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Stimulation of phosphoinositide hydrolysis by oxytocin and the mechanism by which oxytocin controls prostaglandin synthesis in the ovine endometrium

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Slices of caruncular endometrium from steroid-treated ovariectomized sheep were incubated with *myo*-[2-^{3}H]inositol to label tissue phosphatidylinositol. Effects of oxytocin were determined on the rate of incorporation of radioactivity into phosphatidylinositol and on the hydrolysis of phosphoinositides to inositol phosphates and diacylglycerol. Incorporation of radioactivity into phosphatidylinositol was linear during 2 h incubations; 10^{-7} M (100 nM)-oxytocin caused a 2.8-fold increase in the rate of incorporation. In the presence of Li^{+}, addition of 10^{-7} M-oxytocin to slices in which phosphatidylinositol was pre-labelled caused mean increases of 40-fold in the incorporation of radioactivity into inositol mono-, bis- and tris-phosphates. Inositol 1,3,4-trisphosphate was quantitatively the major trisphosphate formed. The action of oxytocin on phosphoinositide hydrolysis was dose- and time-dependent, occurring at concentrations within the range observed in plasma during episodes of secretion *in vivo*, and with a time course comparable with that of the action of oxytocin on uterine prostaglandin production. The effect of oxytocin on incorporation of radioactivity into inositol phosphates was not affected by inhibitors of prostaglandin synthesis. Diacylglycerol 1- and 2-lipases in caruncular endometrium converted up to 72% of added 2-[^{3}H]arachidonyldiacylglycerol into [^{3}H]arachidonic acid during 30 min incubations at pH 7.0. Caruncular endometrium contained 1.49 μmol of phosphatidylinositol/g, representing approx. 0.2 μmol/g of phosphatidylinositol arachidonic acid. It is proposed that the stimulation of endometrial prostaglandin synthesis by oxytocin is accounted for by increased hydrolysis of phosphoinositides to diacylglycerol and inositol phosphates with subsequent release of arachidonic acid from diacylglycerol.

INTRODUCTION

Oxytocin is involved in controlling oestrous cyclicity in ruminants. Oxytocin causes premature regression of the corpora lutea on administration to heifers (Armstrong & Hansel, 1959) and goats (Cooke & Knifton, 1981), and is released into the circulation simultaneously with $PGF_{2\alpha}$ at luteolysis in sheep (Fairclough *et al.*, 1980; Flint & Sheldrick, 1983). Uterine concentrations of the oxytocin receptor rise at luteolysis, to peak at oestrus (Roberts *et al.*, 1976; Sheldrick & Flint, 1985), and immunization against oxytocin delays luteolysis in sheep (Sheldrick *et al.*, 1980; Schams *et al.*, 1983) and goats (Cooke & Homeida, 1985).

These actions of oxytocin can be explained by its stimulatory effect on uterine secretion of the luteolysin, $PGF_{2\alpha}$. Administration of oxytocin at an appropriate stage of the oestrous cycle or pregnancy stimulates uterine production of $PGF_{2\alpha}$ both *in vivo* and *in vitro* (Sharma & Fitzpatrick, 1974; Mitchell *et al.*, 1975; Roberts *et al.*, 1976). The effect of oxytocin on $PGF_{2\alpha}$ secretion is rapid and, because of this, it has been suggested that the role of the peptide in luteolysis may be to ensure that $PGF_{2\alpha}$ secretion is episodic (Flint & Sheldrick, 1983; Flint *et al.*, 1986).

Despite its apparently important role in the regulation of the oestrous cycle, there is little information on the mechanism by which oxytocin stimulates uterine $PGF_{2\alpha}$ secretion. In contrast, it is known that vasopressin, a related peptide, interacts with two types of receptor, one (termed 'V1') exemplified by the receptor in liver and arterial smooth muscle, the other ('V2') by that in renal tissue. The V1 receptor is thought to act through stimulation of the hydrolysis of phosphoinositides, whereas the V2 receptor controls adenylate cyclase (Michell *et al.*, 1979; Thomas *et al.*, 1984). A product of the hydrolysis of phosphoinositides catalysed by phospholipase C, the enzyme responsible for production of the inositol phosphates, is diacylglycerol, and this may in turn be hydrolysed by lipases to fatty acids and glycerol. If the liberated fatty acids include arachidonate, this pathway would be expected to lead to increased prostaglandin synthesis; generation of arachidonate from phosphoinositides has been suggested to be responsible for agonist stimulation of prostanoid synthesis in platelets (Mauco *et al.*, 1978; Bell *et al.*, 1979; Rittenhouse-Simmons, 1979; Chau & Tai, 1981) and pancreas (Bauduin *et al.*, 1981; see also Irvine, 1982).

A similar mechanism may account for the stimulation of prostaglandin production by oxytocin (see Michell *et al.*, 1977). The experiments described here were therefore undertaken to determine whether oxytocin stimulates hydrolysis of phosphoinositides in the sheep uterus. The animals were treated *in vivo* with progestagen and oestrogen, in order to raise uterine oxytocin receptor concentrations (Sheldrick & Flint, 1985), and caruncular

Abbreviations used: $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; DHO-$F_{2\alpha}$, 13,14-dihydro-15-oxoprostaglandin $F_{2\alpha}$.

endometrium was used because it is reported to have a higher rate of prostaglandin production than other uterine tissues in experiments carried out *in vitro* (Findlay *et al.*, 1981).

Part of this work has previously been reported at a meeting of the Society for Endocrinology, held in London on 27–29 November 1985 (Flint *et al.*, 1985).

