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DIVERSE EFFECTS OF Ca^{2+} ON THE PROSTACYCLIN AND CORTICOTROPIN MODULATION OF ADENOSINE 3′: 5′-MONOPHOSPHATE AND STEROID PRODUCTION IN NORMAL CAT AND MOUSE TUMOR CELLS OF THE ADRENAL CORTEX

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Abstract—Isolated cat adrenocortical cells exposed to corticotropin (ACTH) (2–50 μU) and prostacyclin (PGI_2) (10^{-8} to 10^{-4} M) demonstrated dose- and time-dependent increases in cyclic AMP formation and steroid production. In the absence of extracellular Ca^{2+}, ACTH (5 μU) did not augment cyclic AMP levels and steroid release. In contrast, Ca^{2+} deprivation did not affect the ability of PGI_2 (10^{-6} M) to augment cyclic AMP levels and only partially depressed PGI_2-induced steroid release, indicating that ACTH and PGI_2 affect the adenylate cyclase–cyclic AMP system differently. The distinctive effects of PGI_2 and ACTH were further demonstrated in mouse adrenal tumor (Y-1) cells, which responded to ACTH and cholera extertoxin (choleragen) (10^{-9} M) with increases in cyclic AMP formation and steroidogenesis, whereas PGI_2 elicited a profound decrease in cyclic AMP levels and failed to enhance steroid production. The basis of these characteristic effects of ACTH and PGI_2 on normal and adrenal tumor cells appears to be their particular actions on membrane receptors, which modulate cyclic AMP metabolism through different Ca^{2+}-mediated mechanisms.

Existing evidence supports the hypothesis that corticotropin (ACTH) binds to membrane receptors in close association with adenylate cyclase on the surface of the adrenocortical cell, enhancing the activity of this enzyme and elevating intracellular levels of cyclic AMP [1]. The particular mechanism(s) by which this cyclic nucleotide interacts with other biological components to synthesize and extrude steroid from the cell remains uncertain, although it appears that cellular components such as Ca^{2+} and the prostaglandins must be brought into play for the elevation of cyclic AMP to be coupled effectively to steroid production [2]. Ca^{2+} deprivation depresses ACTH-induced cyclic AMP production and steroid release by intact cat adrenal glands [3] and by isolated rat cortical cells [4] and agents that are known to alter Ca^{2+} metabolism (D-600, ruthenium red and A23187) inhibit the steroidogenic response to ACTH in Y-1 adrenal tumor cells [5]. An important role for the prostaglandins in steroidogenesis is indicated by our previous findings that these unsaturated fatty acids are present in adrenocortical cells [6] and that when added exogenously they are capable of increasing cyclic AMP and enhancing steroid production and release in bovine and feline cortical cells respectively [7, 8]. Prostacyclin (PGI_2), a vasodilator and inhibitor of platelet aggregation [9], is by far the most active prostaglandin in promoting steroidogenesis in cat cortical cells [8]. The steroidogenic action of prostaglandins has been attributed to interactions with specific membrane receptors coupled to the adenylate cyclase–cyclic AMP system [8, 10, 11].

Previous studies had demonstrated the presence of multiple specific receptors in normal and neoplastic adrenal tissue [12–15]. The various adrenal tumors are markedly heterogeneous with regard to their responses to several stimulating agents, including ACTH and certain prostaglandins [11, 14, 16]. Using Y-1 functional mouse adrenal tumor cells and isolated cat adrenal cells, we have examined the relative activities of PGI_2 and ACTH in relation to their abilities to increase cyclic AMP levels and to enhance steroid release. The importance of Ca^{2+} to some of these responses was also assessed. This comparative approach was adopted to provide additional information concerning the properties of the cortical adenylate cyclase–cyclic AMP system and thereby lend additional insight into the molecular mechanisms implicated in the steroidogenic process.

