Stimulation of phospholipid hydrolysis and arachidonic acid mobilization in human uterine decidua cells by phorbol ester

Michael P. SCHREY,*† Alison M. READ* and Philip J. STEER†

*Department of Chemical Pathology and †Department of Obstetrics and Gynaecology, St. Mary's Hospital Medical School, University of London, London W2 1PG, U.K.

Vasopressin and oxytocin both stimulated inositol phosphate accumulation in isolated uterine decidua cells. Pretreatment of cells with the phorbol ester 12-*O*-tetradecanoylphorbol 13-acetate (TPA) prevented this agonist-induced phosphoinositide hydrolysis. TPA (0.1 μ M) alone had no effect on basal inositol phosphate accumulation, but stimulated phosphoinositide deacylation, as indicated by a 2-fold increase in lysophosphatidylinositol and glycerophosphoinositol. TPA also stimulated a dose-related release of arachidonic acid from decidua-cell phospholipid [phosphatidylcholine (PC) $\gg$ phosphatidylinositol (PI) > phosphatidylethanolamine]. The phorbol ester 4 β -phorbol 12,13-diacetate (PDA) at 0.1 μ M had no effect on arachidonic acid mobilization. The TPA-stimulated increase in arachidonic acid release was apparent by 2½ min (116 % of control), maximal after 20 min (283 % of control), and remained around this value (306 % of control) after 120 min incubation. TPA also stimulated significant increases in 1,2-diacylglycerol and monoacylglycerol production at 20 and 120 min. Although the temporal increases in arachidonic acid and monoacylglycerol accumulation in the presence of TPA continued up to 120 min, that of 1,2-diacylglycerol declined after 20 min. In decidua cells prelabelled with [³H]choline, TPA also stimulated a significant decrease in radiolabelled PC after 20 min, which was accompanied by an increased release of water-soluble metabolites into the medium. Most of the radioactivity in the extracellular pool was associated with choline, whereas the main cellular water-soluble metabolite was phosphorylcholine. TPA stimulated extracellular choline accumulation to 183 % and 351 % of basal release after 5 and 20 min respectively and cellular phosphorylcholine production to 136 % of basal values after 20 min. These results are consistent with a model in which protein kinase C activation by TPA leads to arachidonic acid mobilization from decidua-cell phospholipid by a mechanism involving phospholipase A-mediated PI hydrolysis and phospholipase C-mediated PC hydrolysis, coupled with further hydrolysis of the 1,2-diacylglycerol product.

INTRODUCTION

The uterotonic actions of oxytocin and vasopressin in women during parturition and menstruation respectively are thought to be partly mediated by increases in uterine prostaglandin production (Fuchs *et al.*, 1982; Strömberg *et al.*, 1983). The hydrolysis of cellular phospholipid and accompanying release of unesterified arachidonic acid is of primary importance in regulating the production of prostaglandins and other eicosanoids (Irvine, 1982), and considerable efforts have been made to identify the various pathways involved. The phosphoinositides constitute an important source of arachidonic acid; however, whether fatty acid mobilization involves phospholipase A or the concerted action of phospholipase C and diacylglycerol lipase is uncertain.

We have previously reported that vasopressin and oxytocin stimulate phospholipase C-mediated phosphoinositide hydrolysis in isolated uterine decidua cells (Schrey *et al.*, 1986). Furthermore, the release of arachidonic acid from phosphatidylinositol (PI) by decidua homogenates was characteristic of a sequential action of phospholipase C and diacylglycerol lipase (Schrey *et al.*, 1986).

An alternative fate of the diacylglycerol product of phosphoinositide hydrolysis is the stimulation of protein kinase C activity (Nishizuka, 1984). The phorbol ester 12-*O*-tetradecanoylphorbol 13-acetate (TPA) binds to and activates protein kinase C, thereby mimicking the action of diacylglycerol (Kikkawa *et al.*, 1983). The purpose of the present study was to investigate a potential role for protein kinase C in the regulation of arachidonoyl-phospholipid metabolism in uterine decidua cells by measuring the effect of TPA on certain parameters of phospholipid turnover in these cells. TPA has been reported to have a variety of effects on cellular phospholipid metabolism. It stimulates phosphoinositide deacylation and arachidonic acid mobilization in mouse macrophages (Emilsson *et al.*, 1986), phospholipid hydrolysis, arachidonic acid release and prostaglandin synthesis in dog kidney (MDCK) cells (Daniel *et al.*, 1981), and phospholipase-C-mediated hydrolysis of phosphatidylcholine (PC) in certain cell types (Guy & Murray, 1982; Besterman *et al.*, 1986), as well as inhibiting agonist-stimulated phospholipase-C-mediated phosphoinositide hydrolysis in a variety of cell types (Labarca *et al.*, 1984; Brock *et al.*, 1985; Monaco & Mufson, 1986).

Abbreviations used: TPA, 12-*O*-tetradecanoylphorbol 13-acetate; PDA, 4 β -phorbol 12,13-diacetate; PI, phosphatidylinositol; PC, phosphatidylcholine; IP, inositol 1-phosphate; IP₂, inositol bisphosphate; IP₃, inositol trisphosphate; DMSO, dimethyl sulphoxide.

† To whom correspondence should be addressed.

We report here that pretreatment of decidua cells with TPA inhibits vasopressin- and oxytocin-stimulated inositol phosphate production. TPA also stimulated diacylglycerol production as well as arachidonic acid mobilization, primarily from PC and PI. The TPA-induced hydrolysis of PC appears to be mainly mediated by phospholipase C, whereas PI is hydrolysed by an increase in phospholipase A activity. It therefore appears that protein kinase C activation in decidua cells has the potential to mediate arachidonic acid release from cellular phospholipids by more than one pathway.

