Influence of oestrogens on muscarinic receptor density and contractile response in the guinea-pig uterus

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The objective of this study was to investigate the contractile response *in vitro* of uterine and myometrial strips from control and oestrogen-treated guinea-pigs to carbachol. In addition, the muscarinic receptor parameters in subcellular preparations from the myometrium of the same animals were evaluated. Strips of guinea-pig uterus contract in response to carbachol in a concentration dependent manner and the effect of carbachol is independent of the presence of an intact endometrium. However, treatment with oestrogen reduces the response to carbachol, when expressed with respect to the effect of KCl. Measurement of the specific binding of [³H]quinuclidinyl benzylate ([³H]QNB) to myometrial membranes from control and oestradiol-treated immature guinea-pigs showed that oestradiol significantly decreased the number of specific binding sites of [³H]QNB, but did not change the apparent affinity of specific [³H]QNB binding. Moreover, the apparent affinity constants for the different antagonists showed a close similarity to those obtained at muscarinic receptors present in the myometrium from control and oestradiol-treated guinea-pigs.

Introduction

Uterine activity is regulated by complex and mutual interactions among sex steroids, neurohypophyseal hormones and neurotransmitters (Maggi *et al.*, 1994a, b). Oestrogens increase the myometrial responsiveness to neurohypophyseal hormones (Maggi *et al.*, 1988), endothelin-1 (Maggi *et al.*, 1991), serotonin (Ichida *et al.*, 1983) and α -adrenergic agonists, without affecting the β -adrenergic response (Roberts *et al.*, 1977). Hence, it is generally accepted that oestrogens sensitize the myometrium to the effect of uterotonic substances. The effect of oestrogen, which alters sensitivity to hormones and neurotransmitters, is apparently mediated by a specific upregulation of the related receptors.

Cholinergic drugs stimulate uterine contraction in several mammals, including rats (Ichida *et al.*, 1983, 1984; Leiber *et al.*, 1984; Varol *et al.*, 1989), rabbits (Riemer *et al.*, 1987a) and guinea-pigs (Garris *et al.*, 1984; Marc *et al.*, 1986). Their effect is mediated by specific interactions with muscarinic receptors present on smooth muscle fibres (Crankshaw, 1984). In guinea-pig myometrium, muscarinic receptors are coupled to an increase in inositol phosphate turnover (Marc *et al.*, 1986) and apparently belong to the M₂ receptor subtype (Eglen *et al.*, 1989; Doods *et al.*, 1993). Conversely, in rat myometrium the M₃ receptor subtype is predominant (Varol *et al.*, 1989).

Although the uterus is relatively sensitive to cholinergic stimulation, little is known about the effect of oestrogen on muscarinic receptors. Treatment with oestrogen increases

(Parkington and Lipton, 1976), decreases (Garris *et al.*, 1984) or does not affect (Riemer *et al.*, 1987b) cholinergic responsiveness in the uterus. This apparent discrepancy could be the result of species-related differences or to differential expression of muscarinic receptor subtypes. Because myometrial muscarinic receptors in guinea-pigs have been extensively characterized using radioligand and cDNA probes (Eglen *et al.*, 1989; Doods *et al.*, 1993), we investigated the effect of administration of oestrogen in guinea-pigs. We evaluated whether subacute oestrogen treatment affects muscarinic receptor density, ligand specificity or myometrial responsiveness. Our results suggest that oestrogen inhibits the expression of muscarinic receptors without changing the subtype of muscarinic receptor expressed.

Materials and Methods

Materials

17 β -Oestradiol-3-benzoate, benzamidine hydrochloride, trypsin inhibitor (type I-S from soybean), DL-dithiothreitol, bacitracin zinc salt and S-(–)-hyoscyamine were obtained from Sigma Chemical Company, St Louis, MO. [³H]Quinuclidinyl benzylate ([³H]QNB, specific activity 32.9 Ci mmol^{–1}) was from New England Nuclear, Boston, MA. Hexahydro-sila-difenidol hydrochloride *p*-fluoro analogue (*p*-F-HHSiD), ($\pm$)-quinuclidinyl- α -hydroxydiphenyl acetate (QNB) and 4-diphenylacetoxy-*N*-methylpiperidine methiodide (4-DAMP) were from Research Biochemicals Inc., Natick, MA. Pirenzepine

