Effects of oestradiol and tamoxifen on oxytocin-induced phospholipase C activation in human myometrial cells

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The role of oestradiol in the control of uterine responsiveness to oxytocin was investigated by measuring oxytocin-induced phospholipase C activation in [³H]inositol-labelled cultured human myometrial cells. Addition of oestradiol to steroid-free culture medium (10% (v/v) fetal calf serum treated with dextran-coated charcoal in phenol red-free medium) enhanced formation of inositol phosphates and this effect was completely abolished by the anti-oestrogen tamoxifen. The inhibitory effect of tamoxifen on oxytocin-induced phospholipase C activation occurred in both steroid-free and complete culture medium; it was time- and concentration-dependent and was only partly reversed by oestradiol. When phospholipase C was activated with PGF_{2α} or fluoroaluminate instead of oxytocin, oestradiol and tamoxifen had the same stimulatory and inhibitory effects, respectively. The inhibitory effect of tamoxifen could not be prevented by treating the cells with pertussis toxin. Moreover, the effect of tamoxifen was not mediated by inhibition of protein kinase C, since the use of staurosporine (a protein kinase inhibitor) resulted in potentiation of phospholipase C activation by oxytocin. Both oestradiol and tamoxifen increased [³H]inositol incorporation into cellular lipids and cell proliferation. These results suggest that oestradiol enhances myometrial responsiveness to oxytocin and other agonists by facilitating phospholipase C activation at a post-receptor level. This effect is antagonized by tamoxifen; however, tamoxifen also has oestrogen-independent inhibitory effects.

Introduction

There is considerable evidence that oestrogens produce profound changes in uterine tissue, including changes in contractility. Recent reports from animal experiments suggest that uterine responsiveness to hormonal stimulation may be controlled by oestrogens. Myometrial activation by oxytocin and prostaglandin F_{2α} (PGF_{2α}) and gap junction formation are increased by oestradiol treatment in rats (Puri and Garfield, 1982; Mackenzie *et al.*, 1983; Garfield, 1984). Oestradiol may play a role in the regulation of contractile activity in rhesus monkeys late in gestation (Figueroa *et al.*, 1989), and may also modulate receptor–G-protein and G-protein–effector coupling, thereby affecting second messenger formation. Oestrogens increase α_1 -adrenergic-stimulated inositol 1,4,5-trisphosphate (IP₃) formation and reduce β -adrenergic stimulated cAMP formation in the rabbit uterus in the absence of changes in receptor density, effector enzyme activity or substrate availability (Roberts *et al.*, 1989). Oestrogen treatment also increases the number of α_2 -adrenoceptors linked to cAMP inhibition and the number of oxytocin receptors (Roberts *et al.*, 1989; Maggi *et al.*, 1991). Moreover, oestrogen treatment in

rabbits results in decreased concentrations of G_{as} in the uterus (Riemer *et al.*, 1988). In rats, there is differential modulation of G_{ai2} and G_{ai3} concentrations in the uterus, in association with changes in steroid concentrations during gestation (Tanfin *et al.*, 1991). However, there is little information on the role of oestrogens in the functional regulation of human myometrium.

In the present study, we used human myometrial cells in culture to investigate the potential role of oestradiol in increasing oxytocin-induced phospholipase C activation and to determine its sites of action. We report here that oestradiol increases oxytocin responsiveness by acting at a post-receptor level. We also show that the anti-oestrogen tamoxifen reduces oxytocin-induced second messenger formation through an effect on phospholipase C activity or G-protein coupling which is partly reversed by oestradiol.

Materials and Methods

Materials

Materials used in this study were purchased from the following sources. [³H]myo-inositol was obtained from Amersham International plc (Amersham); oxytocin was

purchased from Calbiochem (Nottingham); pertussis toxin, tamoxifen, oestradiol, staurosporine, PGF_{2α} and fatty acid-free BSA were from Sigma Chemical Co. (Poole); the oestradiol solid-phase [¹²⁵I] radioimmunoassay kit was purchased from Diagnostic Products Ltd (Caernarfon); tissue culture reagents were from Gibco BRL (Inchinnan). All other reagents were commercial preparations of the highest available purity.

