J. Steroid Biochem. Molec. Biol. Vol. 39, No. 3, pp. 337-342, 1991
Printed in Great Britain

0960-0760/91 $3.00 + 0.00
Pergamon Press plc

EFFECT OF ESTRADIOL AND PROGESTERONE ON PHOSPHATIDYLINOSITOL METABOLISM IN THE UTERINE EPITHELIUM OF THE MOUSE

JANE E. BOURKE,[1] S. DANK,[1] P. A. WILCE[1] and L. MARTIN[2]*
Departments of [1]Biochemistry and [2]Physiology and Pharmacology, University of Queensland, Queensland, Australia 4072

(Received 29 January 1991)

Summary—The effect of estradiol and progesterone on uterine phosphatidylinositol (PtdIns) metabolism was examined in whole uteri and separated uterine luminal epithelium of ovariectomized mice. Incorporation of [^{3}H]*myo*-inositol *in vitro*, into inositol-containing phospholipids extracted from whole uteri, increased in mice injected with estradiol, with maximal incorporation at 9–12 h. The breakdown of PtdIns to inositol polyphosphates was also stimulated in whole uteri by estrogen, with an abrupt increase between 6 and 9 h. Comparable increases in both processes occurred in the uterine epithelium after estrogen stimulation and were inhibited by progesterone pretreatment which by itself had little or no effect. These results suggest that PtdIns metabolism is involved in the stimulation of uterine epithelial cell proliferation by estrogens, and its inhibition by progesterone.

INTRODUCTION

A major pathway of receptor signal transduction in the stimulation of cell growth, involves phosphoinositide metabolism [1, 2]. In this pathway, phosphatidylinositol 4,5 bisphosphate [PtdIns(4,5)P_2] a low abundance membrane phospholipid is cleaved by receptor-mediated activation of a specific phospholipase C, resulting in the generation of two second messengers, inositol (1,4,5)-trisphosphate [Ins(1,4,5)P_3] and diacylglycerol (DAG). The former controls the release of Ca^{2+} from non-mitochondrial intracellular stores, while the latter is the physiological activator of the calcium-activated phospholipid-dependent protein kinase C. In addition, as a substrate for diacylglycerol lipase, DAG releases archidonate for synthesis of prostaglandins [3, 4]. Both inositol phosphates (IPs) and DAG are metabolized and re-used in the synthesis of new phosphatidylinositol (PtdIns). The receptor-stimulated hydrolysis of PtdIns(4,5)P_2 to form the two second messengers has been implicated in a number of cellular processes including secretion, platelet aggregation and cell proliferation. A number of reports have emphasised the rapid and dramatic change in lipid synthesis and mobilization resulting from estrogenic stimulation of the uterus [5, 6]. Grove and Korach [7], showed an estrogenic stimulation of the incorporation of precursors into PtdIns(4,5)P_2 in whole-uterus of ovariectomized mice. Freter *et al.* [8] demonstrated elevation of steady-state levels of PtdIns turnover in cultured MCF-7 mammary tumour cells stimulated to proliferate by estradiol: the increase was blocked by the competitive anti-estrogen LY117018 and the growth-inhibitory transforming growth factor *b*.

The uterine epithelium of the ovariectomized mouse is a useful *in vivo* model system for the study of hormone-stimulated cell proliferation and differentiation. In response to a single injection of estrogen the epithelium undergoes a highly synchronized wave of cell division [9, 10]. The cells can readily be removed from the whole organ with a high degree of purity [11]. Biochemical changes can therefore be precisely related to events associated with S phase and mitosis. DNA synthesis in the epithelial cells is completely inhibited by pretreatment with progesterone [12, 13] although estrogen-binding and other estrogen-stimulated events e.g. protein synthesis are not [14–18]. Previously we reported a marked stimulation by estrogen of incorporation of acetate into PtdIns of the uterine epithelium of the mouse [19]. We have now extended these studies to investigate IP accumulation during estrogen-induced cell proliferation and its inhibition by progesterone.

*To whom correspondence should be addressed.

