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# PARTIAL PURIFICATION AND CHARACTERISATION OF TWO COMPOUNDS FROM AMNIOTIC FLUID WHICH INHIBIT PHOSPHOLIPASE ACTIVITY IN HUMAN ENDOMETRIAL CELLS

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SUMMARY. Two inhibitors of PGF synthesis in human endometrial cells were found in amniotic fluid from term non-labouring patients. Both inhibitors at $10^{-9}$M reduced the amount of arachidonic acid released from the cells but had no effect on PGF synthesis stimulated by arachidonic acid. The molecular weights were 150-165,000 and 70-80,000 daltons as estimated from gel filtration in two systems. Both compounds precipitated with ammonium sulfate, appeared thermostable and lost activity with trypsin digestion. Both inhibited phospholipase activity and had identical retention times with HPLC. We conclude that the compounds are novel endogenous proteins which inhibit endometrial cell phospholipase. 

The onset of parturition is associated in many mammalian species with the release from uterine tissues of substantial quantities of prostaglandins, particularly prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and prostaglandin $E_2$ ($PGE_2$), which activate the uterine smooth muscle and relax the cervical connective tissue (see 1 for review). In certain species such as the sheep, cow and pig, the stimulus to prostaglandin synthesis is a sharp increase in the estrogen: progesterone ratio at term (2).

In primates, including man, the estrogen:progesterone ratio is constant in late pregnancy and the physiological stimulus to prostaglandin synthesis is unknown. A widely held hypothesis proposes that specific non-steroidal substances emanating from the conceptus inhibit uterine prostaglandin synthesis until term when either their concentration falls or they are replaced by specific stimulators (3). If such substances exist, they are likely to be present in amniotic fluid which contains the products of many fetal tissues including kidneys, lungs and placental membranes. In the present study, partially-purified fractions of amniotic fluid from women in late pregnancy were assayed in a preparation of dispersed endometrial cells that recognises the presence of inhibitory activity at any point in the biosynthetic pathway of $PGF_{2\alpha}$.

0006-291X/85 $1.50

METHODS

1. Cell isolation and culture. Endometrial cells were isolated and cultured as previously described (4) and treated with test substances during either incubation or perfusion.

Prior to perfusion the cells were incubated with [$^{3}$H]-arachidonic acid for one hour and then pipetted onto a filter (pore size 0.45µ, diameter 2.5 cm) and perfused at 37°C with G199 medium containing Hank's salts and 1 mg/ml BSA at a rate of 6 ml/h. The system has been described in detail (4). Amniotic fluid components were added as appropriate before the start of perfusion. Radioactivity was measured in each fraction by a standard scintillation counting technique. When a stable base-line had been reached (approximately 1 h from the start of the perfusion) 0.3 ml of medium containing either histamine ($10^{-5}$M) or bradykinin ($10^{-6}$M) was injected into the system via an injection loop. This resulted in sharply increased release of radioactivity which was identified as [$^{3}$H-] arachidonic acid by high performance liquid chromatography (HPLC).

The supernatant from cell incubation experiments was diluted to 2 ml with glass distilled water and extracted with 2 ml ethyl acetate at neutral pH and then at pH 3.0. The second ethyl acetate extract was evaporated to dryness under a stream of $N_2$ and redissolved in 0.5 ml Tris buffer (0.1M, pH 8.0). Duplicate samples of 100 µl were assayed for PGF by RIA as described previously (5). Protein was detected by absorbance at 280 nm and measured by the Lowry method (6).

2. Partial purification of amniotic fluid components. A pool of whole amniotic fluid was obtained from patients with minor complications of pregnancy undergoing amniotomy for induction of labor within two weeks of term. Fluid was collected from 19 patients with a mean age of 26.1 years and a range of 19-42y. It was stored at -20°C prior to pooling, centrifugation (27000g, 15 min, 4°C) and lyophilisation of supernatant. Two additional samples of amniotic fluid were processed separately.

A Sephacryl S300 column, (1.5x35 cm) was equilibrated and run in 0.03M $NH_4HCO_3$ at 4°C. The 70-100% $(NH^4)_2SO_4$ precipitate from 15 ml reconstituted amniotic fluid was eluted at a flow rate of 0.5 ml/min. This was repeated with a Sephadex G200 column (92.5 x 30 cm). Molecular weights were determined in both systems using catalase, ovalbumin and myoglobin as standards.