EXPERIMENTAL

Materials

myo-[2-³H]inositol (15.8 Ci/mmol), [1-¹⁴C]arachidonic acid (59.6 mCi/mmol) and [*methyl*-³H]choline chloride (80 Ci/mmol) were all purchased from Amersham International. Medium 199 was supplied by Flow Laboratories.

Vasopressin, oxytocin, phorbol esters, collagenase, phospholipid and neutral lipid standards and water-soluble choline metabolites were all purchased from Sigma. Precoated silica-gel 60 t.l.c. plates were obtained from Merck, and Dowex 1 × 8 (200–400 mesh) was from Sigma. Organic solvents and scintillation cocktails Fisofluor 2 and 3 were obtained from Fisons.

Decidua-cell preparation

Foetal membranes with attached decidua tissue were obtained from women undergoing elective caesarean section at term before the onset of labour. Decidua tissue was separated from the chorion by sharp dissection, washed twice in Medium 199 containing 0.5% bovine serum albumin and 20 mM-Hepes, pH 7.4, and disaggregated into isolated cells by collagenase digestion, as in the method of Olson *et al.* (1983). Briefly, 1 cm² pieces of the washed tissue were incubated in 20 ml of Medium 199 containing collagenase (0.05%), trypsin inhibitor (0.05%) and deoxyribonuclease (0.05%) for 30 min at 37 °C. Medium was separated from tissue by filtering through coarse nylon gauze, and any adherent blood clots were removed and the medium was discarded. The tissue was then incubated in fresh collagenase-containing medium for 45 min, with vigorous shaking every 10 min. Dispersed cells were separated from any residual tissue by filtering through nylon gauze, and the cell suspension was sedimented by centrifugation (200g for 5 min). Cell pellets were washed by resuspension in Medium 199, centrifuged again (150g for 2 min) and resuspended in Medium 199 (containing 0.5% albumin and 0.01% kanamycin sulphate) at a concentration of 4 × 10⁵ cells/ml. Cell viability, assessed by Trypan Blue exclusion staining, was routinely greater than 90%.

Incubation conditions

The metabolic fate of radiolabelled phospholipid precursors in response to TPA was assessed in decidua cells prelabelled with [³H]inositol, [¹⁴C]arachidonic acid and [³H]choline. TPA was added from a stock solution (10 mM) dissolved in dimethyl sulphoxide (DMSO); control incubations contained the appropriate concentration of (DMSO) alone.

[³H]Inositol metabolism

Decidua cells were prelabelled with [³H]inositol by incubation in Krebs–Ringer medium containing 0.5% albumin, 0.1% glucose, 20 mM-Hepes (pH 7.4), 1 mM-MnCl₂, no added Ca²⁺, and [³H]inositol (15.8 Ci/mmol; 50 μCi/ml) for 2 h at 37 °C under O₂. Cells were then washed in radioisotope-free Medium 199 containing unlabelled inositol (1 mM), centrifuged (200g for 5 min), resuspended (approx. 4 × 10⁵ cells/ml) and incubated for 5–30 min in the presence or absence of oxytocin, vasopressin or TPA in 1 ml of Medium 199 containing inositol (1 mM) and LiCl (10 mM). In some experiments, [³H]inositol-labelled cells were preincubated in the presence or absence of TPA for 30 min before incubation in the presence or absence of oxytocin or vasopressin. Incubations were terminated and inositol metabolites were extracted by addition of 6 vol. of acidified (0.1 M-HCl) methanol/chloroform (2:1, v/v).

[¹⁴C]Arachidonic acid metabolism

Isolated decidua cells (approx. 0.5 × 10⁶ cells/ml) were incubated in Medium 199 containing [¹⁴C]arachidonic acid (58.3 mCi/mmol; 0.5 μCi/ml) for 18 h at 37 °C under O₂. Cells were then centrifuged (200g for 5 min) and resuspended in radioisotope-free medium. This washing was repeated four times before incubation of the resuspended prelabelled cells (approx. 0.5 × 10⁶ cells per incubation) in the presence or absence of TPA in 1 ml of Medium 199 at various times and concentrations (see individual legends for details). Incubations were terminated by the addition of 6 vol. of chloroform/methanol (1:2, v/v). The release of radiolabelled arachidonic acid and its distribution in decidua-cell neutral and phospholipid fractions was then determined.

[³H]Choline metabolism

Decidua cells were prelabelled in Medium 199 containing [³H]choline (80 Ci/mmol) at 5 μCi/ml for 18 h at 37 °C under O₂. After centrifugation as above, the medium was removed and the cells were washed in radioisotope-free medium. This washing procedure was repeated four times before incubation of the prelabelled cells (0.5 × 10⁶ cells/incubation) for 5 or 20 min at 37 °C in the presence or absence of TPA (0.2 μM) in 0.4 ml of medium. Incubations were terminated after chilling on ice, by centrifugation of the cell suspension at 4 °C (200g for 2 min). The medium was removed, and the cell pellet was then resuspended in fresh chilled medium and extracted with 6 vol. of chloroform/methanol (2:1, v/v). The distribution of radiolabelled choline metabolites released into the medium and associated with the cell pellet was then determined as described below.

