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STEROIDOGENIC PROPERTIES OF PROSTANOID STRUCTURES ON BOVINE ADRENOCORTICAL CELLS IN PRIMARY CULTURE

STRUCTURE–ACTIVITY RELATIONSHIP

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Abstract—Reticulo-fasciculata bovine adrenocortical cells grown in monolayer primary culture were used to examine the steroidogenic properties of a series of twenty-six prostanoid structures, including prostacyclin, two endoperoxide analogs and various modified primary PGs. The steroidogenic activity of these cultures was examined either in the continuous presence of effector or under short-term stimulation experiments, thus allowing measurement of cAMP in addition to cortisol production. Primary PG of the E series were found to be the most potent effectors of both parameters, whereas prostacyclin was ineffective. All modifications involving the cyclopentane ring of the PG structure appear to be deleterious to the biological activity. Although a disulfide analog of PGH_2 had no effect, two endoperoxide analogs were steroidogenic, U-44069 being highly potent. The structure–activity relationship among the PG structures was similar in long- or short-term experiments. In addition, a good correlation was found between steroidogenic potency and adenylate cyclase activation, either in intact cells or in a purified plasma membrane preparation.

Despite several reports on the effect of prostaglandins (PG) in the regulation of adrenal steroidogenesis, an obligatory role of these substances in the stimulation of this process by adrenocorticotrophic hormone (ACTH) has not been unequivocally established [1–4]. Use of inhibitors of PG biosynthesis has led to the suggestion that endogenous PG may act at the hypothalamo-pituitary level *in vivo* [5–7]. On the other hand, the direct effect of exogenous PG on steroidogenic activities of various adrenocortical preparations *in vitro* has been documented in recent years. A stimulatory effect has generally been observed with primary PG, especially of the E series [8–15] and recently with prostacyclin [16]. However, inconsistency and variability of response from preparation to preparation have been emphasized by several research groups [10, 12, 13, 15, 16], especially when using highly sensitive enzymatically-dispersed cells [13, 15, 16]. In an attempt to use fresh adrenocortical cell suspensions of bovine origin to establish a quantitative structure–activity relationship for a number of natural and modified PGs, we have also observed such a variability, despite a maintained response of these cells to ACTH. Since this difficulty may be related to uncontrolled variations in the metabolic state of freshly obtained cells, we decided to use the same cellular preparations after various times in primary culture, during which the cells may recover their basal metabolic characteristics. This approach provided an homogeneous biological system which appears to represent a well suited tool for the quantitative study of PG steroidogenic properties. This was established upon both short- (2 hr) and long-term (24 hr) incubations. The active PGs stimulated corticosteroid and cyclic AMP productions in a related manner. This relationship was examined further using purified adrenocortical cell membrane preparations enriched in responsive adenylate cyclase activity. Twenty-six prostanoid structures, including prostacyclin and two endoperoxide analogs, were compared as to their effect upon cyclic AMP and cortisol productions in an attempt to establish a possible structure–activity relationship.

MATERIALS AND METHODS

Eagle's minimum essential culture medium (MEM), serum and antibiotics were purchased from Gibco. The culture medium was made of MEM supplemented with 10% foetal calf serum and containing penicillin (100 U/ml), streptomycin (100 μg/ml) and kanamycin (20 μg/ml). Trypsin (TRL) and lima bean trypsin inhibitor were from Worthington and synthetic β 1–24 ACTH from Ciba. [^{3}H]-cortisol (40–60 Ci/mmole) was furnished by New England Nuclear. [^{125}I]-cyclic AMP and cyclic AMP antiserum were from the Institut Pasteur,

Paris. Primary prostaglandins, *thromboxane B_2 and two endoperoxide analogs* (U-44069 and U-46619) were a generous gift from Dr. J. Pike, Upjohn Co. Prostacyclin was kindly donated by Dr. Lagarde (Lyon). The other prostanoid structures* [17–19] were prepared and kindly provided by P. Crabbe (Grenoble).

