Br. J. Pharmac. (1976), **57**, 273–278

INDOMETHACIN-INDUCED ALTERATIONS IN CORTICOSTEROID AND PROSTAGLANDIN RELEASE BY ISOLATED ADRENOCORTICAL CELLS OF THE CAT

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1 The effects of purported prostaglandin synthesis inhibitors on steroid and prostaglandin (E and F) release from trypsin-dispersed cat adrenocortical cells were investigated.

2 Low indomethacin concentrations potentiated adrenocorticotrophin (ACTH)-evoked prostaglandin and steroid release, whereas higher concentrations depressed both responses to ACTH. The steroidogenic response to exogenous prostaglandin E_2 was not markedly altered over a wide range of indomethacin concentrations.

3 Indomethacin enhanced basal steroid release but did not enhance basal prostaglandin E or F release.

4 5, 8, 11, 14-Eicosatetraynoic acid (ETA) elicited a concentration-dependent inhibition of ACTH-induced steroid release, but had little effect on prostaglandin E_2-induced steroid release. A high concentration of ETA inhibited prostaglandin E and F release.

5 These data are discussed in relation to the concept that prostaglandins provide a critical link in ACTH-induced corticosteroidogenesis.

Introduction

Prostaglandins are believed to act as mediators in a variety of cellular functions, including secretion (Horton, 1969; Flack, 1973). Although the adrenal cortex represents a secretory organ which has been studied extensively in regard to the possible roles of calcium and cyclic adenosine-3′,5′-monophosphate (AMP) as potential intermediates in the steroidogenic process (cf. Halkerston, 1975), the participation of prostaglandins in the mechanism of action of adrenocorticotrophin (ACTH) in triggering steroidogenesis is at present unresolved (Flack, 1973; Shaw & Tillson, 1974). Evidence supporting an interrelationship between prostaglandins and steroidogenesis derives, in part, from pharmacological data, which demonstrate that high concentrations of exogenous prostaglandin of the E series are capable of enhancing steroidogenesis *in vitro* (Flack, Jessup & Ramwell, 1969; Warner & Rubin, 1975). Evidence for the involvement of endogenous prostaglandins in the action of ACTH was provided by our own recent studies which showed that in cat isolated adrenocortical cells, ACTH enhances the biosynthesis of prostaglandins E and F from radiolabelled arachidonic acid (Laychock & Rubin, 1975).

Another approach to ascertaining the role of prostaglandins is to study the effects on steroid production of agents whose primary pharmacological action involves an inhibition of prostaglandin synthesis. The antirheumatic agent, indomethacin, is one of the more potent members of this group (Ferreira & Vane, 1974; Flower, 1974). This investigation is concerned with the effects of indomethacin on ACTH-induced steroid and prostaglandin release from isolated adrenocortical cells; it provides additional evidence for the concept that prostaglandins function as mediators of corticosteroidogenesis.

Methods

Cortical cell suspension

Both adrenal glands were excised from cats and the cortical cells dispersed with trypsin by the procedure described by Rubin & Warner (1975). The cells were maintained in Dulbecco's Modified Eagle's medium (GIBCO) (MEM) supplemented with 0.2% bovine serum albumin and 0.04% trypsin inhibitor when prostaglandin analysis was performed, and 0.6% bovine serum albumin and 0.1% trypsin inhibitor when optimum responsiveness to ACTH was desired for measurement of corticosteroid release.

Steroid and prostaglandin analysis

Following their dispersion, the cells, averaging 2.5×10^5 cells/ml, were incubated for 60–90 min at 37°C in the presence or absence of stimulant and/or inhibitor. The cell suspensions were then centrifuged at 6000 rev/min for 10 min at 4°C, and the supernatant assayed for corticosteroid by competitive protein binding (Jaanus, Carchman & Rubin, 1972).

