Indomethacin inhibits the effects of oestrogen in the anterior pituitary gland of the rat

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ABSTRACT

Two inhibitors of prostaglandin synthesis, indomethacin and aspirin, blocked the increase of oestrogen-binding sites in the nuclear subcellular fraction, an increase which occurs after the administration of oestradiol. Consequently the biological effects of oestrogens in the anterior pituitary gland of the rat (prolactin synthesis, concentration of progesterone-binding sites and cell proliferation) are diminished. The anterior pituitary gland synthesized prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), PGE_2 and PGD_2 from arachidonic acid. This synthesis was blocked when indomethacin was added to the culture media. Oestrogen increased the concentration of PGE_2: an increase that was partially prevented by indomethacin. Prostaglandins may have an important role on the effects of oestrogen in the anterior pituitary gland of the rat.

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INTRODUCTION

Prostaglandins are involved in the regulation of DNA synthesis, and in cell proliferation and differentiation in different tissues (Taylor & Polgar, 1977; O'Farrell, Clingan, Rudland & Jimenez de Asua, 1979; Jimenez de Asua, Otto, Lindgren & Hammaström, 1983). The effect may be stimulatory (Andreis, Whitfield & Armato, 1981; Otto, Nilsen-Hamilton, Boss *et al.* 1982; Burch, Luini, Mais *et al.* 1986) or inhibitory (Bregman, Sander & Meyskens, 1982; Sakai, Yamaguchi, Shiroko *et al.* 1984; Ohno, Fujiwara, Fukushima & Narumiya, 1986) but depends on the prostaglandin and cellular system studied. These compounds are also involved in the synthesis and release of pituitary hormones (Ojeda, Jameson & McCann, 1973; Sundberg, Fawcett, Illner & McCann, 1975).

The secretion of several pituitary hormones is accompanied by increased cell proliferation (Burdman, Calabrese & MacLeod, 1983; Jahn, Burdman & Deis, 1984; Romano, Machiavelli, Perez *et al.* 1984). In addition to the release of prolactin, oestrogen is also required for prolactin cells to proliferate (Burdman, Szijan, Jahn *et al.* 1979; Burdman, Calabrese, Romano *et al.* 1984).

It has been reported that indomethacin, an inhibitor of prostaglandin synthesis, diminishes cell growth in acidophilic and chromophobe cells and completely blocks the stimulation of cell proliferation by oestrogens (Pawlikowski, 1981; Pawlikowski, Kunert-Radek, Lewinski & Karasek, 1981). However, we have found in our laboratory that the administration of indomethacin and aspirin to rats increases DNA synthesis and cell proliferation in the anterior pituitary gland. Cell growth is not localized to any particular type of cell within the heterogeneous population of cells of the anterior pituitary gland, is dose-dependent and also occurs in other tissues such as liver and kidney (D. G. Rosental, M. I. Romano, G. A. Machiavelli, R. L. Perez, N. Sterin Speziale & J. A. Burdman, unpublished observations). It is well established that oestrogens stimulate the growth of lactotrophs in the anterior pituitary gland (Burdman *et al.* 1979; Burdman *et al.* 1984). When indomethacin and oestrogens are given together, however, the oestrogenic stimulation is apparently blocked. To investigate this discrepancy we studied the effects of indomethacin and aspirin on some stimulatory effects of oestrogens in the anterior pituitary gland. The results indicate that giving indomethacin or aspirin

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decreases some of the effects of oestrogen, such as the stimulation of cell proliferation, and increases the concentration of progesterone receptors and prolactin mRNA. The administration of oestrogens changes the subcellular distribution of the steroid receptor. This modification, which is essential to produce the biological effects of oestrogens, is inhibited by indomethacin and aspirin.

MATERIALS AND METHODS

Animals

Male rats of a highly inbred Wistar strain (Comision Nacional de Energia Atomica, Buenos Aires, Argentina) weighing 200–250 g at the start of the experiment were used. They were kept under a lighting regime of 12 h light and 12 h darkness at 20 °C with food and water available *ad libitum*. They were killed by decapitation between 09.00 and 10.00 h.