Extraction and isolation of radiolabelled metabolites

[³H]Inositol metabolites. After extraction at room temperature for 30 min, phase separation was achieved by addition of 2 vol. of chloroform and 3 vol. of EDTA (5 mM). The top aqueous phase was removed and washed with 4 vol. of chloroform. The washed aqueous-phase extracts were then freeze-dried, and the residue was resuspended in 2 ml of water. Water-soluble [³H]inositol metabolites were then separated by anion-exchange chromatography on Dowex 1 (formate form) resin as previously described (Berridge *et al.*, 1982). Samples of the eluate fractions corresponding to glycerophospho-

Fig. 1. Effect of vasopressin and oxytocin on inositol phosphate production

Cells prelabelled with [^3H]inositol were incubated in the absence (■) or the presence (□) of vasopressin (V; $0.1 \mu\text{M}$) or oxytocin (O; $0.1 \mu\text{M}$) in medium containing LiCl (10 mM) for 5 or 30 min. Incubations were terminated and radiolabelled inositol phosphates were extracted and separated as described in the Experimental section. Degrees of significance are: ** $P < 0.01$; * $P < 0.05$.

inositol, inositol monophosphate (IP), inositol bisphosphate (IP₂) and inositol trisphosphate (IP₃) were counted for radioactivity in FisoFluor 2 scintillation cocktail. In experiments where lysophosphatidylinositol production was measured, incubations were terminated by heating in a boiling-water bath for 2 min, and inositol lipids were extracted with an equal volume of butan-1-ol as previously described (Schrey & Montague, 1983). Separation of individual phospholipids was performed by t.l.c. in chloroform/methanol/acetic acid/0.1 M-sodium borate (50:30:8:3, by vol.), polyphosphoinositides, lysophosphatidylinositol and phosphatidylinositol being resolved with respective R_f values of 0, 0.16 and 0.43. Radioactivity corresponding to these inositol lipids was eluted from the gel with 0.4 ml of methanol/water/6M-HCl (80:20:1, by vol.) and measured by scintillation counting in FisoFluor 2.

[^{14}C]Arachidonic acid metabolites. [^{14}C]Arachidonic acid-labelled decidua-cell lipid was extracted into the lower chloroform phase after phase separation after the addition of 2 vol. of chloroform and 3 vol. of 3 M-KCl/5 mM-EDTA. Samples of the chloroform phase were dried under N_2 , resuspended in 50 μl of chloroform/methanol (9:1, v/v), and applied to precoated silica-gel 60 t.l.c. plates (Merck) and developed in light petroleum (b.p. 40–60 $^\circ\text{C}$)/diethyl ether/acetic acid (40:10:1, by vol.) to separate different neutral-lipid fractions as previously described (Schrey & Montague, 1983). Separation of individual phospholipids was also performed by t.l.c., as previously described (Schrey & Rubin, 1979). All lipids were located by exposure to iodine vapour, and the radioactivity present in the gel was measured by liquid-scintillation counting in FisoFluor 3 scintillation cocktail.

[^3H]Choline metabolites. Samples of the chloroform and aqueous-phase extracts of the decidua-cell pellets

Table 1. Effect of TPA on peptide-stimulated IP production

Cells prelabelled with [^3H]inositol were preincubated in the presence of TPA ($0.1 \mu\text{M}$) or vehicle DMSO (0.001 %) for 30 min. The cells were then incubated for a further 30 min with or without vasopressin, ($0.1 \mu\text{M}$) or oxytocin ($0.1 \mu\text{M}$) in the presence of LiCl (10 mM). IP production was determined as described in the Experimental section. Degrees of significance are: * $P < 0.001$; † $P < 0.05$.

Treatment	IP (c.p.m./incubation)	
	DMSO	TPA
Control	1878 $\pm$ 498	2034 $\pm$ 250
Oxytocin	3722 $\pm$ 924†	2025 $\pm$ 98
Vasopressin	6188 $\pm$ 416*	2616 $\pm$ 254

obtained after phase separation were counted for radioactivity, and the remainder was subjected to t.l.c. to determine the distribution of [^3H]choline-labelled lipid and water-soluble metabolites. Phospholipids were separated as described above (Schrey & Rubin, 1979). Water-soluble metabolites were separated by the system of Yavin (1976). Aqueous fractions of the cell extract were freeze-dried, resuspended in 200 μl of ethanol/water (1:1, v/v), applied to silica-gel 60 t.l.c. plates (Merck) and developed in the following solvent system used by Yavin (1976): methanol/0.5 % NaCl/conc. NH_3 (50:50:1, by vol.). Choline, phosphocholine, glycerophosphocholine and CDP-choline were used as standards and separated in this system with respective R_f values of 0.04, 0.25, 0.44 and 0.59. Areas corresponding to these standards were detected by iodine vapour, and their radioactivity was measured by liquid-scintillation counting. Similarly total [^3H]choline metabolites in a sample of the cell medium were measured and their distribution was determined by t.l.c. (Yavin, 1976) after freeze-drying of the remaining medium.

Expression of results

All values are presented as means $\pm$ s.d. ($n = 3$) from single representative experiments repeated two or more times. Statistical evaluation of the data was by Student's t test.

RESULTS

Effects of vasopressin, oxytocin and TPA on phosphoinositide hydrolysis

Vasopressin and oxytocin ($0.1 \mu\text{M}$) both stimulated inositol phosphate (IP) accumulation after 30 min incubation (Fig. 1). Vasopressin exhibited a much greater efficacy and stimulated significant increases in all three inositol phosphates after 5 min. The relative accumulation of IP after 5 and 30 min in the presence of vasopressin suggests a constant rate of phosphoinositide hydrolysis throughout the 30 min incubation period.