Prostaglandin analysis of the supernatant was carried out by radioimmunoassay (RIA) using antiserum to prostaglandin $F_{2\alpha}$ produced in rabbits against prostaglandin $F_{2\alpha}$-bovine serum albumin conjugates (Caldwell, Burstein, Brock & Speroff, 1971). Prostaglandin F determinations were made by direct RIA of 300 µl aliquots of cell incubation medium. For the prostaglandin E determination, 3 ml of supernatant was pooled and acidified to pH 3 with 0.5N HCl and processed through XAD-2 columns, according to the method of Keirse & Turnbull (1973). The ethanol eluate was evaporated to dryness under N_2 at 50°C, resuspended in Na-phosphate buffer (pH 7.4), and subjected to reduction using sodium borohydride (0.02%), which reduces prostaglandins of the E series to prostaglandin F compounds (Levine, 1973). The prostaglandin F equivalents were determined by RIA using anti-prostaglandin $F_{2\alpha}$ antibody. The bound and free tritium labelled prostaglandin F generated during the RIA were separated by nitrocellulose filtration; the filters were dissolved in Bray's solution and counted by liquid scintillation spectrometry.

Since the details of the RIA and antibody characterization are being prepared for a subsequent publication, the prostaglandins in this study will be designated only as prostaglandin F or E equivalents, although differential assays using antisera to prostaglandins $F_{1\alpha}$ and $F_{2\alpha}$ suggest a predominance of prostaglandins E_2 and $F_{2\alpha}$ in the cell incubation medium. Steroid and prostaglandin values were expressed either in ng/2.5×10^5 cells (ng/ml) or as per cent increase relative to control cells.

Drugs and reagents

The following were used: trypsin and lima bean inhibitor (Worthington); bovine serum albumin (fatty acid free) (Sigma); [^{3}H]-corticosterone (42 Ci/mM); [^{3}H]-prostaglandin $F_{2\alpha}$ (175 Ci/mM) (New England Nuclear); non-radiolabelled prostaglandins (Upjohn). β1–24-Adrenocorticotrophin (ACTH) (Ciba); indomethacin (Merck, Sharp & Dohme) and 5,8,11,14-eicosatetraynoic acid (RO-3–1428) (Hoffman-LaRoche) were dissolved in 95% v/v ethanol and added to the final incubation volume (1 ml) in 10 µl aliquots; all other experimental samples received 10 µl ethanol. Concentrations are expressed as mol/l (M).

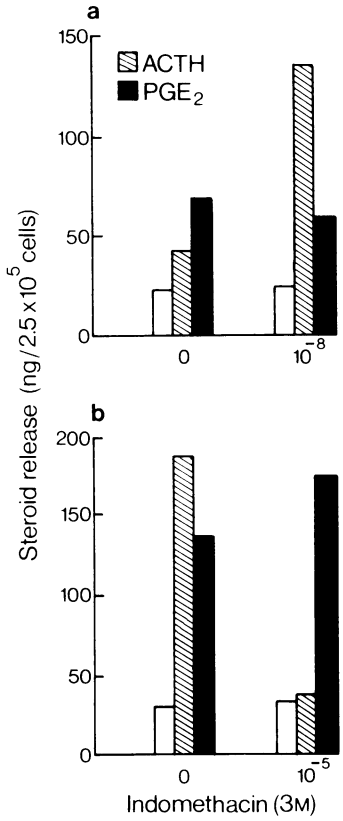

Figure 1 Comparison of the effects of indomethacin on adrenocorticotrophin (ACTH) and prostaglandin E_2 (PGE_2)-induced steroid release from isolated adrenocortical cells. Each vertical column represents the total amount of hormone released by equal number of cells during a 90 min incubation period in the presence or absence of indomethacin plus: (a) ACTH (25 µu) or prostaglandin E_2 (2×10^{-4} M), (b) ACTH (125 µu) or prostaglandin E_2 (3×10^{-5}M). The open columns show basal steroid release from unstimulated cells; hatched columns show effects of ACTH; solid columns show effects of prostaglandin E_2. These results were obtained from 2 different preparations.

