Measurement of phospholipase A₂ activity in human endometrium during the menstrual cycle

R. C. Bonney

Department of Chemical Pathology, St Mary's Hospital Medical School, London W2 1PG

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ABSTRACT

Phospholipase A₂ activity was measured in human endometrium throughout the menstrual cycle using an assay based on the liberation of oleic acid from 1-palmitoyl-2-[¹⁴C]oleoyl phosphatidylcholine. The enzyme was shown to be calcium dependent, to have an optimum pH of 8–9 and an apparent Michaelis constant of 110 μmol/l. Enzyme activity was low in early proliferative-phase tissue (6.08 ± 1.42 (S.E.M.) pmol oleic acid released/mg protein per min) but rose significantly (P<0.01) during the late proliferative phase (10.86 ± 2.79 pmol/mg per min). There was a tenfold increase in activity 2–4 days after ovulation (45.6 ± 13.6 pmol/mg per min) which thereafter declined to reach values which at menstruation were not significantly different from those of the proliferative phase (4.5 ± 1.76 pmol/mg per min). The results indicate that phospholipase A₂ activity in human endometrium is related to the stage of the menstrual cycle and suggest that arachidonic acid release may be influenced by oestradiol and progesterone.

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INTRODUCTION

It has been generally accepted for some years that prostaglandins play an important role in the regulation of menstruation. Early studies by Pickles, Hall, Best & Smith (1965) confirmed the presence of prostaglandins E₂ and F₂α (PGE₂ and PGF₂α) in menstrual fluid and endometrial tissue. Further evidence came from the observation that PGF₂α will induce menstrual bleeding (Wiquist, Bygdeman & Kirton, 1971) and that inhibitors of prostaglandin synthesis, e.g. indomethacin and mefanamic acid, will reduce menstrual blood loss and relieve dysmenorrhoea (Anderson, Haynes, Guillebaud & Turnbull, 1976; Chan, 1983). Prostaglandin F₂α is known to promote vasoconstriction (Malik & McGiff, 1976) whereas PGE₂ has vasodilatory properties (Moncada & Vane, 1978) and it is thought that normal menstrual bleeding is dependent on the balance between the two. More recently, however, other products of the cyclo-oxygenase pathway have been implicated in the mechanism of menstruation, namely, prostaclin (Smith, Abel, Kelly & Baird, 1981) and thromboxane A₂ (Granstrom, Swahn & Lundstrom, 1983). Thromboxane A₂ is a powerful stimulant and prostaclin is the most potent naturally occurring inhibitor of platelet aggregation (Moncada, Gryglewski, Bunting & Vane, 1976). These two substances may have major effects on bleeding and haemostasis.

Although subsequent metabolic events may ultimately determine the amounts of prostaglandins produced, the rate-limiting step in their synthesis is likely to be the release of arachidonic acid from membrane-bound phospholipids by the enzyme phospholipase A₂ (PLA₂). Free arachidonic acid can be metabolized by cyclo-oxygenase to prostaglandin endoperoxides which are then converted to products which include prostacyclin, thromboxane A₂, PGE₂ and PGF₂α.

The endometrium has a considerable capacity to synthesize prostaglandins, particularly during the luteal phase of the menstrual cycle (Singh, Baccarini & Zuspan, 1975; Maathuis & Kelly, 1978), but little is known about the mechanism controlling arachidonic acid release from human endometrium. In the rat, uterine PLA₂ activity can be stimulated in vivo using oestradiol implants and this stimulatory action can be modulated by pretreatment with progesterone (Dey, Hoversland & Johnson, 1982; Pakrasi, Cheng & Dey, 1983). These steroids could therefore be significant in the control of PLA₂ activity, and consequently arachidonic acid metabolites, during the menstrual cycle.
The present study describes an assay for the measurement of PLA₂ in human endometrium and its application to the measurement of the enzyme during the menstrual cycle.

