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Prostaglandin Synthesis in the Feline Adrenal Cortex

Suzanne Gale Laychock
laychock@buffalo.edu

PROSTAGLANDIN SYNTHESIS IN THE FELINE ADRENAL CORTEX

by

Suzanne Gale Laychock

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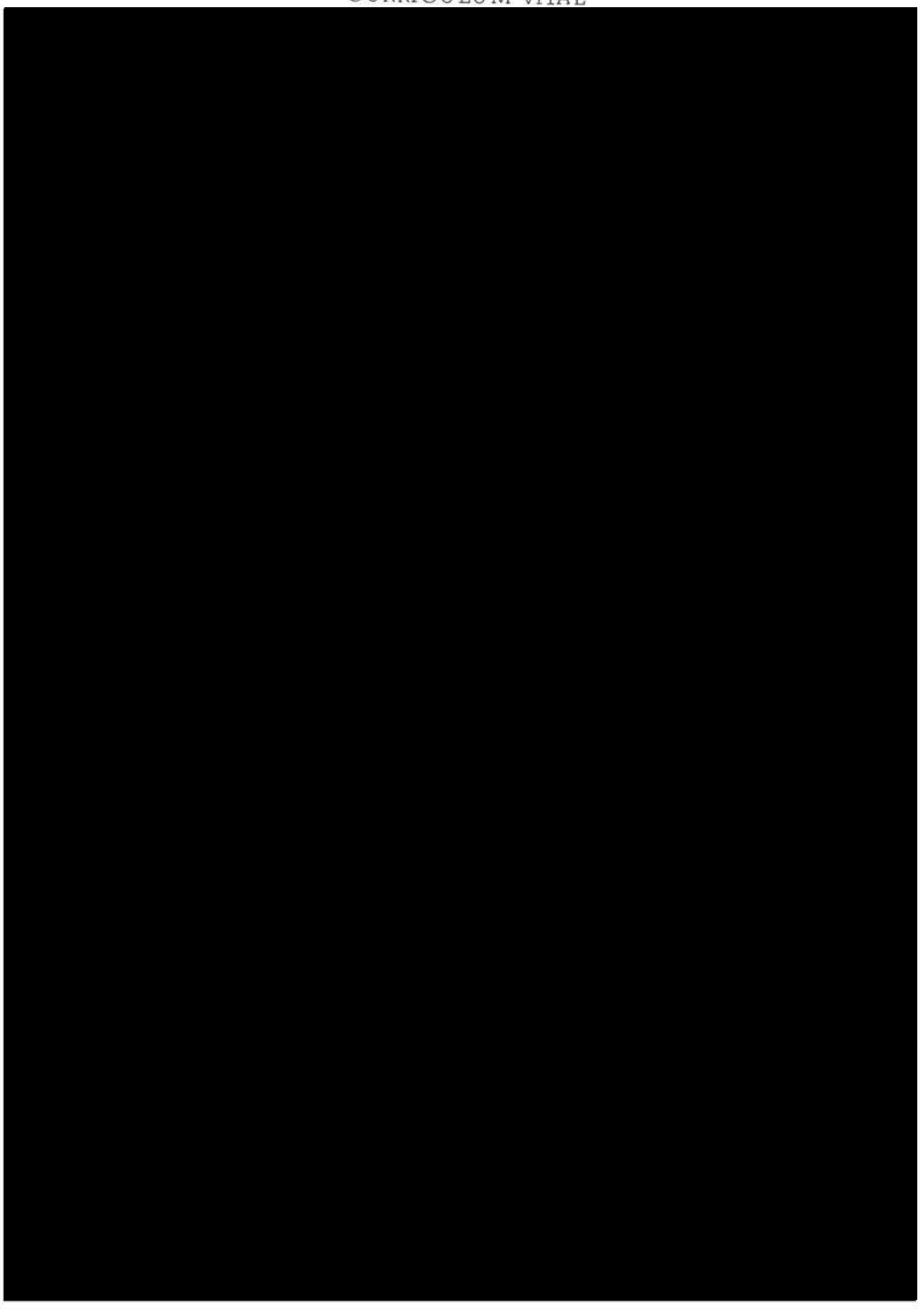