Materials

Indomethacin (Sigma Chemical Co, St Louis, MO, U.S.A.) and aspirin were prepared in ethanol–olive oil (1:6, v/v). Oestra-1,3,5(10)triene-3,17β-diol-di-10 undecylenate (Estrosteron) was kindly supplied by Gador, Buenos Aires, Argentina. [methyl-^{3}H]Thymidine (sp.act. 50·8 Ci/mmol), [17β-2,4,6,7-^{3}H]oestradiol (115 Ci/mmol), [17α-methyl-^{3}H]promegestone (R5020) (87 Ci/mmol) and [1-^{14}C]arachidonic acid (60 mCi/mmol) were obtained from New England Nuclear Co., Boston, MA, U.S.A. Non-radioactive 17β-oestradiol and nordihydroguaiaretic acid were from Sigma Chemical Co.

Treatments

To determine [^{3}H]thymidine incorporation, the concentration of progesterone receptors, prolactin mRNA and prostaglandins, animals received five s.c. injections of indomethacin, aspirin or vehicle at 12-h intervals. The last injection was given 12 h before decapitation of the rats. The doses of indomethacin and aspirin were 2·5 mg/rat and 20 mg/rat in 250 µl vehicle respectively.

Oestradiol diundecylenate was injected 48 h before the animals were killed by decapitation as an s.c. injection of 300 µg in 250 µl vehicle.

For the oestrogen receptor assay, animals received three s.c. injections of indomethacin, aspirin or vehicle at 12-h intervals. Oestradiol diundecylenate was injected 1 h after the last injection and 2 h before decapitation of the rats. The doses used were the same as those described above.

Incorporation of [^{3}H]thymidine

A detailed description of the procedure for the measurement of the incorporation of [^{3}H]thymidine has been described previously (Kalberman, Szijan, Jahn *et al.* 1979). Briefly, fresh tissue (6–8 mg) was incubated under 95% O_2–5% CO_2 in 0·5 ml Medium 199 in the presence of 2 µCi [^{3}H]thymidine for 60 min at 37 °C. At the end of the incubation, the glands were homogenized in 10% trichloroacetic acid (TCA), centrifuged, and the radioactivity was measured in samples of the residue and supernatant fractions. The results are expressed as relative specific radioactivity (d.p.m. per mg DNA in the TCA-insoluble fraction/d.p.m. per mg anterior pituitary gland in the TCA-soluble fraction) × 10. The radioactivity in the TCA-soluble fraction was similar in all the groups studied.

Steroid-binding assays

Oestrogen receptor assay

A detailed description of tissue preparation and determination of cytosolic and nuclear oestrogen receptor has been described elsewhere (Alonso, Burdman & Szijan, 1981). Briefly, anterior pituitary glands were homogenized in TE buffer (0·01 mol Tris/l, pH 7·4; 0·0015 mol disodium EDTA/l; 0·002 mol 2-mercaptoethanol/l). The homogenates were centrifuged at 900 ***g*** to separate the nuclei. The supernatant was centrifuged at 105 000 ***g*** to obtain the cytosol. Aliquots of cytosol (150–200 µg protein) were incubated with [^{3}H]oestradiol (15 nmol/l) and a 500-fold excess of unlabelled oestradiol (non-specific binding) at 15 °C for 16 h. The oestradiol–receptor complex was separated from unbound oestradiol with a charcoal-dextran (1% (w/v)–0·01% (w/v)) suspension and centrifuged at 2000 ***g*** for 10 min. An aliquot of the supernatant was pipetted into a scintillation vial containing 3 ml scintillation fluid (toluene, PPO 12 mg, POPOP 0·3 mg) and the radioactivity determined. To determine the nuclear oestrogen receptor an aliquot of nuclear suspension (150–200 µg protein) was incubated in the presence of [^{3}H]oestradiol (15 nmol/l) with or without a 500-fold excess of unlabelled oestradiol at 4 °C for 1 h followed by 45 min at 30 °C. At the end of the incubation, 1 ml TE buffer was added to each tube and centrifuged. The pellet obtained was extracted with ethanol and treated in the same way as the cytosolic samples.