**MATERIALS AND METHODS**

**Clinical material**

Endometrial tissue was obtained in theatre from women (mean age 36.6 ± 6.0 years, range 24–47 years) who were undergoing curettage during the course of laparoscopic clip sterilization or for investigatory purposes unassociated with malignancy. All had given consent as approved by the local ethical committee (Paddington and North Kensington Health Authority). Only women with regular cycles and no endometrial pathology were included in the study. The tissue was dated by histological examination and assigned to the following phases: (1) early proliferative (days 5–8), (2) late proliferative (days 9–14), (3) transition to early secretory phase (determined by histology), (4) early secretory (days 2–4 postovulation), (5) mid-secretory (days 6–10 postovulation), (6) late secretory (days 10–12 postovulation), (7) transition to menstrual phase (determined by histology) and (8) menstrual phase.

Endometrial tissue was transported on ice (30 min) and stored at −20°C until required for assay. Storage for at least 1 month under these conditions had no effect on enzyme activity although, in practice, samples were assayed within 1 week of collection. No difference was observed between tissues frozen in theatre and those transported as described above.

**Preparation of tissue homogenate**

The tissue was washed, weighed and homogenized (using a Polytron homogenizer; Kinematica GmbH, Lucerne, Switzerland) at a concentration of 80–100 mg/ml in Tris–HCl buffer (0.1 mol/l; pH 8.0) containing calcium (5 mmol/l) and sucrose (0.25 mol/l) followed by centrifugation at 800 g for 10 min. The supernatant was decanted and retained for the assay of PLA₂. Protein concentration of the supernatant was determined according to the method of Lowry, Rosebrough, Farr & Randall (1951).

**Phospholipase A₂ assay**

The substrate used was 100 000 d.p.m. 1-palmitoyl-2[1-14C]oleoyl phosphatidylcholine, (Amersham International plc, Bucks) together with unlabelled 1-palmitoyl-2-oleoyl phosphatidylcholine (Sigma Chemical Co. Ltd, Poole, Dorset) prepared directly in the tube in 0.2% Triton X-100 in Tris–HCl buffer (0.1 mol/l; pH 8.0) containing calcium (5 mmol/l). Unless otherwise stated the final concentration of substrate used was 254 µmol/l. For the majority of studies 100 µl enzyme preparation containing 4–500 µg protein was incubated with 100 µl substrate at 37°C in a shaking water bath for 20 min. [3H]Oleic acid (20 000 c.p.m.; Amersham) was present in the incubation mixture to monitor procedural losses. The reaction was terminated by the addition of 2 ml chloroform : methanol (2 : 1, v/v).

The extraction of oleic acid was completed by the addition of 0.2 ml KCl (2 mol/l)/EDTA (0.5 mmol/l) and the chloroform layer removed by aspiration. A further extraction with 1 ml chloroform ensured a satisfactory recovery of oleic acid (65–70%). The chloroform extracts were then evaporated to dryness under nitrogen and oleic acid was isolated by chromatography on silica-gel thin-layer plates (E. Merck, Darmstadt, F.R.G.) using the system petroleum ether (boiling range 60–80°C) : diethyl ether : acetic acid (80 : 20 : 2, by vol.). The area corresponding to oleic acid was located by a radio-chromatogram scanner (ESI-Panax, Bournemouth, Dorset) and scraped into a scintillation vial containing 100 µl methanol. Radioactivity was counted in a Beckman LS7500 dual-label scintillation counter with a counting efficiency of 50% for 14C using 10 ml OptiScint T scintillation fluid (LKB Instruments Ltd, Croydon, Surrey). Results were expressed as pmol oleic acid released/mg protein per min and for the menstrual cycle study the data were analysed by Student’s t-test. A typical within assay coefficient of variation as calculated from duplicates was 4.6% (n = 14).

**Assay linearity**

The linearity of the assay was ascertained by incubating aliquots of enzyme preparation for 5–120 min under the conditions described above.

**Enzyme concentration**

Linearity with respect to enzyme concentration was determined by incubating aliquots of enzyme preparation containing 0.125–2.0 mg protein for 20 min under standard assay conditions.