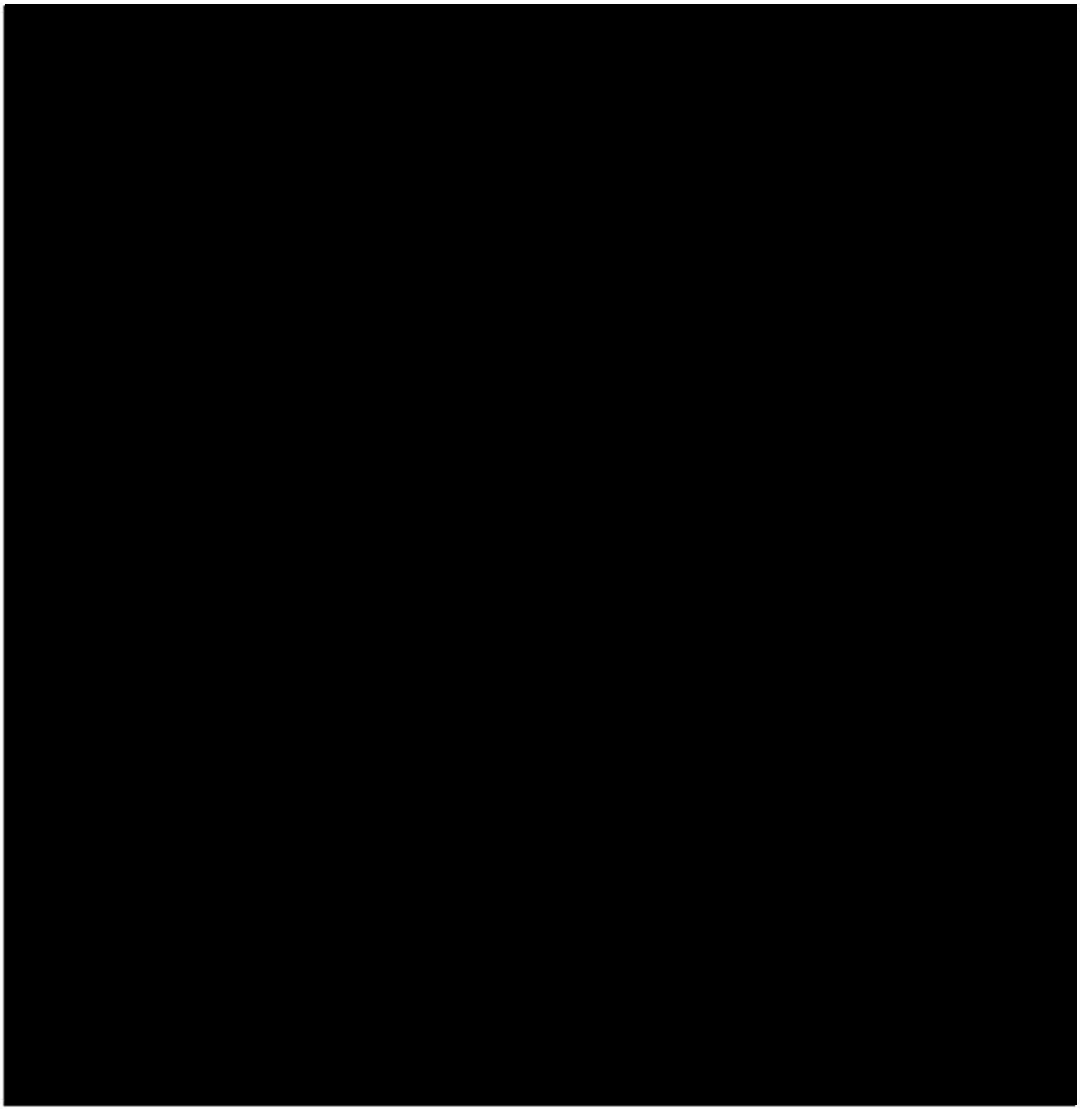

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ACTH	adrenocorticotropic hormone
BCAMP	$N,^{6}$monobutyryl adenosine-3',5'-cyclic monophosphoric acid
BSA	bovine serum albumin
cyclic AMP	adenosine-3',5'-cyclic monophosphate
cyclic GMP	guanosine-3',5'-cyclic monophosphate
EDTA	disodium ethylenediamine tetraacetate
EGTA	ethyleneglycol-bis-(β-aminoethyl ether) N,N'-tetraacetic acid
ETA	5,8,11,14-Eicosatetraynoic acid
MEM	Modified Eagle's Medium
$NaBH_4$	sodium borohydride
NPS-ACTH	o-nitrophenyl sulphenyl ACTH
ng	nanogram
pg	picogram
PG	prostaglandin
RIA	radioimmunoassay
TLC	thin layer chromatography
TSH	Thyroid Stimulating Hormone

INTRODUCTION

The prostaglandins are a family of unsaturated 20-carbon fatty acids, which are ubiquitously distributed throughout virtually all animal tissues. Four series of natural prostaglandins are commonly synthesized in biological systems, and have been designated by the letters E, F, A, and B, which correspond to differences in their degrees of hydroxylation and unsaturation, with the latter denoted by a subscript numeral after the letter (Horton, 1972). The diverse pharmacological activity of these 20-carbon unsaturated fatty acids was originally established by U.S. von Euler (1934), who observed that human semen and extracts of sheep vesicular glands lowered arterial blood pressure and stimulated numerous isolated intestinal and uterine smooth muscle preparations. More recently, the ubiquitous prostaglandins have been implicated in such physiological processes as reproduction, hormone secretion, nerve transmission, lipolysis, vasoconstriction and vasodilation, gastric secretion, and platelet aggregation (Bergstrom, 1967; Horton, 1972).

Most cell types possess a prostaglandin synthetase which is responsive to hormones or other stimuli; through this multi-enzyme complex prostaglandins can be formed as potential intracellular messengers (Ramwell, 1973). The prostaglandins, thus formed may influence cellular function either by directly affecting the activity of a given enzyme or by modulating the levels of other biologically active substances like cyclic nucleotides or calcium ions. Alternatively, the prostaglandins synthesized may be secreted by stimulated cells and influence nearby cells or tissues thus acting as local hormones. Finally, those prostaglandins released into the general circulation may serve as classical hormones at distant target tissues.

Although prostaglandins have been implicated in many diverse cellular functions, the large number of prostaglandins and their physio-

logically active endoperoxide intermediates has complicated the definition of their role as intracellular regulators (Silver and Smith, 1975). Steroidogenic tissues are known to synthesize several types of prostaglandins, and although a substantial amount of evidence has accrued regarding the putative role of prostaglandins in gonadal steroidogenesis (Shaw and Tillson, 1974), much less is known about their role in the action of ACTH on adrenal steroidogenesis. However, the participation of prostaglandins in the regulation of corticosteroid production and release is suggested by the pharmacologic evidence that exogenous prostaglandins elicit steroidogenic activity in systems such as the rat decapsulated adrenal cortex (Flack et al., 1969) and the feline adrenal cortical suspension (Rubin and Warner, 1975). The latter test preparation was used in the present study.

In order to assess more clearly the relationship of prostaglandins to adrenal steroidogenesis, the identity and metabolism of prostaglandins in the adrenal cortex must be ascertained since heretofore this information has been lacking. Hence, the purpose of the present investigation was to elucidate further the role of prostaglandins as an intermediate in the action of ACTH on the adrenal cortex by a comprehensive study of adrenocortical prostaglandins and their functional relationship to steroid production and release.

I. Steroidogenesis in the adrenal cortex

The ultimate cellular response of the adrenal cortex to ACTH stimulation is the synthesis and secretion of various steroid compounds. Unlike most other endocrine organs, the adrenal cortex does not store corticosteroid in anticipation of a releasing stimulus, but rather ACTH enhances steroid production and causes its immediate extrusion from the cell (Jaanus et al., 1970). Morphological studies of the adrenal cortex confirm the absence of intracellular structures which might be correlated with secretory granules storing steroid prior to exocytotic release (Malamed, 1975). Thus, ACTH augments the synthesis and release of corticosteroid by the activation of specific biochemical pathways.

The synthesis of corticosteroids in the adrenal cortex is accomplished by several enzymes located in different organelles. Cholesterol appears to be the initial substrate in the production of corticosteroids by the adrenal gland (Garren, 1968). Cellular esters and fatty acids contribute to the formation of free cholesterol (Macho and Saffran, 1967) which can participate in other cellular reactions culminating in the synthesis of corticosteroids. In the cell, most of the cholesterol exists as esters of unsaturated fatty acids contained within cytoplasmic lipid droplets (Grant, 1968). Before this steroid precursor pool can be utilized, the cholesterol must be liberated by the action of a sterol esterase, which hydrolyzes the cholesterol ester and converts it to a compound available for mitochondrial metabolism (Batta, 1975). Numerous investigations have demonstrated not only that the electron-dense lipid bodies lose their density and acquire a crinkled outline indicative of a loss of lipid when cells are exposed to ACTH (Luse, 1967), but also that on stimulation with ACTH the more unsaturated fatty acid esters of cholesterol are depleted from the human adrenal cortex (Grant et al., 1968).