MATERIALS AND METHODS

Cell preparations. Cat adrenal glands were perfused *in situ* with Locke's solution to wash out residual blood from the glands. The adrenals were removed from the animal, the cortical tissue was separated from the medulla as completely as possible, and the cortical cells were isolated by a previously described trypsin-dispersion procedure [17]. The final incubation medium contained modified Eagle's Minimal Essential Medium supplemented to

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contain 5 mM Hepes* (pH 7.5), 25 mM $NaHCO_3$, 0.04% lima bean trypsin inhibitor, and 0.2% bovine serum albumin. After trypsin treatment, the cortical cells (2.5×10^5 cells/ml) were incubated for various time intervals at 37° in 1 ml of medium in the presence or absence of PGI_2 (10^{-8} to 10^{-4} M) or ACTH (2–50 μU). The incubation was terminated by the addition of 400 μl of cold trichloroacetic acid (17.5%); the samples were chilled on ice and decanted into small centrifuge tubes. The beakers were washed once with 5% trichloroacetic acid (100 μl); the tubes were then centrifuged at 15,000 *g* for 20 min; and the supernatant fluid was removed, placed in a chilled test tube, and frozen for subsequent cyclic nucleotide assay. The assay, performed in this manner, measured cyclic AMP in both the cells and medium. In experiments involving Ca^{2+} deprivation, Eagle's Minimal Essential Medium with Spinner's salts minus Ca^{2+} (F-18, GIBCO, Grand Island, NY) was used and was supplemented with 25 mM $NaHCO_3$, 5 mM Hepes (pH 7.5), 0.04% trypsin inhibitor, 0.2% bovine serum albumin, and 0.2 mM EGTA. The pH of this medium was adjusted to 7.4 with 8% $NaHCO_3$. When required, $CaCl_2$ was added to give a final concentration of 2 mM. Functional mouse adrenal tumor cells (Y-1) were obtained from the American Type Culture Collection, Rockville, MD, and maintained in a growth medium of Hams F-10 (GIBCO) supplemented with glutamine (an additional 2 mM), pencillin (50 I.U./ml), streptomycin (50 μg/ml), fetal calf serum (5%) and horse serum (12.5%) (GIBCO). Cells were grown in 25 cm^2 Falcon tissue culture dishes at 37° in a mixture of 95% air and 5% carbon dioxide at 100% humidity. Experiments were conducted with a cell density of approximately 1×10^6 per dish. Immediately prior to an experiment, cells were washed three times with serum-free incubation medium. The Y-1 cells contained in the culture dishes were incubated under various conditions, the media decanted, and 1 ml of 5% trichloroacetic acid (4°) was added immediately to each dish. Cells treated in this manner were removed with a Teflon scraper, transferred to individual centrifuge tubes, and centrifuged at 15,000 *g* for 20 min. The supernatant fluid was then frozen for subsequent cyclic nucleotide assay. The assay performed in this manner reflects cellular levels of cyclic AMP.

Steroid assay. After a 60-min incubation, the steroid was extracted from cat cortical cells with methylene chloride and assayed by competitive protein binding using human corticosteroid-binding globulin [18]. Values are expressed as nanograms of corticosteroid released by 2.5×10^5 cells/1 hr. The end product of the steroidogenic pathway in Y-1 cells is 20-α-dihydroprogesterone, which was extracted from the medium and assayed by the fluorescence method of Kowal and Fiedler [19], using 20-α-dihydroprogestrone as the standard. Steroid content of the medium was expressed as micrograms of steroid released by 10^6 cells during the 2-hr incubation (μg/10^6 cells/2 hr).

* Abbreviations used: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; EGTA, ethyleneglycol-bis(aminoethylether)tetra-acetate; and MES, 2[*N*-morpholino]ethanesulfonic acid.