Results

Effects of indomethacin on ACTH-induced steroid production

Strikingly low concentrations of indomethacin caused marked effects on ACTH-induced steroidogenesis in isolated cortical cells. Results from a single experiment using a low ACTH concentration (25 µu/ml) are depicted in Figure 1a; they show a marked facilitation of steroid release in the presence of 0.03 µM

(3×10^{-8} M) indomethacin. More detailed analysis of this potentiation using near maximal ACTH concentrations (75–250 μu) demonstrated that 3 nM (3×10^{-9}M) indomethacin augmented ACTH-induced steroid release almost two-fold (Figure 2a). This facilitatory action of the drug was transformed into an inhibitory action as its concentration was increased. ACTH-evoked steroid release in the presence of indomethacin (0.3 μM; 3×10^{-7} M) was less than in its absence (Figure 2a) and in the presence of 30 μM (3×10^{-5} M) indomethacin, a greater inhibition was detected (Figure 1b and 2a). Despite the fact that low and high concentrations of indomethacin caused considerable alterations in the steroidogenic response to ACTH, these same indomethacin concentrations did not elicit similar alterations in the steroidogenic response to exogenous prostaglandin E_2 (Figure 1).

Indomethacin action on basal steroid levels

Indomethacin in the concentration range of 3 nM to 30 μM (3×10^{-9} to 3×10^{-5} M) was capable of augmenting basal steroid release (Table 1). The stimulant effect was small and inconsistent at the lowest concentration tested (3 nM; 3×10^{-9} M), but increased approximately two-fold with 0.3 μM (3×10^{-7}) and 30 μM (3×10^{-5} M) indomethacin, respectively. This facilitatory action of indomethacin was not demonstrable to the same degree in every experiment, but was generally manifest in those preparations showing a greater responsiveness to ACTH.

Effects of indomethacin on prostaglandin release

Steroidogenic concentrations of ACTH elicited dose-related increases in release of prostaglandins E and F (Table 2–expt. 1). An indomethacin concentration (3nM; 3×10^{-9} M) that facilitated ACTH-induced steroid release, likewise potentiated the effects of ACTH on prostaglandin F and E release (Table 2–expt. 2); in the same experiment this facilitatory effect of indomethacin on prostaglandin release was converted to inhibition by a high concentration of indomethacin

Table 1 The effect of indomethacin on basal corticosteroid and prostaglandin (PG) release

Indomethacin (3 M)	*Steroid (% of basal)*	*PGF*	*PGE*
		(% of basal)	
10^{-9}	113 ± 8 (4)	—	—
10^{-7}	215 ± 65 (5)	78 ± 23 (4)	75 ± 14 (3)
10^{-5}	174 ± 35 (5)	87 ± 20 (6)	93 ± 35 (3)

Mean values (± s.e. mean) are expressed as % increase over values obtained in the absence of drug. Number of observations are indicated by number in parentheses; each value was obtained from a different preparation.

Table 2 Prostaglandin release from unstimulated cortical cells, and from cells exposed to ACTH with or without indomethacin

Expt.	*ACTH (μu)*	*Indomethacin (3 M)*	*PGF*	*PGE*
			(pg/2.5 × 10^5 cells)	
1	—	—	270	1515
	25	—	301	2201
	250	—	652	2737
	425	—	960	7426
2	—	—	179	1300
	25	—	165	1363
	25	10^{-9}	213	2645
	25	10^{-5}	79	828
3	—	—	107	633
	250	—	230	1257
	250	10^{-7}	55	524
4	—	—	72	< 6
	250	—	182	873
	250	10^{-7}*	< 6	< 6

Values for each experiment were obtained from medium incubating equal numbers of cells for 1 h at 37°C.
* Indomethacin present for 1 h prior to addition of ACTH and then removed from the medium.

