J. Steroid Biochem. Molec. Biol. Vol. 40, No. 1–3, pp. 465–471, 1991
Printed in Great Britain.

0960-0760/91 $3.00 + 0.00

RELEASE OF ARACHIDONIC ACID AND THE EFFECTS OF CORTICOSTEROIDS ON STEROIDOGENESIS IN RAT TESTIS LEYDIG CELLS

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Summary—The release of arachidonic acid by luteinizing hormone (LH) and the effects of inhibiting phospholipase A_2 (PLA_2) *in vivo* and *in vitro* on LH stimulated steroidogenesis in rat testis Leydig cells has been investigated. It was found that arachidonic acid is rapidly incorporated into phospholipids and is released within 1 min after addition of LH. The effects of treating adult rats with dexamethasone and human chorionic gonadotropin (hCG) *in vivo* on steroidogenesis and prostaglandin synthesis in Leydig cells isolated 6 h later were determined. It was found that hCG caused a marked increase in prostaglandin $F_{2\alpha}$ formation which was inhibited by treatment with dexamethasone. LH-stimulated testosterone production was inhibited in the hCG treated rats and dexamethasone caused a further decrease. Treatment with dexamethasone alone also caused a decrease in the response to LH. HCG, but not dexamethasone, had similar inhibitory effects on LH-stimulated cyclic AMP production. Similarly, the PLA_2 inhibitors quinacrine, dexamethasone and corticosterone, added to the Leydig cells *in vitro*, inhibited LH-stimulated testosterone production but not cyclic AMP production. 11-Dehydrocorticosterone also inhibited LH-stimulated testosterone production, but higher concentrations were required to give 50% inhibition compared to corticosteone (50 and 25 μM, respectively). Ring A-reduced metabolites of corticosterone and progesterone were also found to inhibit LH-stimulated steroidogenesis. The results obtained in this and previous studies are consistent with the activation of PLA_2, (either directly by LH and/or via cyclic AMP), which results in the release of arachidonic acid and the formation of leukotrienes, which stimulate steroidogenesis in the Leydig cell. This study also indicates that corticosteroids and their metabolites may exert inhibitory effects at other sites in the steroidogenic pathways, in addition to PLA_2.

INTRODUCTION

It has been established that cyclic AMP is a second messenger in the action of luteinizing hormone (LH) on testosterone formation in testis Leydig cells. It has also been shown that other second messenger systems may be involved including the phospholipase A_2 (PLA_2) derived arachidonic acid metabolite system [1], calcium [1] and efflux of chloride ions [2]. Arachidonic acid released from phospholipids by the action of PLA_2 or phospholipase C and diglyceride lipase can be metabolized via 3 independent pathways: the cyclooxygenase pathway, the lipoxygenase pathway and the cytochrome *P*-450 dependent epoxygenase pathway [3]. Previous studies from this laboratory demonstrated that lipoxygenase inhibitors (nordihydroguaretic acid: NDGA: 3-amino-1-(3-trifluoromethyl phenyl)-2-pyrazoline hydrochloride: BW 755c) but not cyclooxygenase inhibitors (indomethacin and aspirin) of arachidonic acid metabolism inhibited LH-induced testosterone production by rat Leydig cells [4]. Similar findings have been reported in a variety of other endocrine tissues; Islets of Langerhans [5–7], gonadotrophs [8], corticotrophs [9] and the adrenal cortex [10–12].

In this study we have investigated further the role of arachidonic acid and PLA_2 by measuring the incorporation of arachidonic acid into phospholipids and its release by LH. We have determined the effects of PLA_2 inhibitors *in vitro* (quinacrine and corticosterone) and *in vivo* (dexamethasone). Finally, we have measured the effects of the metabolites of corticosteroids and progesterone on LH-stimulated steroidogenesis to determine if the effects of the corticosteroids could be mimicked by their metabolites.

Proceedings of the VIIIth International Congress on Hormonal Steroids, The Hague, The Netherlands, 16–21 September 1990.

MATERIALS AND METHODS

Materials

Ovine LH (oLH) was obtained from the Endocrinology Study Section, NIH (U.S.A.). The steroids, quinacrine, and bovine serum albumin (fraction V: BSA) were obtained from Sigma Chemial Co. (U.K.), Dulbecco's Modified Eagle's minimum medium (DMEM) was purchased from GIBCO (U.K.), [^{14}C]-arachidonic acid (1.9 GBq/mmol) was purchased from NEN (U.K.). The solvents used for lipid extractions and HPLC were all HPLC grade (BDH Ltd, U.K.).