Cyclic nucleotide assay. Approximately 10^4 cpm of [^{3}H]cyclic AMP (38 Ci/mmole) (Amersham/Searle Co., Arlington Heights, IL), repurified over Dowex 50 resin, were added to each sample in order to monitor recovery of cyclic AMP. The trichloroacetic acid-precipitated samples obtained from normal and tumor cells were then centrifuged at 15,000 *g* for 20 min and the supernatant fluid (1 ml) was applied to a AG 1X8 column (formate form, 200–400 mesh) (Bio-Rad Laboratories, Richmond, CA). The column was washed with 10 ml of 0.1 N formic acid; cyclic AMP was eluted with 10 ml of 2 N formic acid, shell frozen, and lyophilized. The lyophilized residue was then brought up to 500 μl in 0.05 M MES buffer (pH 6.2). Various aliquots of the samples were then assayed using the radioimmunoassay method of Steiner *et al.* [20]. Cyclic GMP was also determined in the same samples analyzed for cyclic AMP. Approximately 10^4 cpm of [^{3}H]cyclic GMP (21 ci/mmoles), repurified as for cyclic AMP, were added so that cyclic GMP recovery could be monitored. Following the elution of cyclic AMP (10 ml of 2 N formic acid), 10 ml of 4 N formic acid was added to elute cyclic GMP. Samples were then assayed according to the method of Steiner *et al.* [20]. Determinations were done in duplicate with an experimental variability of <10 per cent. Cyclic nucleotide values are expressed as pmoles/10^5 cells, or values for drug-treated cells are expressed as a percentage of values derived from corresponding untreated cells. The paired Student's *t*-test was applied to assess statistical significance.

RESULTS

Effects of ACTH and PGI_2 on cyclic AMP and steroidogenesis in cat adrenocortical cells. The effect of various concentrations of ACTH on cyclic AMP levels in cat cortical cells is shown in Fig. 1a. ACTH (2 μU) evoked a transient increase in cyclic AMP levels only at 5 min (150 ± 10 per cent of control) ($P < 0.05$). ACTH (5 μU) and (50 μU) significantly increased cyclic AMP levels at 2 min to 135 ± 9 per cent of control respectively. Whereas 50 μU ACTH elevated cyclic AMP levels even at 60 min (550 ± 43 per cent of basal), with 5 μU ACTH, cyclic AMP levels had returned to control values by 60 min. ACTH also elicited graded increases in the amount of steroid released into the medium during a 60 min incubation, which paralleled the increases in tissue cyclic AMP levels (Table 1). PGI_2 was tested during 60-min incubations at three concentrations (Fig. 1b). PGI_2 (10^{-8} M) produced a relatively small increase in cyclic AMP at 5 min (150 ± 6 per cent of control) with levels remaining significantly above basal levels for 30 min ($P < 0.05$). PGI_2 (10^{-6} M) produced a larger increase in cyclic AMP that was evident at 2 min (300 ± 25 per cent of control) and that decreased to 150 per cent of control by 60 min ($P < 0.05$). PGI_2 (10^{-4} M) produced an early increase (150 ± 9 per cent) in endogenous cyclic AMP levels that reached a peak response (400 ± 53 per cent of control) at 30 min and then declined to 160 ± 12 per cent of control by 60 min (Fig. 1b). These three concentrations of PGI_2 also elicited graded increases

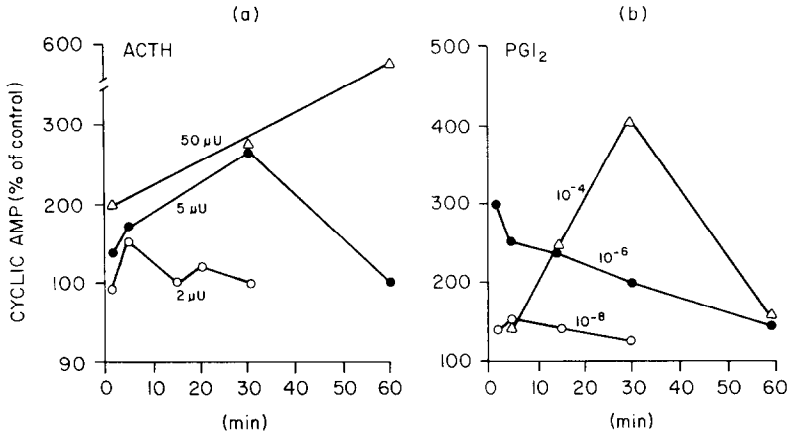

Fig. 1. Effects of ACTH (a) and PGI_2 (b) on cyclic AMP levels in cat adrenocortical cells. Cells were incubated at 37° for up to 60 min in the presence or absence of various concentrations of ACTH or PGI_2. Each point is the mean value obtained from at least four different preparations. Average basal cyclic AMP values after a 60-min incubation were: 0.68 ± 0.10 pmole/10^5 cells (N = 7) and 0.61 ± ± 0.02 pmole/10^5 cells (N = 4) in (a) and (b), respectively.

in steroid release (Table 1). Ethanol, the PGI_2 vehicle, had no significant effect on either basal cyclic AMP levels or steroid release.