**Substrate concentration**

The apparent Michaelis constant (Km) was determined by the method of Lineweaver & Burk (1934) using a range of concentrations of substrate and an incubation time of 15 min under standard assay conditions.
Determination of optimum pH

In this study variations in pH ranging from 4.0–10.0 were obtained with the following buffers: sodium acetate (pH 4.0–5.5), sodium phosphate (pH 6.0–7.0), Tris–HCl (pH 7.5–9.0) and sodium carbonate/bicarbonate (pH 9.5–10.0). The enzyme was prepared in sucrose (0.25 mol/l) without buffer and a modification was made to the volumes of enzyme and substrate used to allow for the addition of 100 µl buffer (0.2 mol/l). The standard procedure was then followed.

Requirement for calcium

The dependence of the enzyme on calcium for maximum activity was demonstrated by incubating aliquots of enzyme in the presence of varying amounts of calcium (0–10 mmol/l). Enzyme and substrate were prepared without calcium and the incubation volumes of each adjusted to allow for the addition of 50 µl buffer containing the required concentration of calcium. In one experiment EGTA at a final concentration of 0.5 mmol/l was incubated with the enzyme preparation before assay without calcium.

RESULTS

Time-linearity of the assay

The release of oleic acid from 1-palmitoyl-2-oleoyl phosphatidylcholine was linear and directly related to incubation time (Fig. 1) for up to 30 min. This pattern of activity was verified in two studies and thus all subsequent assays were conducted with an incubation time of 20 min.

Enzyme concentration

A linear relationship between the amount of enzyme present and the release of oleic acid was demonstrated up to 0.5 mg protein/tube (Fig. 2). Consistent results were obtained in two further studies.

Substrate concentration

The apparent K_m was determined using duplicate measurements of the initial velocity over a range of substrate concentrations (4–1000 µmol/l) at a single time point of 15 min. A typical Lineweaver–Burk plot for oleic acid release from 1-palmitoyl-2-oleoyl phosphatidylcholine is shown in Fig. 3. The K_m value obtained was 110 µmol/l. Successive estimations gave closely similar values (100 and 125 µmol/l).

FIGURE 2. Human endometrial phospholipase A_2 activity, measured by the release of oleic acid from 1-palmitoyl-2-oleoyl phosphatidylcholine, as a function of enzyme concentration.

FIGURE 3. Lineweaver–Burk plot of the initial velocity (V) of oleic acid release at various concentrations of 1-palmitoyl-2-oleoyl phosphatidylcholine between 4 and 1000 µmol/l. The apparent Michaelis constant was calculated to be 110 µmol/l using duplicate measurements of the initial velocity over the range of substrate (S) concentrations at a single time-point.

FIGURE 1. Time-course of human endometrial phospholipase A_2 activity as measured by the release of oleic acid from 1-palmitoyl-2-oleoyl phosphatidylcholine.
Optimum pH

The pH profile as depicted in Fig. 4 demonstrated that the optimum pH for PLA₂ in human endometrium is 8.0–9.0. A minor peak was apparent at pH 6.5. Activity was low at extreme acid and alkaline conditions.

Calcium requirements

Figure 5 illustrates the dependence of the enzyme on calcium. In the absence of calcium the release of oleic acid was only 60% of that in the presence of 2 mmol calcium/l. No difference in activity was observed at concentrations between 2 and 6 mmol/l but at higher concentrations (8–10 mmol/l) activity was slightly reduced (91 and 89% of the activity measured in the presence of 2–6 mmol calcium/l for 8 and 10 mmol calcium/l respectively). Incubation of the enzyme preparation with EGTA before assay in the absence of calcium did not further reduce the amount of activity measured.

Phospholipase A₂ activity in endometrium during the menstrual cycle

The profile of PLA₂ activity in endometrial tissue from women at different stages of the menstrual cycle is presented in Fig. 6. A small but significant (P < 0.01, t = 3.2, d.f.13) increase in enzyme activity from early to late proliferative phase was followed by an approximate tenfold increase 2–4 days after ovulation.

Figure 4. Effect of pH on the release of oleic acid from 1-palmitoyl-2-oleoyl phosphatidylcholine by phospholipase A₂ activity in human endometrium.

Figure 5. Effect of calcium on phospholipase A₂ activity, measured by the release of oleic acid from 1-palmitoyl-2-oleoyl phosphatidylcholine, in human endometrium.