Myocyte isolation and culture

Human myometrial cells were dispersed and cultured as described by Phaneuf *et al.* (1993). Briefly, myometrium was obtained, with the approval of the Central Oxfordshire Research Ethics Committee, from premenopausal women at hysterectomy. All patients gave informed consent. Myocytes were prepared by enzymatic dispersion. The cells were plated in plastic flasks or 24-well plates in Waymouth MB 752/1 containing 10% (v/v) fetal calf serum (FCS), 10 000 iu penicillin ml⁻¹ and 10 mg streptomycin ml⁻¹. Experiments were carried out on primary cultures and on subculture numbers 1–4 from two to five patients. Since phenol red, commonly used in tissue culture media, has oestrogenic actions, for some experiments we cultured myometrial cells in phenol red-free medium made up of RPMI and M199 media (1:32, v:v) supplemented with dextran-coated charcoal-treated-FCS. Oestradiol was measured in FCS and phenol red-free medium using a solid-phase [¹²⁵I] radioimmunoassay kit. The same batch of FCS was used for all experiments.

[³H]inositol labelling

Labelling of phosphoinositides was performed by incubating confluent myocytes grown in 24-well plastic plates (approximately 2.5×10^4 cells cm⁻²) with 2.5 μCi [³H]myo-inositol per well in culture medium complemented with penicillin, streptomycin and 2% (v/v) FCS or dextran-coated charcoal-treated-FCS for 48 h or 50 h. Unless otherwise stated, stimuli were added for 30 min in Hank's buffered saline solution supplemented with 1 mg fatty acid-free BSA ml⁻¹ and 12 mmol LiCl l⁻¹ in a final volume of 250 μl. The reaction was stopped with 1 ml chloroform:methanol:hydrochloric acid (50:100:1, v:v:v). The wells were washed with 0.3 ml chloroform and 0.3 ml water, which were then added to the corresponding extracts. Aliquots (700 μl) of the upper phase were applied to Dowex resin and [³H]inositol phosphates (IPs) were resolved and quantified according to Bone *et al.* (1984). Radioactivity of aliquots (300 μl) of the organic phase was measured to detect [³H]inositol labelling of phospholipids. The concentrations of IPs produced in cells from different experimental conditions were normalized using the formula:

$$\text{normalized IPs d.p.m.} = \frac{\text{d.p.m. of IPs in well} \times \text{mean d.p.m. of lipids in 24 wells}}{\text{d.p.m. of lipids in well}}$$

Statistical analyses

Estimations were carried out with a minimum of four replicates and experiments were performed at least twice

Fig. 1. Effect of serum on oxytocin-stimulated formation of [³H]inositol phosphates (IPs) in human myometrial cells. Cells were incubated for 5 days in the absence or presence of 10% (v/v) fetal calf serum (FCS) in Waymouth medium or dextran-coated charcoal-treated-FCS in phenol red-free (DCC-PRF) medium prior to the stimulation of IPs with 1 μmol oxytocin l⁻¹ (■) or carrier (□). Results are means ± SEM of three experiments performed on cells from different donors. *Significantly different from cells in 0% FCS ($P < 0.001$); **significantly different from cells in 10% normal FCS ($P < 0.001$).

using myocytes obtained from different donors. Statistical comparisons were performed using Student's *t* test or one-way analysis of variance using the Bonferroni multiple comparisons test. Differences were considered significant if $P < 0.05$.

Results

Preliminary experiments tested the effect of FCS on oxytocin-induced IP formation in myometrial cells. Cells grown for 5 days and labelled for 48 h with [³H]inositol in the absence of FCS showed an 8.8-fold increase in the formation of IPs over basal values (Fig. 1). Growing and labelling the cells in 10% FCS increased the oxytocin response by 244%. Phenol red-free medium (containing 10% steroid-free FCS) gave an oxytocin response that was less than that in complete medium, but was still higher than in FCS-free medium (Fig. 1). Oestradiol concentration in normal FCS was 101 ± 5 pmol l⁻¹ ($n = 3$), whereas it was not detected in dextran-coated charcoal-treated-FCS or phenol red-free medium (sensitivity of assay: 25 pmol l⁻¹). Cells grown in the absence of FCS incorporated only $38 \pm 6\%$ of the [³H]inositol of that of cells grown in the presence of 10% FCS ($n = 3$).