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hydrochloride and 11-[[2-[(diethylamino)methyl]-1-piperidiny]-acetyl]5,11-dihydro-6H-pyrido[2,3-b][1,4]benzodiazepine-6-one (AF-DX116) were obtained from Thomae, Biberach. Methoctramine tetrahydrochloride was a generous gift from C. Melchiorre, Bologna. Telenzepine hydrochloride was kindly donated by Byk Gulden Italia (Milan). *R*-(+)-hyoscyamine was prepared in the Department of Pharmaceutical Sciences of the University of Florence, according to the method described by Gualtieri *et al.* (1991). All other chemicals were of analytical grade and were purchased from commercial sources.

Animals

Virgin immature female Dunkin–Hartley guinea-pigs (approximately 200 g body mass, 4–5 weeks old) were obtained from Rodentia (Torre Pallavicina, Bergamo). They were housed six to a cage with free access to food and water and maintained in a 12 h light:12 h dark cycle at an ambient temperature of 22°C.

17 β -Oestradiol-3-benzoate (40 μ g per 200 g) or vehicle were administered once a day for 4 consecutive days and animals were killed on the following day. Steroid hormones were dissolved in absolute ethanol, brought to a final concentration of 1.5% (v/v) ethanol in peanut oil, and injected s.c.

This investigation conformed to the rules for the care and use of laboratory animals of the European Community (86/609/CEE).

Recording of mechanical activity

Strips of guinea-pig uterus (2 mm wide, 15 mm long) were obtained from immature control or oestradiol-treated guinea-pigs. The endometrium was carefully removed by rubbing with gauze; strips were suspended in an organ bath under 1 g resting tension, and superfused with Sund's physiological solution of the following composition (mmol l⁻¹): NaCl 154, KCl 5.63, CaCl₂ 0.54, MgCl₂ 0.95, NaHCO₃ 5.95, glucose 2.78. The solution was oxygenated with 95% O₂ and 5% CO₂ and maintained at 30°C. Isometric contractions were recorded by means of an isometric transducer on a chart polygraph (Battaglia Rangoni KV 135). After an equilibration period of at least 60 min, during which the solution was replaced every 15 min, cumulative concentration–response curves to carbachol were obtained. Since the guinea-pig uterus showed a loss of sensitivity in successive curves of carbachol, only one concentration–response curve was determined in a single preparation. The response to 60 mmol KCl l⁻¹, a nonspecific activator of contraction, was determined for each strip, 15–30 min after washing out the carbachol. The response to carbachol was evaluated as net increase in force, measured as mg and as percentage response in comparison with the contractile effect of KCl (E-carbachol: E-KCl).

Membrane preparation

Uteri were removed, washed in saline solution and freed from fat and loosely bound connective tissue. For binding studies, the myometrium was prepared free of endometrium by scraping with the handle of the scalpel (Riemer *et al.*, 1986).

Membranes were prepared as described by Maggi *et al.* (1988). Myometrium was weighed and minced with fine scissors in 5 volumes of buffer 1 (10 mmol Tris–HCl l⁻¹, pH 7.4, containing 1.5 mmol EDTA l⁻¹, 0.5 mmol dithiothreitol l⁻¹, 1 mmol benzamidine l⁻¹, 0.01% (w/v) bacitracin, 0.002% (w/v) soybean trypsin inhibitor). Thereafter it was homogenized with an Ultra-Turrax apparatus, filtered through two layers of cheese cloth and then homogenized again using a glass–teflon homogenizer. The homogenate was centrifuged at 1000 g for 10 min and the pellets were discarded; the supernatants were then centrifuged at 105 000 g for 45 min. Pellets from this spin were dispersed in buffer 2 (50 mmol Tris–maleate l⁻¹, pH 7.6, containing 10 mmol MgSO₄ l⁻¹, 1 mmol benzamidine l⁻¹, 0.01% (w/v) bacitracin and 0.002% (w/v) soybean trypsin inhibitor) with a glass–teflon homogenizer and centrifuged again at 105 000 g for 45 min.