Bovine adrenocortical cell preparation and culture conditions

Isolated adrenal cell suspensions were prepared according to Kloppenborg *et al.* [20] as modified by Sayers *et al.* [21]. Bovine adrenal glands obtained from the local slaughterhouse were kept in ice-cold MEM before processing within 1 hr after slaughter under sterile conditions. The glands were defatted, halved and the medulla was carefully scraped off. The adrenal cortex was cut into 0.5 mm thick slices with a Stadie–Riggs microtome. The first (inner) slice was discarded; the two following slices, representing mainly fasciculoreticulata cells, were collected (3–4 g) and transferred into a siliconized 60-ml flask containing 40 ml sterilized MEM and 50 mg trypsin. After 30 min at 37° with stirring, the supernatant fluid was discarded and the procedure repeated (6–8 times) with 20 min of stirring. The corresponding supernatants were pooled in a plastic flask at 4° and then centrifuged at 100 *g* for 10 min at room temperature. The cell pellet was resuspended in 50 ml of sterile MEM containing 50 mg of trypsin inhibitor. After a second centrifugation at 100 *g* (10 min), the final pellet was resuspended in culture medium and the cells were counted using a hematimeter. Usually, 10^6 cells were seeded in 5 ml of culture medium in stoppered Falcon culture bottles (25 cm^2 area) at 37° after equilibration with air–CO_2 (95%:5%). Thereafter, the medium was changed every 24 hr.

Cell counting. The culture medium was withdrawn and 1 ml of a 1% aqueous solution of Triton X-100 was added. After standing for 5 min, the nuclei were counted using a Neubaeur hematimeter.

Measurement of cortisol production. The culture medium was extracted with 5 vol. dichloromethane. Cortisol was measured by radioimmunoassay, using a specific rabbit antiserum obtained in the laboratory.

Cyclic AMP determination. Following withdrawal of the culture medium, 2.5 ml of MEM containing 1 mM of methylisobutyl xanthine (and effectors as indicated) were added. After 15 min at 37°, the incubation was stopped by 5 ml of absolute ethanol at −20°. Cells were scraped from the culture bottle with a rubber policeman; the mixture was kept at −20° for at least 2 hr. After centrifugation, the supernatant was collected and an aliquot evaporated to dryness. After acetylation [22], the cyclic AMP content was measured by radioimmunoassay [23].

Adrenal cortex plasma membrane preparation. Plasma membranes were prepared from bovine adrenal glands by the method of Schlegel and Schwyzer [24].

For determination of adenylate cyclase activity, the assay medium contained ATP (1 mM), $MgCl_2$ (6 mM), phosphoenolpyruvate (20 mM), pyruvate kinase (0.2 mg/ml), theophylline (7 mM) in pH 7.4, 20 mM Tris–HCl buffer. When used, sodium fluoride was present at a 10 mM final concentration. Plasma membrane suspensions containing 0.1–0.2 mg of protein were added to initiate the reaction. Following incubation for 15 min at 30°, the reaction was stopped upon addition of absolute ethanol. After 2 hr at −20° and centrifugation, the cyclic AMP content was measured as described above.

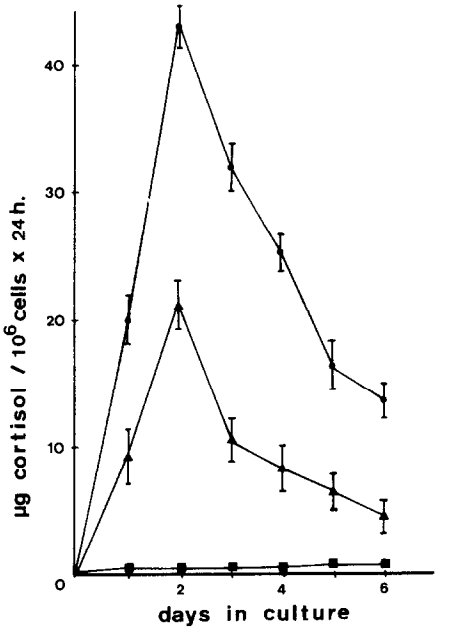

Fig. 1. Cortisol production by bovine adrenocortical cells in monolayer culture with the continuous presence of ACTH or PGE_1, as a function of time. ■, control (no effector added); ●, ACTH (100 mU/ml); ▲, PGE_1 (10^{-5} M). Each point is the mean of six independent observations.