These results suggest that factors present in FCS play an important role in modulating the response of myometrial cells to oxytocin.

Effect of oestradiol on oxytocin-stimulated formation of IPs

The possibility that oestrogens are important factors in serum for myometrial responsiveness was tested using cells grown in steroid-free medium. Adding oestradiol to phenol red-free medium had a small but significant effect, increasing the responsiveness of the cells to oxytocin (Fig. 2). Combination of the anti-oestrogen tamoxifen (1 μmol l⁻¹) to oestradiol completely abolished the effect induced by oestradiol alone, and further inhibited oxytocin responsiveness. Treatment of cells with oestradiol in phenol red-free medium

Fig. 2. Effect of oestradiol on oxytocin-stimulated formation of [³H]inositol phosphates. Cells were incubated in phenol red-free medium complemented with 10% (v/v) dextran-coated charcoal-treated fetal calf serum for 5 days. For the last 3 days, the cells were incubated with 10 nmol oestradiol l⁻¹ (▨), 10 nmol oestradiol l⁻¹ + 3 µmol tamoxifen l⁻¹ (■), or with carrier only (□). Oxytocin was used at 1 µmol l⁻¹. Results are means ± SEM of quadruplicate determinations and are representative of two other experiments performed on cells obtained from different donors. * and **, significantly different from nontreated cells (**P* < 0.05; ***P* < 0.01).

Fig. 3. Reversal of tamoxifen-induced inhibition by oestradiol. Cells were incubated for 48 h in the absence (□) or presence of 3 µmol tamoxifen l⁻¹ (■) in Waymouth medium containing 10% (v/v) fetal calf serum. When combined with tamoxifen, oestradiol was used at 100 nmol l⁻¹ (▨). Oxytocin concentration was 1 µmol l⁻¹. Results are means ± SEM of quadruplicate determinations and are representative of two other experiments carried out on cells obtained from two different donors. *Significantly different from nontreated cells (*P* < 0.001); +significantly different from cells treated with tamoxifen alone (*P* < 0.001).

increased [³H]inositol incorporation into cellular lipids by 26 ± 4% (*n* = 6), probably as a consequence of increased number of cells. Higher concentrations of oestradiol did not increase this effect further (data not shown). Finally, addition of oestradiol to phenol red-free medium increased cell growth by 58% (evaluated by cell count using a Coulter counter). Because, over the same period, the number of cells increased as well as the incorporation of [³H]inositol into cellular lipids, the IPs formed were normalized with the mean labelling of the lipids. Therefore, the results presented in Fig. 2 represent the true increase of formation of IPs in addition to the increase in the number of cells. In contrast, when myocytes were grown in the presence of normal FCS, oestradiol did not modify activation of phospholipase C, or cell proliferation. The data in Figs 1 and 2 are consistent with the hypothesis that the stimulatory effect of FCS is due, in part, to the presence of oestradiol.

Effect of tamoxifen on oxytocin-stimulated IP formation

To obtain more information on the importance of oestradiol in myometrial cell responsiveness to oxytocin, we investigated the effects of tamoxifen alone, and in combination with oestradiol on cells grown and labelled in normal FCS. Treatment with 3 µmol tamoxifen l⁻¹ for 48 h reduced the stimulation of IP formation induced by oxytocin by more than 60% (Fig. 3). This inhibition was partially reversed by combining oestradiol and tamoxifen in the culture medium (Fig. 3). The reversal was never complete, even with concentrations of oestradiol from 1 to 100 nmol l⁻¹ (data not shown), which suggests that tamoxifen affects an oestrogen-independent pathway in addition to an oestrogen-dependent pathway. Evidence of an oestrogen-independent effect of tamoxifen was obtained by using tamoxifen in steroid-free medium. In this medium, tamoxifen partially inhibited the stimulated formation of IPs, but to a lesser extent than in normal FCS (data not shown).