MATERIALS AND METHODS

Animals and hormone treatment

Female Quackenbush mice were housed, fed and ovariectomized as described previously [19, 20]. They were primed for three days with drinking water containing 200 ng/ml estradiol (Sigma, St Louis, MO) and killed by cervical dislocation on day 5 after priming, at various times after the following treatments. Control and estrogen-treated animals received a single subcutaneous (s.c.) injection of 0.1 ml arachis oil, or 50 ng estradiol in arachis oil, respectively on day 5. Progesterone was given as daily s.c. injections of 1 mg, on days 3, 4 and 5 after priming. Uteri were removed, trimmed of mesentery and slit longitudinally, before being placed into ice-cold Krebs bicarbonate buffer.

Incorporation of [^{3}H]D-myo-inositol

Uteri were transferred to vials (10 horns/2.5 ml) containing Krebs buffer containing 5 mM LiCl with 5 μCi/ml [^{3}H]D-*myo*-inositol (Amersham International, Amersham, U.K.), gassed with carbogen (5% CO_2 in oxygen) and kept in a shaking waterbath for 2 h at 37°C. After incubation, the vials were placed on ice, and the horns washed three times with ice-cold buffer.

Tissue extractions

Whole uteri. Uteri were transferred to sucrose buffer A (100 mM sucrose, 50 mM KCl, 40 mM KH_2PO_4 and 30 mM EDTA, pH 7.6) and homogenized (10 horns/ml) using a Polytron homogenizer at a setting of 5 for 30 s. Aliquots were taken for protein estimation [21]. The homogenate was extracted in chloroform–methanol–concentrated HCl–butylated hydroxytoluene (BHT) (2:1:0.01:0.005, v/v), before being filtered through a Whatman No. 1 filter into a separating funnel containing 0.9% NaCl. The two phases were allowed to separate for 1–2 h, after which the lipid phase was collected and the solvent removed by rotary evaporation. The sample was then resuspended in a small volume of chloroform–methanol–BHT (2:1:0.005, v/v) and stored at −20°C under N_2, prior to scintillation counting and thin layer chromatography (TLC). The interface of the two phases was discarded and the upper phase collected for neutralization and separation of IPs.

Epithelial cells. Epithelial cells were isolated using the method of Fagg *et al.* [11]. Briefly, uterine horns were transferred to glass tubes containing 5 mm glass balls and sucrose buffer A (5 horns, 5 balls/ml). Tubes were then flushed with N_2 sealed with parafilm and vortexed for 2 min. Samples were taken for protein estimation. The epithelial fraction was then filtered through a wire gauze filter and extracted as above.

Phospholipid separation

Phospholipids were separated as described by Pollack *et al.* [22]. Briefly, lipid samples were dried under a stream of N_2 and dissolved for application to TLC plates (Merck, Darmstadt, Fed. Rep. Germany) which had previously been saturated with 1 mM EDTA, pH 5.5, and dried prior to activation overnight at 120°C. Standards and samples were applied 1.5 cm from the base of the plate and developed for approx. 1 h, using chloroform–methanol–H_2O–acetic acid (65:50:2:5, v/v), until the solvent front was 12–14 cm from the origin. Lipids were visualized using iodine vapour and identified by co-migration with standards. The band corresponding to PtdIns and its derivatives was scraped from the plate and extracted using chloroform–methanol (1:1). These samples were then divided for phosphorus assays [23] and scintillation counting.

Separation of IPs

Aqueous-phase fractions were neutralized with 200 mM $NaHCO_3$, and processed for analysis of the total [^{3}H]IP fraction, using anion exchange chromatography on AG1X8 columns (200–400 mesh, formate form, Bio-Rad, Sydney, Australia). Routinely, [^{3}H]inositol was washed from the columns with 10 ml H_2O and glycerophosphatidylinositol/lysophosphatidylinositol with 10 ml 5 mM sodium tetraborate/30 mM sodium formate: total IPs were batch eluted with 1.0 M ammonium formate in 0.1 M formic acid as described previously [24]. In some experiments, inositol (4)-monophosphate [Ins(4)P], inositol (1,4)-diphosphate [Ins(1,4)P_2] and Ins(1,4,5)P_3 were eluted stepwise from 5 mM formic acid/2 mM ammonium formate, 0.1 M formic acid/0.2 M ammonium formate, and 0.1 M formic acid/1.0 M ammonium formate, respectively.