RESULTS

Steroidogenic activity of bovine adrenocortical cells in the continuous presence of prostanoids. The steroidogenic properties of the bovine adrenocortical cell system in primary culture were characterized in

* Trivial names and abbreviations used: Primary prostaglandins (PG): PGA_1, PGA_2, PGE_1, PGE_2, $PGF_{1\alpha}$, $PGF_{2\alpha}$, PGB_1; 15(S)-15 methyl- $PGF_{2\alpha}$, 15 Me-$PGF_{2\alpha}$; Thromboxane B_2, TxB_2; prostacyclin, PGI_2; 15(S)-hydroxy-9α,11α-(epoxymethano)prosta 5-Z,13 E-dienoic acid, U-44069; 15(S)-hydroxy-11α,9α-(epoxymethano) prosta 5-Z, 13 E-dienoic acid; U-46619. Modified PG [17–19] were: 11-deoxy PGE_1; 11α-methyl-11-deoxy PGE_2, 11-Me PGE_2; 11α-butyl-11-deoxy PGE_2, 11-butyl PGE_2; 11α-phenyl-11-deoxy PGE_2, 11-Phe PGE_2; 11α-benzyl-11-deoxy PGE_2, 11-benzyl PGE_2; 11-deoxy $PGF_{2\alpha}$; 11-deoxy $PGF_{2\beta}$; disulfide analog of PGH_2, SS-PGH_2; 9α,11α-trithiocarbonate of 9,11-bisdeoxy PGF_2; 11α-mercapto-11-deoxy $PGF_{2\alpha}$; 11α-mercapto-11-deoxy $PGF_{2\beta}$; 9α,11α-dimercapto-9,11-bisdeoxy PGF_2; 15α-hydroxy-11α-methyl-10-oxo-9-aza-10-homo-prosta-5,13-dienoic acid; 15α-hydroxy-10-oxo-9-aza-10.homo-prosta-5,13-dienoic acid; 3′,5′,cyclic adenosine monophosphate, cAMP.

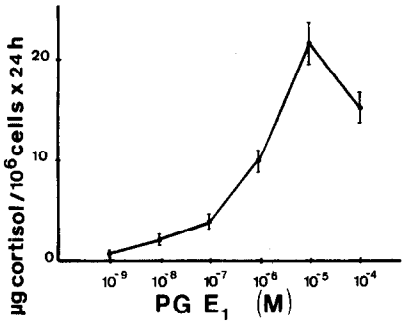

Fig. 2. Effect of increasing PGE_1 concentration on cortisol production by bovine adrenocortical cells in monolayer culture. Cells were grown in the continuous presence of various PGE_1 concentrations; cortisol production during day 2 of culture was measured. Each point is the mean ± S.E.M. of four experiments.

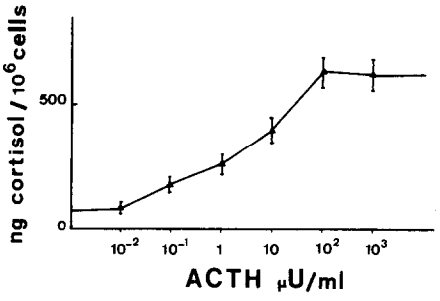

Fig. 3. Cortisol production during a 2-hr exposure to various ACTH concentrations by cultures previously grown for five days in the absence of effector. Each point is the mean ± S.E.M. of three observations.