Samples were prepared by ammonium sulfate precipitation and Sephadex G200 or Sephacryl S300 gel filtration. An RPSC column was used with a gradient of 60% acetonitrile in 0.1% trifluoroacetic acid (solvent A) against 0.1% trifluoroacetic acid (solvent B). Flow rate was 1 ml/min and the initial buffer concentration was 40%A into 60%B. A gradient of 85%A into 15%B over thirty minutes was used. Peaks were detected at 280 nm.

3. Assay of Phospholipase Activity. Phospholipase $A_2$ activity was measured by the method of Ballou and Cheung (7), using L-3-phosphatidyl choline, 2 [1-$^{14}$C] oleoyl-1-palmitoyl as substrate. The radioactivity associated with free oleic acid amounted to A2175 dpm and 4383 dpm in the absence and presence respectively of phospholipase $A_2$.

Phospholipase C activity was measured by the method described by Rittenhouse (8), using L-3-phosphatidyl [N-methyl-$^{14}$C] choline 1,2 dipalmitoyl as substrate. Radioactive phosphoryl choline released measured 181 and 2080 dpm in the absence and presence of enzyme respectively.

RESULTS

Fig 1 shows a typical profile of obtained after Sephadex G200 gel filtration. Three inhibitory regions are apparent. The estimated molecular weights of the inhibitory compounds were 150-165,000 (Inhibitor I), 70-80,000 (Inhibitor II), and 18,000 as calculated from Sephadex G200 and Sephacryl S300 gel

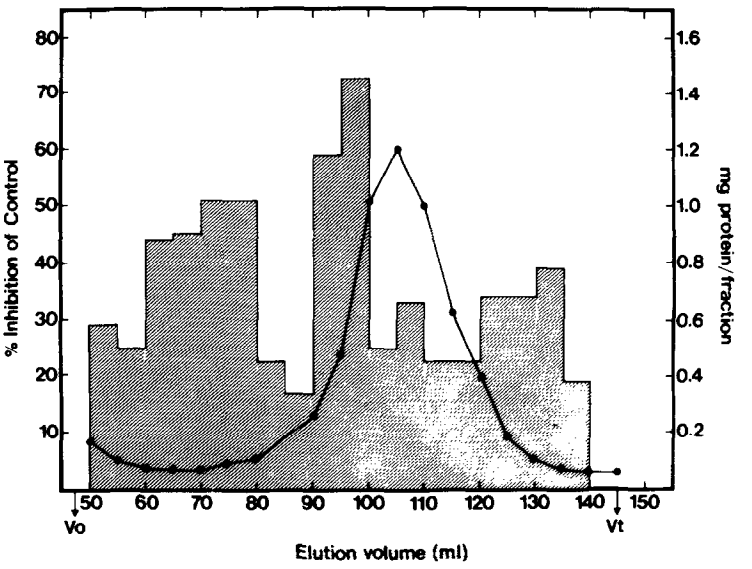
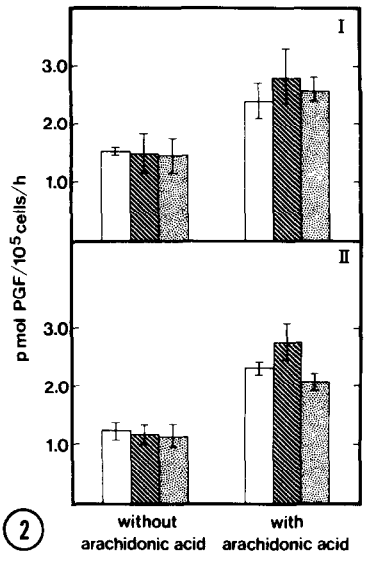



Fig 1
Sephadex G 200 gel filtration. The 70-100% ammonium sulfate precipitate from 25 ml whole amniotic fluid from non-laboring patients was eluted thorugh a G200 Sephadex column with 0.03M ammonium bicarbonate. The protein content of the fraction was measured by the Lowry method. Each fraction was lyophilised, redissolved in 100 µl glass distilled water and added to 199 medium (10 µl/ml). Washed endometrial cells ($10^5$) were added to 1 ml aliquots of medium and incubated with and without histamine as described in the text. The PGF content of each fraction was measured by RIA after extraction and expressed as percentage inhibition according to the formula: 100-[(G200 fraction with histamine-G200 fraction without histamine) x 100/(control with histamine-control without histamine)]. The controls contained 10 µl glass distilled water per ml 199 medium.