MATERIALS AND METHODS

Animals

Clun Forest ewes were ovariectomized under pentobarbitone/halothane anaesthesia by using aseptic surgical techniques. At various times after ovariectomy (not less than 6 weeks) the animals received medroxyprogesterone acetate by intravaginal sponge (Veramix sheep sponge; Upjohn Co., Crawley, Sussex, U.K.) for 11 days; on removing the sponge, oestradiol-17β was administered intramuscularly (100 μg in sterile arachis oil), on days 11 and 12. The animals were killed by overdose of barbiturate on day 13 and the uteri immediately cooled on ice.

For determination of the time course of the response of the uterus to oxytocin, a PVC [poly(vinyl chloride)] catheter was inserted into one jugular vein in each of three ovariectomized steroid-treated ewes, under local anaesthesia, by the method of Seldinger (1953). Oxytocin (1 μg; Bachem U.K., Saffron Walden, Essex, U.K.) was administered via the catheter in 1 ml of 0.9% NaCl, and blood samples were withdrawn at intervals before and after oxytocin treatment for measurement of the prostaglandin $F_{2\alpha}$ metabolite DHO-$F_{2\alpha}$ in plasma.

Incubation of tissue slices

Individual endometrial caruncles dissected with scissors from the uterus were collected into ice-cold 0.9% NaCl. Slices (approx. 0.3 mm thick) were cut by hand by using a razor blade while the tissue was held between a rigid clear plastic sheet and wetted filter paper. Slices were collected into ice-cold Krebs–Ringer bicarbonate-buffered saline containing 10 mM-glucose (Buffer 1), blotted, weighed and transferred to glass scintillation vials. After addition of incubation medium, each vial was gassed individually with O_2/CO_2 (19:1), closed with a rubber serum stopper, and incubated with shaking (90 cycles/min) at 37 °C.

For determination of incorporation of labelled inositol into phosphatidylinositol, slices were incubated for up to 2 h in 1 ml of Buffer 1 containing 10 μM-*myo*-[2-^{3}H]inositol (10 μCi/ml; Amersham International, Amersham, Bucks, U.K.), with or without 10^{-7} M (100 nM)-oxytocin. Incubations were terminated by adding 10 ml of unacidified chloroform/methanol (2:1, v/v) and homogenization.

To investigate the effect of oxytocin on accumulation of inositol phosphates, slices were incubated as above for 2 h in the absence of oxytocin. In order to remove unincorporated inositol, the medium was then removed, the slices rinsed with 1 ml of Buffer 1, and the medium replaced with 2 ml of Buffer 1 containing 10 μM-unlabelled inositol. After gassing, incubation was continued for a further 30 min. The medium was then removed, and the slices rinsed with 1 ml of Buffer 1 before replacing the medium with 1 ml of Buffer 1 with or without 10 mM-LiCl, as required. Incubation was continued for a further 10 min before addition of oxytocin in 10 μl of Buffer 1; incubations were subsequently terminated by addition of 1 ml of ice-cold 15% (w/v) trichloroacetic acid. All rinses and additions of fresh medium were made with gassed media maintained at 37 °C.

Separation of labelled products

Phosphatidylinositol. Neutral chloroform/methanol extracts were separated into aqueous and organic phases by addition of 2 M-NaCl (2 ml/10 ml of extract). For determination of incorporation of [^{3}H]inositol into phosphatidylinositol, the chloroform layer (plus the interface; see Fain & Berridge, 1979) was then washed four times with 2 ml of NaCl and dried; radioactivity was counted after adding scintillation fluid containing 4 g of PPO (2,5-diphenyloxazole)/litre of toluene. T.l.c. of the chloroform extract on silica-gel G plates (Kieselgel 60 F_{254}; Merck, Darmstadt, Germany) developed in chloroform/methanol/acetic acid/water (65:50:1:4, by vol.), followed by radiochromatogram scanning showed that, under these conditions, more than 95% of extracted radioactivity migrated with authentic phosphatidylinositol (Sigma, Poole, Dorset, U.K.)

Inositol phosphates. After trichloroacetic acid had been added to incubated slices and the incubation vials kept on ice for 15 min, the slices were removed by centrifugation, and the supernatants decanted and extracted five times with 5 ml of water-saturated diethyl ether. Ether extracts were discarded, residual diethyl ether removed from the aqueous phases under a stream of N_2 at 40 °C, and the extracts neutralized (to pH 7.0–8.5) by addition of 0.5 M-NaOH in 0.5 M-Tris before storage at −20 °C.

For separation of [^{3}H]inositol and [^{3}H]inositol mono-, bis- and tris-phosphates, neutralized extracts were applied to columns (6 cm × 0.5 cm) of Dowex-1 exchange resin (X8; 100–200 mesh; formate form; Sigma; prepared from the chloride form by washing sequentially with HCl, aq. NH_3 and formic acid), which were eluted sequentially with: 80 ml of water; 25 ml of 25 mM-disodium tetraborate containing 60 mM-sodium formate; 25 ml of 0.1 M-formic acid/0.2 M-ammonium formate; 25 ml of 0.1 M-formic acid/0.4 M-ammonium formate; 25 ml of 0.1 M-formic acid/1.0 M-ammonium formate (Richards *et al.*, 1979; Downes & Michell, 1981). Peaks of radioactivity were identified as described by Berridge *et al.* (1983). By pooling fractions and repeating the separation, it was shown that the columns were not overloaded with extract; 100% of the radioactivity eluted in the third fraction (inositol monophosphate) was eluted in the same fraction after repeated chromatography; for radioactivity in the fourth fraction, this value was 93%. Insufficient material was available from the final fraction (inositol trisphosphate) to allow an accurate determination in this way. For measurement of radioactivity, samples (2 ml) taken from each pooled fraction were counted for radioactivity in 15 ml of aqueous-miscible scintillation fluid (Packard 299) and count rates were corrected for counting efficiency (external standardization). After use, Dowex columns were washed with, and stored in, 20 ml of 0.1 M-formic acid/2.0 M-ammonium formate; they were extensively washed with distilled water immediately before re-use.