When [^3H]inositol-labelled decidua cells were preincubated with the phorbol ester TPA ($0.1 \mu\text{M}$) for 30 min, subsequent stimulation of inositol phosphate production by vasopressin or oxytocin was inhibited (Table 1), suggesting that TPA might inhibit peptide activation of decidua-cell phospholipase C.

Table 2. Effect of TPA on inositol lipid metabolism

Cells prelabelled with [^3H]inositol were incubated in the presence of TPA (0.1 μM) or vehicle DMSO (0.001 %) for 20 min in medium containing LiCl (10 mM). Incubations were terminated by boiling for 2 min and inositol phospholipids were extracted into butan-1-ol. Phospholipids were separated by t.l.c., and water-soluble metabolites by Dowex anion-exchange chromatography. Degrees of significance are: * $P < 0.001$; † $P < 0.01$.

Metabolite	Treatment ...	Distribution of radioactivity (%)	
		DMSO	TPA
Polyphosphoinositides		3.0 $\pm$ 0.2	2.1 $\pm$ 0.1†
Lysophosphatidylinositol		1.1 $\pm$ 0.1	2.1 $\pm$ 0.1*
PI		87.0 $\pm$ 13.4	83.2 $\pm$ 5.4
Glycerophosphoinositol		3.9 $\pm$ 0.1	8.1 $\pm$ 0.9†
IP		2.8 $\pm$ 0.4	2.7 $\pm$ 0.7
Inositol polyphosphates		2.3 $\pm$ 0.2	1.9 $\pm$ 0.3

Treatment of [^3H]inositol-labelled cells with TPA (0.1 μM) for 30 min also stimulated phosphoinositide deacylation, as evidenced by a 2-fold increase in production of lysophosphatidylinositol and glycerophosphoinositol (Table 2). However, no changes in phospholipase C hydrolysis products were observed (Table 2). Despite this, TPA caused a significant decrease in [^3H]polyphosphoinositides (Table 2), with a small non-significant decrease in PI.

Effect of TPA on arachidonic acid mobilization

Decidua cells prelabelled with [^{14}C]arachidonic acid exhibited a dose-related release of radiolabel in response to a 2 h incubation with TPA from PC, PI (Fig. 2) and phosphatidylethanolamine (results not shown), the respective values for radiolabelled phosphatidylethanolamine in control and TPA (10 μM) incubations being 14083 $\pm$ 595 and 11791 $\pm$ 405 c.p.m. (mean $\pm$ S.D., $n = 3$, $P < 0.01$). A corresponding dose-related increase in free arachidonic acid was also observed in response to TPA (Fig. 2), the effect being half maximal at 20 nM.

Most of the released arachidonic acid originated from PC, followed by PI and then phosphatidylethanolamine. A phospholipase- A_2 -mediated mobilization of arachidonic acid from PI by TPA is consistent with our findings regarding TPA-stimulated deacylation of [^3H]inositol-labelled PI; however, the mechanisms mediating PC and phosphatidylethanolamine hydrolysis are uncertain.

The phorbol ester 4 β -phorbol 12,13-diacetate (PDA), which has a much lower affinity for the phorbol ester receptor (Leach *et al.*, 1983), was without effect at 0.1 μM on arachidonic acid release from prelabelled cells. Thus, after a 2 h incubation the amount of radiolabelled arachidonic acid released into and associated with the medium was 2789 $\pm$ 152, 4002 $\pm$ 154 and 2750 $\pm$ 199 c.p.m./incubation under basal conditions and in the presence of TPA (0.1 μM) and PDA (0.1 μM) respectively.

The time course of TPA-induced arachidonic acid release in prelabelled decidua cells is shown in Fig. 3. TPA (0.2 μM) caused a significant increase in fatty acid release after 2½ min (116 $\pm$ 7 % of basal release; $n = 3$, $P < 0.02$), the magnitude of this effect increasing to 283 % and 306 % of basal release after 20 and 120 min respectively (Fig. 3). Interestingly, TPA also stimulated significant increase in 1,2-diacylglycerol and monoacyl-

glycerol production, to 172 % and 131 % of basal values respectively after 20 min incubation (Fig. 3). Furthermore, the relative changes in 1,2-diacylglycerol, monoacylglycerol and free arachidonic acid at 20 and 120 min would be consistent with a respective precursor-product relationship for these three lipids, and indeed high activity of diacylglycerol lipase has been previously reported in human decidua tissue (Okazaki *et al.*, 1981).

Fig. 2. Effect of TPA concentration on arachidonic acid mobilization from cellular phospholipid

Cells prelabelled with [^{14}C]arachidonic acid (AA) were incubated with increasing concentrations of TPA for 2 h. Incubations were terminated, and lipids (cells plus medium) extracted and separated as described in the Experimental section. Degrees of significance are: ** $P < 0.01$.

Fig. 3. Time course of TPA-stimulated arachidonic acid release and 1,2-diacylglycerol and monoacylglycerol production

Cells prelabelled with [14 C]arachidonic acid were incubated for various times in the presence of TPA (0.2 μ M) (○, □, △) or vehicle DMSO (0.002%) (●, ■, ▲). Cells and medium were extracted together and lipids separated as described in the Experimental section: □, ■ monoacylglycerol; ○, ●, 1,2-diacylglycerol; ▲, △, arachidonic acid. Degrees of significance are: * P < 0.05; ** P < 0.02; *** P < 0.001.