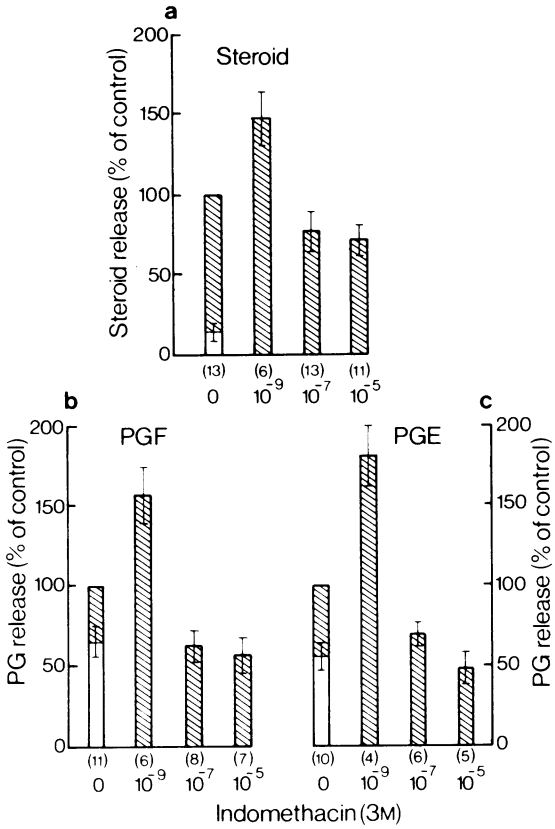

Figure 2 The effects of indomethacin on adrenocorticotrophin (ACTH)-induced steroid and prostaglandin release. Prostaglandin (PGE and PGF) and steroid determinations were made on aliquots of incubation medium following incubations of 60 and 90 min, respectively. Each vertical column represents the mean response to ACTH (±s.e.) elicited in the presence of a given indomethacin concentration; the values are expressed as a percent of the response to ACTH in the absence of drug. Mean values (±s.e.) obtained in the absence of both ACTH and indomethacin are also depicted (open columns) to illustrate the stimulant effects of ACTH in the absence of indomethacin. The number of experiments is indicated by the figure in parentheses under each column.

(30 μM; 3×10^{-5} M) (Table 2–expt. 2). Experiment 3 of Table 2 illustrates the potent inhibitory action of indomethacin even in the concentration of 0.3 μM (3×10^{-7}M). The irreversible nature of this inhibition is shown by the finding that after a 1 h exposure to indomethacin, cells reincubated in an inhibitor-free medium were unable to release measurable amounts of prostaglandin E or F in response to ACTH (Table 2–expt. 4).

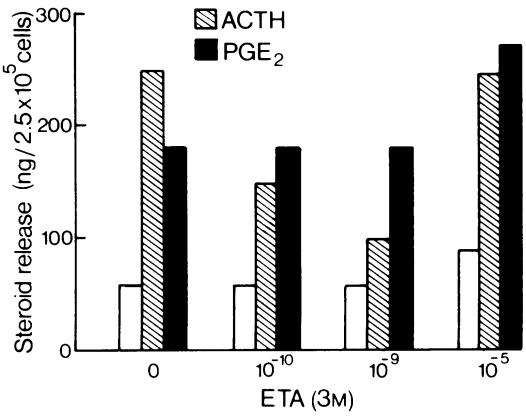

Figure 3 Comparison of the effects of 5,8,11,14-eicosatetraynoic acid (ETA) on adrenocorticotrophin (ACTH)- and prostaglandin E_2 (PGE_2)-evoked steroid release. The columns represent the total amount of steroid released by a single preparation during a 90 min incubation period with varying indomethacin concentrations, in the presence of either ACTH (50 μu, hatched columns) or prostaglandin E_2 (2×10^{-4}M, solid columns), or in their absence (open columns).

Figure 2 summarizes the results of all experiments, including those presented in Table 2. Indomethacin at a concentration of 3 nM (3×10^{-9} M) potentiated ACTH-evoked release of prostaglandins by almost two-fold. Higher indomethacin concentrations (0.3 μM and 30 μM; 3×10^{-7} and 3×10^{-5} M) depressed ACTH-evoked prostaglandin E and F values to below basal levels; with these same indomethacin concentrations, ACTH still increased steroid release by six-fold or more above basal levels (Figure 2a).