Determination of diacylglycerol lipase activities

Preparation of 2-[^{3}H]arachidonyl 1,2-diacylglycerol. 2-[^{3}H]Arachidonyl 1,2-diacylglycerol was prepared from 2-[^{3}H]arachidonyl phosphatidylcholine. Reaction conditions for synthesis of 2-[^{3}H]arachidonyl phosphatidylcholine were as described by Irvine & Dawson (1979); different quantities of [^{3}H]arachidonic acid were used, depending upon the specific radioactivity of labelled diacylglycerol required. Lysophosphatidylcholine (2.5 mg; Sigma) and [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid [85 Ci/mmol; New England Nuclear Corp. (Nuclear Research Products, Stevenage, Herts., U.K.) 12.5 or 125 μCi] were dissolved in 1 ml of 50 mM-sodium phosphate buffer, pH 7.4, containing 68 mM-NaF, 4 mM-$MgCl_2$, 20 mM-ATP, 0.5 mM reduced coenzyme A and 0.5 ml of a microsomal fraction prepared from rat liver as described by Higgins & Dawson (1977). After incubation under N_2 for 1 h at 37 °C the mixture was extracted with 20 ml of chloroform/methanol (2:1, v/v) and the phases separated by adding 5 ml of 0.9% NaCl. The chloroform phase was dried, and the resulting 2-[^{3}H]acyl phosphatidylcholine separated by t.l.c. on silica-gel G plates developed in chloroform/methanol/acetic acid/water (65:50:1:4, by vol.) containing 0.005% butylhydroxytoluene. After radiochromatogram scanning and elution of the major radioactive band, which migrated with phosphatidylcholine, the labelled product (2-[^{3}H]arachidonyl phosphatidylcholine) was incubated in 1 ml of 50 mM-sodium phosphate buffer, pH 7.4, with phospholipase C (200 units, from *Bacillus cereus*; Sigma) for 30 min at 37 °C, under N_2. The resulting [^{3}H]diacylglycerol was extracted with chloroform/methanol (2:1, v/v) and separated from residual phosphatidylcholine by chromatography on columns (7 cm × 0.5 cm) of silicic acid (Mallinkrodt) in chloroform; [^{3}H]diacylglycerol was eluted in the second and third 1 ml fractions, phospholipids being retained. The specific radioactivity of the product was determined by counting, and spectrophotometric measurement of glycerol after saponification in alcoholic KOH, using glycerol kinase (Denton & Randle, 1967). The lowest specific radioactivity of the 2-[^{3}H]arachidonyl diacylglycerol used as substrate for the lipase determinations described in the present paper was 5.5 mCi/mmol.

Incubation conditions. Activities of diacylglycerol 1-lipase and monoacylglycerol 2-lipase were determined by incubating total homogenates of caruncular endometrium, or subcellular fractions derived from them, with 2-[^{3}H]arachidonyl diacylglycerol. Labelled products ([^{3}H]monoacylglycerol and [^{3}H]arachidonate) were separated by t.l.c., and radioactivity was measured by liquid-scintillation counting.

Preparation of homogenates and subcellular fractions (95000 *g* supernatant and pellet) was carried out as described by Sheldrick & Flint (1985). The 95000 *g* pellet is referred to hereafter as the 'microsomal fraction'. [^{3}H]Diacylglycerol substrate was solubilized before incubation by sonication in 2 mM-NaCl. Lipase assay mixtures contained 100 μl of tissue preparation (9–900 μg of protein), 0.5 ml of 100 mM-Tris/HCl, pH 7.0, and 0.6 ml of 2 mM-NaCl containing up to 4.5×10^4 d.p.m. of substrate. Incubations were at 37 °C (with shaking) for up to 30 min, and were terminated by the addition of 200 μl of 1 M-HCl and cooling to 0 °C. After incubation the reaction mixtures were extracted by vigorous mixing with 6 ml of chloroform/methanol (2:1, v/v), and after separating the phases by adding 1 ml of 0.12 M-KCl, the chloroform phases were dried, taken up in chloroform/methanol and applied to silica-gel G plates, which were developed in diethyl ether/light petroleum (b.p. 100–120 °C)/NH_3 (sp.gr. 0.880) (360:40:1, by vol.). Radioactive products, identified on the plates by radiochromatogram scanning and by comparison with the mobilities of authentic [^{3}H]arachidonate, monoacylglycerol and diacylglycerol, were counted after scraping into scintillation vials and adding 0.5 ml of methanol and 5 ml of toluene/PPO scintillation fluid. Recoveries of [^{3}H]arachidonate, [^{3}H]monoacylglycerol and [^{3}H]diacylglycerol after extraction and t.l.c. were $73.5 \pm 1.3\%$, and results have been corrected for extraction losses.

The chromatography system used to separate reaction products resulted in arachidonic acid remaining at the origin (R_F values: arachidonic acid, 0.0; monoacylglycerol, 0.3; diacylglycerol, 0.6). Products of the further metabolism of arachidonic acid (e.g. prostaglandins) also remained at the origin. Thus metabolism of arachidonic acid released by 2-lipase did not affect values obtained for 2-lipase activity.