RESULTS

Preliminary experiments established optimal conditions for assessment of incorporation of [^{3}H]*myo*-inositol into lipids and IPs. The

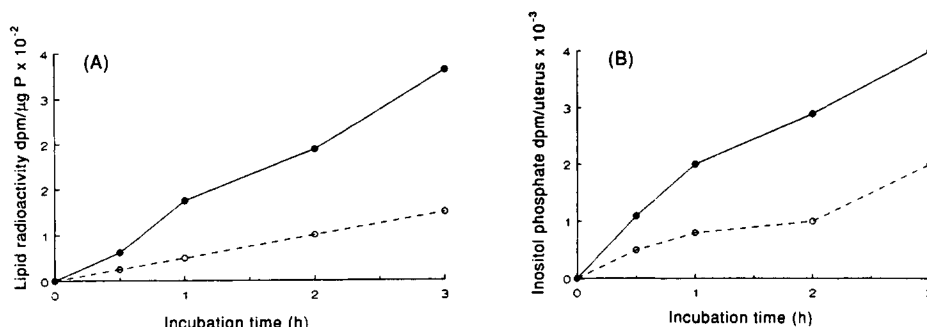

Fig. 1. Incorporation of [^{3}H]*myo*-inositol into whole uterine inositol-containing phospholipids and IPs. Each point represents an incubation with 6 uterine horns. Solid lines: uteri stimulated with estradiol 18 h previously. Dotted lines: vehicle injected controls. Panel A—inositol containing phospholipids, panel B—IPs.

specific radioactivity of PtdIns, PtdIns(4)P and PtdIns(4,5)P_2 and accumulation of IPs in the whole uterus with time of incubation are shown in Fig. 1. Both increased linearly with time in control uteri and those stimulated with estrogen 18 h previously. Rates of incorporation were markedly higher in the estrogen treated uteri.

Fractionation of the IPs indicated that the predominant radioactive species was Ins(4)P, which constituted approx. 60% of the total IP fraction. Ins(1,4)P_2 and Ins(1,4,5)P_3 represented 20 and 10%, respectively (data not shown). Figure 2 shows changes in rates of incorporation of precursor into IPs and PtdIns and its derivatives isolated from whole uterus after a single estrogen injection. Specific radioactivity of the PtdIns fraction (panel A) increased steadily to a peak between 9 to 12 h after estrogen injection and declined thereafter. At the peak, the specific radioactivity was some 7 times that of the control group. Accumulation of whole uterus IPs is shown in panel B. This too increased steadily after estrogen stimulation until 6 h when it jumped abruptly to a peak at 9 h and then declined. The increase in specific activity of the IP fraction was much smaller than that of the lipid fraction, the maximum increase being only 2.5 times the control value.

Separation of the epithelium from the residual tissues (glandular epithelium, connective tissue stroma and myometrium) revealed that the IP radioactivity was distributed approximately equally between the two fractions. Since epithelial cells comprise only 5% of total uterine tissues [9] the IP content of the epithelial cells is approx. 20 times higher than that of the remnants. However, the specific radioactivity of the inositol containing phospholipids was of the same order in both fractions.

The effects of estrogen and progesterone on incorporation into inositol containing phospholipids and accumulation of IP was monitored in the uterine epithelium and residual tissues 4, 9 and 18 h after estrogen stimulation (Fig. 3). For the epithelium in animals given estrogen alone, these times correspond, respectively to the prereplicative (late G_1) phase, the early S phase and what is best described as post S phase, when