Progesterone receptor assay

All procedures were carried out at 0–4 °C. The anterior pituitary glands were homogenized in ice-cold TE buffer at a concentration of 25 ml/g tissue. The homogenates were centrifuged for 1 h at 105 000 ***g***. The supernatant cytosolic fractions were

decanted and adjusted to a concentration equivalent to 1·8 mg protein/ml with TE buffer. The resulting cytosol was then used for the measurement of cytoplasmic receptor. Aliquots (200 µl) of the cytosol fractions were incubated with 5 nmol [^{3}H]R5020/l with or without unlabelled R5020 at 2–4 °C for 3 h (Pasqualini & Nguyen, 1980). Separation of bound and unbound [^{3}H]R5020 was performed by the addition of 100 µl charcoal-coated dextran (3% (w/v)–0·3% (w/v)) suspension (Horwitz & McGuire, 1978), which was mixed for 3 s and incubated for 5 min. Subsequently, the tubes were centrifuged at 2000 *g* for 10 min. A 150 µl aliquot of the supernatant was pipetted into a scintillation vial containing 3 ml scintillation fluid (toluene, PPO 12 mg, POPOP 0·3 mg) and the radioactivity determined.

Radioconversion of [1-^{14}C]arachidonic acid (AA) into prostaglandins

The anterior pituitary glands were incubated *in vitro* with indomethacin (1 mmol/l), aspirin (6 mmol/l) or nordihydroguaiaretic acid (10 mmol/l). For each experiment five glands were incubated in 1 ml Krebs–Ringer bicarbonate buffer containing 0·25 µCi [1-^{14}C]AA for 1 h at 37 °C in a metabolic shaking bath under an atmosphere of 95% O_2–5% CO_2. At the end of the incubation period, the reaction was stopped by adding 0·1 ml citric acid (1 mol/l).

Extraction, separation and quantification of radiolabelled prostaglandins

The lipids were extracted three times with 2 ml chloroform. The organic solvent was dried under nitrogen at 25 °C. The lipid residues were dissolved in a mixture of chloroform:methanol (2:1), applied to silica gel thin-layer chromatography (TLC) plastic plates (0·25 mm thick) and developed twice in a solvent system consisting of the upper phase from a mixture of ethylacetate:2,2,4-trimethylpentane:acetic acid:water (6·6:3·0:1·2:6·0, by vol.).

During the chromatographic separation, phospholipids remained at the origin and the R_F values were 6-keto-prostaglandin $F_{1\alpha}$ ($PGF_{1\alpha}$) 0·16, $PGF_{2\alpha}$ 0·27, PGE_2 0·42, PGD_2 0·56 and AA 0·73.

Standards of the various prostaglandins were subjected to simultaneous chromatography and were visualized by spraying the corresponding areas of the plate with a 10% solution of phosphomolybdic acid in ethanol and heating at 110 °C. The silica gel from the TLC areas of the specific AA products were transferred to liquid scintillation vials and the radioactivity determined. The results are expressed as c.p.m. because there were no significant differences either in counting efficiency or in the weight of the incubated anterior pituitary glands. Extraction efficiency of prostaglandins was 85%.

TABLE 1. Effect of indomethacin, aspirin and oestradiol on the incorporation of [^{3}H]thymidine into rat pituitary DNA. Values are means ± S.E.M. of five separate experiments

Treatment	Incorporation of [^{3}H]thymidine (relative specific radioactivity)
Vehicle	98 ± 27
Indomethacin	222 ± 66*
Aspirin	176 ± 43*
Oestradiol	680 ± 160*
Oestradiol + indomethacin	334 ± 96*†
Oestradiol + aspirin	288 ± 79*†

*P < 0·01 compared with values for vehicle-treated rats; †not significant compared with values for indomethacin- or aspirin-treated rats (Student's t-test).
One pituitary gland (6–8 mg) was used in each experiment.
Relative specific radioactivity is expressed as the ratio of (d.p.m./mg DNA in the trichloroacetic acid (TCA)-insoluble fraction to d.p.m./mg anterior pituitary gland in the TCA-soluble fraction) × 10. Treatments are described in Materials and Methods.

Determination of prostaglandin E_2

Prostaglandin E_2 levels were measured by radioimmunoassay using a New England Nuclear Kit (Boston, MA, U.S.A.).