Determination of the tissue content of phosphatidylinositol

Caruncular endometrium was extracted by homogenization in neutral chloroform/methanol (2:1, v/v), using 10 ml/g of tissue, and phases separated by addition of 2 M-NaCl. The lower phases (chloroform plus interface) were dried and applied to silica-gel G thin-layer plates, which were developed in chloroform/methanol/acetic acid/water (65:50:1:4, by vol.). Phosphatidylinositol standards run on the same plates were identified by exposure to I_2 vapour; plates were cut so that the part of the plate carrying the sample was not exposed to I_2. After chromatography the area of the plate bearing the sample which corresponded to phosphatidylinositol was scraped, the silica gel eluted with chloroform/methanol (2:1, v/v), and a portion used for assay of phospholipid phosphorus by the method of Bartlett (1959) as modified by Boettcher *et al.* (1961).

Determination of arachidonic acid content of phosphatidylinositol

The remainder of the extract of phosphatidylinositol was evaporated almost to dryness under a stream of N_2, and then refluxed with 5% (w/v) H_2SO_4/methanol to form the methyl esters, which were separated on a 50-metre support-coated open tubular column of SP1000 in a Pye 104 chromatogram linked to a DP88 integrator. Peaks were identified by comparison with the retention time of standards.

Determination of oxytocin receptor concentrations

Concentrations of oxytocin receptor were measured in membrane fractions prepared from homogenates of caruncular endometrium by differential centrifugation, as described by Sheldrick & Flint (1985). Briefly, membrane fractions prepared from 2000 *g* supernatants by centrifugation at 95000 *g* were incubated (50 μg of protein) in 0.1 ml of 25 mM-Tris/HCl, pH 7.6, containing 0.1% (w/v) bovine serum albumin, 1 mM-$MnCl_2$ and 5 nM-[*tyrosine*-3,5-^{3}H]oxytocin (27 Ci/mmol; Cambridge Research Biochemicals, Harston, Cambs., U.K.). Non-specific binding was measured in the presence of

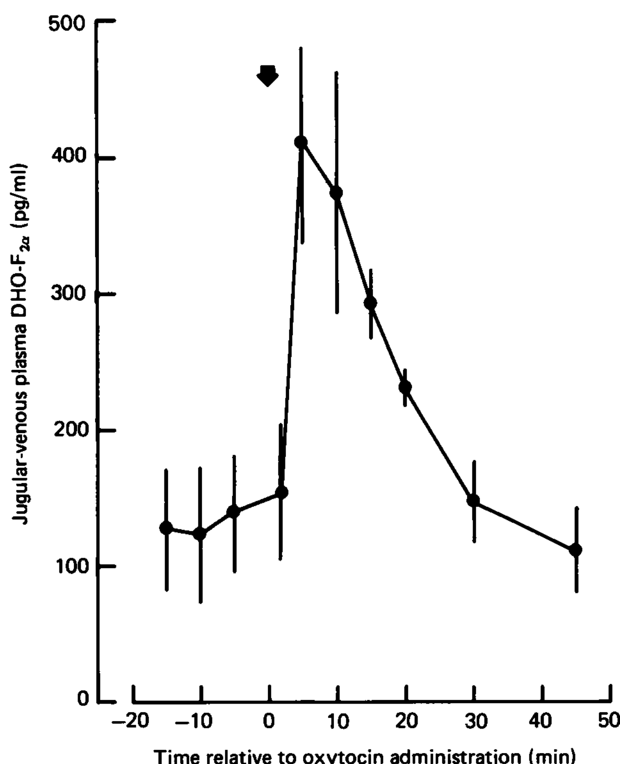

Fig. 1. Time course of effect of oxytocin on uterine secretion of $PGF_{2\alpha}$ in vivo

Concentrations of DHO-$F_{2\alpha}$ were measured in jugular-venous plasma before and after administration of 1 μg of oxytocin at zero time (arrowed). Values are for three ewes; bars indicate S.E.M.

100 pmol of unlabelled oxytocin. After incubation for 15 min at 25 °C, the bound oxytocin was separated from unbound oxytocin by filtration under vacuum through Durapore membranes type GVWP (Millipore U.K., Harrow, Middx., U.K.). The labelled oxytocin was dissolved in 1 ml of 2-ethoxyethanol before liquid-scintillation counting. Values are expressed as fmol of [^{3}H]oxytocin bound/mg of protein and are corrected for non-specific binding.

Radioimmunoassay of DHO-$F_{2\alpha}$

DHO-$F_{2\alpha}$ was measured in plasma samples by the method of Mitchell *et al.* (1976). For the batch of assays in which the samples reported here were measured, mean sensitivity (determined from 2 × S.D. below the zero-binding point) was 20.8 pg/ml; intra- and inter-assay coefficients of variation were 9.5 and 13.1% respectively (determined at 25 pg/tube). Values were corrected for extraction recoveries for each assay; mean recovery was 79.3%.

Statistics

Variances are expressed as S.E.M. Differences between groups were tested by using a one-tailed Student's *t* test.

RESULTS

Effect of oxytocin on uterine prostaglandin production

In order to confirm that the uteri of the progestagen- and oestradiol-treated sheep used in the present studies were responsive to oxytocin, receptor concentrations were measured in samples of the tissues used. To determine the time course of the action of oxytocin, peripheral circulating concentrations of DHO-$F_{2\alpha}$ were determined in three steroid-treated sheep given oxytocin.

