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# EFFECT OF PROSTAGLANDIN SYNTHETASE INHIBITORS ON BASAL AND ACTH-STIMULATED STEROID SYNTHESIS BY SEPARATED ADRENOCORTICAL ZONES

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SUMMARY

The effect of the prostaglandin synthetase inhibitors aspirin and indomethacin on steroid synthesis by the capsular (zona glomerulosa) and decapsulated adrenal gland (z. fasciculata-reticularis) obtained from hypophysectomized rats were studied *in vitro*. No unambiguous indication was obtained for a role of prostaglandins in the unstimulated state. ACTH-stimulated corticosterone production rate was reduced in the capsular but enhanced in the decapsulated gland by the inhibitors. The reduction of aldosterone production rate was disproportionately slight as compared to that of capsular corticosterone. The results are compatible with the view that prostaglandins play an essential role in the mediation of ACTH stimulation to glomerulosa cells and also allow the assumption of the existence of prostaglandin inhibitor sensitive and insensitive pathways or pools in adrenal steroid biosynthesis.

## INTRODUCTION

Synthetic prostaglandins (PG) of the E-type were reported to stimulate aldosterone and corticosterone production rate by the zona glomerulosa [1, 2] as well as corticosterone production rate by the zona fasciculata-reticularis [3] of the rat's adrenal gland. Similar stimulation of corticosteroid synthesis was observed using bovine [4] and cat [5] adrenal preparations. The rapid metabolism of E-type PGs in the pulmonary circulation [6–8] excludes the role of circulating PGEs in the regulation of adrenocortical steroid synthesis. Considering, however, that ACTH was reported to stimulate PGE synthesis by rat adrenal homogenates [9] and release of immunoreactive $PGF_{2\alpha}$ (albeit not so immunoreactive $PGE_2$) by feline adrenocortical cells [10] an intracellular modifier function may be attributed to PGs. In an attempt to test this assumption we examined the effect of PG synthetase inhibitors on basal and ACTH-stimulated steroid synthesis by the rat's adrenal capsular gland (zona glomerulosa) and decapsulated gland (zona fasciculata-reticularis), respectively.

## MATERIALS AND METHODS

*Materials.* Synthetic adrenocorticotropin (ACTH) (Synacthen, Ciba) was dissolved either in acidified physiological saline (pH 3.5) or in Krebs–Ringer bicarbonate glucose solution containing 0.5% (w/v) human serum albumin. All glass surfaces coming into contact with ACTH were coated with dichlorodimethylsilane–trichloromethylsilane (95:5) (Merck). Indomethacin (Chinoin) was freshly dissolved in warm physiological saline adjusted to pH 7.4. Acetylsalicylic acid (aspirin) (Biogal) was dissolved in Krebs–Ringer-bicarbonate solution.

Human serum albumin (fraction V, Humán) was used for tissue incubation while bovine serum albumin (fraction V, Calbiochem) and gamma globulin (Serva) were used in steroid binding assays.

[1,2-$^3$H]-aldosterone (57 Ci/mmol) was purchased from New England Nuclear, Boston, MA, [1,2-$^3$H]-corticosterone (34 Ci/mmol) was obtained from The Radiochemical Centre, Amersham.

Organic solvents, all analytical grade, with the exception of acetic acid and those used for scintillation solution, were further purified according to Bush[11].

Antibody to aldosterone (088) was prepared in the laboratories of Prof. and Mrs J. F. Tait at the Worcester Foundation for Experimental Biology, Shrewsbury, MA. The original sample (1:100 dilution in 4% BSA in 0.15 M potassium phosphate buffer pH 7 containing 0.1% (w/v) sodium azide) was divided and stored in ampoules under nitrogen at −20°C. Stock solutions (1:500) were kept at +4°C.

*Animals, tissue preparation, incubation technique.* Male Sprague–Dawley (CFY) rats, weighing 130–155 g, were kept on a standard semisynthetic diet (sodium: 165 mequiv./kg, potassium: 145 mequiv./kg) with free access to tap water. Light and temperature of the animal house was controlled. The animals were hypophysectomized via the transauricular route by means of a stereotaxic equipment [12] 16–20 h before decapitation. Tap water was replaced by 5% glucose solution after hypophysectomy. Completeness of hypophysectomy was checked by *post-mortem* inspection of the sella turcica.