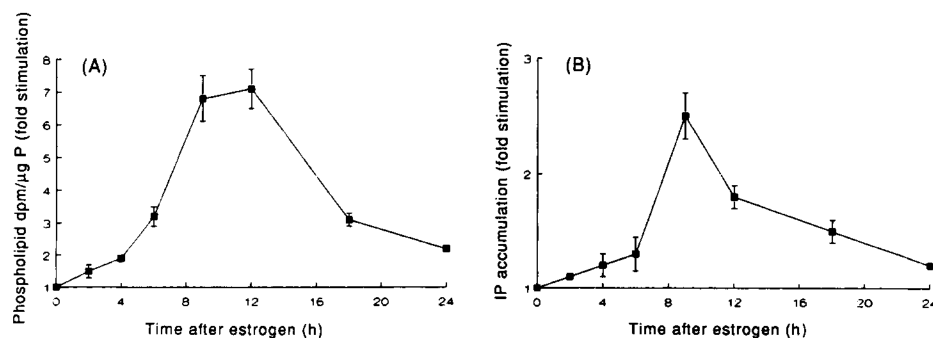

Fig. 2. Incorporation of [^{3}H]*myo*-inositol into whole uterine inositol-containing phospholipids and IPs after a single injection of estrogen. The results are expressed as mean increases (× control = 1) ± SEM or range. Each point is based on 2–6 replicate 2 h incubations of 5 uterine horns. Panel A—inositol containing phospholipids, panel B—IPs.

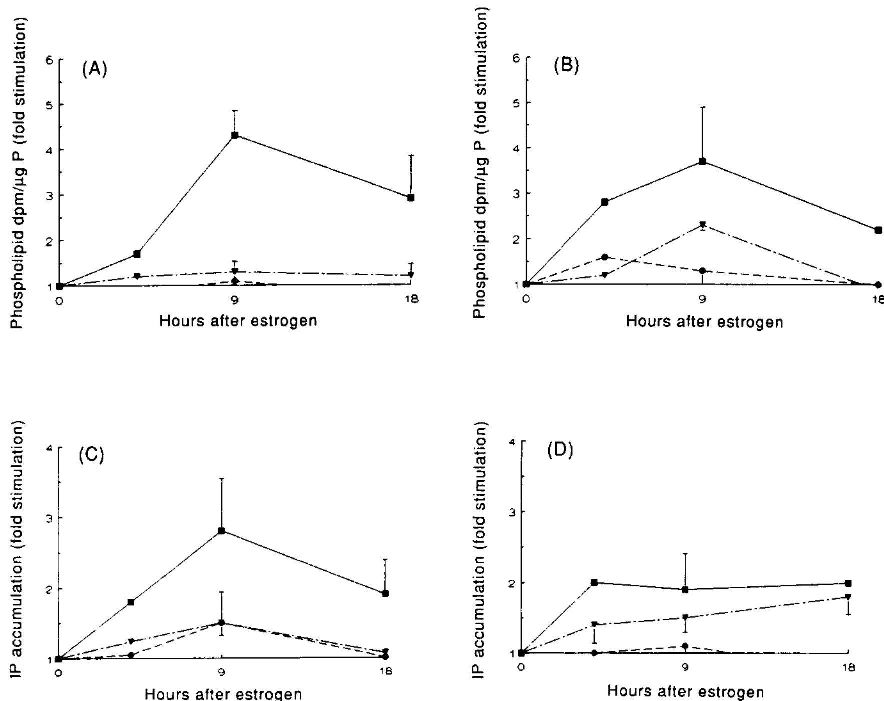

Fig. 3. Effects of estradiol and progesterone on incorporation of [^{3}H]*myo*-inositol into inositol-containing phospholipids and IPs in epithelial (panel A and C, respectively) and residual tissues (panel B and C, respectively) of mouse uterus. Results are expressed as mean increases (× control = 1) ± SEM, or range, with each experiment replicated 2–3 times. Data were analysed by 2 way ANOVA. In the epithelial fraction, estrogen alone (—■—) produced highly significant increases in the specific activity of the PtdIns fraction and in IP accumulation compared to progesterone alone (—●—) and progesterone plus estrogen (—▼—) (F ratios = 24.5 and 49.3 with 1 vs 15 and 11 degrees of freedom (d.f.), respectively: $P < 0.001$ for both). There were no significant differences between progesterone and estradiol (F ratios = 0.2 and 1.1, respectively with d.f. as above). The pattern was seen in PI accumulation in the remnants: estrogen vs progesterone and progesterone and estrogen ($F = 26.4$, 1 vs 11 d.f., $P < 0.001$); progesterone vs progesterone + estrogen ($F = 1$, 1 vs 11 d.f.). IP accumulation in uterine residual tissue was significantly increased by estrogen when given alone (estrogen vs progesterone, $F = 31.5$, 1 vs 15 d.f., $P < 0.001$) and to a lesser extent when given after progesterone ($F = 10.6$, 1 vs 15 d.f., $P < 0.001$). Progesterone alone had no significant effect.