The effect of tamoxifen on formation of IPs was time- and concentration-dependent (Fig. 4). Similar results were obtained with ICI 182,780 (data not shown), which is a potent and specific inhibitor of oestrogen action (Wakeling *et al.*, 1991; Wakeling and Bowler, 1992).

In contrast to its effect on oxytocin-stimulated formation of IPs, tamoxifen (3 µmol l⁻¹) increased [³H]inositol incorporation in the lipid fraction of cells grown in normal FCS (Fig. 5). Similar results were obtained in cells grown in phenol red-free medium complemented with 10% (v/v) dextran-coated charcoal-treated FCS (Fig. 5). This was paralleled by an increase of 57% in the number of cells. The addition of oestradiol or tamoxifen to dextran-coated charcoal-free-FCS stimulated cell proliferation to 95% of that obtained in normal FCS, confirming that both compounds stimulate myocyte proliferation.

Effects of oestradiol and tamoxifen on AlF₄⁻ and PGF_{2α}-stimulated formation of IPs

The site of action of oestradiol and tamoxifen was studied using AlF₄⁻ and PGF_{2α} as stimulants to determine whether they acted at the post-receptor level. Adding oestradiol to the medium increased the responsiveness of the cells to AlF₄⁻ (Fig. 6), which activates G-proteins directly in the absence of any receptor activation. A similar effect was observed when cells were treated with PGF_{2α} (Fig. 6a). The effects of tamoxifen on both stimuli were comparable to that observed on oxytocin, that is the stimulated formation of IPs was decreased (Fig. 6b). Moreover, for both stimuli, oestradiol induced a partial reversal of the tamoxifen-induced inhibition to a level similar to that obtained with oxytocin (data not shown).

These results indicate that oestradiol stimulates the activation of myometrial cells whatever the agonist is, suggesting an action at a post-receptor level.

Fig. 4. Effect of tamoxifen on oxytocin-induced formation of $[^3\text{H}]$ inositol phosphates (IPs). (a) Time course of the tamoxifen-induced inhibition of the production of IPs stimulated by $1 \mu\text{mol oxytocin l}^{-1}$. Tamoxifen ($1 \mu\text{mol l}^{-1}$) was added during the last 6–50 h of the $[^3\text{H}]$ inositol-labelling period. The basal disintegrations per minute (d.p.m.) were 385 ± 13 . (b) Concentration-response curve of the tamoxifen-induced inhibition of the formation of IPs stimulated by $1 \mu\text{mol oxytocin l}^{-1}$. Tamoxifen was present for 48 h before stimulation with oxytocin. Results are means $\pm$ SEM of quadruplicate determinations and are representative of another experiment performed on cells from a different donor.

Effect of pertussis toxin

To determine which class of G-proteins was affected by tamoxifen, we used pertussis toxin on tamoxifen-treated cells grown in medium containing normal FCS. Pertussis toxin reduced oxytocin-induced stimulation in human myometrial cells by approximately 50% (Fig. 7), suggesting that G-proteins of the G_i and G_o families (pertussis toxin-sensitive and insensitive G-proteins, respectively) are probably involved in this pathway (Phaneuf *et al.*, 1993). Tamoxifen reduced oxytocin stimulation in both pertussis toxin-treated and control cells. This finding, together with results obtained on AlF_4^- (Fig. 6), suggests that the expression of a protein downstream of the receptor (for example,

Fig. 5. Concentration-response curve of tamoxifen-induced increase in $[^3\text{H}]$ inositol incorporation into cellular lipids. The cells were grown for 5 days in 10% (v/v) fetal calf serum (FCS) in Waymouth medium (●) or dextran-coated charcoal-treated FCS in phenol red-free medium (○) before treatment in the absence or presence of tamoxifen. Results are means $\pm$ SEM of quadruplicate determinations and are representative of two other experiments performed on cells from different donors.