filtration. The profile shown was obtained five times from the pooled amniotic fluid and twice from two separate amniotic fluids. As the inhibitory activity of the fraction corresponding to peak 3 was less intense and more erratic than that of peaks 1 (Inhibitor I and 2 (Inhibitor II), further work was restricted to characterisation of the latter compounds.

Depression by Inhibitors I and II of histamine-stimulated release of PGF could be caused by a reduction either in the liberation of free arachidonic acid or in the conversion of arachidonic acid to PGF metabolism. The alternatives were investigated separately by determining the effects of the inhibitors first on PGF synthesis stimulated by addition of arachidonic acid, and second on the release of arachidonic acid stimulated by histamine or bradykinin. No inhibition of basal or arachidonic acid-stimulated synthesis of PGF was observed (Fig 2).

Typical profiles of the release of arachidonic acid in the perfusion system are shown in Fig 3. The peak/noise ratio was high even when the response to histamine was reduced by Inhibitors I and II. Results summarised in



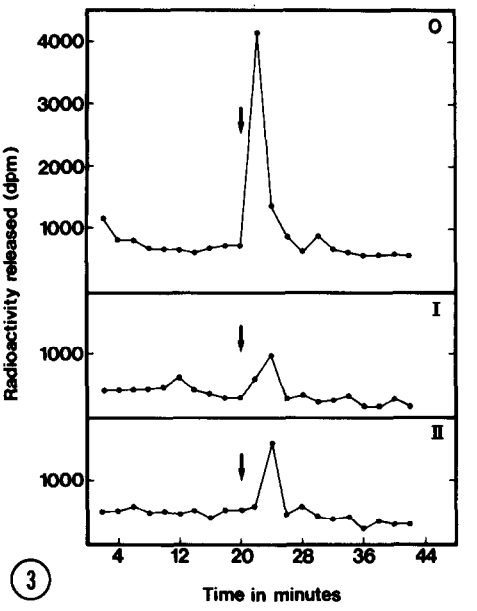




Fig 2
The effect of Inhibitors I and II on arachidonic acid stimulated PGF synthesis rate. Fractions from gel filtration corresponding to Inhibitors I (60-90 ml eluate) and II (90-100 ml eluate) were pooled, lyophilised, weighed and dissolved in glass distilled water, and added to 199 medium in the concentrations shown. Cells were incubated in this medium with and without arachidonic acid and PGF was measured by RIA after extraction. The experiment was performed four times and results are expressed as the mean ± SE. I and II refer to Inhibitors I and II.

Control 0.2 µg/ml inhibitor 2.0 µg/ml inhibitor

Fig 3
Perfusion of endometrial cells. Endometrial cells labelled with [$^3$H-] arachidonic acid were perfused with 199 medium for one hour with (I and II) and without (O) the addition of inhibitory material. Inhibitors I and II were used at a concentration of 0.2 µg equivalents/ml medium. Each perfusion system contained $10^5$ cells. After a further 20 min, 0.3 ml histamine ($10^{-5}$M) was added to the cells via an injection loop at the times indicated by the arrows.

Table 1 show highly significant activity of Inhibitors I and II on [$^3$H-] arachidonic acid release by endometrial cells.

When the dose response relationship was examined it was found that the effect of Inhibitors I and II increased with concentration up to a level of about 70% inhibition (Fig 4). Dose dependent inhibition of phospholipase $A_2$ (from porcine pancreas) was observed in the presence of both inhibitors (Fig 5) but there was no effect on phospholipase C (from Bacillus cereus) at the concentrations tested. Activity was lost by heating the inhibitors to 121°C for 20 min but the compounds remained active after heating to 80°C for 15 min. Incubation with trypsin (1 µg/10 µg inhibitor) for 1 h at 37°C completely destroyed activity of the inhibitors.