many cells are in G_2 and mitosis although some remain in late S phase [9, 10]. In mice given progesterone the epithelial cells are blocked in G_1 [12] and do not enter S phase or mitosis after estrogen stimulation. Estrogen alone stimulated incorporation of precursor into inositol phospholipids and accumulation of IP in the epithelial cell fraction. The time-courses of the responses were similar to those in whole uterus, with peaks of comparable magnitude at 9 h. In both cases progesterone pretreatment had no significant effect in itself, but completely inhibited the increases produced by estrogen. In the residual tissues, estrogen alone again stimulated incorporation of precursor into inositol phospholipids and accumulation of IP and progesterone alone had no significant effect. Progesterone pretreatment inhibited the increases produced by estrogen, but not completely.

DISCUSSION

Almost every aspect of lipid metabolism that we have investigated in the uterine epithelium is markedly and acutely stimulated by estrogen treatment, e.g. HMG CoA reductase activity [20] cholesterol synthesis, acetate incorporation into all classes of phospholipids and depletion of phospholipid arachidonic acid content [19]. With the exception of arachidonic acid depletion, all of the above responses were inhibited by progesterone pretreatment, and this was particularly striking for HMG CoA reductase activity, cholesterol synthesis and acetate incorporation into PtdIns. The present results are entirely consistent with this picture. Estrogen stimulated incorporation of [^{3}H]inositol into PtdIns and its subsequent hydrolysis to IPs, and progesterone completely inhibited both responses.

The increase in accumulation of IPs induced by estrogen was substantially less than the increase induced in the specific activity of PtdIns. We have reported only the specific activity of the total PtdIns fraction, of which PtdIns(4,5)P_2, the immediate precursor for IP, constitutes only a minor part, which was below the sensitivity of our assays. It is a distinct possibility therefore that estrogenic stimulation of the epithelium actually leads to inhibition of IP production. The sensitivity of present techniques do not allow the experiments required to exclude this possibility for the uterine epithelium. However, such inhibition seems unlikely given the general observation that IP production is usually increased in cells stimulated to proliferate by growth factors [25–27] including estrogen [8].

There are several other explanations for the anomaly. Thus, most of the increased incorporation into the total PtdIns fraction would reflect increased membrane synthesis associated with the epithelial cell hypertrophy and proliferation induced by estrogen. The time course of the PtdIns incorporation response to estrogen is entirely consistent with this view. Furthermore, there is likely to be intracellular compartmentalization of IPs, in that areas of maximum membrane growth may not correspond to areas of maximum inositide turnover. Alternatively, the differential increase in specific activity of PtdIns, relative to accumulation of IP, may result from estrogenic stimulation of the putative inositol exchange enzyme [28, 29].

Our results agree with those of Toth and Hertelendy [30] in that estrogen stimulated, and progesterone inhibited, incorporation of inositol into uterine PtdIns, but are not consistent with their observation that progesterone synergized with estrogen in stimulating incorporation of ^{32}P into uterine PtdIns via the putative 'exchange' enzyme. However Toth and Hertelendy [30] used minces of whole uterus, and the differential effects of progesterone observed in their study may reflect different enzyme populations normally compartmentalized in different uterine tissues. Furthermore, the treatment regimens used by Toth and Hertelendy involved chronic simultaneous administration of estrogen and progesterone. Such regimens result in major alterations in the cellular constitution of the endometrium and the responsiveness of the endometrial cells to estrogen [9].