Cortical cells that were incubated in Ca^{2+}-free medium did not respond to ACTH (5 μU) with an increase in cyclic AMP at 5 min (Fig. 2a); a 20–30 per cent decrease was observed. ACTH (50 μU) increased cyclic AMP levels at 15 min in the presence and absence of Ca^{2+} to 600 ± 23 and 200 ± 35 per cent of control, respectively. PGI_2 (10^{-6} M), which exhibited steroidogenic activity comparable to that of ACTH (5 μU), by contrast, was still able to increase cyclic AMP levels at 5 min to 415 ± 45 per cent in the absence of Ca^{2+}, which compares favourably with an increase to 450 ± 55 per cent in the presence of Ca^{2+} (Fig. 2b). After a 15-min exposure to PGI_2 in the absence of Ca^{2+}, cyclic AMP levels rose to 290 ± 22 per cent of control, which was similar to the 320 per cent increase obtained in the presence of Ca^{2+}.

ACTH (2–50 μU) did not augment steroid release from cells incubated in a Ca^{2+}-free medium (Table 1). In contrast, the effect of PGI_2 as a steroidogenic agent was depressed but not completely abolished in the absence of Ca^{2+} (Table 1); Ca^{2+} lack reduced the steroidogenic effects of 10^{-6} and 10^{-4} M PGI_2 by 70 and 51 per cent respectively.

In contrast to their clearcut actions on cyclic AMP metabolism, ACTH and PGI_2 exerted variable effects on cyclic GMP formation. With ACTH (5 μU), maximal effects were observed at 5 min, the cyclic GMP levels rising to 146 ± 32 per cent of control (N = 7) (P > 0.5). Similarly, PGI_2 (10^{-6} M) also manifested a peak effect after 5 min, with cyclic GMP levels reaching 141 ± 22 per cent of control (N = 7) (P > 0.5).

Effects of ACTH, choleragen and PGI_2 on cyclic AMP and steroidogenesis in adrenal tumor cells. The effect of a steroidogenic concentration of ACTH

Table 1. Effect of Ca^{2+} deprivation on the steroidogenic response of feline adrenocortical cells to PGI_2 and ACTH and the relationship of the cyclic AMP response to steroidogenesis*

Addition		Corticosteroid (ng/2.5 × 10^5 cells) No Ca^{2+}	Corticosteroid (ng/2.5 × 10^5 cells) 2 mM Ca^{2+}	Average & increase in cyclic AMP†
None		5 ± 2	7 ± 2	
PGI_2	10^{-8} M	7 ± 2	11 ± 2	130
	10^{-6} M	24 ± 5	83 ± 20	228
	10^{-4} M	58 ± 8	119 ± 14	233
ACTH	2 μU	8.3‡	76 ± 17	114
	5 μU	4 ± 1	114 ± 8	165
	50 μU	6 ± 2	199 ± 30	350

* Experiments were carried out as described in Fig. 2. Values are means ± S.E. each derived from at least six to eight different preparations

† These numbers represent the average per cent increase in cyclic AMP during a 60-min exposure. Data were taken in part from Fig. 1.

‡ N = 2.