Cell isolation and purification

Rat testis Leydig cells were obtained from 200–300 g Sprague–Dawley rats by collagenase dispersion [13]. Leydig cells of density 1.07 g/ml were obtained using a combination of centifugal elutriation and density gradient centrifugation as described previously [14]. This methodology routinely yielded a Leydig cell rich preparation (>95% purity) as assessed by 3β-hydroxysteroid dehydrogenase histochemistry [13].

Cell incubations

Purified Leydig cells were suspended in DMEM containing 0.1% BSA and plated out into 24-well Costar culture plates at a density of 100,000–150,000 cells/500 μl/well. The cells were preincubated for 90 min at 32°C in an air incubator and then for a further 30 min with inhibitors of PLA_2 (corticosteroids or quinacrine) followed by incubation for a further period of 120 min in the presence or absence of LH (0.1–100 ng/ml). Incubations were terminated by the addition of $HClO_4$ (final concentration 0.5 M) and neutralized with K_3PO_4 (final concentration 0.23 M). Quinacrine was made up in DMEM while the steroids were made up in ethanol. Additions were made as 5 μl aliquots and where ethanol was used as the solvent the final ethanol concentration was 1%. Appropriate additions were made to control incubations.

In the experiments where the rats (6 per group) were treated with hCG (100 IU) and dexamethasone (1 mg/kg), the cells were isolated and purified, as described above, 6 h after injection of the hCG. Dexamethasone was given 1 h before the hCG. The isolated cells 400,000/well) were incubated as described above except that they were preincubated for 2 h and then incubated for 3 h with medium alone or different concentrations of LH. There was no difference in the results obtained from the control rats injected with the vehicle (saline) compared with untreated controls. Cell viability was determined by diapharose histochemistry [15].

Radioimmunoassays (RIAs)

Testosterone was determine by RIA [16]. Cyclic AMP was also measured by RIA [17] modified by the acetylation procedure [18]. Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) was assayed by RIA [19] using the antiserum provided to Professor A. P. Flint (Institute of Zoology, London) by Dr R. Kellie (M.R.C. Reproductive Biology Unit, Edinburgh).

Incorporation and release of arachidonic acid

Cells were plated at a density of up to 1×10^6 cells/ml and incubated with [^{14}C]arachidonic acid (0.1–0.2 μCi/dish) for up to 24 h at 34°C in an air incubator. For each time course of arachidonic acid incorporation into phospholipids, cells were removed from the dishes at different times for up to 24 h using trypsin (0.2%) and EDTA (0.4%) and then extracted with chloroform/methanol. A rapid normal phase HPLC method using a solvent mixture of acetonitrile/methanol/phosphoric acid (85%) (130:5:1.5 v/v) was used to separate the major phospholipids both by u.v. (203 nm) and radiometric detection [20].

To measure the release of arachidonic acid, the Leydig cells (0.5×10^6/ml) were plated out in 24-well Costar plates and incubated with 0.1 μCi of [^{14}C]arachidonic acid for up to 4 h in an air incubator at 34°C. Cells were washed twice with 1 ml DMEM containing 0.5% fatty acid free BSA to remove the free arachidonic acid and then incubated in this medium for 15 min at 34°C before adding the LH. Incubations were terminated at the times indicated by addition of 1 ml ice cold methanol. The supernatant was separated from the cells and either counted for radioactivity released from the cells or extracted with chloroform/methanol. Extracts were analysed on reverse phase HPLC to measure quantitatively [^{14}C]arachidonic acid released from the cells.

Analysis of data

Results are expressed as means ± SEM. The significance of the differences was assayed by Student's t-test. Differences were considered to be significant when $P < 0.05$.

RESULTS

Incorporation and release of arachidonic acid

Over 70% of the added [^{14}C]arachidonic acid was incorporated rapidly into three phospholipids; phosphatidyl inositol (PI); phosphatidyl ethanolamine (PE) and phosphatidyl choline (PC). Figure 1 shows a typical chromatograph of the separated endogenous phospholipids and the phospholipids labelled with [^{14}C] arachidonic acid. It can be seen that the latter reflected the endogenous levels. Figure 2 shows the rate of incorporation. The incorporation into PI and PE reached a maximum within 2–5 h whereas the incorporation into PC showed a continuous increase throughout the period of incubation (25 h). The highest incorporation was also into PC.