The mean ($\pm$S.E.M.) concentration of oxytocin receptor in membrane fractions prepared from the samples of caruncular endometrium used in the present experiments was 211 $\pm$ 23 fmol/mg of protein. This was close to the concentration observed in caruncular endometrium in spontaneously cyclic ewes on day 15 after oestrus, the mean oestrous-cycle length for the flock being 16.5 days (Sheldrick & Flint, 1985), and was raised relative to the concentration in ovariectomized ewes that did not receive steroid treatment (34 fmol/mg of protein; E. L. Sheldrick, unpublished work).

Administration of oxytocin to ovariectomized steroid-treated ewes caused a rapid increase in circulating concentrations of DHO-$F_{2\alpha}$, the principal pulmonary metabolite of $PGF_{2\alpha}$ secreted by the uterus (Fig. 1); peripheral concentrations were raised within 5 min of administering oxytocin and remained elevated for 15 min after treatment. Maximal concentrations were raised at least 3-fold relative to basal levels.

Establishment of optimal incubation conditions for labelling of phosphatidylinositol: effect of oxytocin

In initial experiments, slices of caruncular endometrium were incubated for up to 3.5 h in the presence of 0.5, 10 and 100 μM-[^{3}H]inositol in order to determine optimal

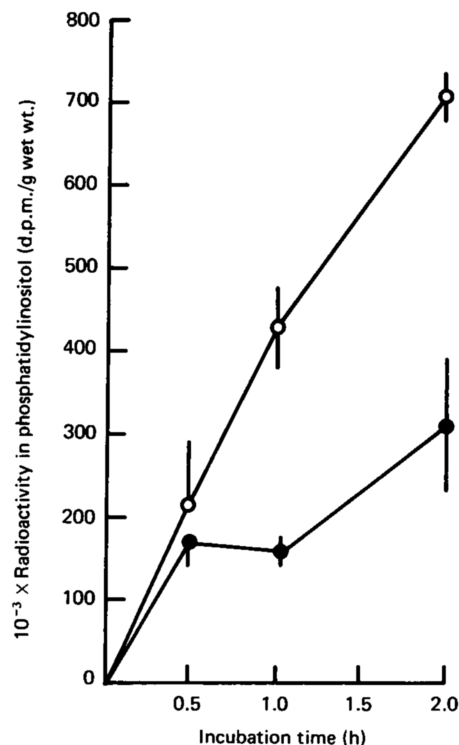

Fig. 2. Effect of oxytocin on incorporation of [^{3}H] from *myo*-[2-^{3}H]inositol into phosphatidylinositol in slices of caruncular endometrium incubated with 10 μM-[^{3}H]-inositol (10 μCi/ml) for various lengths of time

●, Control slices; ○, slices incubated with 10^{-7} M-oxytocin; $n = 3$ in each case.

Table 1. Effects of oxytocin (10^{-7} M) and LiCl (10 mM) on accumulation of inositol metabolites in slices of caruncular endometrium

Incubations were determined at 15 s or 20 min after addition of oxytocin.

Additions	10^{-3} × Accumulation of ^{3}H (d.p.m./g wet wt.) in: Inositol	Glycerophospho-inositol	Inositol monophosphate	Inositol bisphosphate	Inositol trisphosphate
−LiCl					
None	6300±450	11±2.7	20±5.4	7.7±0.9	2.9±1.3
Oxytocin					
15 s	7300±320	9.7±0.4	20±4.2	8.0±1.1	4.7±0.8
20 min	4800±380*	9.5±0.5	37±3.0*	63±7.3**	32±2.4**
+LiCl					
None	9300±850	13±2.4	36±2.2	9.6±1.6	4.1±0.8
Oxytocin					
15 s	9400±1100	13±4.0	22±3.3*	6.3±2.1	3.9±0.7
20 min	7500±2200	9.4±2.5	270±68*	310±100*	89±25*

*$P < 0.05$; **$P < 0.005$ compared with incubations without oxytocin ($n = 3$ in each case).

Table 2. Accumulation of inositol phosphates in slices of caruncular endometrium incubated for various times after addition of oxytocin (10^{-7} M)

All incubations were in the presence of 10 mM-LiCl.

Time after addition of oxytocin (min)	10^{-3} × Accumulation of ^{3}H (d.p.m./g wet wt.) in: Inositol	Glycerophospho-inositol	Inositol monophosphate	Inositol bisphosphate	Inositol trisphosphate
0	5300±390	3.1±0.8	9.6±0.7	2.0±0.7	2.1±1.0
1	4900±410	4.8±1.4	20±5.6	10±2.1*	5.6±2.5
3	5000±900	6.4±1.9	26±4.1*	16±5.5*	10±4.1
10	4900±580	8.7±1.1*	43±5.6**	29±8.6*	18±0.9**
30	4000±320*	7.1±1.4*	205±4.6**	94±16 **	51±6.4**

*$P < 0.05$; **$P < 0.005$ compared with zero-time controls ($n = 3$ in each case).

incubation conditions for the incorporation of radioactivity into phosphatidylinositol. Labelling of phosphatidylinositol was maximal using 10 μM-inositol, and in the presence of 10 μM-[^{3}H]inositol, maximum labelling was reached after 2 h incubation. Subsequent incubations were therefore carried out under these conditions. Uptake of radioactivity/g wet wt. of tissue was constant when 75–225 mg of tissue slices were incubated/ml of medium, and labelling of phosphatidylinositol was unaltered when slices were washed twice or three times with 10 mM-inositol instead of once.