The final pellet was resuspended in buffer 2 to give protein concentrations of 2–5 mg ml⁻¹ and stored frozen at –80°C in small fractions until used for ligand binding studies.

The protein concentration of each membrane fraction was determined using the Pierce protein assay reagent (Pierce Chemicals, Rockford, IL), based on the method of Bradford with BSA as standard (Bradford, 1976).

The DNA content was measured according to the method of Labarca and Paigen (1980).

Cholinergic receptor binding assays

All binding assays were performed in triplicate in buffer 2 using [³H]QNB with a specific activity of 32.9 Ci mmol⁻¹. Incubations were carried out in a final volume of 300 μ l at 25°C for 30 min, unless otherwise indicated.

In homologous competition curves [³H]QNB was present at 0.2 nmol l⁻¹ in tubes containing increasing concentrations (0.3–10 000 nmol l⁻¹) of unlabelled QNB, and at 0.01–0.2 nmol l⁻¹ in tubes without unlabelled ligand. In the heterologous competition curves fixed (0.2 nmol l⁻¹) concentrations of the tracer were displaced by increasing concentrations of several unlabelled ligands (1–100 000 nmol l⁻¹). Muscarinic receptor subtypes were characterized by examining [³H]QNB binding in the presence of several muscarinic antagonists such as the M₁ selective antagonists, pirenzepine and telenzepine, the M₂ selective antagonists, AF-DX116 and methoctramine, and 4-DAMP and p-F-HHSiD, which exhibit high affinity for the M₃ muscarinic receptor.

After incubation, the reaction was stopped using 5 ml 50 mmol Tris–HCl l⁻¹ cold buffer, pH 7.4; the reaction mixture was then drawn under vacuum through GF/B filter papers using a Brandel M-48 cell harvester. The glass-fibre filter papers were presoaked in 0.1% (w/v) polyethylenimine to reduce nonspecific binding to the filters. Then the filters were washed twice with 5 ml ice-cold buffer and partially dried under vacuum. The filter papers were placed in polyethylene vials with 4 ml Filter-Count (Packard Instrument, Groningen) and the amount of radioactivity recorded in a TRI-CARB 1900TR (Packard).

Histology

Uterine and myometrial specimens derived from control and oestradiol-treated guinea-pigs were fixed by immersion in Bouin's solution. After fixation, the tissues were embedded in paraffin wax and sections of about 5 μm thickness were cut. Paraffin wax was removed from the sections, and they were stained with haematoxylin-eosin and observed and photographed using a Nikon MICROPHOT-FX microscope (Nikon, Kogaru, Tokyo).

Statistical analyses

Data were analysed by the weighted least-squares iterative curve fitting LIGAND program (Munson and Rodbard, 1980) to obtain the dissociation constants (K_i) of the six muscarinic antagonists. The ALLFIT program (De Lean *et al.*, 1978) was used for the analysis of sigmoidal dose-response curves obtained in the binding studies. This program uses the constrained four-parameter logistic model to obtain the half-maximal ineffective concentration (IC_{50}) values and the logit-log slope (pseudo Hill coefficient, nH).

pD_2 values of carbachol ($-\log$ of the half-maximal effective concentrations) were evaluated according to the GRAPHPAD program, edited by Harvey Motulsky (University of San Diego, 1985).

All statistically significant differences were assessed using Student's t test, and $P < 0.05$ was considered significant. All values quoted are means $\pm$ SEM.

Results

Effect of the injection of oestradiol

An increase in uterine wet mass was observed in tissue from oestradiol-treated animals compared with controls. In fact, 40 μg oestradiol per 200 g of body mass daily for 4 days caused more than a 300% increase in the wet mass of the uteri compared with the control group (control: 218 ± 17 mg, $n = 22$; oestradiol-treated: 676 ± 24 mg, $n = 19$, $P < 0.001$). In a limited number of guinea-pigs, there was a significant decrease in the DNA:protein ratio (control: 0.85 ± 0.06 , $n = 5$, oestradiol-treated: 0.46 ± 0.09 , $n = 5$, $P < 0.001$).