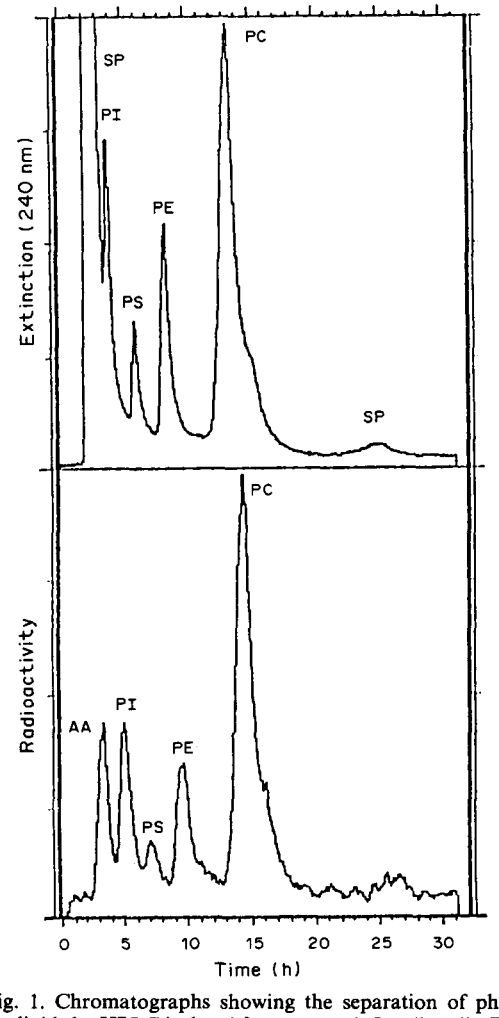

Fig. 1. Chromatographs showing the separation of phospholipids by HPLC isolated from rat testis Leydig cells. The cells were labelled with [^{14}C]arachidonic acid as described in Materials and Methods. The upper chromatogram is the extinction of the phospholipids at 203 nm and the lower chromatogram is the radioactivity of the same extract. The phospholipids were identified by their retention times compared with standards, SF, solvent front; PS, phosphatidyl serine, SP, sphingomyelin.

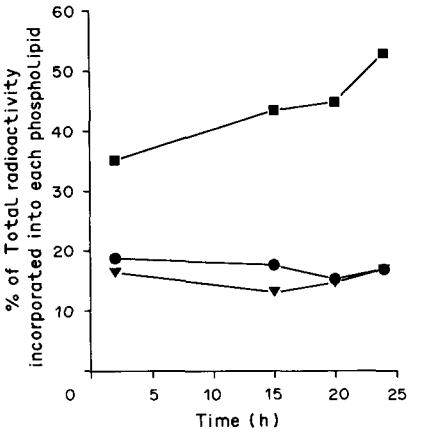

Fig. 2. Time course of [^{14}C]arachidonic acid incorporated into phospholipids in rat testis Leydig cells. The cells were incubated with [^{14}C]arachidonic acid for the times indicated as described in Materials and Methods, ■, PC; ●, PI and ▲, PE.

Figure 3 shows the time course of the [^{14}C]arachidonic acid released from the prelabelled cells when challenged with LH compared to the control Two separate sets of incubations with LH are shown. Because large numbers of cells were required only single incubations were possible for each time point. The results are representative of three separate experiments in which the stimulation of [^{14}C]arachdonic acid release from the cells was 2–3 fold above the controls and was at a maximum within 60 s after LH addition.

Effect of treatment with quinacrine on cyclic AMP and testosterone production by rat testis Leydig cells

We have previously shown [21] that basal testosterone production (4.73 ± 1.5 ng/10^6 cells/2 h) and cyclic AMP accumulation (0.47 ± 0.12 pmol/10^6 cells/2 h) are unaffected by treatment with quinacrine. Treatment with LH significantly increased both testosterone production (121.6 ± 22.5 ng/10^6 cells/2 h) and cyclic AMP accumulation (162.5 ± 7.0 pmol/10^6 cells/2 h). Increasing concentrations of quinacrine (10^{-8}–10^{-5} M) had an inhibitory effect on LH-induced testosterone production. The minimum effective dose was 2.5 μM and 50% inhibition was obtained with 7.2 μM [21]. The inhibitory effect on testosterone was not accompanied by a concomitant decrease in LH-induced cyclic AMP accumulation demonstrating a clearcut dissociation between testosterone production and cyclic AMP accumulation. At