Despite the fact that indomethacin in the concentrations of 0.3 μM and 30 μM (3×10^{-7} and 3×10^{-5}M) enhanced basal steroid release, these same concentrations were unable to elicit a consistent or significant change in basal prostaglandin E or F release (Table 1).

Effects of 5,8,11,14-eicosatetraynoic acid

Although this study was primarily concerned with the action of indomethacin, several experiments were also carried out with 5,8,11,14-eicosatetraynoic acid (ETA). ETA in concentrations as low as 0.3 nM (3×10^{-10}M) impaired the steroidogenic response to ACTH (Figure 3). The inhibition was concentration-dependent over the range of 0.3 nM to 0.3 μM (3×10^{-10} to 3×10^{-7} M). However, at the highest ETA concentration tested (30 μM; 3×10^{-5} M), the inhibitory action was reversed (Figure 3). The stimulant action of exogenous prostaglandin E was

not markedly affected by an ETA concentration as high as 0.3 μM (3×10^{-7} M), but its action, like that of ACTH, was potentiated by 30 μM (3×10^{-5} M) ETA (Figure 3). Basal steroid release was increased by ETA. In concentrations of 0.3 μM and 30 μM (3×10^{-7} and 3×10^{-5} M), ETA augmented basal steroid release by $121 \pm 7\%$ and $155 \pm 7\%$, respectively (three experiments each).

In two separate experiments, ACTH-facilitated prostaglandin F release was reduced by ETA (30 μM; 3×10^{-5}M), from 1660 and 92 pg to 578 and 37 pg, respectively. This depression of the ACTH response produced by ETA was of a sufficient magnitude to cause prostaglandin E and F release to fall below basal levels. In a third experiment where ETA (30 μM; 3×10^{-5}M) failed to depress ACTH-evoked prostaglandin F release from a value of 447 pg, it decreased ACTH-induced prostaglandin E release from 1089 pg to below measurable levels.

Discussion

In assessing the potential significance of cyclic AMP in a particular physiological function, Sutherland and his associates presented certain criteria which had to be considered (cf. Robison, Butcher & Sutherland, 1971). With these criteria in mind, we have been investigating the postulate that prostaglandins provide an important link in the steroidogenic action of ACTH on isolated adrenocortical cells of cats. Previous findings show that exogenous prostaglandin E_2 is capable of augmenting steroidogenesis, and that this stimulant action resembles in several ways the action of ACTH (Warner & Rubin, 1975). In addition, ACTH enhances the conversion of radiolabelled prostaglandin precursor, arachidonic acid, into prostaglandin E and F (Laychock & Rubin, 1975). The present study provides further support for this postulate by demonstrating: (a) the presence of endogenous prostaglandins (E and F) in cat cortical cells by radioimmunoassay, (b) that ACTH enhances their release in a concentration-dependent manner and, (c) that indomethacin and ETA, agents that alter prostaglandin synthesis, cause profound changes in ACTH-evoked steroid release.

The ability of indomethacin in low concentrations to produce a parallel potentiation of steroid and prostaglandin release provides strong support for the concept that prostaglandins play a key physiological role in ACTH-induced steroidogenesis. The unexpected finding that indomethacin, a prototype inhibitor of prostaglandin synthesis, enhances release in low concentrations may relate to an ability to impair the catabolism of endogenous prostaglandins. For example, prostaglandin dehydrogenase, which catalyses the conversion of prostaglandin to a keto derivative, appears susceptible to inhibition by indomethacin (cf. Flower, 1974). Although another action not related to prostaglandin metabolism, such as inhibition of phosphodiesterase (Flores & Sharp, 1972), could account for the facilitatory action of indomethacin on ACTH-evoked steroid release, the fact that the steroidogenic action of exogenous prostaglandin E_2 was not greatly altered by a low indomethacin concentration is in harmony with the notion that indomethacin is indeed exerting its principal action on steroid release through an alteration in endogenous prostaglandin levels.