Incorporation of [^{3}H]inositol into phosphatidylinositol increased with time over a 2 h incubation period and was stimulated 2.8-fold by addition of 10^{-7} M (100 nM)-oxytocin to the incubation medium (Fig. 2).

Effect of oxytocin on hydrolysis of phosphoinositides

Radioactivity was detectable in inositol phosphates when endometrial slices were extracted with trichloroacetic acid after incubation with [^{3}H]inositol, and inositol phosphate labelling was enhanced by addition of Li^+ or oxytocin to the incubation medium (Table 1). Oxytocin was effective at 20 min, but not at 15 s. In view of the action of Li^+, subsequent experiments were conducted with 10 mM-LiCl. The action of oxytocin was dose- and time-dependent (Tables 2 and 3); increased labelling of all three inositol phosphates was detectable within 1 min of addition of 10^{-7} M-oxytocin to slices, and a similar effect was seen with 10^{-9} M (1 nM)-oxytocin after 20 min. Increased radioactivity was detected in glycerophosphoinositol in the time-course and dose–response experiments. Oxytocin had no effect at 10^{-11} M (10 pM).

Separation of isomers of inositol trisphosphate by h.p.l.c. revealed little inositol 1,4,5-trisphosphate in extracts of endometrium that had been incubated in the presence of 10^{-7} M-oxytocin for 20 min (R. F. Irvine & A. J. Letcher, unpublished work). By using a Partisil SAX 10 column eluted with a stepwise ammonium formate gradient (Irvine *et al.*, 1985; Batty *et al.*, 1985), 86–91% of radioactivity in inositol trisphosphates was eluted with inositol 1,3,4-trisphosphate. Between 22 and 30% of the summed radioactivity in the tris- plus tetrakis-phosphate fractions was eluted with inositol 1,3,4,5-tetrakisphosphate.

Table 3. Accumulation of inositol phosphates in slices of caruncular endometrium incubated with various doses of oxytocin

Incubations were for 20 min after addition of oxytocin. All incubations contained 10 mM-LiCl.

[Oxytocin] (M)	10^{-3} × Accumulation of ^{3}H (d.p.m./g wet wt.) in: Inositol	Glycerophospho-inositol	Inositol monophosphate	Inositol bisphosphate	Inositol trisphosphate
0	5500±170	6.2±1.1	13±1.7	3.0±0.9	0.1±0.1
10^{-11}	5100±310	4.6±0.7	12±0.5	2.2±0.8	0.1±0.1
10^{-9}	5600±680	11±1.8	37±6.4*	14±2.6*	6.0±3.7
10^{-7}	4300±640	11±1.0*	100±9.3**	49±1.7**	31±3.5**
10^{-6}	3800±190**	9.2±2.4	150±23**	73±15*	39±8.6*

*$P < 0.05$; **$P < 0.005$ compared with incubations without oxytocin ($n = 3$ in each case).

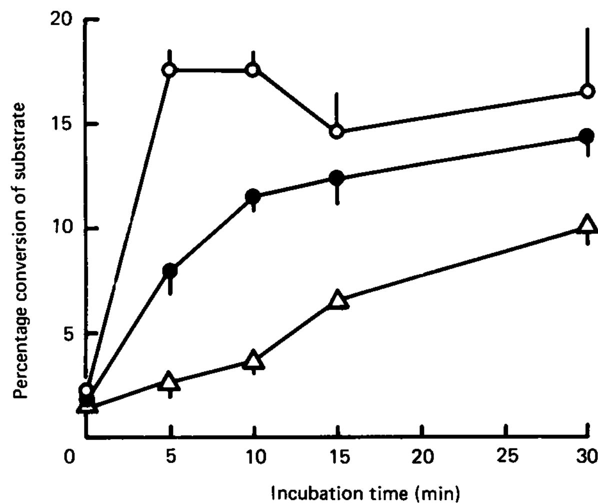

Fig. 3. Activity of diacylglycerol 1-lipase assayed by measuring incorporation of label from 2-[^{3}H]arachidonyl diacylglycerol into 2-[^{3}H]arachidonyl monoacylglycerol

Unfractionated homogenates of caruncular endometrium were incubated for various times with 4.5×10^4 d.p.m. of 2-[^{3}H]arachidonyl diacylglycerol (sp. radioactivity 5.5 mCi/mmol) at pH 7.0. Results are presented as percentage conversion of substrate to monoacylglycerol with various concentrations of homogenate; △, 9 μg of protein; ●, 30 μg of protein; ○, 90 μg of protein. Vertical bars indicate S.E.M.; $n = 3$ for each point.

Because of the possibility that the action of oxytocin on phosphoinositide hydrolysis was mediated indirectly through increased prostaglandin synthesis, the effect of oxytocin was examined in the presence of inhibitors of prostaglandin synthesis. Accumulation of radioactivity in inositol phosphates was unaffected by addition of either meclofenamic acid (0.1 mM) or indomethacin (0.05 mM) to the incubation medium when additions were made 10 min before oxytocin; radioactivity in inositol monophosphate increased between 16- and 18-fold both in the absence of inhibitors and in the presence of either meclofenamic acid or indomethacin.

Activities of diacylglycerol lipases

Diacylglycerol 1-lipase was present in unfractionated homogenates of caruncular endometrium. At pH 7.0, 15±3% of 2-[^{3}H]arachidonyl diacylglycerol was converted into [^{3}H]monoacylglycerol during 30 min incubations with 90 μg of protein. Under these conditions, initial rates of reactions were proportional to enzyme concentration (Fig. 3).