TABLE 1

The effect of amniotic fluid components on the release of arachidonic acid from endometrial cells

| Treatment | n | Radioactivity released (dpm) | % Inhibition of control |
|---|---|---|---|
| Nil | 7 | 451 ± 81 | |
| Histamine (Control) | 7 | 1424 ± 270 | 0 |
| Histamine + Inhibitor I | 7 | 751 ± 124 | 65.6*** ± 9.1 |
| Histamine + Inhibitor II | 6 | 680 ± 59 | 61.0** ± 12.8 |
| Nil | 3 | 236 ± 37 | |
| Bradykinin (Control) | 3 | 650 ± 70 | 0 |
| Bradykinin - Inhibitor I | 3 | 287 ± 65 | 82.7*** ± 17.3 |
| Bradykinin - Inhibitor II | 3 | 321 ± 20 | 88.5*** ± 9.8 |

Radioactively-labelled endometrial cells were perfused in 199 medium for one hour with and without the addition of inhibitory material. Each perfusion system contained $10^5$ cells. After obtaining a steady baseline, histamine (0.3 ml $10^{-5}$M) or bradykinin (0.3 ml $10^{-6}$M) was introduced to the system via an injection loop. Results are expressed as mean ± SE. To obtain the % stimulation, the baseline dpm was subtracted from the dpm obtained in the presence of histamine. The stimulation in the presence of each inhibitor was then calculated as a proportion of its control.

Radioactivity released is expressed as mean dpm ± SE
*** p < .001 compared to 0 ** p < .01 compared to 0

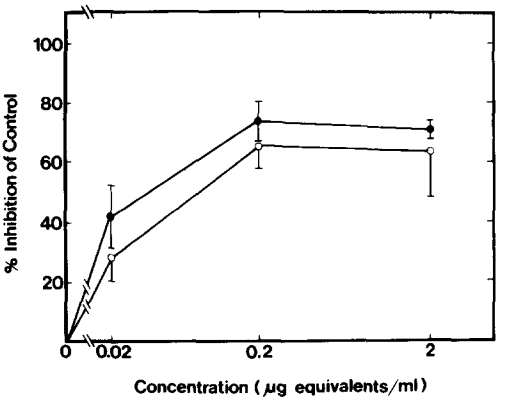



Fig 4
Dose response relationship of Inhibitors I and II. The active material eluted from the Sephadex G200 column was subjected to HPLC under conditions described in the Methods section. As the lyophilised fractions obtained from HPLC were too small to weigh acurately, concentration is expressed in terms of µg weight before HPLC. The HPLC-purified material was diluted with G199 medium and used in the perfusion of [$^3$H] arachidonic acid labelled cells. After an initial 1.3 h perfusion, histamine (0.3 ml, $10^{-5}$M) was introduced into the system via an injection loop. The percentage inhibition was calculated according to the formula 100-(dpm in the presence of histamine and inhibitor-dpm in the absence of histamine and inhibitor) x 100/(dpm in control in the presence of histamine -dpm in control in the absence of histamine). The controls contained 10 µl glass distilled water per ml 199 medium. Each point was obtained 3 or 4 times and is expressed as a percentage of its own control ± SE.

○ Inhibitor I ● Inhibitor II

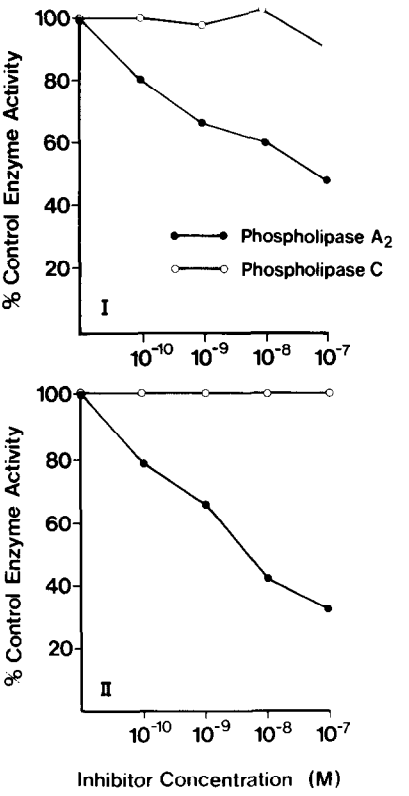



Fig 5
Effect of Inhibitors I and II on Phospholipase A and C activity. Inhibitors I and II were partially purified by ammonium sulfate precipitation, gel filtration and HPLC. As the lyophilised fractions obtained from HPLC were too small to weigh accurately concentration is expressed in terms of weight before HPLC.
Values for Inhibitor I are the means of duplicate experiments (range < ±16% of the mean) and values for Inhibitor II are the means of triplicate experiments (S.E.< ±8.6 for all values).
I - Inhibitor I II - Inhibitor II