The adrenals, excised immediately after decapitation, were separated into capsular (fibrous capsule

plus zona glomerulosa) and decapsulated gland (zona fasciculata, reticularis and the medulla) under a binocular magnifier according to the method of Giroud *et al.* [13]. Histological examination showed that the capsular gland was contaminated with a small amount of fasciculata cells. During preparation, the tissue was kept at room temperature in a solution of NaCl–KCl–Hepes (144:5.9:20 mM, pH 7.4).

Incubation of 1–2 pairs of adrenals was carried out in 4 ml of Krebs–Ringer bicarbonate solution ([K*] = 4.5 mM) containing 2 g of glucose and 5 g of albumin per l. under 95% $O_2$ : 5% $CO_2$ at 37°C, according to the method of Saffran and Schally [14]. The medium used for the first 30 min of incubation was discarded and the medium used for the consecutive 60 min was analysed for steroid concentration. PG synthetase inhibitors were present in both periods while ACTH was added in the second (31–90 min) period only. In the experiment examining the effect of aspirin contralateral glands were incubated in separate vessels, one exposed to aspirin while the other served as a control.

*Steroid analysis.* Extraction and partition-type purification of 0.5 ml vols of incubation medium were executed after the addition of 3,500–5,000 c.p.m. of [$^3$H]-corticosterone, as previously described [15]. The dry residue of the organic extracts was redissolved in 1 ml of ethanol. An aliquot of 0.25 ml was used for determining [$^3$H]-corticosterone recovery, 0.1 ml was used for aldosterone and the remaining part for corticosterone determination.

The ethanolic aliquot used for aldosterone estimation was completed with 3,500–4,500 c.p.m. of [$^3$H]-aldosterone, evaporated *in vacuo* and transferred to methanol-prewashed Schleicher–Schüll 2043b chromatographic paper in methylene chloride–acetic acid (2,000:1, v/v). Aldosterone was isolated with Bush B5 chromatography at 37°C. Prednisone was used for reference standard. The spots running parallel to prednisone as localized at 254 nm, were eluted with methylene chloride–methanol (1:1 by vol.). The eluting solvent was evaporated in silanized tubes under nitrogen and redissolved in 1 ml of ethanol. One 0.25 ml aliquot was taken for [$^3$H]-aldosterone recovery while 2–3 different aliquots were taken for aldosterone assay.

Aldosterone was estimated by means of radioimmunoassy. A 1:200,000 dilution of antiserum was prepared with a binding solution which contained 1 mg of albumin, 1 mg of gamma globulin, 1 mg of sodium azide and 3 nCi of [$^3$H]-aldosterone per ml of 0.05 M borate buffer pH 8.0. The sample extracts and duplicate calibration standard samples (0–300 pg) were transferred into small centrifuge tubes. Ethanol was evaporated *in vacuo* and 250 µl of the binding solution were added. The tubes were incubated at room temperature for 30 min and at 4°C overnight. Cold dextran-coated charcoal (25 mg of Dextran T 70 and 250 mg of Norit A in 30 ml borate buffer pH 8.0), 250 µl, were then added to each tube. The tubes were swirled on a vortex mixer and centrifuged at 5,000 *g* for 2 min at 1°C. Three hundred µl of supernatant was transferred into a PPO–POPOP–toluene–ethanol–dioxane scintillation solution for tritium counting.

Tritium activity was counted for 50 min (or 10,000 counts) in a Beckman LS 254 liquid scintillation spectrometer using automatic external standardization. The computation of the bound fraction of [$^3$H]-aldosterone involved correction for the residual activity (d.p.m.) of tritium used for recovery estimation. Corrections were done for [$^3$H]-aldosterone as well as [$^3$H]-corticosterone recovery. The calculations were carried out with a Wang 2100 computer.