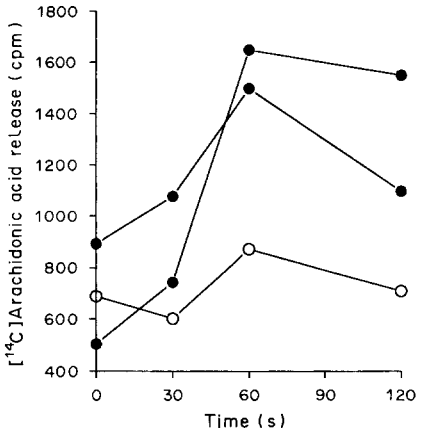

Fig. 3. Release of [^{14}C]arachidonic acid from Leydig cells. The cells were incubated for 4 h with [^{14}C]arachidonic acid, washed and then challenged with LH or fresh medium as described in Materials and Methods. After termination of the incubations at the times indicated, the medium was extracted and analysed for [^{14}C]arachidonic acid by HPLC. ●, LH and ○, control.

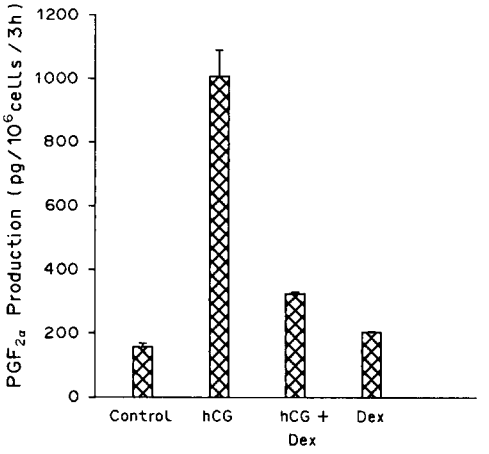

Fig. 4. The effects of hCG and dexamethasone *in vivo* on $PGF_{2\alpha}$ production in Leydig cells *in vitro*. The rats (5 per group) were treated with vehicle (saline), hCG (100 IU) hCG plus dexamethasone (1 mg/kg) or dexamethasone alone. 6 h Later the animals were sacrificed and the Leydig cells were isolated and then incubated for 3 h as described in Materials and Methods. The medium was then collected and analysed for $PGF_{2\alpha}$.

the highest concentration of quinacrine used (10^{-4} M) cyclic AMP was only inhibited by 30% while testosterone was inhibited by 85%. Even at the highest concentration quinacrine had no adverse effect on cell viability.

Effects of hCG and dexamethasone in vivo

Adult male rats were treated with hCG, hCG and dexamethasone, or dexamethasone alone and the animals were killed 6 h later and the Leydig cells isolated. The responses to various doses of LH were measured. Also in order to detemine the effectiveness of dexamethasone in inhibiting the PLA_2, $PGF_{2\alpha}$ was measured. It was found that hCG caused a marked increase in $PGF_{2\alpha}$ levels which was inhibited by dexamethasone (Fig. 4). In these cells isolated from hCG-treated animals the response to added LH was markedly inhibited compared with the controls, although the basal levels were higher (Fig. 5). Treatment with hCG and dexamethasone further decreased the testosterone production. Dexamethasone by itself also caused a decrease in the response to LH. The cells from hCG-treated rats were also desensitized with respect to the effects of LH on cyclic AMP production. They also had a high basal level of cyclic AMP compared with the controls. The high basal level was not found when the rats were treated with hCG and dexamethasone, indicating that the latter inhibited "basal" cyclic AMP formation. Treatment with dexamethasone alone did not affect cyclic AMP production stimulated with LH with the high concentrations used, although a small decrease did occur with the lower amounts.

Effects of corticosteroids in vitro

Previously we have shown that dexamethasone had similar effects to quinacrine [21] i.e. it dose-dependently inhibited LH-stimulated testosterone production but not cyclic AMP when added to the cells *in vitro*. The concentration that gave 50% inhibition was 36 μM.