G-proteins or a phospholipase C isoform) may be affected by tamoxifen.

Lack of involvement of protein kinase C in the mechanisms of action of tamoxifen

It has been proposed that tamoxifen exerts some of its actions by inhibiting protein kinase C (O'Brian *et al.*, 1990). We tested this possibility by looking at the effect of a 5 min incubation with staurosporine, a protein kinase inhibitor, on oxytocin-induced formation of IPs in cells grown in medium containing normal FCS. Phospholipase C activation was amplified following inhibition of protein kinase C by staurosporine (Fig. 8). This was opposite to the inhibitory effect obtained with tamoxifen. Moreover, tamoxifen reversed the effect of staurosporine. Similar findings were obtained with a specific protein kinase C inhibitor, RO 318220 (kind gift of the Roche Foundation) (data not shown). Hence, these data indicate that tamoxifen does not produce its effects on myometrial cells by inhibiting protein kinase C.

Discussion

This study provides evidence that, in human myometrial cells, oestrogens enhance agonist-stimulated phospholipase C activation. In addition they stimulate cell proliferation and $[^3\text{H}]$ inositol incorporation into cellular lipids. We have demonstrated that the antioestrogen tamoxifen exhibits an agonist effect on cell proliferation and $[^3\text{H}]$ inositol incorporation. However, it exerts a strong inhibitory effect on the formation of IPs stimulated by oxytocin, $\text{PGF}_{2\alpha}$ or AlF_4^- . Oestradiol partially reverses the inhibitory effect obtained with tamoxifen, suggesting that a significant portion of this effect is through

Fig. 6. Effect of oestradiol and tamoxifen on PGF_{2α}- and AIF₄⁻-induced formation of inositol phosphates (IPs). (a) The cells were incubated in phenol red-free medium complemented with 10% (v/v) dextran-coated charcoal-treated fetal calf serum (FCS) for 5 days. For the last 3 days, the cells were treated with 10 nmol oestradiol l⁻¹ (■) or carrier (□). (b) The cells were incubated in Waymouth medium complemented with 10% (v/v) FCS in the absence (□) or presence (■) of 1 μmol tamoxifen l⁻¹ for the last 3 days. PGF_{2α} was used at 1 μmol l⁻¹ and AIF₄⁻ at 10 μmol l⁻¹. Results are means ± SEM of quadruplicate determinations and are representative of two other experiments performed on cells obtained from different donors. Statistical difference from nontreated cells: **P* < 0.05; ***P* < 0.01 and ****P* < 0.001.

an interaction of tamoxifen with the oestrogen receptor pathway.

The lack of effect of oestradiol in medium complemented with normal FCS is most probably due to full activation by the oestradiol already present in FCS. This is consistent with the inhibitory effect obtained with tamoxifen in FCS medium. Adding oestradiol in dextran-coated charcoal-treated-FCS medium without phenol red resulted in increased cell proliferation and overall increased metabolism of polyphosphoinositides. This is also observed in oestrogen-positive cells (Schrey *et al.*, 1992; Etindi *et al.*, 1992). As in MCF-7 cells stimulated with bombesin (Schrey *et al.*, 1992), oestradiol does not appear to exert a permissive effect on oxytocin action in myometrial cells, but positively affects the incorporation of [³H]inositol into inositol lipids. This may reflect oestrogenic effects on phospholipid synthesis rather than a direct effect on oxytocin receptors. Other likely targets for oestradiol action include the expression of G-protein and phospholipase C isoforms. We have shown that oestradiol increases both oxytocin- and AIF₄⁻-stimulated formation of IPs, suggesting an action at the post-receptor

Fig. 7. Effect of pertussis toxin treatment on tamoxifen-induced inhibition of oxytocin-stimulated formation of [³H]inositol phosphates (IPs). Cells grown in medium containing normal fetal calf serum were incubated in the absence (□) or presence (■) of 3 μmol tamoxifen l⁻¹ for 48 h and/or 2.5 μg pertussis toxin ml⁻¹ for the last 24 h of the [³H]inositol-labelling period. Basal formation of IPs was 425 ± 53 and 477 ± 46 d.p.m. in the absence or presence of pertussis toxin, respectively. Results are the means ± SEM of three experiments performed on cells obtained from two different donors. *Significantly different from nontreated cells (*P* < 0.01).