Histological studies

Histological sections of myometrial preparations confirmed the presence of two distinct muscular layers (longitudinal and circular), free of endometrium, both in control and in oestradiol-treated animals (Fig. 1c, d). Morphological details of intact uterine tissues are shown (Fig. 1a, b).

Contractility studies

In uterine strips derived from immature guinea-pigs, carbachol (50 nmol l^{-1} – $10 \mu\text{mol l}^{-1}$) produced a concentration-dependent increase in contraction. The maximum effect was obtained with $10 \mu\text{mol carbachol l}^{-1}$, and consisted of a net increase in tension of 2023 ± 223 mg. In the

same preparations, $60 \text{ mmol KCl l}^{-1}$ produced a contraction of 863.0 ± 105.1 mg. The concentration-response curve to carbachol, expressed as the ratio of different responses to carbachol to the response to $60 \text{ mmol KCl l}^{-1}$ (E-carbachol:E-KCl), is shown Fig. 2). The pD_2 value for carbachol is 6.49 ± 0.07 . Similar results were obtained in endometrium-free uterine strips (data not shown). In these preparations, the maximum response to carbachol was 1738 ± 280 mg, and the E-carbachol:E-KCl ratio was 2.34 ± 0.08 .

Strips obtained from oestrogen-treated guinea-pigs contracted at higher concentrations of carbachol, since the first concentration of carbachol that could induce a measurable response was $0.2 \mu\text{mol l}^{-1}$. The net increases in tension produced by $10 \mu\text{mol carbachol l}^{-1}$ and by $60 \text{ mmol KCl l}^{-1}$ were 3357 ± 166.3 mg and 2308 ± 88 mg, respectively. Hence, the concentration-response curve is expressed as E-carbachol:E-KCl. The pD_2 value in these preparations was 6.37 ± 0.11 (Fig. 2). Again, removal of endometrium in oestrogen-treated animals did not modify either the response to carbachol or to KCl. The effect of $10 \mu\text{mol carbachol l}^{-1}$ was a contraction of 3395 ± 259.8 mg and the effect of KCl was a contraction of 2430 ± 243.8 mg.

Radioligand binding studies

The specific binding of [^3H]QNB to guinea-pig myometrial membranes was linear with protein concentrations ranging from 20–270 μg per tube (data not shown), with a correlation coefficient of 0.999. For binding experiments, a membrane protein concentration of about 60 μg per tube was chosen.

Binding reactions of [^3H]QNB to myometrial membranes from control and oestradiol-treated guinea-pig uteri were time and temperature dependent. The time course of [^3H]QNB specific binding revealed a rapid rise in [^3H]QNB binding, which reached a plateau at 5–10 min at 25 and 37°C, while 120 min was not enough to reach the plateau if the membranes were incubated at 0–4°C. The results of time and temperature dependence of [^3H]QNB binding (2 nmol l^{-1}) to membranes derived from control and oestradiol-treated guinea-pigs, respectively, are shown (Fig. 3).

Scatchard analysis of [^3H]QNB saturation curves indicated a linear relationship in both cases, suggesting the presence of a single class of high affinity, high capacity binding sites. A representative Scatchard plot (Fig. 4) shows that oestradiol treatment greatly affected binding capacity, without changing affinity. Indeed in five separate experiments, the B_{max} was significantly blunted in oestradiol-treated uteri ($1390.2 \pm 63.9 \text{ fmol mg}^{-1}$ protein versus $701.9 \pm 33.2 \text{ fmol mg}^{-1}$ protein, $P < 0.001$), while the K_d value was unchanged ($0.29 \pm 0.04 \text{ nmol l}^{-1}$ versus $0.22 \pm 0.02 \text{ nmol l}^{-1}$).

The [^3H]QNB binding was inhibited by pirenzepine and telenzepine (M_1 selective antagonists; Hammer *et al.*, 1980; Hammer and Giachetti, 1982), AF-DX116 and methoctramine (M_2 selective antagonists; Melchiorre *et al.*, 1987; Micheletti *et al.*, 1987) and 4-DAMP and p-F-HHSiD (M_3 selective antagonists; Barlow *et al.*, 1976; Lambrecht *et al.*, 1989).