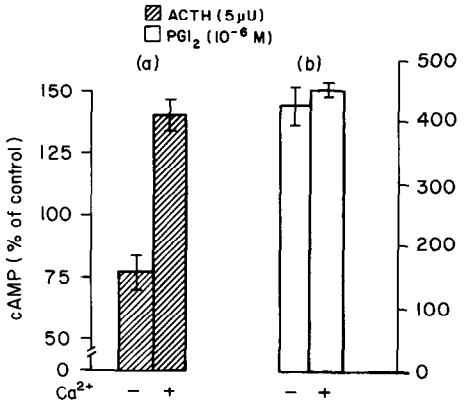

Fig. 2. Comparative effects of Ca^{2+} deprivation on cyclic AMP levels in response to ACTH and PGI_2. Cat cortical cells were prepared as described in Materials and Methods and washed twice with Ca^{2+}-free medium. The cells (2.5×10^5/ml) were incubated for 5 min in the presence or absence of Ca^{2+} with or without ACTH (5 μU) or PGI_2 (10^{-6} M). Cyclic AMP levels were determined in triplicate from three different preparations.

(250 μU) on cyclic AMP levels in Y-1 functional adrenal tumor cells is depicted in Fig. 3. Exposure to this concentration of ACTH caused cyclic AMP levels to increase from 1.98 ± 0.24 pmoles/10^5 cells for control cells to 160 ± 10 per cent of this value at 5 min ($P < 0.05$); cyclic AMP returned to control levels over the ensuing 25 min (Fig. 3). In contrast to the change in cyclic AMP levels produced by ACTH, cyclic GMP formation was not altered significantly from control levels (0.234 ± 0.034 pmole/10^5 cells) during the course of the 60-min incubation. ACTH (250 μU) produced a 2.5 ± 0.3-fold increase in the amount of steroid released during a 60-min incubation as compared to control cells (0.15 ± 0.02 μg/2 hr/10^6 cells) (N = 3).

Studies were also carried out to determine whether the bacterial enterotoxin choleragen (10^{-9} M) could elevate endogenous cyclic AMP levels in Y-1 adrenal tumor cells. Choleragen had a latent period of more than 60 min before producing a significant increase (180 ± 6 per cent) in cyclic AMP (Fig. 3) ($P < 0.05$). Cyclic AMP levels remained elevated for the duration of the incubation (4 hr; data not shown). Under the conditions of this study, no effect of choleragen on cellular cyclic GMP levels was observed. The amount of steroid released into the medium after a 120-min exposure to choleragen was 0.89 ± 0.03 μg/2 hr/10^6 cells, compared to an average basal value of 0.22 ± 0.012 μg/2 hr/10^6 cells (N = 2). Cell viability during the course of this study was >95 per cent, when determined by trypan blue exclusion.

PGI_2 (10^{-5} M) also altered cyclic AMP levels in Y-1 cells (Fig. 4). The overall pattern of cyclic AMP concentrations with respect to time was a marked depression that was manifest within the first 5 min after exposure to PGI_2 and that reached a nadir over the ensuing 60 min (10 per cent of control) (Fig. 4), followed by a return to control levels after 120 min (data not shown). In addition, cyclic AMP levels in the medium did not show any significant changes under conditions where cellular nucleotide levels were depressed (data not shown). No statistically significant change in basal endogenous cyclic GMP levels (0.17 ± 0.015 pmole/10^5 cells) was observed during the 120-min incubation period in the presence of 10^{-5} M PGI_2 (N = 3). The levels of both cyclic nucleotides remained essentially unchanged in naive (untreated) and ethanol-treated (PGI_2 vehicle) control cells during the incubation period (0–120 min), while steroid levels in the medium or in cells were unaltered by the addition of PGI_2 (data not shown). The viability of PGI_2-treated cells at the termination of an experiment was >95 per cent.

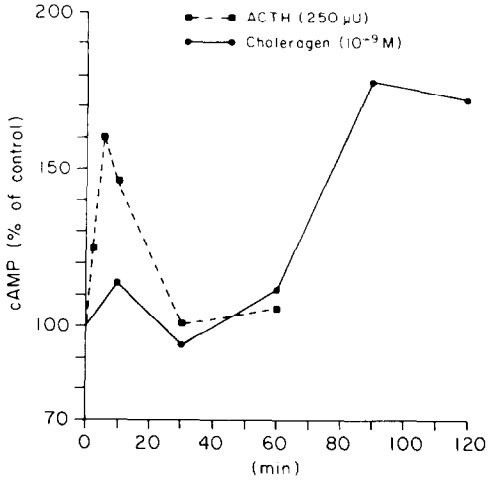

Fig. 3. Time course of cyclic AMP formation in Y-1 adrenal tumor cells during exposure to ACTH or choleragen. Y-1 adrenal cells were grown in tissue culture dishes as described in Materials and Methods and incubated at 37° for up to 120 min in the presence or absence of ACTH (250 μU) or choleragen (10^{-9} M). Each point is the mean value derived from three different preparations.