Samples were obtained from the incubation media of two anterior pituitary glands which were incubated in 1 ml Krebs–Ringer bicarbonate buffer for 1 h at 37 °C in a metabolic bath under an atmosphere of 95% O_2–5% CO_2. At the end of the incubation period, the reaction was stopped by adding 0·1 ml citric acid (1 mol/l). The prostaglandins were extracted three times with 2 ml chloroform. The organic solvent was dried under nitrogen at 25 °C. The extracts were resuspended in assay buffer and an aliquot was used for the determination of the prostaglandins. Extraction efficiency of PGE_2 was 85%.

Dot hybridization analysis of rat pituitary cytoplasmic preparations

A detailed description of pituitary cytoplasmic preparations has been given elsewhere (White & Bancroft, 1982). Briefly, anterior pituitary glands were homogenized in Tris (10 mmol/l, pH 7·0), EDTA (1 mmol/l) and 0·5% NONIDET P-40, and centrifuged at 900 *g* to separate the nuclei. The supernatant was centrifuged at 15 000 *g* to obtain the cytosol, which was incubated for 15 min at 60 °C with 7·4% formaldehyde for denaturation; it was then serially diluted and applied with suction onto a nitrocellulose sheet. The nitrocellulose sheet was then baked (80 °C, 90 min) to fix cytoplasmic macromolecules; it was then

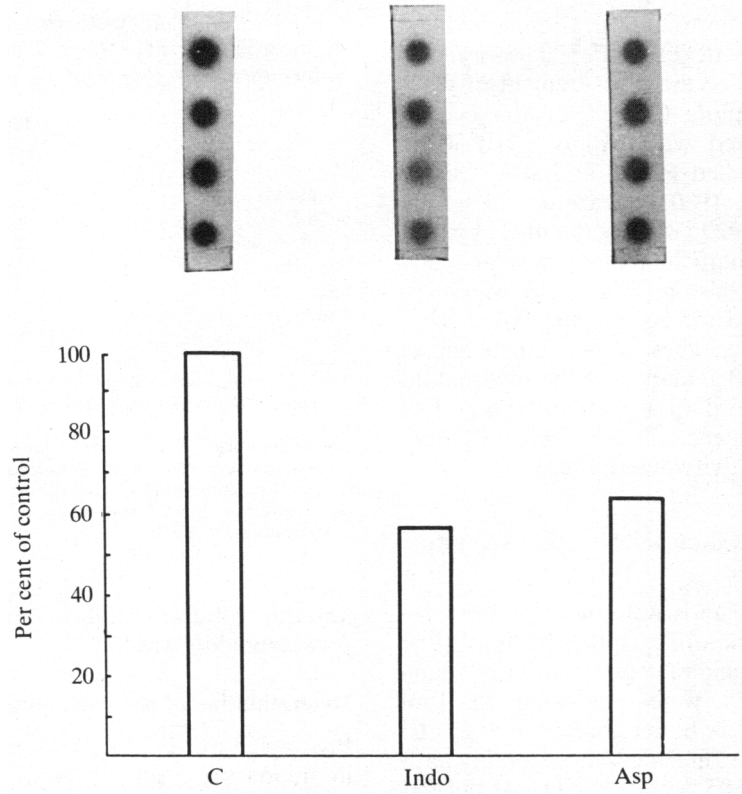

FIGURE 1. Dot blot analysis of pituitary cytoplasmic preparations from rats treated with five s.c. injections at 12-h intervals of vehicle, indomethacin or aspirin. Samples were dotted onto nitrocellulose filters under a moderate vacuum. Filters were then hybridizated with ^{32}P-labelled cDNA of rat prolactin (pPRL-1). Washed filters were autoradiographed for 48 h at −80 °C with intensifying screens. Dots contain, from top to bottom, 10, 7·5, 5 and 3·75 μg protein. Values for each cascade (control, indomethacin, aspirin) were determined by integrating the values under the curve from densitometric analysis. Control values were defined as 100%. Histograms show 100% in controls (C), 55% indomethacin (Indo)-treated and 64% aspirin (Asp)-treated. The results are from one experiment performed with two pituitaries for each experimental condition. A separate experiment showed similar results.