Figure 6. Phospholipase A₂ activity in human endometrium with respect to the stage of the menstrual cycle. Values are means ± S.E.M. The value for each individual subject was determined in duplicate and the number of subjects per group is indicated in parentheses. Where only two observations were available the individual values are indicated. EP, early proliferative phase (days 4–8); LP, late proliferative phase (days 9–14); P/ES, transition between proliferative and early secretory phase (determined by histological examination); ES, early secretory phase (days 2–4 post-ovulation); MS, mid-secretory phase (days 6–10 postovulation); LS, late secretory phase (days 10–12 postovulation); LS/M, transition to menstrual phase (determined by histological examination); M, menstrual phase. Mean values were all significantly different from each other (Student’s t-test) excluding EP vs M and MS vs P/ES (P < 0.01 with the exception of EP vs ES, LP vs ES and LP vs MS when P < 0.001).
(P < 0.001, t = 5.7, d.f.10 and t = 8.3, d.f.17 for early and late proliferative phases respectively). The mean activity of the enzyme in tissue in transition between proliferative and early secretory phase was double that of late proliferative phase and half that of the early secretory-phase tissue. The means were significantly different (P < 0.01, t = 3.7, d.f.14 and t = 3.5, d.f.11 for late proliferative and early secretory phases respectively). Throughout the secretory phase, mean enzyme activities declined to reach values which at menstruation approximated to those of the early proliferative phase and were not significantly different (t = 1.32, d.f.5).

**DISCUSSION**

The aim of the present study was to determine the characteristics of PLA₂ in human endometrium and to measure the activity during the menstrual cycle. In proliferative-phase endometrium the enzyme was found to be calcium dependent and to have a pH optimum of 8–9 which is indicative of membrane-bound PLA₂ (Blackwell & Flower, 1983). However, a minor peak at pH 6–5 and some residual activity in the absence of calcium suggests that some lysosomal activity may be present and the likelihood of two PLA₂ enzymes with separate pH optima should be considered. A pH optimum of 8 with a secondary peak of activity at pH 6 and a requirement for calcium was also reported for PLA₂ of guinea-pig endometrium (Downing & Poyser, 1983). In contrast, lysosomal PLA₂ of mid-term human decidua showed optimum activity at pH 5 and was inhibited by calcium (Akesson & Gustavii, 1975). In human endometrium, in the present study, an apparent Kₐp of 110 µmol substrate/l was found. No published data are available for comparison but Dey et al. (1982) reported saturation of the rat uterine enzyme at concentrations of 120 µmol substrate/l which approximates to the findings reported here.

The activity of PLA₂ in human endometrium, as measured under the conditions established in this study, was found to be within the range 2–62.5 pmol/mg protein per min depending on the stage of the menstrual cycle. These values can be compared with measurements of PLA₂ activity obtained by other workers and are lower than those reported for human endometrium of pregnancy. The average activity measured by Grieves & Liggins (1976) in term decidua was 351 pmol/mg protein per min. Different activities might be expected if the marked physiological and endocrinological changes which take place during pregnancy are considered.

Information derived from animal studies supports the view that uterine PLA₂ may be regulated by the action of ovarian steroids. Dey et al. (1982) and Pakrasi et al. (1983) showed that when hypophysectomized rats were treated with implants of oestradiol for 5 days, there was a substantial increase in uterine PLA₂ activity which could be inhibited by dexamethasone. Furthermore, administration of progesterone for 5 days had no effect, but enzyme activity was enhanced by injection of oestradiol at the end of the treatment period. The response was maximal at 12 and 24 h following oestradiol injection and was inhibited by cycloheximide which suggests that protein synthesis is involved. A dexamethasone-inducible protein, termed macrocortin, which inhibits PLA₂ activity has already been described (Blackwell, Carnuccio, Di Rosa et al. 1980) but whether a similar mechanism operates for the action of oestradiol or whether de-novo enzyme synthesis is induced remains to be established.