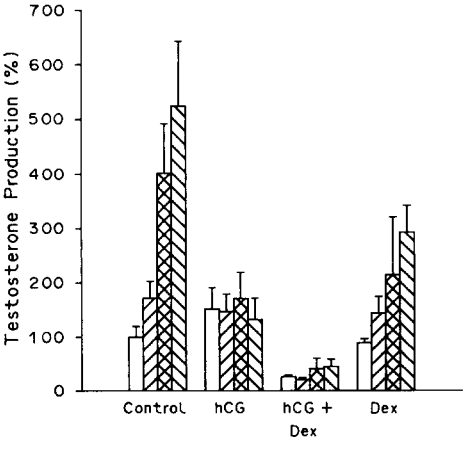

Fig. 5. The effects of hCG and dexamethasone *in vivo* on testosterone production in Leydig cells *in vitro*. The animals were treated as described in the legend to Fig. 4. The cells were then incubated without or with different concentrations of LH (□, control; ▨, 0.1 ng/ml; ⊠, 1 ng/ml; ▧, 100 ng/ml). The medium was then analysed for testosterone as described in Materials and Methods. The results are means +SD for triplicate incubations expressed as a percentage of the control. The latter was 6.7 ± 1 ng testosterone/10^6 cells/2 h.

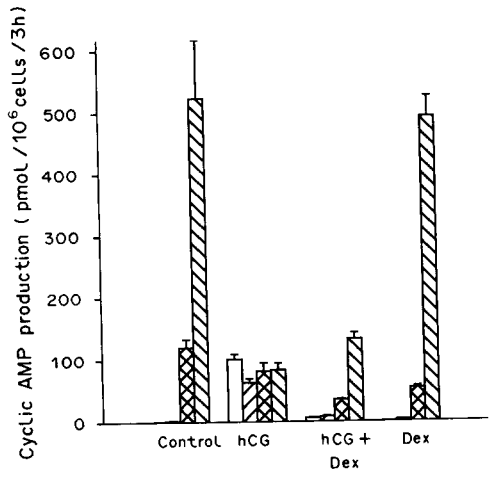

Fig. 6. The effects of hCG and dexamethasone *in vivo* on cyclic AMP production in Leydig cells *in vitro*. The animals were treated as described in the legend to Fig. 4. The cells were then incubated without or with different concentrations of LH (□, control; ▨, 0.1 ng/ml; ⊠, 1 ng/ml; ▧, 100 ng/ml). The medium was then analysed for cyclic AMP as described in Materials and Methods. The results are means ± SD for triplicate incubations.

The natural hormone in the rat, corticosterone, also inhibited LH-stimulated testosterone production when added to the Leydig cells (Fig. 7); 50% inhibition was obtained when 25 μM was added. 11-Dehydrocorticosterone also inhibited LH-stimulated steroidogenesis, although 50 μM was required to give 50% inhibition (Fig. 7).

Similar experiments were also carried out with corticosteroid metabolites. It was found that 3β- and 3α-, 11β,21-trihydroxy-5α-pregnane-20-one caused a dose-dependent inhibition

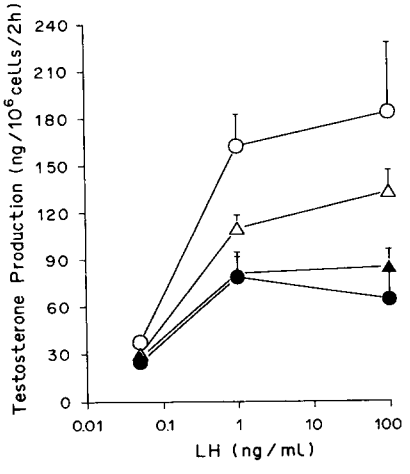

Fig. 7. The effects of corticosterone and 11-dehydrocorticosterone on LH-stimulated testosterone production in testis Leydig cells. The cells were incubated with different concentrations of LH and with; no further additions ○, corticosterone, ● 25 μM and 11-dehydroxycorticosterone, △, 25 μM, ▲, 50 μM. The results are means ± SD for triplicate incubations.