Within the adrenal cortical mitochondrion the side chain of cholesterol is cleaved, forming pregnenolone by a sequence of enzymatic reactions (Fig. 1). This cleavage of the side chain from cholesterol

BIOSYNTHESIS OF CORTICOSTEROIDS

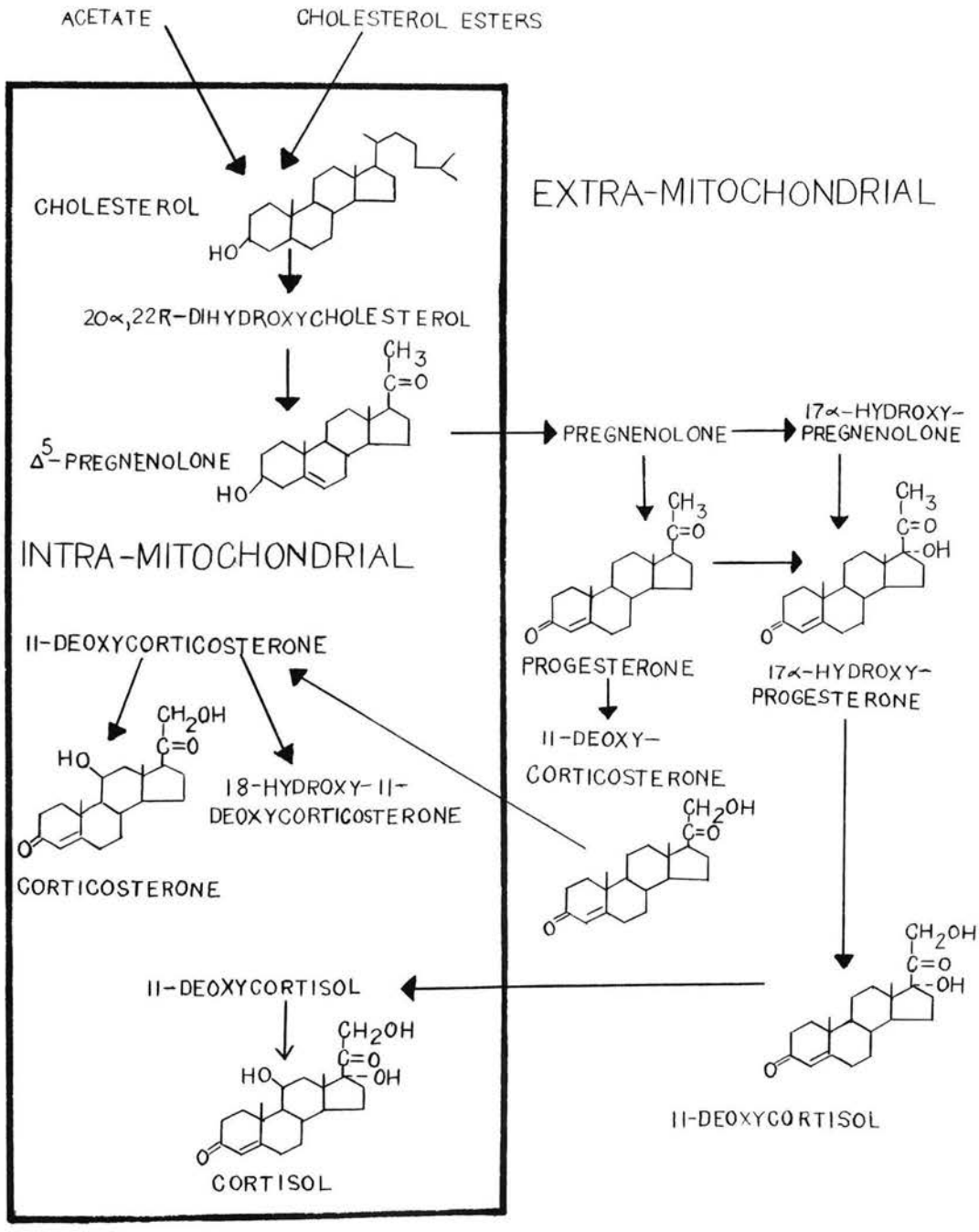


FIG. 1

depends upon a desmolase complex found within the mitochondria (Halkerston et al., 1961). This enzyme complex appears to convert cholesterol to 20α-hydroxycholesterol, then to 20α-22ξ-dihydroxycholesterol, and finally to pregnenolone and isocaproic acid (Garren, 1968; Constantopoulos et al., 1962, 1966). But, uncertainty remains as to the identity of some intermediates involved in the conversion of cholesterol to pregnenolone. While Roberts et al. (1969) were able to isolate radiolabeled 20α-hydroxycholesterol from bovine adrenal tissue, the conversion of cholesterol to 20α, 22R-dihydroxycholesterol and 22-R hydroxycholesterol has also been established (Burstein et al., 1970a, b) using acetone-dried powder preparations of human, bovine, and guinea pig adrenals.

The mitochondrial enzyme complex responsible for cleavage of the cholesterol side-chain requires NADPH and oxygen and utilizes cytochrome P_{450} as a terminal oxidase (Schulster, 1974). In addition to these cofactors, the regulation of pregnenolone synthesis is also at least partly dependent upon a modification of mitochondrial membrane structure. Changes in the permeability properties of the mitochondrial membrane might enhance the entry of NADPH used by mitochondrial cholesterol-cleaving enzymes, or might allow the exit of pregnenolone from the organelle (Schulster, 1974). The latter possibility received support when, using an acetone powder of adrenal mitochondria, pregnenolone was demonstrated to cause an allosteric inhibition of the conversion of cholesterol to pregnenolone (Koritz and Hall, 1964). This observation is the basis of the Koritz and Hall model of ACTH action which postulates that ACTH indirectly affects the mitochondrial membrane so that exit of pregnenolone from within the mitochondria is enhanced (Koritz, 1968). The enhanced removal of pregnenolone stimulates its own synthesis in the absence of feedback inhibition and results in an overall increase in the rate of steroidogenesis. This central facet of the Koritz-Hall model has, however, been challenged as a mechanism

of action of ACTH, since inhibition of the conversion of pregnenolone to progesterone by cyanoketone in rat adrenal sections (Farese, 1971a) did not block ACTH stimulation of pregnenolone synthesis despite the accumulation of pregnenolone within the gland. It is possible that experimental results obtained from acetone powder mitochondrial preparations do not pertain to whole cell preparations, or that intact cells have a mechanism for the removal of pregnenolone from its inhibitory site.