The increased accumulation of IP induced in the uterine epithelium between 6 and 9 h appears to be closely linked with the cellular hypertrophy and proliferation induced by estrogen [10]. Precursor incorporation into epithelial PtdIns closely parallels the number of epithelial cells in S phase, the maximal increase in IP production occurs when the rate of entry of cells into S phase is maximal, and both inositide and proliferative responses are completely inhibited by progesterone. Nevertheless, altered inositide metabolism does not appear to play a major early role in the initiation of epithelial cell proliferation. Nor do our results enable us to link altered inositide metabolism exclusively to proliferative responses, since similar, but smaller, changes were seen in the remnant tissues of the uterus. These comprise: a population of epithelial gland cells, approximately equal in size to the luminal epithelium, which undergo a minor proliferative response to estrogen that is inhibited by progesterone; about 45% stromal cells, of which about 30% are stimulated by progesterone and estrogen to enter DNA synthesis, with the same time-course as the luminal epithelium stimulated by estrogen alone; about 45% myometrial cells, which do not proliferate in response to either progesterone or estrogen, but whose contractile responses to polypeptide hormones such as oxytocin, are known to involve the inositide signal pathway. Thus increased inositide metabolism induced by estrogen could be associated with glandular or stromal cell proliferation, or increased myometrial sensitivity to spasmogens, while the inhibition of the increase by progesterone could be associated with inhibition of gland proliferation or its well-known inhibition of myometrial contractility. These issues have largely been ignored by previous workers who have studied the effects of estrogen and progesterone on inositide metabolism of whole uterine minces.

It is not clear if the changes in inositide metabolic responses are a direct consequence of estrogenic stimulation or responses to the autocrine or paracrine secretion of growth factors known to be induced in the uterus by estrogen, e.g. EGF [31] and IGF-1 [32] both of which may utilize inositide breakdown as the second messenger pathway in their target cells.

REFERENCES

1. Berridge M. J. and Irvine R. F.: Inositol phosphates and cell signalling. *Nature* **341** (1989) 197–204.
2. Shears S. B.: Metabolism of the inositol phosphates produced upon receptor activation. *Biochem. J.* **260** (1989) 313–324.