*Reliability of aldosterone determination.* Tritium *recovery* was 75.3 ± 2.2 (S.E. %) ($n$ = 48). *Accuracy* of aldosterone determination from albumin containing phosphate buffer was examined in the range of 75–800 pg per sample: pg measured equalled 1.049 (±0.031) pg added + 0.98 (±13.28) pg, ($r$ = 0.996, $n$ = 12). *Precision* of the method was examined by duplicate determination of serum samples (1–3 ml each). Coefficient of variation for intra-assay duplicates was 17.3% (range 25–206 pg/ml, $n$ = 9 pairs), while that for inter-assay duplicates was 9.5% (range: 47–255 pg/ml, $n$ = 4 pairs). *Blank* values for 2.5 ml of albumin containing phosphate buffer amounted to 8.6 ± 3.7 pg ($n$ = 7). (This value was corrected for the loss of tritiated internal standard in course of the procedure). *Sensitivity*, i.e. the smallest amount of steroid in the original sample significantly differing from zero, was computed on the basis of the lower confidence limit of the regression line obtained in the accuracy examinations and the upper confidence limit of the blank and may be set at 35 pg. *Specificity* of the antibody was tested for corticosterone, DOC, progesterone, 18-hydroxy-DOC and 18-hydroxycorticosterone. On the bases of 50% displacement of [$^3$H]-aldosterone the cross-reactivity of all these compounds was below 0.1% (taking the reactivity of aldosterone as 100%). Specificity of the overall assay was examined by analyzing buffer samples containing progesterone, DOC, and corticosterone, one µg of each. Virtual aldosterone content was estimated between 29 and 92 pg ($n$ = 4). Half µg of 18-hydroxycorticosterone was quantified as 68, 72 and 102 pg, resp., of aldosterone.

*Corticosterone* was estimated by means of competitive protein binding assay as previously described [15]. Modifications were as follows: (i) chromatography was omitted, (ii) the binding solution contained 1.5 ml mouse serum and 0.1 g gamma globulin per 100 ml. Specificity of the murine serum applied was estimated as for the aldosterone antiserum. Taking the displacing effect of corticosterone for 100%, DOC displayed 15%, progesterone 1%, 18-hydroxycorticosterone 1% and aldosterone far less than 1% efficiency.

*Statistical analysis.* Mean values and S.E.M. have been expressed. Significance of differences was esti-

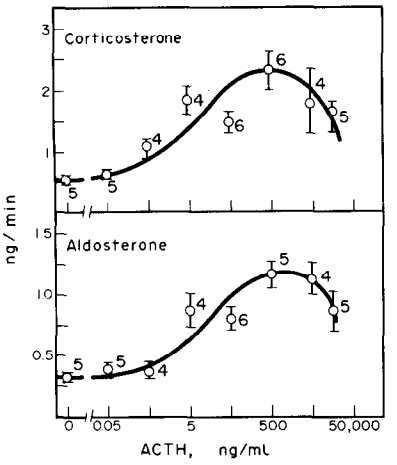


Fig. 1. Corticosterone (above) and aldosterone (below) production rate (ng per gland per min) of the capsular gland as a function of ACTH concentration.

mated by Student's paired or unpaired *t*-test, depending on the experimental arrangement. The *t*-test was replaced by a *d*-test in cases when the *F*-test revealed significant differences between the corresponding variances.

## RESULTS

### *Response of the capsular and decapsulated gland to ACTH*

To test the responsiveness of the tissue preparations used, the response to ACTH was examined. Five ng/ml ($1.7 \times 10^{-9}$ M) of ACTH brought about a significant increase in capsular aldosterone ($P < 0.05$) and corticosterone production rate ($P < 0.02$) (Fig. 1). Maximum production rate of either hormone was observed at 500 ng/ml. Above this concentration a gradual fall occurred. Corticosterone production rate by the decapsulated gland (Fig. 2) displayed a significant increase from an ACTH concentration of 5 ng/ml ($P < 0.02$). A further stimulation was observed up to 50 μg/ml. Although the threshold of stimulation for the two tissue preparations was the same (5 ng/ml),

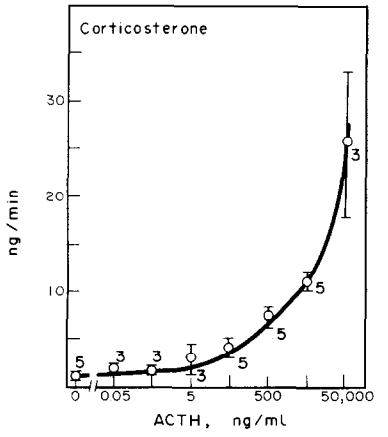


Fig. 2. Corticosterone production rate (ng/gland/min) of the decapsulated gland as a function of ACTH concentration.