Pregnenolone leaves the mitochondrion and is converted into progesterone by the action of an NAD-dependent 3β-hydroxysteroid dehydrogenase and a proton-transfer enzyme in the microsomal fraction (Beyer and Samuels, 1956; Ewald et al., 1964). This reaction does not appear to be stimulated directly by ACTH since studies show that high concentrations of cyclic AMP, a proposed second-messenger of ACTH action, inhibit the conversion of pregnenolone to progesterone in rat adrenal homogenates and in isolated beef adrenocortical mitochondria (Koritz et al., 1968), while physiological concentrations of the cyclic nucleotide had no effect on the conversion process.

The conversion of progesterone and 11α-hydroxyprogesterone to deoxycorticosterone and 17-hydroxydeoxycorticosterone occurs in the microsomal fraction of the cell where enzymes catalyze the introduction of a hydroxy group into the 21 position of the steroid nucleus (Garren, 1968). The 21-hydroxylase in adrenal microsomes requires NAD^+ and atmospheric oxygen, as is common for reactions in which cytochrome P_{450} is the oxygen-activating enzyme (Ryan et al., 1957; Estabrook et al., 1963). If the 17α-hydroxylating enzyme system is active, the predominant steroid secreted will be cortisol rather than corticosterone, as is the case in the feline adrenal cortex (Bush, 1953; Jaanus et al., 1970).

Deoxycorticosterone is transformed into corticosterone, and 17-hydroxydeoxycorticosterone is transformed into cortisol by the

introduction of the hydroxyl group in the 11β position of the steroid molecule (see Fig. 1). Once again, the enzymes responsible for this conversion are present in the mitochondria of the adrenal cortex and require NADPH and oxygen and mitochondrial cytochrome P_{450} (Garren, 1968). The fact that high concentrations of deoxycorticosterone are formed from (20S)-20-hydroxycholesterol in isolated rat adrenal cells, suggests that the synthesis of corticosterone from deoxycorticosterone is a relatively slow reaction not under the control of ACTH (Sharma, 1973).

II. Mechanism of ACTH action

A. ACTH binding

In order to appreciate fully the possible roles which prostaglandins may play in the steroidogenic process, it is necessary to provide background information regarding what is already known about the mechanism of action of ACTH. The action of ACTH on the adrenal cortex is typified by the hormone complexing with its specific receptor system in order to initiate regulatory and metabolic processes which culminate in the enhanced synthesis and secretion of corticosteroid. The binding of ACTH to the adrenal cortical cell has been described for cortical tissue slices (Golder and Boyns, 1972) and membrane preparations from rat and mouse adrenal tissue (Hofmann et al., 1970; Lefkowitz et al., 1971), suggesting that ACTH binds at the cortical cell surface (Pastan et al., 1966; Richardson and Schulster, 1972).

The binding data accrued from studies using receptor extracts of adrenal cortical tumor homogenates (Lefkowitz et al., 1971) or intact isolated adrenal cells (McIlhinney and Schulster, 1974) indicate that two sets of ACTH receptors are present which differ in their equilibrium and kinetic constants. Low affinity binding sites are in the majority, while the high affinity binding sites are in the minority and appear to be responsible for steady, sustained ACTH responses. Two populations of binding sites may provide greater flexibility in the cellular response

to changing levels of ACTH at the target tissue, but whether there are any other specialized functions associated with the different receptor sites is a matter of speculation. Studies using the ACTH analogue, o-nitrophenylsulphenyl ACTH (NPS-ACTH) (Moyle et al., 1973), however, suggest that the two receptor sites for ACTH may actually differ as to their role in reading, translating and amplifying the hormone-carried message. The studies by Moyle and co-workers demonstrate the competitive binding of NPS-ACTH to an ACTH receptor site, which when activated is capable of increasing steroidogenesis in the absence of increases in cyclic AMP. It appears, however, that a second type of ACTH binding site, insensitive to NPS-ACTH, stimulates steroidogenesis subsequent to increasing cyclic AMP levels within the adrenal cortical cell. Thus, the differences among receptors may lie in their potential for activating adenylyl cyclase or other membrane-associated enzymes (Lefkowitz et al., 1971; Moyle et al., 1973).

B. Proposed mediators for expression of hormone-carried message

1. Cyclic AMP. The ACTH hormone-receptor complex activates a specific plasma membrane enzyme of the target tissue, and it is this enzyme which is responsible for discriminating environmental signals and generating the translated form of the hormone-carried message (Torda, 1971). The translated form of the message is the second-messenger hypothesized by Sutherland and co-workers (Robison et al., 1971); and it has generally been demonstrated that polypeptide hormones modify tissue function by altering tissue levels of cyclic AMP through activation of a plasma membrane associated adenylyl cyclase (Shaw and Tillson, 1974).

The theory that ACTH action is mediated through the activation of adenylyl cyclase and accumulation of cyclic AMP in adrenal cells was substantiated by the studies of Haynes (1958) using adrenal cortical slices to demonstrate the synthesis of cyclic AMP in response to ACTH. The establishment of cyclic AMP as a second messenger in ACTH action

has since been corroborated by numerous investigators using as evidence the accumulation of cyclic AMP in vitro and in vivo (Grahame-Smith et al., 1967), as well as the stimulated conversion of ATP to cyclic AMP after the addition of ACTH to adrenal homogenates (Taunton et al., 1967). The location of adenylyl cyclase within the plasma membrane of several cell types (Sutherland et al., 1965), including the adrenal cortical cell (Lefkowitz et al., 1971), suggests an ACTH-receptor-adenylyl cyclase complex for translating the hormonal message.

Controversy has arisen, however, regarding the obligatory role of cyclic AMP in ACTH-induced steroidogenesis. As the studies previously described by Moyle illustrate , NPS-ACTH caused increases in adrenal steroidogenesis without augmenting adrenal cyclic AMP levels, and in fact, ACTH-induced cyclic AMP accumulation was inhibited by NPS-ACTH (Moyle et al., 1973; Kong et al., 1972). In addition, studies with isolated rat adrenal cells have shown that low concentrations of ACTH stimulate steroidogenesis without causing detectable changes in the concentration of cyclic AMP (Beall and Sayers, 1972). It is possible that low but steroidogenic concentrations of ACTH fail to induce detectable increases in the level of cyclic AMP due to the limits of the analytic techniques, since high ACTH concentrations increase cyclic AMP in the rat adrenal preparation in which adenylyl cyclase was not detectably responsive to low ACTH concentrations (Beall and Sayers, 1972).