Table 3. Effect of TPA on the distribution of [14 C]arachidonic acid in prelabelled decidua cells

Cells prelabelled with [14 C]arachidonic acid were incubated with TPA (0.2 μ M) or its vehicle DMSO (0.002%) for 20 min. Incubations were terminated and cellular lipids were extracted and separated by t.l.c. as described in the Experimental section. The percentage distribution of radioactivity in the different individual lipid fractions were determined, the total lipid values (mean $\pm$ S.D.) being 108460 ± 1525 and 105605 ± 5166 c.p.m./incubation in the presence of DMSO and TPA respectively. Degrees of significance are: * P < 0.001; † P < 0.01.

Lipid fraction	Treatment ...	(%) Distribution of radioactivity	
		DMSO	TPA
Phospholipid		76.85 $\pm$ 0.30	72.82 $\pm$ 0.30*
Monoacylglycerol		0.26 $\pm$ 0.01	0.34 $\pm$ 0.02†
1,2-Diacylglycerol		0.47 $\pm$ 0.05	0.81 $\pm$ 0.04*
Arachidonic acid		1.83 $\pm$ 0.07	5.32 $\pm$ 0.09*
Triacylglycerol		20.59 $\pm$ 0.25	20.72 $\pm$ 0.26

The effect of TPA (0.2 μ M) on the distributions of [14 C]arachidonic acid between total phospholipid and neutral lipid fractions after 20 min incubation of prelabelled decidua cells is shown in Table 3. Clearly the observed increases in monoacylglycerol, 1,2-diacylglycerol and free arachidonic acid in the presence of TPA are a consequence of arachidonoyl-phospholipid hydrolysis, with no redistribution of radiolabel occurring within the triacylglycerol pool after 20 min (Table 3). However, after 120 min TPA induced a $22 \pm 4\%$ ($n = 3$, $P < 0.01$) increase in radiolabel associated with the triacylglycerol pool (results not shown).

Since PC is the major source of TPA-induced arachidonic acid release, the question arises of the relative roles of phospholipase A_2 and phospholipase C/diacylglycerol lipase in the hydrolysis of this phospho-

lipid. The TPA-mediated increase in diacylglycerol production provides circumstantial support for a role for phospholipase-C-mediated hydrolysis of PC rather than PI, since TPA was without effect on inositol phosphate production (Table 2).

In some experiments decidua cells were prelabelled with [14 C]stearic acid for 18 h before incubation with TPA. In these studies TPA (0.2 μ M) stimulated an increase ($162 \pm 22\%$ of basal; $n = 3$, $P < 0.02$) in [14 C]stearate-labelled diacylglycerol after 20 min, similar in magnitude to that observed in [14 C]arachidonate-labelled cells (Fig. 3). However, no significant changes in the labelled lysophosphatidylcholine pool were observed after 2 or 20 min (results not shown), thus arguing against a role for phospholipase A_2 in the release of arachidonic acid from PC.

Table 4. Effect of TPA on the release of water-soluble [³H]choline metabolites

Cells prelabelled with [³H]choline were incubated with either TPA (0.2 μ M) or its vehicle DMSO (0.002%) for 5 or 20 min. Medium and cells were separated by centrifugation. Choline metabolites were extracted from the cells, and the radioactivity in the medium, the water-soluble cellular fraction and the cellular phospholipid were determined as described in the Experimental section. Degrees of significance are: * P < 0.001, † P < 0.01, ‡ P < 0.02, § P < 0.05.

Fraction	Time ... Treatment ...	Radioactivity (c.p.m./incubation)			
		5 min		20 min	
		DMSO	TPA	DMSO	TPA
Medium		10848 $\pm$ 100	16864 $\pm$ 408*	11402 $\pm$ 732	31168 $\pm$ 818*
Cellular water-soluble		36187 $\pm$ 819	39408 $\pm$ 1906	36507 $\pm$ 1818	44501 $\pm$ 1212†
PC		140931 $\pm$ 28258	142634 $\pm$ 18035	137851 $\pm$ 11088	109174 $\pm$ 5422‡
LysoPC		784 $\pm$ 27	754 $\pm$ 53	712 $\pm$ 16	818 $\pm$ 48§

Table 5. Distribution of water-soluble [³H]choline metabolites

Cells prelabelled with [³H]choline (5 μ Ci/ml) for 18 h were washed four times and incubated in Medium 199 for 20 min. Cells and medium were separated by centrifugation and the cellular water-soluble choline metabolites extracted. The distribution of radioactive water-soluble choline metabolites in the medium and cells was then determined by t.l.c. as described in the Experimental section.

	Distribution of radioactivity (%)	
	Medium	Cell-associated
Choline	79.6 $\pm$ 2.6	9.3 $\pm$ 5.6
Phosphocholine	12.2 $\pm$ 4.3	46.7 $\pm$ 1.0
Glycerophosphocholine	7.7 $\pm$ 1.4	35.5 $\pm$ 0.2
CDP-choline	0.5 $\pm$ 0.5	8.5 $\pm$ 4.4

Effect of TPA on [³H]choline metabolism

Previous studies have reported evidence for a TPA-stimulated phospholipase-C-mediated hydrolysis of PC in certain cell types (Guy & Murray, 1982; Besterman *et al.*, 1986). The present study employed decidua cells

prelabelled with [³H]choline after 18 h to investigate the effect of TPA on the fate of various choline metabolites. The effect of TPA (0.2 μ M), during incubation for 5 and 20 min, on the release of radioactivity into the medium and the amount of radioactivity associated with the cellular pools of phospholipid and water-soluble metabolites is shown in Table 4. TPA stimulated a mean 56% increase in the accumulation of radiolabel in the medium after 5 min, which rose to 273% of control values after 20 min. These changes were accompanied by smaller parallel increases in the cellular water soluble pool in the presence of TPA (Table 4). Furthermore, TPA also caused a significant loss of radiolabel from the PC pool after 20 min incubation, suggesting that the net increases in intra- and extra-cellular [³H]choline metabolites were due to TPA-stimulated PC hydrolysis. A small (15%) but significant increase in lysophosphatidylcholine was also observed in the presence of TPA after 20 min, suggesting an increase in phospholipase A activity directed toward PC. Assuming isotopic equilibrium between the various [³H]choline pools, the PC pool is 200 times that of lysophosphatidylcholine. If all the TPA-induced increase in water-soluble choline metabolites were generated via lysophosphatidylcholine (i.e. via phospholipase A and lysophospholipase action), this latter pool would be turning over approx. 40 times during a 20 min incubation. Despite this, no significant changes in the glycerol