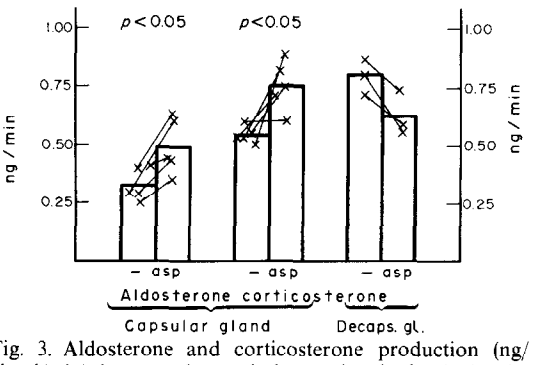


Fig. 3. Aldosterone and corticosterone production (ng/gland/min) by capsular and decapsulated glands in the absence and presence of aspirin (100 μg/ml). Matched incubates are connected with lines.

maximal stimulation for the capsular gland was four fold while it attained a factor of 24 for the decapsulated gland.

### *Effect of PG synthetase inhibitors on unstimulated tissue*

Aspirin significantly enhanced aldosterone and corticosterone production rate by the capsular gland (Fig. 3). The other inhibitor applied, indomethacin (1 or 10 μg/ml) failed to exert a significant effect on basal steroid output (data not shown).

The response of the decapsulated gland to these agents differed from that of the capsular tissue. All three matched-pair incubations exhibited a decrease in corticosterone output in response to aspirin (Fig. 3) while a tendency for increase was observed in response to indomethacin (data not shown).

### *Effect of PG synthetase inhibitors on the function of ACTH-stimulated capsular gland*

The effect of inhibitors on the stimulated capsular gland was examined at an ACTH concentration of 50 as well as 500 ng/ml. Aldosterone output was not significantly affected by indomethacin while corticosterone response was reduced at both concentrations of ACTH. The reduction attained the 0.05 significance level with an indomethacin concentration of 10 μg/ml (Fig. 4).

The effect of aspirin (5–1,000 μg/ml) in the presence of 50 ng/ml ACTH was examined in six experiments only. In all experiments corticosterone production rate was below the mean of the corresponding control (data not shown). After stimulation with 500 ng/ml of ACTH, 100 μg/ml aspirin failed to affect aldosterone production rate while an aspirin level of 500 μg/ml partially reduced aldosterone response to ACTH ($P < 0.1$), (Fig. 5). In contrast to aldosterone, corticosterone response by the same tissue to ACTH was gradually reduced by increasing levels of aspirin (at 100 μg/min: $P < 0.1$; at 500 μg/ml: $P < 0.05$) (Fig. 5).

### *Effect of PG synthetase inhibitors on the function of the ACTH-stimulated decapsulated gland*

The effect of the inhibitors was tested at ACTH concentrations of 50, 500 and 5,000 ng/ml. The re-

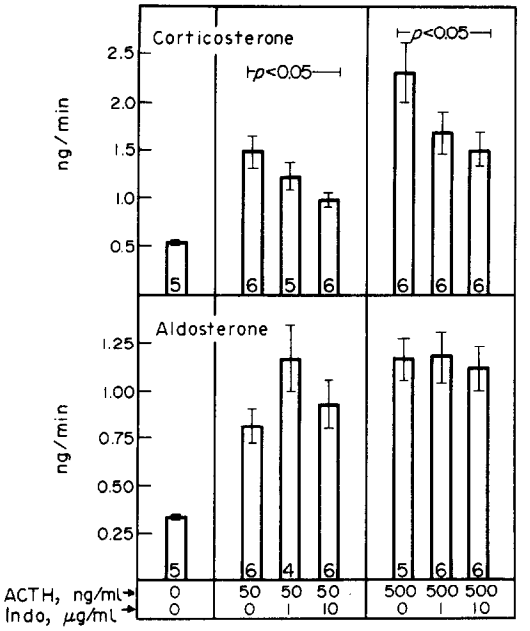


Fig. 4. Effect of ACTH and indomethacin on capsular corticosterone (above) and aldosterone (below) production rate (ng/gland/min).