If cyclic AMP is a mediator of ACTH action on the adrenal cortex, then according to the criteria set forth by Sutherland and co-workers (Robison et al., 1971) to describe second messengers of hormone action, not only should cyclic AMP be synthesized by this tissue in response to ACTH, but exogenous cyclic AMP should mimic the steroidogenic action of ACTH. Since the pioneer work of Haynes et al. (1959) and Birmingham et al. (1960) which demonstrated the steroidogenic capacity of this nucleotide in rat adrenal sections, many workers have shown that exogenous cyclic AMP, or its mono- or dibutyryl-derivative, is able to enhance

corticosteroid synthesis in a manner not unlike ACTH. Rat adrenal cell suspensions are responsive to micromolar amounts of cyclic and dibutyryl-cyclic AMP in synthesizing corticosteroid (Haksar et al., 1973; Sayers et al., 1971). In addition, dibutyryl-cyclic AMP (the more lipid soluble cyclic AMP analogue) was effective in inducing two- to seven-fold increases in steroid output when added in millimolar concentrations to monolayer cultures of mouse adrenal cortex tumors (Kuo et al., 1975). Cyclic GMP, another naturally occurring cyclic nucleotide, is also capable of enhancing steroidogenesis when added in vitro to rat adrenal cell suspensions (Kitabchi and Sharma, 1971), but it is generally much less potent in this regard than cyclic AMP.

Evidence has accumulated to suggest that cyclic GMP is involved in promoting cellular events that are antagonistic to those mediated through changes in cyclic AMP levels (Goldberg et al., 1973). Since the unitary role of cyclic AMP as the second messenger of the ACTH-adrenal interaction has been questioned (Moyle et al., 1973), the failure of cyclic AMP to fully reproduce adrenal response to ACTH (Rubin et al., 1973) and the observation that elevated cyclic GMP levels in the rat adrenal are synonomous with decreased cyclic AMP levels (Whitley et al., 1974, 1975) lends credence to an hypothesized role for cyclic GMP in mediating ACTH-induced adrenal steroidogenesis. However, the precise regulatory relationship between the two cyclic nucleotides has not been elucidated.

As a second messenger of ACTH action in the adrenal cortical cell, cyclic AMP should be responsible for certain metabolic or molecular changes which manifest themselves during corticosteroid synthesis. The delayed onset of steroidogenesis following exposure of the adrenal gland (Rubin et al., 1973) and isolated adrenal cells (Beall and Sayers, 1972) to cyclic AMP allows time for the nucleotide to activate enzymes participating in the induction of steroidogenesis. Likewise, the increase in adrenal cyclic AMP induced by ACTH reaches maximum levels prior

to the attainment of maximum steroid release (Grahame-Smith et al., 1967; Rubin et al., 1972). Specifically, cyclic AMP activates the synthesis of a protein essential for converting cholesterol to the corticoid precursor, pregnenolone. During the interval between cyclic AMP generation and steroidogenesis, cyclic AMP binds to a protein receptor found in the soluble cytoplasmic fraction derived from adrenal cortical tissue (Gill and Garren, 1969). Associated with the cyclic AMP receptor protein there appears to be a protein kinase whose activity is stimulated by cyclic AMP (Gill and Garren, 1970).

The complex of cyclic AMP-receptor and protein phosphokinase was demonstrated by the studies of Gill et al. showing that both binding and phosphorylation activities sediment as a single peak in the analytical centrifuge and migrate as a single band after polyacrylamide gel electrophoresis. Similar methodology was instrumental in illustrating the dissociation of the nucleotide receptor and kinase components following incubation of the microsomal cell fraction with cyclic AMP, and the concomitant stimulation of protein kinase (Gill and Garren, 1971). From these studies a model emerges suggesting that the cyclic AMP receptor protein represses the protein kinase when they are associated as a complex, and that cyclic AMP binding dissociates the receptor protein from the kinase which is then fully activated (Schulster, 1974).

Phosphoprotein kinase is therefore another mediator in the tropic mechanism of action of ACTH. The studies of Walton et al. (1971) demonstrate that ribosomes may be the endogenous substrate for this enzyme in bovine adrenal glands since it catalyzed the phosphorylation of a protein associated with 80S sedimentable ribosomes. A similar activity for the kinase has been demonstrated in rat liver microsomal preparations (Loeb and Blat, 1970). The ribosomal phosphorylation has been postulated to modulate the translation of stable messenger RNA, leading to increased synthesis of a regulator-protein facilitating the translocation of cholesterol to the mitochondrion for conversion to

pregnenolone and ultimately to corticosteroid (Garren et al., 1971). Protein kinase has also been implicated in activating enzymes associated with the conversion of cholesterol esters to free cholesterol (Schulster, 1974). The generation of an active protein kinase not only appears to be a critical precedent event for steroid production and release but has the potential for being a powerful cellular mediator regulating a variety of metabolic processes.

The role of protein kinase in activated steroidogenesis has been defined as the capacity to phosphorylate and activate a stable messenger RNA which codes for the translation of a protein active in steroidogenesis. Studies utilizing several protein synthesis inhibitors have helped to elucidate the mechanism of action of ACTH in this regard (Rubin et al., 1973).

2. Protein synthesis and steroidogenesis. The increase in protein synthesis due to ACTH does not appear to be dependent upon RNA synthesis, since actinomycin D is ineffective in blocking steroidogenesis (Sato, 1965). Cycloheximide, which is a pharmacological agent responsible for inhibiting the translation of cytoplasmic RNA into protein, effectively inhibits ACTH stimulated steroidogenesis (Garren et al., 1971; Rubin et al., 1973). On the other hand, cycloheximide is ineffective in preventing the accumulation of cyclic AMP in response to ACTH. These results suggest that a newly synthesized protein is essential to steroidogenesis at some point after the generation of cyclic AMP.

Studies by Sharma (1973) and Garren (1968) illustrate that the ACTH stimulated conversion of (20S)-20-hydroxycholesterol into corticosterone and the synthesis of deoxycorticosterone from this precursor are not inhibited by cycloheximide. Thus, these enzymatic transformations are not dependent upon protein synthesis. The results indicate that cycloheximide inhibits ACTH action by preventing the conversion of cholesterol to its hydroxy derivative through a mechanism involving microsomal, rather than mitochondrial, protein synthesis

(Garren, 1968). Attention has also been directed toward the desmolase enzyme complex in an attempt to establish the regulatory site for the rapid steroidogenic effect of ACTH, since it is the first in a series of enzymatic conversions of cholesterol and its metabolites to steroid. Studies illustrated, however, that the half-life of the cholesterol side-chain desmolase complex in hypophysectomized rats was 3-4 days, whereas the rapid drop in the steroidogenic capacity of these rats decayed with a half-life of 6-7 hours (Mostafapour and Tchen, 1973). Therefore, the decay in the steroidogenic capacity after hypophysectomy was attributed to the decay of an RNA with a half-life of 6-7 hours, and it was hypothesized that this RNA might be responsible for the synthesis of a labile steroidogenic protein mediating the ACTH response (Garren et al., 1965).