TABLE 2. Effect of indomethacin and aspirin on the concentration of progesterone-binding sites in the anterior pituitary gland of the rat. Values are means ± S.E.M. of six separate experiments

Treatment	Progesterone-binding sites (fmol/mg protein)
Vehicle	10·5 ± 1·0
Aspirin	8·4 ± 2·0
Indomethacin	8·0 ± 2·2
Oestradiol	31·2 ± 5·0*
Oestradiol + indomethacin	14·0 ± 4·0†
Oestradiol + aspirin	12·8 ± 3·0†

*$P < 0·01$ compared with values for vehicle-treated rats; †not significant compared with values for vehicle-treated rats (Student's *t*-test). Treatments are described in Materials and Methods.

prehybridized, hybridized and washed (Maniatis, Frisch & Sambrook, 1982). Prolactin cDNA (pPRL-1) was kindly supplied by R. Maurer, Department of Physiology and Biophysics, University of Iowa, Iowa, U.S.A. ^{32}P-Labelled pPRL-1 DNA probe (sp. act. $1–2 \times 10^8$ c.p.m./μg) was prepared by nick translation (Maniatis *et al.* 1982).

RESULTS

The administration of oestradiol diundecylinate, a long-acting oestrogen, stimulated the incorporation of [^{3}H]thymidine into pituitary DNA and this stimulation was blocked when the rats received indomethacin or aspirin (Table 1). Other effects of

TABLE 3. Effect of indomethacin and aspirin on nuclear and cytosolic oestrogen-receptor concentration in the anterior pituitary gland of the rat. Values are means ± S.E.M. of three separate experiments

	Oestradiol receptor (fmol/mg protein)	
	Cytosolic	Nuclear
Treatment		
Vehicle	165 ± 65	76 ± 13
Indomethacin	227 ± 51†	106 ± 4†
Oestradiol	98 ± 31*	206 ± 41*
Oestradiol + indomethacin	211 ± 71†	104 ± 14†
Aspirin	164 ± 53†	78 ± 13†
Oestradiol + aspirin	148 ± 19†	105 ± 7†

*$P < 0.05$ compared with values for vehicle-treated rats; †not significant compared with values for vehicle-treated rats (Student's *t*-test).
Treatments are described in Materials and Methods.

TABLE 4. Radioconversion of [1-^{14}C]arachidonic acid into prostaglandins (PG) in anterior pituitary gland. The results are from one experiment

	$PGF_{2\alpha}$ (c.p.m.)	PGE_2 (c.p.m.)	PGD_2 (c.p.m.)
Treatment			
Vehicle	1679	2011	1310
Indomethacin	120	150	100
Aspirin	260	305	170
Nordihydroguaiaretic acid	1700	2100	1290

For each experiment five anterior pituitary glands were incubated in 1 ml Krebs–Ringer bicarbonate buffer in the presence of [1-^{14}C]arachidonic acid, indomethacin, aspirin or nordihydroguaiaretic acid.

TABLE 5. Effect of indomethacin and oestradiol on prostaglandin E_2 (PGE_2) content in the anterior pituitary gland of the rat. Values are means ± S.E.M. of four individual determinations

	PGE_2 content (pg/mg tissue)
Treatment	
Vehicle	3·3 ± 1·8
Oestradiol	53·6 ± 12·3*
Oestradiol + indomethacin	17·8 ± 6·1*
Oestradiol + aspirin	22·6 ± 9·0*

*$P < 0.01$ compared with values for vehicle-treated rats (Student's *t*-test).
Treatments are described in Materials and Methods.

oestrogen such as the increase in the concentration of prolactin mRNA (Fig. 1) and the increase in progesterone-binding sites (Table 2) were also blocked by these drugs. In rats treated with indomethacin or aspirin, there was a marked decrease in the concentration of prolactin mRNA in the pituitary gland (Fig. 1). Injection of estradiol increased the concentration of progesterone receptors in the cytosolic fraction of the anterior pituitary gland and this effect was abolished by indomethacin or aspirin (Table 2). Thus, three different effects of oestradiol were inhibited by these two inhibitors of prostaglandin biosynthesis.