Table 6. Effect of TPA on choline release and intracellular phosphocholine production

Cells prelabelled with [³H]choline were incubated with either TPA (0.2 μ M) or its vehicle DMSO (0.002%) for 5 or 20 min. Cells and medium were separated by centrifugation. Water-soluble choline metabolites were extracted from the cells, and the radioactivity associated with the phosphocholine fraction was determined after t.l.c. as described in the Experimental section. Similarly, the radioactive choline fraction associated with the medium was determined after freeze-drying the medium and analysis by t.l.c. Degrees of significance are: * P < 0.001; † P < 0.01.

	Time ... Treatment ...	Radioactivity (c.p.m./incubation)			
		5 min		20 min	
		DMSO	TPA	DMSO	TPA
Extracellular choline		2888 $\pm$ 201	5283 $\pm$ 676†	4031 $\pm$ 137	14158 $\pm$ 1015*
Intracellular phosphocholine		12686 $\pm$ 1492	13973 $\pm$ 1594	15077 $\pm$ 314	20547 $\pm$ 1448†

phosphocholine pool were apparent after 5 or 20 min exposure to TPA (results not shown).

The percentage distribution of [^3H]choline between the various water-soluble metabolites within the extracellular and intracellular pools is shown in Table 5. Most of the radiolabel in the medium is associated with choline, whereas that of the cellular pool is present mainly as phosphocholine and glycerophosphocholine. A similar distribution of radioactivity between phosphocholine and glycerophosphocholine has been reported in [^3H]choline-labelled neuroblastoma cells (Liscovitch *et al.*, 1987). The effect of TPA on [^3H]choline and [^3H]phosphocholine contents in the medium and cells respectively is shown in Table 6. A significant 36% increase in cellular phosphocholine after 20 min (Table 6) in conjunction with the significant decrease in PC seen after 20 min (Table 4) is consistent with a TPA-stimulated phospholipase-C-mediated hydrolysis of PC. Subsequent hydrolysis of phosphocholine to choline and leakage of the latter into the medium would also account for the increased extracellular accumulation (351% of basal after 20 min) of choline in the presence of TPA (Table 6).

DISCUSSION

In this study we confirm our previous observation that vasopressin and oxytocin stimulate phosphoinositide hydrolysis in human uterine decidua cells (Schrey *et al.*, 1986). Vasopressin-stimulated phosphoinositide hydrolysis has been reported in a number of cell types, including liver (Creba *et al.*, 1983), vascular smooth muscle (Takhar & Kirk, 1981) and mammary tumour cells (Monaco & Mufson, 1986). Although the physiological significance of this phenomenon in decidua cells is uncertain, the mobilization of intracellular Ca^{2+} by IP_3 (Berridge & Irvine, 1984), the activation of protein kinase C by diacylglycerol (Nishizuka, 1984) and the hydrolytic release of prostaglandin precursor from the arachidonate-rich diacylglycerol product (Irvine, 1982) represent the potential involvement of three signal pathways. This third pathway is the mechanism by which oxytocin appears to stimulate endometrial prostaglandin production in sheep (Flint *et al.*, 1986). We have proposed a similar mechanism for the mediation of the decidua-cell prostaglandin biosynthesis in response to oxytocin and vasopressin (Schrey *et al.*, 1986).

Since the phorbol ester TPA is a potent activator of protein kinase C, we have used it to investigate the potential role of this signal pathway in the control of phospholipid turnover in uterine decidua cells. Pretreatment of decidua cells with TPA blocked vasopressin- and oxytocin-induced increases in inositol phosphate accumulation. Whether this phenomenon mimics a physiologically relevant mechanism mediating feedback inhibition of the inositol-lipid signalling system is unknown. However, in the present study vasopressin-induced phosphoinositide hydrolysis did not exhibit any refractoriness during a 30 min incubation, thus the rate of inositol phosphate production in decidua cells in the presence of vasopressin ($0.1 \mu\text{M}$) was the same at 5 and 30 min. Although a small degree of refractoriness was apparent with oxytocin, the difference in the rate of inositol phosphate accumulation at 5 and 30 min was not statistically significant.

A similar inhibition of agonist-stimulated phosphoinositide hydrolysis by TPA has been observed in other systems (Labarca *et al.*, 1984; Brock *et al.*, 1985; Monaco & Mufson, 1986). A role for TPA-mediated receptor down-regulation has been eliminated in the case of vasopressin-stimulated WRK-1 cells (Monaco & Mufson, 1986) and angiotensin II-stimulated vascular smooth-muscle cells (Brock *et al.*, 1985), suggesting that the inhibitory mechanism operates after agonist binding. In the present study we have observed a significant decrease in decidua-cell polyphosphoinositides after exposure to TPA ($0.1 \mu\text{M}$) for 20 min, accompanied by the accumulation of PI deacylation products. It is thus interesting to speculate whether TPA causes substrate depletion of a hormone-sensitive PI pool, resulting in impaired agonist-stimulated phosphoinositide hydrolysis by phospholipase C. However, this interpretation is not consistent with studies on vascular smooth-muscle cells, where TPA (0.1 mM) pretreatment for 5 min inhibited angiotensin II-stimulated inositol phosphate accumulation without affecting the polyphosphoinositide contents (Brock *et al.*, 1986).