Recent evidence substantiates the presence of a heat-stable protein in rat adrenal fractions which functions similarly to liver sterol carrier proteins (SCP) (Kan et al., 1972) in that it appears to be active in the synthesis, transport, and metabolism of cholesterol. The cholesterol SCP complex could be responsible for the delivery of cholesterol to the mitochondria where cholesterol is enzymatically converted to pregnenolone. Thus, cycloheximide appears to either prevent the translocation of cholesterol from the cytoplasm into the mitochondrion, or acts directly upon a mitochondrial function essential for the transformation of the sterol. It remains to be determined whether other mediators of cholesterol permeance of the mitochondrial membrane, such as calcium or prostaglandins, might not be affected by the presence of the antibiotic and account for the observed inhibition of cholesterol metabolism.

3. Calcium, ACTH, and steroidogenesis. The critical role of calcium in the steroidogenic process is underscored by the numerous studies in a variety of preparations that calcium deprivation leads to a profound depression of ACTH-induced steroid production and release

(Halkerston, 1975). The participation of calcium in the activation of adenylyl cyclase, as well as an involvement of the cation in steroidogenic events subsequent to the activation of adenylyl cyclase, are important events in the tropic action of ACTH. The studies of Lefkowitz et al. (1970) demonstrate that while the adrenal ACTH-receptor interaction does not require calcium, ACTH stimulation of adenylyl cyclase does require calcium, as evidenced by the fact that EGTA, a chelator of calcium, inhibited the cyclase stimulation. Thus, there appears to be a calcium requiring step between the binding of ACTH to its receptor and the subsequent activation of adrenal adenylyl cyclase. Similar calcium requirements for ACTH sensitive adenylyl cyclase have been demonstrated in the bovine adrenal cortex and ghosts from rat fat cells (Bar and Hechter, 1969), rat adrenal cell suspensions (Haksar et al., 1972, 1973; Sayers et al., 1972), and the perfused feline adrenal gland (Rubin et al., 1972).

Several studies illustrating the steroidogenic capacity of exogenous cyclic AMP disclosed yet another role for calcium in this process, for corticosterone synthesis induced by this nucleotide seems to depend upon the availability of the cation (Birmingham et al., 1960; Haksar et al., 1972; Rubin et al., 1972; Kuo et al., 1975; Warner and Rubin, 1975). Thus, while increasing levels of calcium cause parallel increases in the steroidogenesis elicited by ACTH and cyclic AMP in isolated rat adrenal cells (Sayers et al., 1972), the deletion of calcium from this system as well as from rat adrenal slices in vitro (Farese, 1971a)showed comparable decreases in the steroidogenic action of ACTH and exogenous cyclic AMP. It appears, therefore, that calcium is involved in the process of steroidogenesis subsequent to the activation of adenylyl cyclase itself.

The mechanism of action of ACTH in the adrenal cortex is such that calcium is in some manner accumulated, mobilized, or redistributed intracellularly (Jaanus and Rubin, 1971; Leier and Jungmann,

1973) enabling it to participate in such varied processes associated with the synthesis and release of corticosteroids as the facilitation of protein synthesis, increasing the rate of hydroxylation of corticoid intermediates, and ultimately enhancing the release of corticosteroid from the cell (Berridge, 1975).

The multitude of second-messenger roles for calcium are ultimately related to the synthesis and activities of other second messengers functioning during cell activation. It has been shown in several physiological preparations that ACTH or cyclic AMP can modulate calcium homeostasis by stimulating the uptake of calcium by the sarcoplasmic reticulum (Entman et al., 1969), increasing the influx of ^{45}Ca into the adrenal cortex (Leier and Jungmann, 1971), and playing a role in the redistribution of internal calcium in the adrenal cortex (Jaanus and Rubin, 1971). The ability of dibutyryl cyclic AMP to stimulate minimal steroidogenesis in the absence of calcium (Haksar and Peron, 1972; Birmingham et al., 1973; Warner and Rubin, 1975) might be explained by release of calcium from some intracellular pool. In addition, the steroidogenic response of isolated rat adrenal cells to high levels of ACTH showed a substantial synthesis of corticosterone in the absence of added calcium (Haksar and Peron, 1973). Perhaps this is indicative of a cellular reserve of calcium utilizable for ACTH stimulation of steroidogenesis, or suggests an alternate route of stimulation by ACTH which is not dependent upon calcium and is only manifest at very high concentrations of ACTH. Studies by Rubin et al. (1972) show that cyclic AMP alone is not capable of redistributing adrenal cortical cellular calcium, whereas ACTH has this capacity; therefore the proposal that ACTH may not only activate a calcium dependent adenylyl cyclase but also affect alternate metabolic pathways concerned with calcium mobilization appears plausible.

It has been suggested that the ACTH-mobilized calcium exerts effects beyond cyclic AMP production (Sayers et al., 1972), perhaps by redistributing to an active site such as the endoplasmic reticulum

or mitochondria (Rubin et al., 1972). The locus of calcium actions at the mitochondrial level may be related to an enhanced permeability of the organelle's membrane to extramitochondrial NADPH with a consequent stimulation of the hydroxylation reactions occurring during steroidogenesis (Matsuba et al., 1970; Peron et al., 1965). In addition, the calcium-induced conformational change in beef adrenal mitochondrial membranes and the demonstration of at least two calcium-binding sites in the intact cristae membrane (Matsuba et al., 1970), suggests that ACTH may release intracellular calcium which in turn binds to acidic phospholipid sites on the cristae membrane resulting in a conformational change in the membrane related to enhanced corticosteroidogenesis.

Another membrane associated event of calcium redistribution in response to ACTH concerns the plasma membrane and release of corticosteroid. The accumulation of intracellular steroid in adrenal glands perfused with calcium free medium (Jaanus et al., 1970), despite the diminished synthesis of steroid from ^{3}H-acetate precursors (Jaanus et al., 1972), indicated that steroid release was dependent upon the availability of calcium. An exocytotic mechanism of release for adrenal steroids has been hypothesized to occur in conjunction with the release of newly synthesized protein (Rubin et al., 1974; Laychock and Rubin, 1974). In other secretory systems such as the adrenal medulla (Douglas and Rubin, 1961), synaptic nerve endings (Simpson, 1968; Miledi, 1973), and endocrine pancreas (Malaisse et al., 1971), calcium has been demonstrated to facilitate secretion.