A relationship with ovarian steroids is further indicated by the work of Downing & Poyser (1983) who measured endometrial PLA₂ activity in guinea-pig endometrium on days 7 and 16 of the oestrous cycle and noted an increase in activity on day 16. They suggested that the rise in plasma concentrations of oestradiol which occurs after day 10 might be responsible for the increase in enzyme activity. These findings lend weight to the concept of a similar control mechanism for PLA₂ activity during the human menstrual cycle.

In the present study PLA₂ activity in human endometrium was shown to be lower in the proliferative phase than in the secretory phase of the cycle. There was a significant increase (80%) in the mean activity in late proliferative-phase endometrium compared with that of the early proliferative phase which is in accordance with the rise in plasma concentrations of oestradiol, characteristic of the follicular phase of the cycle. The preovulatory surge in oestradiol acting in concert with secretion of progesterone from the newly formed corpus luteum may subsequently be responsible for the dramatic rise in PLA₂ activity which occurs 2–4 days after ovulation. Evidence for this is also apparent in the transition between proliferative and early secretory phases where the activity of the enzyme is double that of the late proliferative phase. As progesterone secretion increases so PLA₂ activity is progressively inhibited until at menstruation, when both ovarian steroid concentrations are low, enzyme activity is reduced to proliferative-phase values.

In the absence of any other influencing factors, the pattern of PLA₂ activity should determine the availability of arachidonic acid for prostaglandin production. If this were so, the prostaglandin content of the endometrium might be expected to follow a similar pattern to that of PLA₂ activity. There is, however,
general agreement that secretory-phase tissue concentrations of prostaglandins are substantially higher than those of the proliferative phase, with the highest levels of both prostaglandins occurring at menstruation (Downie, Poyser & Wunderlich, 1974; Singh et al. 1975; Willman, Collins & Clayton, 1976). These findings conflict with the results of the present study and suggest that other factors modulate prostaglandin synthesis. It is possible that in the cycle, leukocyte infiltration gives rise to a predominance of lysosomal \( \text{PLA}_2 \) with a low optimum pH which has not been detected under the present assay conditions.

To assume a direct relationship between endometrial \( \text{PLA}_2 \) activity and prostaglandin concentrations is an over simplification of a complex situation. The origin and fate of arachidonic acid must be considered. The action of phospholipase \( C \) and diglyceride lipase may also contribute to the generation of free arachidonic acid. Furthermore, the availability of free arachidonic acid will be influenced by uptake into phospholipids. Downing, Hutchon & Poyser (1983) found that normal secretory endometrium esterified significantly more arachidonic acid into phospholipids than did proliferative-phase tissue. Similarly, in the guinea-pig, arachidonic acid uptake into endometrium is enhanced at the time of maximum prostaglandin biosynthesis (Ning, Leaver & Poyser, 1983). This increase in uptake could increase the amount of substrate available for degradation to free arachidonic acid and thus increase the quantity of prostaglandins synthesized.

Prostaglandin concentrations in endometrium may be further regulated by the action of the enzyme 15-hydroxyprostaglandin dehydrogenase which catalyses the metabolism of prostaglandins to inactive metabolites. Prostaglandin dehydrogenase has been studied throughout the menstrual cycle by Casey, Hemsell, MacDonald & Johnston (1980) who demonstrated a pattern of activity which was similar to that of the present study for \( \text{PLA}_2 \) that is maximum activity in early and mid-secretory endometrium and low activity in menstrual and premenstrual endometrium. While, as the above authors suggest, progesterone may stimulate prostaglandin dehydrogenase, it appears from their data that enzyme activity is already increasing in the late proliferative-phase tissue and it may well be oestradiol which is the effective steroid. Progesterone may subsequently inhibit prostaglandin metabolism thus permitting a rise in prostaglandins towards menstruation. Since hormonal stimulation of \( \text{PLA}_2 \) and prostaglandin dehydrogenase will result in opposing effects on prostaglandin production, the final concentrations present will reflect a balance between the activities of the two enzymes. However, modulation of cyclo-oxygenase activity could also play a significant part.

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**REFERENCES**


that generates from prostaglandin endoperoxides the substance (prostaglandin x) which prevents platelet aggregation. Prostaglandins 12, 715–737.