detail in separate experiments, using synthetic ACTH as the typical effector added to the culture medium. It was observed that the cell population responded to ACTH in a dose-dependent manner and that 100 mU/ml in the culture medium elicited a maximal cortisol production, as measured after 24 hr incubations, for 10^6 cells plated as described above. These conditions were thus taken as a reference to study the effect of the available prostanoids. Figure 1 shows that when ACTH was present in the culture medium (100 mU/ml) from the beginning of the culture, the cortisol production measured every 24 hr sharply increased during the second and third days. Despite the continuous presence of ACTH, the steroid production thereafter declined, in agreement with data from another group [25], from day 7 to 12. Cortisol production was measured each 24 hr in the continuous presence of PGE_1 (10^{-5} M), added at the beginning of the culture. Figure 1 shows that maximal release of cortisol in the medium was observed (as with ACTH) during days 2 and 3. The steroidogenic activity thereafter decreased, despite the continuous presence of PGE_1. The same pattern was observed with all other potent prostanoids mentioned. It was concluded, therefore, that the most sensitive test of stimulation would be the determination of cortisol released during the second day of culture, the effector being present from zero time. These conditions were then used to choose the most convenient prostanoid concentration to be employed. Figure 2 gives the dose response obtained when increasing PGE_1 concentrations (10^{-9}–10^{-4} M) were present in the culture medium. Whereas a significant stimulatory effect on cortisol release was detected at 10^{-8} M concentration, maximal stimulation was observed at 10^{-5} M PG. At higher doses, the stimulatory effect was impaired, suggesting a toxic effect on the cell functions. Consequently, the prostanoids to be studied were all introduced in the culture medium at zero time and 10^{-5} M concentration; cortisol released in the medium during the second day of culture was measured as the index of steroidogenic activity of the system.

Table 1. Effect of prostanoids on cortisol production by adrenocortical cells during day 2 of culture in the continuous presence of the effector (10^{-5} M PG) from zero time, as compared to that of ACTH (100 mU/ml)*

	Cortisol ($\mu g/10^6$ cells × 24 hr)	% ACTH
Control	0.061 ± 0.002	—
ACTH (100 mU/ml)	43.2 ± 3.1	100
PGE_1	23.3 ± 2.4	54
PGE_2	19.4 ± 2.3	45
PGA_1	17.3 ± 1.5	40
PGA_2	19.4 ± 1.8	45
$PGF_{1\alpha}$	12.1 ± 0.9	28
$PGF_{2\alpha}$	10.4 ± 0.7	24
15-Me $PGF_{2\alpha}$	13.4 ± 1.2	31
11-deoxy PGE_1	8.6 ± 0.8	20
U-44069	21.6 ± 2.3	50
U-46619	10.8 ± 1.5	25
PGB_1	15.1 ± 2.1	35

* Means and standard deviations were calculated from triplicates (quadruplicates for the control). Other prostanoids tested and showing no significant effect have been omitted.

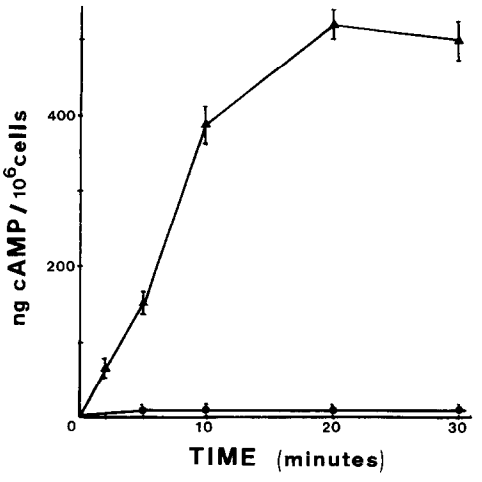

Fig. 4. Cyclic AMP level as a function of time in the culture medium of cells previously grown as in Fig. 3. ●, in the absence of effector; ▲, after addition of ACTH (1 mU/ml) at zero time, together with methylisobutylxanthine (1 mM). Each point is the mean ± S.E.M. of three observations.

Table 1 illustrates the data obtained under these conditions with all the available PG structures. PGE_1 appeared to be the most effective molecule of the series, although its effect remained about half that of a maximal dose of ACTH. The other primary PG were also active. No significant difference was observed in the activity with increasing unsaturation of the side chains (1 and 2 series). TxB_2 was totally ineffective. Introduction of an alkyl substituent on the PG ring totally blunted the steroidogenic effect of the corresponding primary PG. Changing the C-15 hydroxy group of $PGE_{2\alpha}$ to a tertiary alcohol (15-methyl $PGF_{2\alpha}$) did not enhance significantly the steroidogenesis activators; whereas U-46619 was moderately active, its isomer U-44069 was about as active as PGE_1.