In the previous discussion of the protein kinase associated increase in steroidogenesis, it was noted that ribosomal phosphorylation and newly synthesized protein are critical to the action of ACTH. Studies by Farese (1971a) demonstrated that ACTH and cyclic AMP stimulated steroid release from rat adrenal sections were equally dependent upon calcium, and in calcium free adrenal incubations the incorporation of ^{3}H-leucine into protein was inhibited. Calcium deletion

did not result in a comparable inhibition of RNA synthesis, however, illustrating the metabolic viability of the tissue. Farese (1971b), in later studies demonstrated that calcium stimulated the transfer of amino acids from the amino acyl-tRNA complex to the growing peptide chain, possibly by increasing transfer enzyme activity.

The evidence thus far indicates a second messenger role for calcium and a cooperation with cyclic AMP in mediating the action of ACTH. Whether calcium may in turn enhance the synthesis or activity of other adrenal messengers, such as the prostaglandins or regulatory proteins responsible for mediating the tropic action of ACTH, has not been fully elucidated.

III. Prostaglandins

A. Functions of prostaglandins

In addition to a postulated role for prostaglandins in steroidogenesis, they are thought to participate in a variety of other physiological processes, and they demonstrate a wide spectrum of pharmacological actions on diverse biological systems. In autonomically innervated tissues, results obtained in studies of prostaglandin distribution, release, and action indicate that prostaglandins of the E-type play a physiological regulatory role in sympathetically innervated tissues (Hedqvist, 1973). This action is mediated both by inhibition of norepinephrine release and by alteration of the effector response to norepinephrine, perhaps by an effect upon the adenylyl cyclase-cyclic AMP system (Bergstrom, 1967) and/or calcium availability (Strong and Bohr, 1967). In light of the role of prostaglandins regulating sympathetically innervated tissues, it is not unexpected that the prostaglandins synthesized by the kidney medulla have potent vascular and natriuretic activities contributing to their antihypertensive effect (Lee, 1973). Prostaglandins as smooth muscle active agents also influence pulmonary vascular and bronchial muscle tone (Smith, 1973). The cardiovascular actions of

prostaglandins are widely recognized in myocardial tissue, as well as in the splanchnic, hepatic, gastric, cerebral, ovarian and uterine circulations (Nakano, 1973).

Prostaglandins also play a part in mediating the action of several hormones. Lipolysis in the epididymal fat pad *in vitro* is induced by electrical stimulation of sympathetic postganglionic fibers, epinephrine, norepinephrine, ACTH, thyroid stimulating hormone (TSH), and glucagon, and PGE_1 is able to antagonize these lipolytic effects (Berti and Usardi, 1964; Steinberg *et al.*, 1964). The mode of action of prostaglandins on lipolysis is largely unknown, although it appears to be either competitive or noncompetitive inhibition depending upon calcium availability (Paoletti and Puglisi, 1973). In addition, the prostaglandins are extremely effective inhibitors of cyclic AMP accumulation in fat cells (Butcher and Baird, 1968) and reduce the sensitivity of adenylyl cyclase to many different hormones.

Prostaglandins also exert regulatory effects upon the hypothalamus-pituitary-endocrine system, and investigators have studied the relationship between the levels of cyclic nucleotides and prostaglandins in regard to tropic hormone synthesis. Evidence suggests that while prostaglandins are not the physiological releasing hormones, they may mediate the release of releasing hormones from the hypothalamus by affecting the release of cerebral neurotransmitters and/or the vascular properties of the hypophyseal-portal system (Flack, 1973). Prostaglandins also stimulate adenylyl cyclase and cyclic AMP formation in the anterior pituitary (Zor *et al.*, 1970) and tropic hormone synthesis may be mediated by both agents. Studies on endocrine organs such as the adrenal gland, thyroid gland, ovary, and testis have demonstrated increased hormone synthesis and release in response to exogenously administered prostaglandins (Flack, 1973). An increase in cyclic AMP, however, is not obligatory to the actions of prostaglandins on the adrenal endocrine system (Beall and Sayers, 1972) as it appears to be in the

other systems (Burke, 1970; Kuehl et al., 1970). The observed increases in the biosynthesis of prostaglandins upon hormonal stimulation of fat cells as well as endocrine organs further supports the concept that endogenous prostaglandins may serve some function in regulating hormone action at the tissue level (Bergstrom, 1967).

The synthesis of prostaglandins is also associated with the mediation of the inflammatory response. Prostaglandins are involved in hyperalgesia, pain production, erythema, and edema (Ferreira et al., 1974). In addition, fever of infectious diseases is brought about by bacterial pyrogens acting on the anterior hypothalamus through an increase in the synthesis and release of prostaglandins of the E series (Feldberg, 1974). Prostaglandins appear to contribute to almost all aspects of the inflammatory reaction and increased vascular permeability, and granuloma formation in the development of fever, seem to be central to their mechanism of action (Hart, 1974).

Prostaglandins have also been implicated in platelet aggregation, and their synthesis in platelets during aggregation has been shown (Smith et al., 1974). It has been postulated that quiescent platelets are characterized by low cytoplasmic calcium concentrations, and that prostaglandins such as PGE_1 enhance the extrusion of calcium via a cyclic AMP-dependent calcium pump. In contrast, reduction in platelet cyclic AMP levels, as by generation of endoperoxide prostaglandin intermediates, leads to calcium influx and ultimately to platelet aggregation (Salzman, 1976).

Thus, the synthesis of prostaglandins and their intermediates plays an important role in the mechanism of many hormones and physiological processes often related to the cellular calcium and cyclic nucleotide levels. In addition, the pronounced effects of prostaglandins upon the cardiovascular system indicates that prostaglandins released from tissues play significant physiological roles. There is little evidence that synthesized prostaglandins accumulate intracellularly, except in

seminal vesicles, but rather they appear to be released from cells soon after de novo synthesis (Piper and Vane, 1969).