At higher indomethacin concentrations, a functional relationship between prostaglandin and steroid release was less clear. Indomethacin caused an irreversible inhibition of ACTH-induced prostaglandin E and F release, accompanied by a more modest impairment of ACTH-evoked steroid release; thus, steroid release was still augmented approximately six-fold despite prostaglandin release being at or near basal levels. This finding suggests that enhanced activity of the prostaglandin system may not be a *sine qua non* for the steroidogenic action of ACTH. However, these experiments are to be interpreted with caution since our conclusions regarding prostaglandin metabolism must be considered from the perspective that a measurement of prostaglandin release is being equated with cellular synthesis and turnover. The validity of this assumption is based upon the fact that increased prostaglandin release is a consequence of an activation of biosynthesis rather than secretion from a preformed store (Ramwell & Rabinowitz, 1972). This assumption seems justified, since in the few experiments in which we have determined intracellular prostaglandin levels by analysis of control and stimulated adrenocortical cells, their values were only 10–20% of those obtained from analysis of the incubation medium (Laychock & Rubin, unpublished observations).

The ability of indomethacin to enhance basal steroid release is also potentially an important finding, since if this increase were the result of an action on prostaglandin synthesis, then it would imply that an alteration in prostaglandin metabolism is capable of triggering steroid release in the absence of the primary stimulus, ACTH. Although this still may be the case, we were unable to detect any consistent or marked changes in basal prostaglandin E or F release caused by indomethacin.

ETA, an acetylenic analogue of arachidonic acid, inhibits the latter's conversion to prostaglandin E and F (Ahern & Downing, 1970); and in the present investigation depressed ACTH-evoked prostaglandin E and F release from cortical cells. The use of this prostaglandin analogue provided further evidence for a physiological role for prostaglandins in the regulation of steroidogenesis by causing, even in very low concentrations, a dose-dependent inhibition of ACTH-induced steroid release. The reversal of the inhibition

seen with a high ETA concentration cannot be explained at present; it may be another consequence of altered prostaglandin metabolism or it may represent an additional action of the drug.

The fact that ETA did not, like indomethacin, potentiate ACTH-induced release in low concentrations, does not necessarily detract from the notion that these two agents exert their primary effects on steroid release through actions on prostaglandin metabolism. There is general agreement that indomethacin and ETA exert their actions at different sites in the biosynthetic pathway (Lands, LeTellier, Rome & Vanderhoek, 1972; Flower, 1974); the particular modification of prostaglandin synthesis elicited by each inhibitor could result in variable effects on the physiological response.

In conclusion, we feel that ample evidence now exists which is compatible with the idea that prostaglandins play some physiological role in the regulation of adrenal steroidogenesis. The extent and manner in which a particular prostaglandin is directly involved cannot be ascertained at present. Only prostaglandins E and F were analyzed in this study; and in general they were altered to a similar extent. It is entirely possible that other prostaglandins, as for example, prostaglandins A and B as well as the more labile endoperoxide intermediates, may also be involved in the action of ACTH; one or more may be facilitatory and others inhibitory. In any event, the accumulating evidence provides encouragement for the continuation of these investigations to assess more precisely the physiological significance of prostaglandins in adrenal steroidogenesis.

We wish to thank the following individuals for generously supplying drugs: Dr J.E. Pike (Upjohn) for prostaglandin E_2; Dr J.J. Chart (Ciba) for β1,24-adrenocorticotrophin; Dr H.D. Brown (Merck, Sharp & Dohme) for indomethacin; and Dr J.R. Paulsrud (Hoffmann-LaRoche, Inc.) for 5,8,11,14-eicosatetraynoic acid (Ro-3-1428). This investigation was supported by USPHS Research Grant AM-18066 (NIAMDD); S.G.L. is an NIH Predoctoral Fellow supported by USPHS Pharmacology Training Grant GM-07111, awarded to the Department of Pharmacology, Virginia Commonwealth University; Medical College of Virginia.

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(Received November 6, 1975. Revised December 15, 1975.)