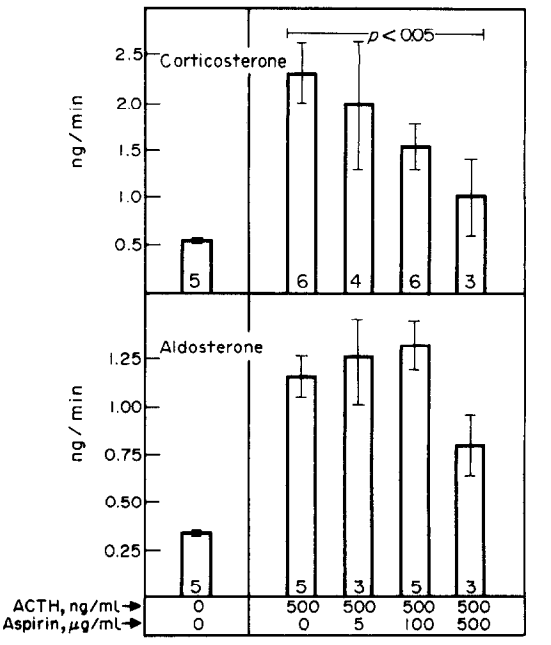


Fig. 5. Effect of ACTH and aspirin on capsular corticosterone (above) and aldosterone (below) production rate (ng/gland/min).

sponse of this tissue differed from that of the capsular gland. While the drugs reduced corticosterone production rate of the capsular gland they failed to exert this effect in the decapsulated gland. More surprisingly, indomethacin brought about a significant enhancement ($P < 0.05$) of the steroid stimulating effect of the highest ACTH concentration (Fig. 6).

## DISCUSSION

The experiments reported here were designed to examine the role of intracellular PGs in ACTH-stimulated steroid synthesis. A comparison of PG function in glomerulosa and fasciculata cells was undertaken with regard to the different mode of control of steroid secretion by these cells. It has been well established that while ACTH is the sole or at least the most important physiological regulator of fasciculata cells, glomerulosa function is specifically controlled by change in salt-water balance. Since these changes are mediated in the z. glomerulosa by factors other than ACTH, the role of intracellular PGs in the control of ACTH-stimulated steroid synthesis may be different in the two kinds of tissue.

The experiments executed on unstimulated tissue yielded rather conflicting results. Aspirin significantly increased steroid production rate by glomerulosa cells while hormone output by the z. fasciculata-reticularis was diminished by this drug. At present we can not explain the difference in the response of the two tissues. Bearing in mind, however, that indomethacin failed to exert a significant effect on steroid produc-

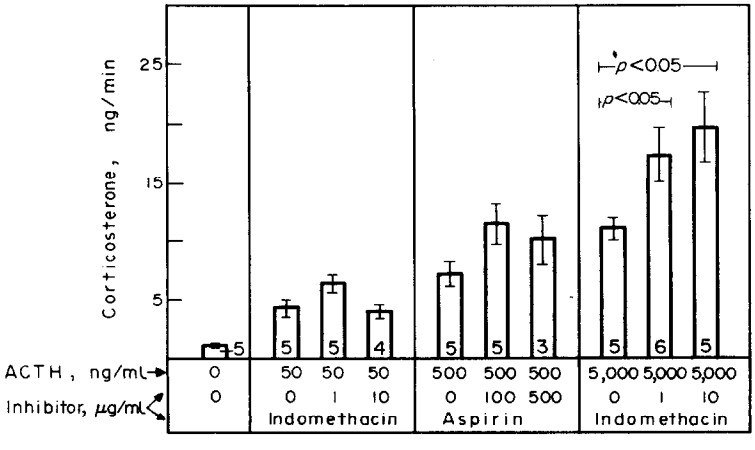


Fig. 6. Effect of ACTH and aspirin or indomethacin on corticosterone production (ng/gland/min) by the decapsulated gland.

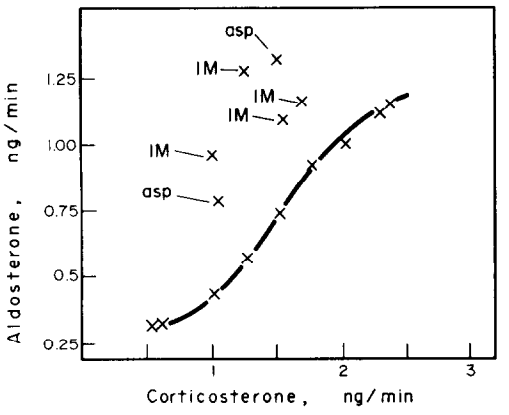


Fig. 7. Aldosterone production rate in function of corticosterone production rate by capsular glands. The curve was constructed on the basis of the visually drawn curves in Fig. 1, in the range of 0 500 ng/ml of ACTH.

tion rate, this effect of aspirin may not be attributed to the inhibition of PG synthesis.