The results of the present study show that TPA is also a potent activator of arachidonic acid release from prelabelled decidua cells (half-maximal effect at 20 nM). The redistribution of arachidonic acid was indicative of a hydrolytic pathway involving phospholipid breakdown rather than triacylglycerol hydrolysis, the major phospholipid species involved being PC and PI. The loss of arachidonic acid from PI correlates with a 2-fold increase in the production of the deacylation products lysophosphatidylinositol and glycerophosphoinositol in response to TPA ($0.1 \mu\text{M}$). A similar phospholipase A-mediated pathway involving phosphoinositide hydrolysis has been previously reported to account for the mobilization of arachidonic acid induced by TPA and diacylglycerol in mouse peritoneal macrophages (Emilsson *et al.*, 1986). The molecular mechanism mediating this TPA-stimulated phosphoinositide deacylation is unknown, but may involve protein kinase C-dependent phosphorylation and deactivation of lipocortin (Hirata *et al.*, 1984), an inhibitory regulator protein of phospholipase A_2 (Blackwell & Flower, 1983). Indeed, a role for protein kinase C in the phosphorylation and suppression of anti-phospholipase A_2 activity in thrombin-stimulated platelets has been demonstrated (Touqui *et al.*, 1986).

The mechanisms involved in the release of arachidonic acid from decidua-cell PC in response to TPA are less clear than those mediating PI hydrolysis. In decidua cells prelabelled with [^{14}C]stearic acid, we did not observe any changes in the amounts of radiolabelled lysophosphatidylcholine in response to TPA, nor did we see any changes in glycerophosphocholine in cells prelabelled with [^3H]choline, although a small (15%) increase in lysophosphatidyl[^3H]choline was detectable after 20 min in the presence of TPA. In contrast with this relative lack of accumulation of PC deacylation products in response to TPA, TPA stimulated significant increases in the accumulation of extracellular choline (351% of control) and intracellular phosphocholine (136% of control) coupled with a decrease in radiolabelled PC in [^3H]choline-labelled decidua cells after 20 min. Similar stimulatory effects of TPA on the release of choline and phosphocholine have been reported previously in various cell lines, and roles for phospholipase C (Guy & Murray,

1982; Besterman *et al.*, 1986) and phospholipase D (Liscovitch *et al.*, 1987) have been proposed. Since TPA is also known to stimulate incorporation of choline into cellular PC (Kinzel *et al.*, 1979; Paddon & Vance, 1980), it is possible, however, that TPA causes a net release of choline metabolites in prelabelled cells, owing to a redistribution of choline into a more metabolically active PC pool with a high basal turnover. Although the molecular mechanisms controlling PC hydrolysis are unknown, the demonstration of a potent inhibitor of phospholipase-C-mediated PC hydrolysis in dog myocardium (Wolf & Gross, 1985) also implies a regulatory role for this enzyme in PC turnover. Indeed, the potential involvement of phospholipase-C-mediated PC hydrolysis in receptor-mediated cell activation has been demonstrated by the ability of platelet-derived growth factor to stimulate phosphocholine release in 3T3-L1 cells (Besterman *et al.*, 1986). Furthermore, vasopressin stimulates the production of diacylglycerol species, in hepatocytes, whose fatty acid composition implicate them to be generated from phospholipids other than the phosphoinositides (Hughes *et al.*, 1984; Bocckino *et al.*, 1985).

In the present study TPA also stimulated diacylglycerol production, which would also be consistent with an increase in phospholipase-C-mediated hydrolysis of PC rather than PI, since TPA had no effect on the production of inositol phosphates. A similar pathway has also been proposed to account for the TPA-induced increases in diacylglycerol production in HL-60 cells (Besterman *et al.*, 1986) and myoblasts (Grove & Schimmel, 1982). Furthermore, in the present study TPA also increased monoacylglycerol accumulation in decidua cells, as well as that of arachidonic acid. We interpret these observations as evidence of diacylglycerol hydrolysis and propose that the rapid turnover of diacylglycerol originating from PC could account for most of the arachidonic acid mobilized from PC in response to TPA. This interpretation is consistent with the previously reported high activity of diacylglycerol lipase in human uterine decidua tissue (Okazaki *et al.*, 1981). In this respect we have also reported a similar sequential action of phospholipase C and diacylglycerol lipase to account for the release of arachidonic acid from PI by decidua homogenates (Schrey *et al.*, 1986).

Despite the present findings on TPA-stimulated arachidonic acid mobilization, PI deacylation and PC hydrolysis, in preliminary studies we have not observed such effects on phospholipid turnover in decidua cells in response to vasopressin or oxytocin (M. P. Schrey, A. M. Reed & P. J. Steer, unpublished work). The reason for this apparent discrepancy is unknown, since TPA is thought to mimic the actions of diacylglycerol, whose endogenous amounts are presumably elevated in response to peptide-induced phosphoinositide hydrolysis in decidua cells. However, a more sustained and potent activation of protein kinase C by TPA compared with diacylglycerol might be expected, in view of the rapid removal of endogenous diacylglycerol by diacylglycerol kinase and diacylglycerol lipase compared with the relatively slower rate of elimination of TPA. Alternatively, the possibility exists that TPA may operate by a pathway different from that of diacylglycerol.