Table 4. Percentage conversion of 2-[^{3}H]arachidonyl diacylglycerol to monoacylglycerol (MG) and arachidonic acid ($C_{20:4}$) by subcellular fractions of caruncular endometrium at pH 3.5 and pH 7.0

All incubations were for 30 min at 37 °C in the presence of 50000 d.p.m. of 2-[^{3}H]arachidonyl diacylglycerol (sp. radioactivity approx. 50 mCi/mmol) and contained: cytosol, 185 μg of protein; 95000 g pellet (microsomal fraction), 150 μg; total homogenates, 900 μg. Values are means±S.E.M. of three determinations (unless otherwise indicated in parentheses).

Fraction	pH 3.5 MG	pH 3.5 $C_{20:4}$	pH 7.0 MG	pH 7.0 $C_{20:4}$
Cytosol	6.3 (2)	9.6 (2)	2.8±0.4	68±8.5
Microsomal fraction	47±6.1	15±0.9	9.4±3.7	64±8.3
Total homogenate	—	—	5.8±2.3	72±5.2

When reaction conditions were optimized for detection of diacylglycerol 1-lipase, there was little accumulation of [^{3}H]arachidonic acid and therefore little apparent 2-lipase activity. However, with a lower concentration of 2-[^{3}H]arachidonyl diacylglycerol and a higher enzyme concentration, unfractionated homogenates converted 72.5% of substrate to [^{3}H]arachidonic acid in 30 min incubations containing 900 μg of protein at pH 7.0 (Table 4). Under these conditions accumulation of labelled monoacylglycerol was reduced to 5.8%.

With microsomal and cytosolic fractions at pH 7.0, 1- and 2-lipase activities were similar to those in unfractionated homogenates (Table 4). At pH 3.5, the 1-lipase activity of microsomal fractions predominated, resulting in accumulation of labelled monoacylglycerol. Little lipase activity was detectable in cytosolic fractions at pH 3.5.

Arachidonic acid content of phosphatidylinositol

Caruncular endometrium contained 1.49±0.34 μmol of phosphatidylinositol/g ($n = 6$). Analysis of the fatty

acid associated with phosphatidylinositol showed that this phospholipid contained $5.6 \pm 0.82\%$ (w/w) arachidonic acid ($n = 5$).

DISCUSSION

These data show (*a*) that oxytocin increases the incorporation of radioactivity into phosphatidylinositol in slices of caruncular endometrium incubated with [^{3}H]inositol, (*b*) that oxytocin increases the hydrolysis of phosphoinositides to diacylglycerol and inositol tris-, bis- and mono-phosphates, (*c*) that the major inositol trisphosphate produced is inositol 1,3,4-trisphosphate, (*d*) that caruncular endometrium contains lipases catalysing the hydrolysis of diacylglycerol to fatty acids and glycerol, and (*e*) that phosphatidylinositol extracted from caruncular endometrium contains arachidonic acid. Taken together, these results indicate that oxytocin stimulates the hydrolysis of phosphoinositides, including presumably phosphatidylinositol mono- and bis-phosphates, in caruncular endometrium and that arachidonic acid may be released from the diacylglycerol so produced. The time course of the response to oxytocin *in vitro* was comparable with that of the effect on prostaglandin synthesis observed *in vivo*, and the minimal effective concentration range *in vitro* ($10^{-11}-10^{-9}$ M) was equivalent to the concentration in peripheral plasma during episodes of secretion at luteolysis (10^{-10} M; Flint & Sheldrick, 1983). Experiments in which endometrial minces were incubated with and without arachidonic acid showed that availability of arachidonic acid is rate-limiting in prostaglandin synthesis in ovine endometrium (Findlay *et al.*, 1981). The hypothesis is therefore proposed that stimulated phosphoinositide metabolism accounts for the effect of oxytocin on prostaglandin synthesis in caruncular endometrium.

Many endocrine and paracrine receptors stimulate rates of phosphoinositide metabolism. Michell (1982) lists 25 such receptors. Examples of peptide hormones generating second messengers in this way are cholecystokinin/pancreozymin, thyroid-stimulating hormone, luliberin ('LHRH'), thyroliberin ('TRH'), corticotropin-releasing factor ('CRF'), angiotensin ('angiotensin II'), bradykinin, substance P, thrombin and vasopressin (the V1 receptor; see the Introduction section). Vasopressin is the most relevant of these hormones to the present discussion because of its similarity to oxytocin and its affinity for the uterine oxytocin receptor (Sheldrick & Flint, 1985). It is of interest, therefore, that the methods used to demonstrate an effect of vasopressin on phosphoinositide metabolism in other tissues are similar to those used here; vasopressin stimulates incorporation of radioactivity into phosphatidylinositol (Michell *et al.*, 1979) and increases hydrolysis of labelled phosphatidylinositol phosphates (Kirk *et al.*, 1981; Charest *et al.*, 1985) in rat hepatocytes, and increases labelling of phosphatidylinositol and hydrolysis of phosphatidylinositol phosphates in adrenal glomerulosa cells (Balla *et al.*, 1985).