The addition of aspirin and indomethacin to the capsular gland exposed to ACTH gave concordant results. Therefore, the observed effects may have been a consequence of the supposed reduction in PG synthesis. Corticosterone production rate by the z. glomerulosa was significantly reduced by both inhibitors. This observation suggests an essential role of PGs in the mediation of ACTH stimulation of the glomerulosa cells. Since the degree of inhibition of PG synthesis was not estimated by us, no conclusion can be drawn concerning the mode of attachment of PGs to the hormone-receptor–adenylate cyclase-protein synthesis system.

While PG synthetase inhibitors significantly reduced corticosterone output by the ACTH-stimulated capsular gland, indomethacin failed to affect aldosterone production rate and only the highest concentration (500 $\mu$g/ml) of aspirin had a reducing effect. In this context it should be mentioned that this high concentration of aspirin decreased the pH of the incubation medium by 0.24. Since the effect of indomethacin on pH was negligible the reduced aldosterone production rate in the presence of 500 $\mu$g/ml of aspirin may have been such a technical artefact. Explanation is required, however, for the discrepancy between aldosterone and capsular corticosterone production rate observed in the other groups. Saturation of the 18-hydroxylase system with its substrate, corticosterone, could bring about such a discrepancy provided that substrate saturation persisted after treatment with PG synthetase inhibitors. However, there is no indication of substrate saturation in the inhibitor-treated groups and mean aldosterone production rates were higher than expected on the basis of corticosterone production rates (Fig. 7).

The possibility of aldosterone biosynthesis without corticosterone as a key intermediary is repeatedly reported in literature [cf. e.g. 16]. The assumption of ACTH-induced capsular corticosterone biosynthesis being sensitive while the major pathway of aldosterone biosynthesis (bypassing corticosterone) being insensitive to PG synthetase inhibitors would provide an explanation for these observations.

Vinson and Whitehouse [17] described separate pools for bound and free steroids in the capsular gland but not so in the decapsulated one. The major product of the bound pool is aldosterone while corticosterone is that in the free pool. Our results would be compatible also with the assumption of inhibitor-sensitivity of the free pool and insensitivity of the other one.

PG synthetase inhibitors failed to reduce but augmented corticosterone production rate in the decapsulated gland. Two questions arise concerning this finding, namely the discrepancy between glomerulosa and fasciculata cells and the mechanism of PG inhibitor induced stimulation of fasciculata hormone production, resp. Our results indicate that endogenous PGs are not obligatory mediators in ACTH-elicited steroid synthesis in fasciculata cells. As to the possible importance of this finding it is worthwhile to recall the functional difference between the two zones: increased secretion of corticosterone by fasciculata cells is the obligatory response to elevated ACTH level in stress but increased output of hormone by glomerulosa cells may depend on the sodium–potassium balance [18]. Provided that the observed decrease in capsular corticosterone production was in fact due to inhibition of PG synthesis such a dependence might be mediated via PGs.

As a mechanism of PG synthetase inhibitor induced hyper-production of corticosterone by the decapsulated gland one could postulate the reduced synthesis of $PGF_{2\alpha}$. A negative interaction between $PGF_{2\alpha}$ and the appropriate trophic hormone has been described in human granulosa cells [19] and in the thyroid gland [20]. Nevertheless, indomethacin inhibits the synthesis of the $PGE_2$ and $PGF_{2\alpha}$ approximately to the same extent [21, 22], moreover exogenous $PGF_{2\alpha}$ does not reduce ACTH-induced corticosteroid synthesis [23]. Therefore, the role of $PGF_{2\alpha}$ in inhibitor induced hyperproduction does not seem to be probable. To reveal the mechanism of this phenomenon the reported side-effects of PG synthetase inhibitors must first be excluded.

In contrast to findings in several species, no specific receptors of $PGE_1$ could be revealed in rat adrenal homogenates [24]. (Receptors in glomerulosa cells may have been masked by the preponderant mass of fasciculata, reticularis and medullary cells.) In view of this report and the present results a physiological role of PGs in the control of fasciculata cell function remains to be established.

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