Finally, it is noteworthy that arachidonic acid has been shown to inhibit the synthesis and release of prolactin from human uterine decidua tissue *in vitro*

(Handwerger *et al.*, 1981). Furthermore, TPA also inhibits decidual prolactin production and secretion in isolated decidua cells (Harman *et al.*, 1986). Our present observations on TPA-stimulated arachidonic acid mobilization in uterine decidua cells therefore provide a mechanistic link between protein kinase C activation, arachidonic acid mobilization and the inhibitory regulation of decidual prolactin production.

This work was supported by a grant from Birthright.

REFERENCES

- Berridge, M. J. & Irvine, R. F. (1984) *Nature* (London) **312**, 315–321
- Berridge, M. J., Downes, C. P. & Hanley, M. R. (1982) *Biochem. J.* **206**, 587–595
- Besterman, J. M., Duronio, V. & Cuatrecasas, P. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 6785–6789
- Blackwell, G. J. & Flower, R. J. (1983) *Br. Med. Bull.* **39**, 260–264
- Bocckino, S. B., Blackmore, P. F. & Exton, J. H. (1985) *J. Biol. Chem.* **260**, 14201–14207
- Brock, T. A., Rittenhouse, S. E., Powers, C. W., Ekstein, L. S., Gimbrone, M. A., Jr. & Alexander, R. W. (1985) *J. Biol. Chem.* **260**, 14158–14162
- Creba, J. A., Downes, C. P., Hawkins, P. T., Brewster, G., Michell, R. H. & Kirk, C. J. (1983) *Biochem. J.* **212**, 733–747
- Daniel, L. W., King, L. & Waite, M. (1981) *J. Biol. Chem.* **256**, 12830–12835
- Emilsson, A., Wijkander, J. & Sundler, R. (1986) *Biochem. J.* **239**, 685–690
- Flint, A. P. F., Leat, W. M. F., Sheldrick, E. L. & Stewart, H. J. (1986) *Biochem. J.* **237**, 797–805
- Fuchs, A.-R., Fuchs, F., Husslein, P., Soloff, M. A. & Fernström, M. J. (1982) *Science* **215**, 1396–1398
- Grove, R. I. & Schimmel, S. D. (1982) *Biochim. Biophys. Acta* **711**, 272–280
- Guy, G. R. & Murray, A. W. (1982) *Cancer Res.* **42**, 1980–1985
- Handwerger, S., Barry, S., Barrett, J., Markoff, E., Zeither, P., Cwikel, B. & Siegel, M. (1981) *Endocrinology* (Baltimore) **109**, 2016–2021
- Harman, I., Costello, A., Ganong, B., Bell, R. M. & Handwerger, S. (1986) *Am. J. Physiol.* **251**, E172–E177
- Hirata, F., Matsuda, K., Notsu, Y., Hattori, T. & Del Carmine, R. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 4717–4721
- Hughes, B. P., Rye, K. A., Pickford, L. B., Barritt, G. J. & Chalmers, A. H. (1984) *Biochem. J.* **222**, 535–540
- Irvine, R. F. (1982) *Biochem. J.* **204**, 3–16
- Kikkawa, U., Takai, Y., Tanaka, Y., Miyake, R. & Nishizuka, Y. (1983) *J. Biol. Chem.* **258**, 11442–11445
- Kinzel, V., Kreibach, G., Hecker, E. & Suss, R. (1979) *Cancer Res.* **39**, 2743–2750
- Labarca, R., Janowsky, A., Patel, J. & Paul, S. M. (1984) *Biochem. Biophys. Res. Commun.* **123**, 703–709
- Leach, K. L., James, M. L. & Blumberg, P. M. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 4208–4212
- Liscovitch, M., Blusztajn, J. K., Freese, A. & Wurtman, R. J. (1987) *Biochem. J.* **241**, 81–86
- Monaco, M. E. & Mufson, R. A. (1986) *Biochem. J.* **236**, 171–175
- Nishizuka, Y. (1984) *Nature* (London) **308**, 693–698
- Okazaki, T., Sagawa, N., Okita, J. R., Bleasdale, J. E., MacDonald, P. C. & Johnston, J. M. (1981) *J. Biol. Chem.* **256**, 7316–7321

- Olson, D. M., Skinner, K. & Challis, J. R. G. (1983) *J. Clin. Endocrinol. Metab.* **57**, 694–699
- Paddon, H. B. & Vance, D. E. (1980) *Biochim. Biophys. Acta* **620**, 636–640
- Schrey, M. P. & Montague, W. (1983) *Biochem. J.* **216**, 433–441
- Schrey, M. P. & Rubin, R. P. (1979) *J. Biol. Chem.* **254**, 11234–11241
- Schrey, M. P., Read, A. M. & Steer, P. J. (1986) *Biosci. Rep.* **6**, 613–619
- Strömberg, P., Åkerlund, M., Forsling, M. L. & Kindahl, H. (1983) *Br. J. Obstet. Gynaecol.* **90**, 332–337
- Takhar, A. P. S. & Kirk, C. J. (1981) *Biochem. J.* **194**, 167–172
- Touqui, L., Rothhut, B., Shaw, A. M., Fradin, A., Vargaftig, B. B. & Russo-Marie, F. (1986) *Nature (London)* **321**, 177–180
- Wolf, R. A. & Gross, R. W. (1985) *J. Biol. Chem.* **260**, 7295–7303
- Yavin, E. (1976) *J. Biol. Chem.* **251**, 1392–1397

Received 30 March 1987/21 May 1987; accepted 11 June 1987