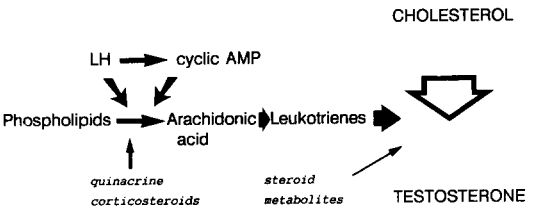

Fig. 8. Scheme for the control of testosterone production via activation of PLA_2 by LH and cyclic AMP. LH- and cyclic AMP-stimulated steroidogenesis is inhibited by PLA_2 inhibitors (quinacrine and corticosteroids), indicating that liberation of arachidonic acid from phospholipids is involved in the control of steroidogenesis. Ring A-reduced metabolites of corticosteroids and progesterone inhibit steroidogenesis, indicating that there may be additional loci for the control of testosterone production by steroids.

of LH-stimulated steroidogenesis. The concentrations that gave 50% inhibition were the same as those obtained with corticosterone. Similarly it was found that all four isomers of pregnanolone inhibited LH-stimulated testosterone production; 5–10 μM was required to give 50% inhibition.

DISCUSSION

The evidence presented in this study supports the concept that the release and action of arachidonic acid and/or its metabolites are involved in the stimulation of steroidogenesis by LH. [^{14}C]arachidonic acid was shown to be incorporated into the three main phospholipids present in the Leydig cells in proportion to their endogenous concentrations. Addition of LH caused a very rapid release of arachidonic acid. Experiments in which the Leydig cells labelled with [^{14}C]arachidonic acid were superfused with LH, also very rapidly released radioactivity (Schulster D. and Cooke B. A., unpublished observations). It remains to be determined which of the phospholipids is the source of the released arachidonic acid. In view of the high incorporation of arachidonic acid into PC, which increased continuously during 25 h incubation, it is possible that this is a rapidly turning over phospholipid and may be an important source of arachidonic acid.

It is interesting to note that both dexamethasone and quinacrine inhibited steroidogenesis maximally without affecting cyclic AMP accumulation. We have also found that cyclic AMP induced steroidogenesis is also inhibited by these compounds [21]. It is possible, therefore, that PLA_2 activation is "down stream" from cyclic AMP formation (Fig. 8). Alternatively, both LH and cyclic AMP may directly activate PLA_2.

Both dexamethasone and mepacrine, two structurally unrelated PLA_2 inhibitors [22, 23], caused a dose-dependent inhibition of LH-induced testosterone formation. The inhibitory effect of dexamethasone on PLA_2 was confirmed (albeit indirectly) in the *in vivo* experiments where it was shown that the stimulation of $PGF_{2\alpha}$ formation by hCG was completely inhibited in the presence of dexamethasone. This effect of hCG on prostaglandin synthesis requires 4–8 h before maximum production is obtained [24]. Previous studies have also shown that hCG stimulates prostaglandin synthesis [25]. The inhibitory effects of dexamethasone administration *in vivo* on testosterone production on the isolated Leydig cells *in vitro* and the effects on prostaglandin formation, are consistent with dexamethasone action being via inhibition of PLA_2.

In agreement with previous *in vitro* and *in vivo* studies [1, 26, 27], it was found that hCG caused desensitization of the subsequent responses to LH in terms of cyclic AMP and testosterone production and that basal productions were increased. The mechanisms involved in this increased basal production are unknown. It is interesting to note that in the present study, the latter did not occur if the animals were treated with dexamethasone prior to hCG.

It has recently been reported that rat Leydig cells contain the enzyme 11β-hydroxysteroid dehydrogenase [28] which presumably protects the testis from the effects of the circulating corticosteroids by converting corticosterone to 11-dehydrocorticosterone. Dexamethasone is not a substrate for this enzyme and would not be inactivated. In order to determine the possible effects of the 11β-hydroxysteroid dehydrogenase, further experiments were carried out with corticosterone. It was found that 25 μM was necessary to give 50% inhibition of steroidogenesis. However, inhibition was also obtained with 11-dehydrocorticosterone although 50 μM was required to give the same inhibition. It is possible that this effect of the 11-dehydrocorticosterone was obtained after conversion to corticosterone because the 11β-hydroxysteroid dehydrogenase is a reversible enzyme. Alternatively, the inhibitory effects of these steroids may be partly non-specific and be acting on other pathways in addition to the PLA_2. The effects of metabolites of the corticosteroids and progesterone were also investigated. It was found that the metabolites added dose-dependently inhibited LH-stimulated steroidogenesis. It is unlikely that these steroids inhibit PLA_2; their locus of action therefore requires further investigation.

Acknowledgements—We are grateful for the financial support of Sandoz Ltd (Basel), the Agricultural and Food Research Council, The Wellcome Trust and the Peter Samuel Royal Free Fund.

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