Measurement of cyclic AMP in the culture medium under these continuous stimulation experiments yielded values not significantly different from those of the blank of the method. This holds for PG as well as for ACTH stimulation and suggests that cAMP was metabolized during these long-term incubations (24 hr). Use of phosphodiesterase inhibitors to block this metabolism was avoided since these drugs could have led to undesirable metabolic effects in these long-term culture experiments. Acute stimulation experiments were therefore designed to take into account both cAMP and cortisol production as cell response parameters to PG challenge.

Acute stimulation of cAMP and cortisol production. Bovine adrenocortical cells were maintained in culture for five days without any effector added to the medium. Thereafter these cells were challenged with the effector under study. As with long-term experiments (see above), the system was first characterized using ACTH in order to define the most sensitive conditions to evaluate its steroidogenic activity. As illustrated in Fig. 3, these cells exhibited a high sensitivity to ACTH as compared to the long-term stimulation conditions: 100 μU/ml in the medium elicited a maximum steroidogenic response over 2-hr incubations. In the presence of ACTH (1 mU/ml), the cAMP level in the culture medium sharply increased, reaching a maximum after 20 min stimulation (Fig. 4) and thereafter declining slowly. It was concluded, therefore, that the best means of evaluating the cell response is to measure cortisol after 2 hr and cAMP after 15 min incubation of these 5-day-old cultures in the presence of the effector under study. When these conditions were used with increasing concentrations of PGE_1, the dose–response obtained for cortisol release showed that 10^{-6} M PG was sufficient to elicit a maximal steroidogenesis (Fig. 5). However, the

Table 2. Effect of prostanoids on cAMP and cortisol production by adrenocortical cells in culture, as compared to that of ACTH*

	Cortisol (ng/10^6 cells × 2 hr)	% ACTH (1mU/ml)	Cyclic AMP (ng/10^6 cells × 15 min)	% ACTH (1mU/ml)
Control	76 ± 5		10 ± 1	
ACTH (1 mU/ml)	712 ± 42	100	720 ± 40	100
PGE_1	715 ± 37	100	231 ± 19	32
PGE_2	603 ± 45	85	211 ± 20	29
PGA_1	503 ± 32	71	125 ± 9	17
PGA_2	528 ± 53	74	118 ± 12	16.3
$PGF_{1\alpha}$	226 ± 26	32	130 ± 14	18
$PGF_{2\alpha}$	202 ± 33	28	103 ± 13	14
15-Me $PGF_{2\alpha}$	203 ± 27	29	105 ± 9	15
11-deoxy PGE_1	145 ± 29	20	53 ± 6	7
U-44069	698 ± 56	98	227 ± 28	31.5
U-46619	218 ± 27	31	115 ± 12	16
PGI_2	13 ± 4	2	15 ± 3	2
PGB_1	450 ± 42	63	95 ± 7	13

* After five days in culture without effector, the cells were challenged by addition of PG (10^{-5} M) or ACTH (1 mU/ml); cortisol was measured in the medium after 2 hr and cAMP after 15 min stimulation. Means and standard deviations were calculated from triplicates (quadruplicates for the control). Other prostanoids tested and showing no significant effect are not included.

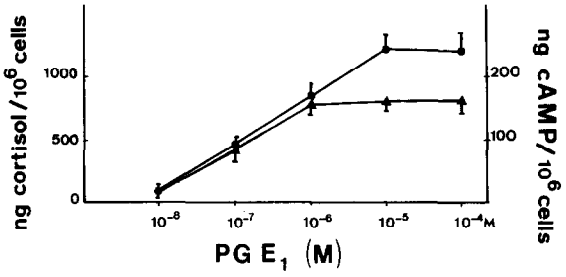

Fig. 5. Response of bovine adrenocortical cells to acute stimulation by PGE_1 after five days of culture without effector. ▲, cortisol production after a 2-hr exposure to PGE_1 at various concentrations; ●, cAMP production after a 15-min exposure to PGE_1 in the presence of methylisobutylxanthine (1 mM). Each point is the mean ± S.E.M. of three determinations.