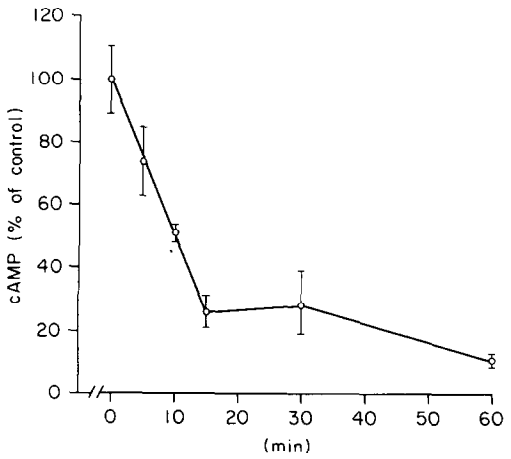

Fig. 4. Effect of PGI_2 on cyclic AMP levels in Y-1 adrenal tumor cells. Cells were incubated for up to 60 min in the presence or absence of PGI_2 (10^{-6} M). Each point is the mean ± S.E. of triplicate plates (N = 4).

DISCUSSION

In trypsin-dispersed cat adrenal cells, graded concentrations of PGI_2 and of ACTH produced changes in intracellular levels of cyclic AMP that were dose- and time-dependent, in that the magnitude and duration of the response were increased with increasing concentrations of PGI_2 and ACTH. The cyclic AMP response mirrored the steroidogenic response in that the magnitude of ACTH and PGI_2 stimulation of cyclic AMP levels was related to the magnitude of steroid release (cf. Table 1). These effects contrast with those on rat adrenal cells, in which PGI_2 increased cyclic AMP levels without discernibly enhancing steroid release [21]. Although the effects of ACTH on cyclic AMP and steroid production were completely depressed in Ca^{2+} depressed cells, the effects of PGI_2 were not blocked completely by a lack of Ca^{2+}. These observations support previous studies of both normal and neoplastic cortical tissue [10, 11, 14], in which it was concluded that ACTH and prostaglandins activate adenylate cyclase by actions on different receptors. The lack of impairment of PGI_2-induced cyclic AMP formation by Ca^{2+} deprivation may explain at least partially the presence of a low level of steroidogenesis under these conditions. The importance of cyclic AMP to Ca^{2+}-deprived cells may be its capacity to regulate Ca^{2+} availability within the cortical cell [22]. The effect of Ca^{2+} deprivation on the response to ACTH cannot be explained by effects on binding of the hormone to its receptor [23, 24], but appears to be localized to the mechanism that couples receptor activity to adenylate cyclase [4]. On the other hand, at the highest dose of ACTH tested, a significant increase in cyclic AMP was seen in the absence of Ca^{2+}, though no steroid was produced. This is consistent with our previous findings on perfused cat adrenal glands [3, 18] in that, although ACTH caused a Ca^{2+}-dependent increase in cortical cyclic AMP, a dissociation of augmented cyclic AMP levels and steroid release was observed with various experimental conditions. These observations also strengthen the argument that Ca^{2+} may affect steroidogenesis at one or more sites distal to cyclic AMP formation [1, 25].

