the difference between these two studies is unclear, although it may reflect the use of different ligands to define M_2 and M_3 receptors. Indeed, in our hands tripitramine has proven to be a highly sensitive ligand to reveal even small (approximately 10%) populations of low affinity binding sites, presumably M_3 receptors (Choppin *et al*., 1998). Therefore, it is a suitable probe to detect even minor changes in M_2 and M_3 receptor proportions under different conditions.

The present study has shown that the pharmacological antagonist profiles (using binding and functional experiments) in tissues from both sham operated and ovariectomized rat isolated uterus equate most closely with the muscarinic M_3 muscarinic receptor. Only tripitramine competition radioligand binding experiments were able to highlight a small M_2 population. Overall, ovariectomy had no effect on the receptor density, proportion of sites or on muscarinic M_3 function in uterine tissue.

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