We next investigated which of the steps required for the effects of oestrogen to occur were modified by indomethacin and aspirin. For this purpose we determined the binding of [^{3}H]oestradiol in different subcellular fractions of the rat pituitary gland. The results demonstrated a similar binding of the tritiated steroid in treated- and non-treated rats (Table 3). Two hours after the injection of oestradiol, the concentration of the oestradiol receptor increased in the nuclear fraction and decreased in the cytosolic fraction of the anterior pituitary gland (Table 3). This modification in the subcellular distribution of the oestradiol receptor produced by the administration of the steroid did not occur in the anterior pituitary gland of rats treated with indomethacin or aspirin (Table 3). *In vivo*, indomethacin may bind competitively to the oestradiol receptor preventing the binding of oestradiol. During the in-vitro assay, the high concentration of oestradiol (15 nmol/l) used may displace the bound indomethacin. To clarify this possibility we reduced the oestradiol concentration (10 and 4 nmol/l) in the assay mixture and again found that indomethacin did not interfere with the binding of oestradiol to its receptor (data not shown). The anterior pituitary gland *in vitro* synthesized PGE_2, $PGF_{2\alpha}$ and PGD_2 at least and their synthesis was inhibited by the addition to the incubation media of indomethacin or aspirin (Table 4). There were no significant differences in the synthesis of prostaglandin when nordihydroguaiaretic acid was added to the incubation media (Table 5). The concentration of PGE_2 increased significantly in the anterior pituitary gland of rats treated with oestrogen and this increase was reduced by indomethacin and aspirin (Table 5).

DISCUSSION

The increased cell proliferation in the anterior pituitary gland produced by oestrogens is different from that induced by inhibitors of prostaglandin synthesis. Oestrogens stimulate DNA synthesis and cell growth in prolactin-secreting cells and prolactin release is required for the stimulation to occur. The increase in cell growth produced by indomethacin and aspirin is not cell-specific and it is not related to hormonal release (D. G. Rosental, M. I. Romano, G. A. Machiavelli, R. L. Perez, N. Sterin Speciale & J. A. Burdman, unpublished observations).

However, when indomethacin and oestrogens are administered together, instead of an additive effect on cell proliferation, the stimulation produced by the

steroid seems to be inhibited. Studies on oestrogen receptors indicate that, in the presence of indomethacin or aspirin and after the administration of oestradiol, the oestradiol–receptor complex does not increase in the nuclear fraction. This indicates a decreased concentration of the receptor bound or capable of binding to the cell chromatin. Because the primary biological effects of oestrogens occur after the binding of the oestrogen–receptor complex to DNA, one can predict that in the presence of indomethacin or aspirin these biological effects should be either diminished or abolished. Indeed, the synthesis of two proteins which are oestrogen-dependent, progesterone receptor and prolactin, was diminished in rats treated with indomethacin or aspirin. It appears that these drugs have a general inhibitory effect on the biological responses to oestrogens.

Oestrogens increase the concentration of prostaglandins in their target tissue. Indomethacin, at the dose employed, partially inhibited this effect in the anterior pituitary gland. Two alternative non-exclusive possibilities can explain this inhibition. The first is a similar mechanism to that which explains the inhibition of the other biological effects of this steroid as discussed above, whereas the second is a direct effect of indomethacin on the stimulation by oestrogens of prostaglandin synthesis at a post-transcriptional level.

The doses of indomethacin and aspirin employed were chosen because they have a clear stimulatory effect on cell proliferation in the anterior pituitary gland of the rat. In previous work (D. G. Rosental, M. I. Romano, G. A. Machiavelli, R. L. Perez, N. Sterin Speziale & J. A. Burdman, unpublished observations) we demonstrated that at this dose indomethacin does not interfere either with the release of pituitary hormones or with the level of cyclic AMP. The main known effect of indomethacin and aspirin is the inhibition of prostaglandin synthesis. It is premature at this time to associate this inhibition with the effect of these drugs on oestrogen receptors and on the stimulatory action of the steroid. An in-vitro system in which the inhibition produced by these drugs can be reversed by the addition of prostaglandins could serve this purpose. Experiments of this type have been employed with cells in culture stimulated to proliferate and *in vivo* with rat colonic epithelium (De Rubertis, Craven & Saito, 1985; Dubois, Bolton & Cuzner, 1986).

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