The Y-1 functional mouse adrenal tumor cell responded to certain steroidogenic agents in a predictable fashion. ACTH and choleragen, which are purported to interact with different cortical membrane receptors [13, 26], enhanced both cyclic AMP production and steroid release in tumor cells, confirming earlier investigations [27–29]. In contrast to its stimulatory action on feline cortical cells, PGI_2 in Y-1 cells caused a profound and prolonged depression of basal cellular cyclic AMP levels. After the cyclic AMP levels returned to control values at 2 hr, the cells were still viable and firmly attached to the plate, but had not produced steroid. This, to the best of our knowledge, is the first account of a depression of basal cyclic AMP formation elicited by PGI_2 in an endocrine cell, although PGI_2 was reported recently to decrease cyclic AMP content of coronary vascular tissue [30], and one or another of the prostaglandins has been shown to be capable of inhibiting basal and stimulated cyclic AMP formation in the adipocyte [31, 32] and blood platelet [33].

The ability of PGI_2 to diminish cyclic AMP levels in adrenal tumor cells shows that the ineffectiveness of PGI_2 in promoting steroidogenesis did not result from a lack of interaction with these cells. Several explanations directly involving adenylate cyclase could account for the dramatic fall in cyclic AMP, although an increase in phosphodiesterase activity cannot be ruled out. Guanyl nucleotide binding sites have been implicated as an essential component in the activation of adrenocortical adenylate cyclase [34]. The previous finding that GTP converts prostaglandin endoperoxide (PGH_2)-induced inhibition of basal adenylate cyclase in fat cell ghosts to stimulation [32] suggests that an important clue to the inhibitory action of PGI_2 on cyclic AMP levels in Y-1 adrenal tumor cells may involve the interaction of guanine nucleotides with adenylate cyclase. Alternatively, PGI_2 may act to enhance directly the availability of free intracellular Ca^{2+}, which in turn could exert an inhibitory action on adenylate cyclase [35]. Finally, the induction of specific Ca^{2+}-dependent regulator proteins by PGI_2 or ACTH to modulate adenylate cyclase activity could explain the enhancement and depression of cyclic AMP accumulation that we observed in normal and tumor cells respectively. Initially we had hoped to explore the role of Ca^{2+} in cyclic AMP and steroid metabolism by Y-1 cells, but due to their absolute Ca^{2+} requirement in order to adhere to the substratum, we were technically unsuccessful in these attempts. Kowal *et al.* [28], using adrenal tumor cells, have previously reported a requirement for Ca^{2+} in the stimulation of steroid and cyclic AMP formation by ACTH.

Based upon these observations and those using other adrenocortical cell systems [10, 11, 14], it seems clear that a variety of steroidogenic agents interact through different membrane receptors to activate adenylate cyclase. The findings that ACTH and PGI_2 stimulate cyclic AMP and steroid production in feline cortical cells, but that PGI_2 inhibits cyclic AMP formation and does not augment steroidogenesis in tumor cells, suggest that adenylate cyclase activation and steroidogenesis are linked somehow. This conclusion is in harmony with previous studies demonstrating that cortical cells that lose the ability to respond to ACTH or prostaglandin with increased adenylate cyclase activity also lose the ability to respond to these substances with increased steroidogenesis [16, 36]. In contrast, our observations do not support a role for cyclic GMP in steroidogenesis elicited by any of the agents used in our experiments; our results essentially support the conclusions of other investigators [29] and of a recent study carried out in isolated rat adrenocortical cells showing that cyclic GMP is not a mediator of steroidogenesis [37].

Although particular attention has been paid to the adenylate cyclase–cyclic AMP system, ACTH may also express its effects in other ways, as for example by its recently demonstrated ability to stimulate prostaglandin synthesis by activation of a Ca^{2+}-dependent phospholipase A_2 [2, 38]. The physiological significance, however, of prostaglandins, such as PGI_2, in adrenocortical function is still unknown and is the

subject of continuing investigation in our laboratory. Nevertheless, the potential importance of PGI_2, particularly, is heightened by its potent steroidogenic activity in cat adrenocortical cells [8] and by the knowledge that its metabolite, 6-keto-$PGF_{1\alpha}$, has been found in rather high concentrations in isolated rat adrenocortical cells [21]. Our preliminary findings, that this metabolite of PGI_2 did not alter basal or ACTH-induced increases of cyclic AMP levels in either Y-1 cells or medium and is much weaker as a steroidogenic agent than PGI_2 in cat cortical cells [8], imply that PGI_2 itself, rather than its metabolite, is the active moiety.

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