3. Michell R. H. and Kirk C. J.: Why is phosphatidylinositol degraded in response to certain stimulation of receptors? *Trends Pharmac. Sci.* **2** (1981) 86–89.
4. Okazaki T., Sagawa N. and Okita J. R.: Diacylglycerol metabolism and arachidonate release in human fetal membranes and decidua vera. *J. Biol. Chem.* **254** (1981) 7316–7321.
5. Aizawa Y. and Mueller G. C.: The effect *in vivo* and *in vitro* of estrogens on lipid synthesis in the rat uterus. *J. Biol. Chem.* **236** (1961) 381–387.
6. Spooner P. M. and Gorski J.: Early estrogen effects on lipid metabolism in the rat uterus. *Endocrinology* **91** (1972) 1273–1283.
7. Grove R. I. and Korach K. S.: Estrogen stimulation of phosphatidylinositol metabolism in mouse uterine tissue. *Endocrinology* **121** (1987) 1083–1088.
8. Freter C. E., Lippman M. E., Cheville A., Zinn S. and Gelmann E. P.: Alterations in phosphoinositide metabolism associated with 17-β estradiol and growth factor treatment of MCF-7 breast cancer cells. *Molec. Endocr.* **2** (1988) 159–166.
9. Martin L., Finn C. A. and Trinder G.: Hypertrophy and hyperplasia in the mouse uterus after oestrogen treatment: an autoradiographic study. *J. Endocr.* **56** (1973) 133–146.
10. Martin L., Pollard J. W. and Fagg B.: Oestriol, oestradiol-17β and the proliferation and death of uterine cells. *J. Endocr.* **69** (1976) 103–115.
11. Fagg B., Martin L., Rogers L., Clark B. and Quarmby V. E.: A simple method for removing the luminal epithelium of the mouse uterus for biochemical studies. *J. Reprod. Fert.* **57** (1979) 335–339.
12. Martin L., Das R. M. and Finn C. A.: The inhibition by progesterone of uterine epithelial proliferation in the mouse. *J. Endocr.* **57** (1973) 549–554.
13. Martin L., Finn C. A. and Trinder G.: DNA synthesis in the endometrium of progesterone-treated mice. *J. Endocr.* **56** (1973) 303–307.
14. Pollard J. W. and Martin L.: The effects of sex steroids on the synthesis of uterine non-histone proteins. *Molec. Cell. Endocr.* **2** (1975a) 183–191.
15. Pollard J. W. and Martin L.: Cytoplasmic and nuclear non-histone proteins and mouse uterine cell proliferation. *Molec. Cell. Endocr.* **6** (1975b) 223–229.
16. Quarmby V. and Martin L.: Effects of progesterone on uptake and metabolism binding in mouse uterine epithelium. *Molec. Cell. Endocr.* **27** (1982a) 317–330.
17. Quarmby V. and Martin L.: Qualitative effects of progesterone on estrogen binding in mouse uterine epithelium. *Molec. Cell. Endocr.* **27** (1982b) 331–342.
18. Smith J. A., Martin L. and King R. J. B.: Effects of oestradiol-17β and progesterone on total and nuclear-protein synthesis in epithelial and stromal tissues of mouse uterus and of progesterone on the ability of these tissues to bind oestradiol-17β. *Biochem. J.* **119** (1971) 773–784.
19. Stacey K., Wilce P. A. and Martin L.: Effects of female sex hormones on lipid metabolism in the uterine epithelium of the mouse. *Int. J. Biochem.* **23** (1990) 371–376.
20. Wilce P. A., Leijten L. and Martin L.: Stimulation of 3-hydroxy-3-methylglutaryl CoA reductase in mouse uterine epithelial cells by oestradiol-17β. *Biochem. J.* **218** (1984) 849–855.
21. Lowry O. H., Rosenbrough N. J., Farr A. L. and Randall R. J.: Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **193** (1951) 265–275.
22. Pollock W. K., Rink T. J. and Irvine R. F.: Liberation of [^{3}H]arachidonic acid and changes in cytosolic free calcium in fura 2-loaded human platelets stimulated by ionomycin and collagen. *Biochem. J.* **235** (1986) 869–872.
23. Chen P. S., Toribara T. Y. and Warner H.: Microdetermination of phosphorus. *Analyt. Chem.* **28** (1956) 1156–1161.
24. Berridge M. J., Dawson M. J. C., Downes C. P., Heslop J. P. and Irvine R. F.: Changes in the levels of inositol phosphates after agonist dependent hydrolysis of membrane phosphoinositides. *Biochem. J.* **212** (1983) 473–451.
25. Habernict A. J. R., Glomset J. A. and King W. C.: Early changes in phosphatidylinositol and arachidonic acid metabolism in quiescent Swiss 3T3 cells stimulated to divide by platelet derived growth factor. *J. Biol. Chem.* **256** (1981) 12329–12335.
26. Sawyer S. and Cohen S.: Enhancement of calcium uptake and phosphoinositol turnover by epidermal growth factor in A-431 cells. *Biochemistry* **20** (1981) 6280–6286.
27. Wakelam M. J. O., Davis S. A., Housley M. D., Mackay I., Marshall C. J. and Hall A.: Normal $p^{21n\text{-}ras}$ couples bombesin and other growth factor receptors in inositol phosphate production. *Nature* **323** (1986) 173–176.
28. Takenawa T. and Egawa K.: Phosphatidylinositol: myoinositol exchange enzyme from rat liver: partial purification and characterisation. *Archs Biochem. Biophys.* **202** (1980) 601–607.
29. Takenawa T., Saito M., Nagai Y. and Egawa K.: Solubilization of the enzyme catalyzing CDP-diglyceride-independent incorporation of myoinositol into phosphatidylinositol and its comparison to CDP-diglyceride: inositol transferase. *Archs Biochem. Biophys.* **182** (1977) 244–250.
30. Toth M. and Hertelendy F.: Differential effect of progesterone on the labelling of phosphatidylinositol with [^{3}H]inositol and [^{32}P]phosphate in the uterus of the estrogen treated ovariectomized rat. *J. Steroid Biochem.* **28** (1987) 629–635.
31. Gozalez F., Lakshman J., Hoath S. and Fisher D. A.: Effect of oestradiol-17β on uterine epidermal growth factor concentration in immature mice. *Acta Endocr. (Copenh)* **105** (1984) 425–428.
32. Murphy L. J., Murphy L. C. and Friesen H. G.: Estrogen induces insulin-like growth factor-1 expression in rat uterus. *Molec. Endocr.* **1** (1987) 445–451.