Fig. 8. Effect of staurosporine and tamoxifen on oxytocin-induced formation of [³H]inositol phosphates. Cells grown in medium containing normal fetal calf serum were treated in the absence or presence of 3 μmol tamoxifen l⁻¹ 3 days before the determination of basal and oxytocin-activated IP formation (1 μmol oxytocin l⁻¹). When present, staurosporine (1 μmol l⁻¹) was added 5 min before oxytocin. Results are the mean (d.p.m.) ± SEM of quadruplicate determinations and are representative of another experiment performed on cells obtained from a different donor. The basal values were 561 ± 71 and 547 ± 58 d.p.m. in the absence or presence of staurosporine, respectively. *Significantly different from control cells (*P* < 0.001); +significantly different from cells treated with staurosporine alone (*P* < 0.001).

level. In rats, the short form of G_{αs}, and in rabbits phospholipase C isoforms γ and δ, can be upregulated following treatment with oestrogens (Bouvier *et al.*, 1991; Phillippe and Saunders, 1992).

In addition to its anti-oestrogenic effect on stimulated [³H]inositol phosphate formation in myometrial cells, tamoxifen exhibits significant oestrogen-independent effects. Our data do not support an action of tamoxifen on protein kinase C as it has been proposed in other systems. Indeed, other oestrogen independent effects have been reported for

tamoxifen, which cannot be accounted for by actions on protein kinase C or calmodulin inhibition. These include inhibition of heat-induced thermoresistance in oestrogen positive MCF-7 cells (protein kinase C-independent; Mikkelsen *et al.*, 1991), synergism of cisplatin with respect to the cytotoxic effect against the human melanoma cell line T-289 (protein kinase C- and calmodulin-independent; McClay *et al.*, 1993), and proliferation of normal and malignant colonic cells (calmodulin-independent; Lointier *et al.*, 1992).

The agonist effect of tamoxifen on myocyte proliferation appears to be relatively specific for uterine tissue. Tamoxifen has been shown to induce DNA synthesis and uterine growth of immature or ovariectomized rats (Nicholson and Griffiths, 1980; Wakeling and Bowler, 1988; Branham *et al.*, 1993), to induce *c-fos* expression on endometrial carcinoma grown in nude mice (Sakakibara *et al.*, 1992), and stimulate proliferation of normal endometrium and endometrial carcinoma (Jamil *et al.*, 1991; Gorodeski *et al.*, 1992; Branham *et al.*, 1993). By contrast, tamoxifen reduces proliferation of non-uterine cells: human malignant glioma tumours (Pollack *et al.*, 1990), normal colonic and LoVo carcinoma cells (Lointier *et al.*, 1992), rat vascular smooth muscle cells and adventitial fibroblasts (Grainger *et al.*, 1993).

Tamoxifen can inhibit both pertussis toxin sensitive and insensitive oxytocin-induced phospholipase C activation, suggesting that G-protein(s) of the G_i and $G_{q/11}$ families might be a target for its action. However, western blotting experiments using anti- G_i and anti- $G_{q/11}$ have shown that the concentration of these G-proteins does not change following a 5-day treatment with tamoxifen (S. Phaneuf, G. N. Europe-Finner, S. P. Watson and A. López Bernal, unpublished results). This indicates that a phospholipase C specifically activated by these G-proteins might be affected by tamoxifen. The agonist and antagonist effects of tamoxifen on IP formation and [3 H]inositol incorporation seem to occur in parallel (Figs 4b and 5) suggesting an effect proximal to phospholipase C. Tamoxifen may also affect a single or several unidentified proteins that, in turn, interact with this pathway. Further studies are needed to identify the proteins that have their expression modified by tamoxifen and oestradiol.

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