cAMP production plateaued at a higher PG concentration (10^{-5} M). It may be noticed that no deleterious effects on cortisol production were observed at this high PG concentration, by contrast to what was seen in long-term experiments. It thus appears that these short-term stimulations of previously unchallenged 5-day-old cell cultures provided a system which exhibited a much higher sensitivity than that given by the long-term (24 hr) stimulation experiments. In addition, this system allowed the examination of two response parameters, namely adenylate cyclase activity and corticosteroidogenesis. This system was therefore used to compare the effect of the available prostanoids, all tested at 10^{-5} M concentrations. Table 2 gives the corresponding data, as well as the maximal effect of ACTH (1 mU/ml) which may be taken as reference. PGE_1 again appears to be the most effective prostanoid and under these conditions exhibits a steroidogenic effect equivalent to that of ACTH. However, PGE_1 is only one-third as potent as ACTH in increasing cAMP level. A similar potency on both response parameters is exhibited by the endoperoxide analog U-44069. Another observation concerns only the short-term stimulations which allowed measurement of cAMP production; although as powerful as ACTH in eliciting steroidogenesis, the most active PG structures exhibited, at best, about 30 per cent of the ACTH effect on adenylate cyclase activity. However, there is no discrepancy between the steroidogenic effect and the stimulation of cAMP production when both parameters are available for a given PG structure (Table 2).

Adenylate cyclase activity of purified membrane preparation. Bovine adrenal cortex plasma membrane preparations were used in looking for a structure–activity relationship of the available prostanoids on adenylate cyclase activity. This activity was measured under basal conditions (control) and in the presence of fluoride, a well known stimulus of the cyclase [26]. ACTH (10 mU) was included as a reference test as in afore-mentioned experiments. All the available prostanoids were tested at 10^{-5} M final concentration in the incubations. Corresponding adenylate cyclase activities and relative potency of PGs on this system with regard to ACTH are given in Table 3. As expected from the data obtained with whole cells, PG of the E series were the most potent primary PG and were as effective as ACTH. A discrepancy appears in the case of PG of the F series, which had no effect on membrane adenylate cyclase activity although they were moderately active on cAMP production by the whole cells (see Table 2). On the other hand, one of the endoperoxide analogs (U-44069) appeared to be the most powerful prostanoid on this isolated system, being more potent than ACTH (135%), while the U-46619 isomer had no detectable effect.

Table 3. Effect of prostanoids on adenylate cyclase activity of adrenocortical plasma membrane preparation, as compared to that of ACTH and sodium fluoride*

	Cyclic AMP (ng/min/mg protein)	% ACTH
Control	4.9 ± 0.4	
NaF (10 mM)	79.7 ± 7.9	440
ACTH (10^{-6} M)	18.1 ± 1.7	100
PGE_1	17.1 ± 1.6	94
PGE_2	16.9 ± 1.5	93
PGA_1	9.9 ± 0.9	55
PGA_2	7.8 ± 1.1	43
$PGF_{1\alpha}$	0.2 ± 0.4	1
$PGF_{2\alpha}$	0.6 ± 0.2	3
15-Me $PGF_{2\alpha}$	0.3 ± 0.3	2
11-deoxy PGE_1	0.1 ± 0.86	0
U-44069	24.4 ± 1.6	135
U-46619	0.9 ± 0.3	5
PGB_1	0.98 ± 1.16	5
PGI_2	1.1 ± 0.6	6

* All prostanoids were used at 10^{-5} M final concentration. Means and standard derivations were calculated from triplicate determinations (quadruplicates for the control). Other prostanoids showing no significant effect are not included.