All different antagonists tested displaced [^3H]QNB with the same high affinity in membranes from both control and

Fig. 1. Photomicrographs of uterine tissue from (a) immature and (b) oestradiol-treated guinea-pigs; myometrial uterine tissue after endometrium removed from (c) immature and (d) oestradiol-treated guinea-pigs are shown (haematoxylin–eosin stained). Scale bars represent 0.16 mm.

oestradiol-treated animals. Computer analysis of competition curves was performed using the method previously described (ALLFIT) and no statistical differences appear between the

parameters of the sigmoidal dose–response curves obtained in binding using the same substance in the two myometrial preparations.

Fig. 2. Isometric contractile response to carbachol. Uterine strips from control (●) and oestradiol-treated (○) guinea-pigs were incubated in Sund's solution at 30°C in the presence of the indicated concentrations of carbachol. The data are means of the pooled responses from at least 15 uterine strips, expressed as the ratio of the response to carbachol: to the response to 60 mmol KCl l⁻¹. No error bars are shown, because the errors were within the size of the symbols.

Fig. 3. Time and temperature dependence of specific [³H]quinclidinyl benzylate ([³H]QNB) binding to membranes prepared from control (closed symbols) and oestradiol-treated (open symbols) guinea-pigs. Myometrial membranes (60 µg per tube) were incubated with 2 nmol [³H]QNB l⁻¹ with or without 10 000 nmol l⁻¹ of the unlabelled QNB (to measure nonspecific binding) for different durations at 0–4°C (●, ○), 25°C (■, □) and 37°C (▲, △).

Fig. 4. Equilibrium binding of quinclidinyl benzylate ([³H]QNB) to myometrial membranes prepared from control (●) and oestradiol-treated (○) guinea-pigs. Membranes were incubated at 25°C for 60 min with fixed concentrations (0.01–0.2 nmol l⁻¹) of [³H]QNB with or without increasing concentrations of unlabelled QNB (0.3–10 000 nmol l⁻¹); this plot was derived from the fit of two typical curves and each point is the mean of triplicate determinations.

Typical examples of multiple competition curves for [³H]QNB obtained with the myometrial membranes of control and oestradiol-treated animals are shown (Fig. 5).

Figure 6 summarizes the relative potencies of the different ligands for [³H]QNB binding to control and oestradiol-treated guinea-pig myometrium. The abscissa shows the *p*K_i value (–log of the K_i) for the control membranes, while the ordinate shows the *p*K_i for the membranes from oestradiol-treated animals. A linear correlation between the *p*K_i values plotted yielded an *r* value of 0.99.

Discussion

This study indicates that biologically active muscarinic receptors are present in the guinea-pig uterus. In addition, it demonstrates for the first time an oestradiol-induced decrease in the number and biological activity of these receptors. Indeed oestrogens stimulate a twofold decrease in [³H]QNB binding, when expressed per mg of protein, and a parallel decrease in uterine responsiveness to cholinergic stimulation, when expressed as a function of maximal KCl-induced contractility. However, when the binding capacity was expressed per DNA, oestradiol had no effect. Similarly, no significant changes induced by oestrogens were found in the contractile force generated by carbachol, as an absolute value. This apparent discrepancy should be interpreted in the light of the real effect of oestradiol on uterine smooth muscle cells: that is, hypertrophy more than hyperplasia. This and previous studies (Michael and Schofield, 1969; Badia *et al.*, 1986) indicate that oestrogens

Fig. 5. Antagonist competition curves for quinuclidinyl benzylate ($[^3\text{H}]\text{QNB}$) binding in myometrial membranes. Membranes from (a) control and (b) oestradiol-treated guinea-pigs were incubated for 60 min at 25°C with 0.2 nmol l^{-1} $[^3\text{H}]\text{QNB}$ in the presence or absence of increasing concentrations of several ligands: pirenzepine ($\blacksquare$), telenezepine ($\bullet$), AF-DX116 ($\blacktriangle$), methoctramine ($\blacktriangledown$), *p*-FHHSiD ($\blacklozenge$), 4-DAMP ($+$), S-($-$)-hyoscyamine ($\times$), and R-($+$)-hyoscyamine ($*$). The data are plotted as percentages of maximal binding and were analysed by interactive nonlinear regression; values are the means of three different experiments, each carried out in triplicate.