Caruncular endometrium contained 1- and 2-diacylglycerol lipases, as demonstrated by conversion of substrate to 2-[^{3}H]arachidonyl monoacylglycerol and [^{3}H]arachidonic acid respectively. In platelets, hydrolysis at *sn*-1 precedes that at *sn*-2 (Chau & Tai, 1981), and the results obtained with endometrial extracts are consistent with this sequence; at high substrate and low enzyme concentrations [^{3}H]monoacylglycerol accumulated and incorporation of label into arachidonic acid was low. At low substrate and high enzyme concentrations, on the other hand, a higher proportion of label was incorporated into [^{3}H]arachidonic acid; these differences in product ratios could result from the 1-lipase having a higher K_m, and a higher $V_{max.}$, than the 2-lipase. Studies on the effect of pH on lipase activity also revealed similarities between endometrium and other organs; in both platelets (Chau & Tai, 1981) and rat brain (Cabot & Gatt, 1976, 1977) a form of the 1-lipase is active at acid pH, whereas the 2-lipase has a pH optimum at neutral pH or above.

The phosphatidylinositol content of the endometrium (1.5 μmol/g) was similar to that in other tissues, which generally contain 0.5–2.5 μmol of phosphatidylinositol/g, representing 2–12% of the total tissue phospholipid (White, 1973; Michell, 1975). However, the arachidonic acid content of endometrial phosphatidylinositol (5.6%) was low; in most tissues between 7 and 40% of the fatty acid contained in phosphatidylinositol is arachidonic acid, of which most is esterified at position 7 (White, 1973; and see Holub *et al.*, 1970). Most of the fatty acids esterified in phosphatidylinositol are long-chain acids with M_r values in the range 256 (palmitic)–304 (arachidonic); it can therefore be calculated that, within a probable error of $\pm 20\%$, caruncular endometrium contained 0.2 μmol of phosphatidylinositol arachidonic acid/g. If substrate for prostaglandin synthesis were derived solely from phosphatidylinositol, the quantity of $PGF_{2\alpha}$ released from the uterus in response to oxytocin would represent a significant proportion of this arachidonic acid; maximum utero-ovarian venous plasma concentrations of $PGF_{2\alpha}$ during the oestrous cycle are approx. 70 pmol/ml (Thorburn *et al.*, 1973), which, corrected for packed cell volume and multiplied by a blood flow of 25 ml/min, would suggest a rate of uterine secretion of 80 nmol/h. This secretion rate occurs into both uterine veins and can be expected to be maintained for 2 h (Flint & Sheldrick, 1983). Thus maximum secretion per episode of release is approx. 0.32 μmol. Given that the weight of caruncular endometrium/uterus is 10 g (A.P.F. Flint, unpublished work), this represents approx. 15% of available phosphatidylinositol arachidonic acid. It should be noted, however, that this conclusion will only apply if the phosphatidylinositol utilized for prostaglandin synthesis is distributed throughout all the cells in the endometrium; if a single cell type is involved, then conclusions based on measuring phosphatidylinositol in the whole caruncle may not be valid. Furthermore, this calculation disregards arachidonic acid esterified in phosphatidylinositol phosphates.

It has been speculated (Brinsfield & Hawk, 1973) that the lipid droplets that accumulate in endometrial epithelial cells exposed to progesterone represent a precursor for prostaglandin synthesis; however, there is no evidence that these droplets contain phosphatidylinositol. The use of phosphatidylinositol as a substrate for prostaglandin synthesis might be expected to involve stored lipid rather than that in the cell membrane, since, when used for prostaglandin synthesis, the diacylglycerol produced on phosphoinositide breakdown becomes unavailable for recycling to phosphatidylinositol. Prostaglandin synthesis might therefore deplete cell membrane

phosphatidylinositol unless an independent store of the lipid was utilized.

Li^+ caused accumulation of inositol phosphates in tissue stimulated with oxytocin, presumably owing to inhibition of inositol-1-phosphatase (EC 3.1.3.25) (Hallcher & Sherman, 1980; Berridge *et al.*, 1982). Rholam *et al.* (1985) have reported that Li^+ causes a conformational change in the oxytocin molecule such that it assumes a vasopressin-like structure and binds to vasopressin–neurophysin with increased affinity. This is unlikely to affect the interpretation of the present results as the concentration of Li^+ required to cause this conformational change is in the molar, rather than in the millimolar, range (Rholam *et al.*, 1985). Furthermore, oxytocin increased inositol phosphate formation in the absence of added Li^+.

In addition to release of arachidonic acid from phosphatidylinositol phosphates catalysed by phospholipase C and di- and mono-acylglycerol lipases, which the present results show to be a potential pathway, arachidonate may also be released from phosphoinositides and other phospholipids through increased phospholipase A_2 activity. In many tissues, including human (Bonney, 1985) and guinea pig (Downing & Poyser, 1983) endometrium, phospholipase A_2 is Ca^{2+}-dependent, and a raised intracellular Ca^{2+} concentration resulting from agonist-stimulated Ca^{2+} mobilization has been suggested to control this enzyme (see Irvine, 1982; Hokin, 1985). The data presented here suggest that the major inositol trisphosphate formed 20 min after oxytocin stimulation is inositol 1,3,4-trisphosphate, which is unlikely to mobilize Ca^{2+} (Irvine *et al.*, 1985); similar data have been obtained in guinea-pig hepatocytes stimulated with angiotensin (Burgess *et al.*, 1985). Although this observation and the time-course data (Table 2) show that phosphoinositide hydrolysis occurs sufficiently rapidly to account for the DHO-$F_{2\alpha}$ response to oxytocin (Fig. 1), they do not preclude transient formation of inositol 1,4,5-trisphosphate, and associated Ca^{2+} mobilization, at earlier times. Ca^{2+}-stimulated phospholipase A_2 activity after exposure to oxytocin therefore represents an additional potential source of arachidonic acid for prostaglandin synthesis, and the increased accumulation of radioactivity in glycerophosphoinositol in response to oxytocin (Tables 2 and 3) suggests that this may occur.

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