## DISCUSSION

Two compounds are present in human amniotic fluid obtained before the onset of labor which inhibit arachidonic acid release and prostaglandin F synthesis in dispersed endometrial cells stimulated by histamine or bradykinin. Evidence consistent with a protein structure included precipitation with ammonium sulfate, high molecular weights, loss of activity on trypsin digestion and absorbance at 280 nm.

Both compounds appear to reduce the rate of synthesis of PGF by inhibiting phospholipase activity. They were detected initially by observing inhibition of histamine-stimulated synthesis of PGF as shown in Fig 1. Histamine is known to increase phospholipase activity (9) and arachidonic acid concentration is held to be rate limiting in prostaglandin synthesis. The possibility that the compounds inhibit cyclo-oxygenase or prostaglandin synthetase was

excluded by observing that archidonic acid-stimulated release of PGF was not significantly decreased by inhibitors I and II even at a concentration of 2 µg/ml (Fig 2). A phospholipase as the site of inhibitory action was supported further when it was found that the release of labeled arachidonic acid stimulated by either histamine, bradykinin or Ca ionophore A23187 was decreased in the presence of the inhibitors (Table 1). The degree of inhibition appears not to increase at concentrations above 0.2 µg equivalents/ml (Fig 4) possibly indicating that more than one pathway is available for release of arachidonic acid from cellular stores.

Both inhibitors and have substantial activity at 0.2 µg/ml as calculated from the gel filtration product (Table 1). The latter has a large serum albumin component and subsequent purification by HPLC resulted in a recovery of around 10%. The concentrations shown in Fig 4 and Fig 5 are calculated from the dry weight of the gel filtration product as at this stage we have not recovered enough of the product to weigh accurately. Approximate estimates of the concentration of Inhibitor I (MW 165,000) and Inhibitor II (MW 80,000) giving 60-70% inhibition are $1x10^{-10}$M and $2x10^{-10}$M respectively. This potency exceeds published values for other phospholipase inhibitors. Synthetic inhibitors are active in the range of $10^{-7}$ to $5x10^{-3}$ (10) and the range of activity for corticosteroids is $2x10^{-8}$M to $10^{-3}$M (11,12). Lipomodulin (13) and macrocortin (12) are known to mediate glucocorticoid effects. Lipomodulin has activity at $5x10^{-8}$M (14).

Inhibitor I may be a dimer of Inhibitor II. The compounds were indistinguishable in their heat-stability, protein-digestibility, active concentrations and HPLC retention times. Molecular weight was the only basis on which they could be separated.

Inhibitors I and II appear to be novel compounds. Other potential endogenous modulators of prostaglandin synthesis in amniotic fluid have been reported, but none inhibits arachidonic acid release which is held to be the rate limiting step of a prostaglandin synthesis (14). Factors described by Billah and Johnson (15) and Casey et al (16) stimulate prostaglandin synthesis and two others have been shown to decrease cell-free prostaglandin synthetase activity (17, 18).

Any change in the rate of synthesis of Inhibitors I and II at term would not be reflected in their concentration in amniotic fluid if the turnover rate is low. Incubation of amniotic fluid at 37°C for 96 h was associated with inactivation of Inhibitor I but no loss of activity of Inhibitor II (data not shown) suggesting that turnover may be relatively slow.

The possibility that the presence of Inhibitors I and II in pooled amniotic fluid represents the contribution of a single atypical sample is unlikely because two single amniotic fluids gave similar profiles to that shown in

Fig 1. We conclude that they are normal constituents of amniotic fluid near term, but further work is required to determine their source. The potency of these compounds and their location suggest a physiological role in the regulation of prostaglandin synthesis during pregnancy.

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