increase the contractile network in the uterus and induce marked changes in protein content and uterine weight more than a clear-cut increase in the number of cells. Hence, the substantial stability of the number of muscarinic receptors per cell corresponds to a functional decrease in the real density and biological activity.

Fig. 6. Graphical representation of the selectivity of several ligands for the binding of quinuclidinyl benzylate ($[^3\text{H}]\text{QNB}$) to membranes prepared from myometrium of control and oestradiol-treated guinea-pigs. Aliquots of membranes were incubated in the presence of 0.2 nmol l^{-1} labelled ligand and increasing concentrations of unlabelled drugs (10^{-10} – $10^{-5}\text{ mol l}^{-1}$). The solid line represents the line of identity ($p\text{Ki control} = p\text{Ki oestradiol-treated}$), whereas the dotted line represents the normal regression line ($r = 0.99$). PIR, pirenzepine; TEL, telenezepine; MET, methoctramine; S-JOS, S-($-$)-hyoscyamine; R-JOS, R-($+$)-hyoscyamine.

Because the rank order of potency of several cholinergic ligands is identical in control and oestradiol-treated animals, we infer that oestradiol induces a real decrease in receptor density more than a switch in receptor subtype expression. On the basis of ligand selectivity of the muscarinic receptor found in the guinea-pig uterus, it is difficult to identify definitively the subtype of receptor present. Nevertheless, our results are compatible with the predominant expression of the M_2 receptor subtype in the guinea-pig uterus (Eglen *et al.*, 1989, 1991; Doods *et al.*, 1993).

The muscarinic receptors identified in the guinea-pig uterus mediate a rather potent stimulation of myometrial contractility that is three times greater than KCl. Since the endometrium contains biologically active muscarinic receptors (Weiss and Gurpide, 1988), and is able to produce uterotonic substances such as oxytocin (Lefebvre *et al.*, 1992) and endothelin-1 (Orlando *et al.*, 1990), we investigated the relative contribution of the endometrial layer to the contractile activity of carbachol. Careful removal of the endometrial layer (as indicated by serial histological evaluation) did not affect maximal myometrial responsiveness to cholinergic stimulation in either treated or untreated animals.

These findings indicate that the uterotonic effect of carbachol is independent of the release of endometrial factors and is mediated by receptors present on smooth muscle fibres. The results are in apparent contrast to a previous study in the rabbit uterus, which showed lower cholinergic sensitivity in

scraped myometrial strips (Riemer *et al.*, 1986). Conflicting results may derive from interspecies variations or from different procedures of endometrial removal.

These results on the negative effect of oestrogens on myometrial muscarinic receptor density are relatively surprising. In fact, oestrogens generally sensitize more than desensitize the uterus to the action of uterotonic substances. However, the results are in agreement with a previous report on a lower responsiveness of uterine strips to acetylcholine in oestrogen-primed ovariectomized guinea-pigs (Garris *et al.*, 1984). In addition, they are in keeping with results obtained in the pregnant rabbit uterus at term: a physiological model of an oestrogen-dominant uterus (Riemer *et al.*, 1986). Indeed, the maximal uterine response to carbachol showed a threefold decrease between day 30 and day 31 of pregnancy (Riemer *et al.*, 1986). These results, together with the demonstration of cholinergic denervation in the pregnant uterus at term (Wikland *et al.*, 1984; Morizaki *et al.*, 1989), indicate that hormonal factors, more than cholinergic transmission, are important in controlling the contractile activity of the oestrogen-dominant uterus. This oestrogen-induced relative insensitivity to cholinergic influence may have physiological significance at the end of pregnancy, when the maternal myometrium should be selectively sensitive to appropriate hormonal messages for the initiation of the labour process.

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