DISCUSSION

A possible solution to the problem of response variability from preparation to preparation when using fresh adrenocortical cell suspensions to test the steroidogenic properties of prostanoid structures [13, 15, 16] was examined using similar cell preparations kept in primary culture. These cultures appeared to meet satisfactorily the first aim of this work: when the cells responded to ACTH by increased steroidogenesis, they were always found to be sensitive to potent prostanoids. When the PG effect was compared to that of ACTH (both under maximal stimulation conditions), a very good reproducibility was obtained with independent cell cultures. The system appears very flexible since its steroidogenic response could be examined either in long-term (continuous presence of the effector studies) or in short-term experiments. Previously unchallenged five-day-old cultures appear to be the most sensitive system in short-term incubations, both with ACTH and PG, and allow the simultaneous assessment of adenylate cyclase and steroidogenesis activations.

The reproducibility of response to PG (and ACTH) observed when adrenocortical cells are kept in culture may be explained by the fact that under culture conditions, the cells recover their metabolic machinery and correct for possible damages due to enzymatic and/or mechanical treatments applied during the cell dispersion procedure. This may be particularly important in the case of PG since adrenocortical prostaglandin receptor has been reported to be destroyed by trypsin treatment, whereas ACTH receptor was preserved under the same conditions [27, 28].

Although previously unchallenged cell cultures appear to be most sensitive to stimulation, this work shows a good correlation between the relative steroidogenic properties of the PG structures under short (2 hr) or prolonged (24 hr) stimulations. Under both conditions PGE_1 and U-44069 endoperoxide analog appear the most potent PG compounds (Tables 1 and 2). There is also a good correlation between the steroidogenic potency of the active PG structures and their ability to stimulate adenylate cyclase in the intact cell. When adenylate cyclase activity of purified adrenocortical membrane preparations was used as the target system, again PGE_1 and U-44069 were the most active PG. However, the potency of these PG relative to ACTH was much higher in this system than in the intact cell. This observation suggests that whereas stimulation of adenylate cyclase in intact cell involves a PG membrane receptor, the enzyme may be exposed to an additional direct effect of PGs in broken cell preparations. The result was particularly striking in the case of U-44069 which was more efficient than ACTH (135 per cent) in activating membranous adenylate cyclase. A yet unexplained finding is the absence of an effect of PGF in this system, while these PG appeared active on adenylate cyclase activity in intact cells. Taken together, these results do not point to any dissociation between steroidogenic and cAMP production under cell stimulation by potent PG. These findings are thus in agreement with the proposal that the initial rise in cAMP is the major intracellular messenger in the steroidogenesis stimulation by exogenous PG in the intact cell [29].

All primary PG (A, E, F) appear to be potent positive effectors of adrenocortical steroidogenic as well as adenylate cyclase activities in culture. Under all conditions, PGE_1 was the most active structure, although it should be noted that evaluation of steroidogenic activity was limited to glucocorticoid (cortisol) measurement. Whereas modification of the side chains (one or two double bonds, methyl at C-15) did not modify the activity of the corresponding primary PG, changes in the ring substitution greatly impaired their potency on our biological system; 11-desoxy PGE_1 was about five times less active than PGE_1 and the PGE_2 structures bearing an alkyl substituent at C-11 were totally inactive. However, the presence of an oxygen at C-11 does not seem mandatory since A and B types primary PG appeared actively steroidogenic. By contrast to data reported by others [16] using feline adrenocortical cells, prostaglandin I_2 was not able to stimulate bovine fasciculoreticulata cells in culture under our conditions. It is difficult to explain this somewhat unexpected finding, although stability of PGI_2 might have been critical in our incubation system. Although a disulfide analog of PGH_2 was ineffective, two epoxymethano-endoperoxide analogs exhibited interesting steroidogenic properties in contrast to the reported ineffectiveness of U-46619 on the feline system [16]. A new observation was the relatively high potency of the 9α,11α-epoxymethano structure (U-44069) on the intact cell activity and most strikingly on the isolated membrane adenylate cyclase activity. These epoxymethano-endoperoxide analogs have been shown to be active on platelet aggregation while a very small increase in cAMP production was observed in this system [30]. This would suggest that endogenous endoperoxides, which could be produced during PG biosynthesis in adrenocortical tissue, may be worth considering as potential modulators of corticosteroidogenesis.

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