B. Prostaglandin metabolism

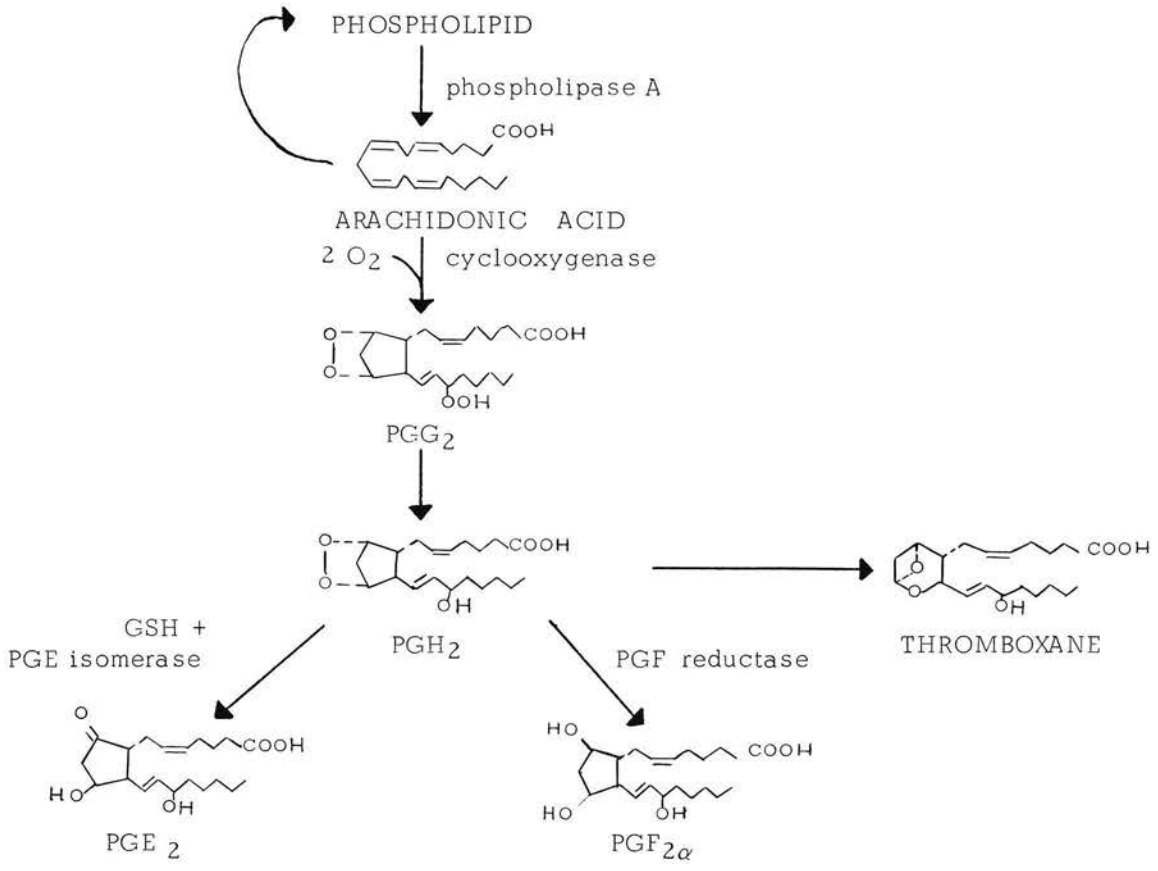
Almost all tissues studied, with the exception of erythrocytes, possess a prostaglandin synthetase complex in association with the cell microsomal membrane fraction (Bergstrom, 1967; Bohman and Larsson, 1975). Suitable substrates for the synthetase include three polyunsaturated fatty acids commonly found in membrane phospholipids: dihomo-γ-linolenic acid, arachidonic acid and 5, 8, 11, 14, 17-eicosapentaenoic acid (Ellis et al., 1975). The availability of the precursor fatty acids has been postulated as the controlling factor in prostaglandin synthetase activity (Lands and Samuelsson, 1968).

The concept that the controlling factor in prostaglandin synthesis is the activation of an enzyme system capable of supplying free fatty acid precursors to the prostaglandin synthetase complex has received wide support. Not only does the addition of arachidonic acid augment prostaglandin synthesis in many tissues (Shio et al., 1971; Kuehl et al., 1973) including human platelets (Silver et al., 1973) and rat stomach homogenates (Cohen and Jaffe, 1973), but the presence of snake venom and phospholipase A enhances the conversion of phospholipids to free fatty acids and thereby increases the synthesis of prostaglandins (Shio et al., 1971; Kunze et al., 1971). A significant proportion of cell membrane phospholipids consist of polyunsaturated fatty acids (Haye et al., 1976) which can be released enzymatically in order to provide substrate for prostaglandin synthetase (Pace-Asciak and Wolfe, 1970). The activation of an acid hydrolase, such as phospholipase A, triglyceride lipase and/or lysophospholipase (Silver et al., 1974) is believed to liberate arachidonic acid from phospholipids, cholesterol esters, and triglycerides in blood and other tissues for prostaglandin formation.

Direct evidence for hormonal activation of prostaglandin

synthesis due to a liberation of fatty acid precursors is derived from the fact that phospholipase A is present in most cell types (Ellis et al., 1975), and hormones such as thyroid stimulating hormone (TSH) liberate arachidonate by enhancing phospholipase A activity (Haye et al., 1976). The studies by Haye and co-workers (1973; 1976) demonstrated that TSH activated thyroid phospholipase A to liberate arachidonate from two distinct pools of precursors - the phospholipids, especially phosphatidyl inositol, and the triglycerides. These studies also showed that phospholipase activation was independent of cyclic AMP when phospholipids served as the substrate, but the release of arachidonate from triglycerides was stimulatable by cyclic nucleotides, suggesting that there may be dual hormonal control of prostaglandin synthesis. Another line of evidence supporting the role of phospholipase in prostaglandin synthesis arises from studies employing mepacrine, an inhibitor of phospholipase A. In guinea-pig lungs, the release of prostaglandins induced by bradykinin was prevented using mepacrine (McGiff et al., 1976b). Since the adrenal gland is richly supplied with cholesterol esters and phospholipids (Grant, 1968), it is possible that ACTH affects prostaglandin synthesis via a mechanism involving one or more acylhydrolases, including phospholipase.

Intermediates in the synthesis of prostaglandins are the endoperoxides, PGG_2, and PGH_2, and the thromboxanes. A microsomal fatty acid cyclo-oxygenase converts arachidonic acid to PGG_2, while a peroxidase converts PGG_2 to PGH_2 (Gorman, 1975). PGG_2 and PGH_2 are rapidly converted to PGE_2 and $PGF_{2\alpha}$, respectively. An isomerase converts PGG_2 to 15-hydroperoxy PGE_2 which is then converted to PGE_2 by a dehydrogenase, while an endoperoxide reductase is responsible for the synthesis of $PGF_{2\alpha}$ (Fig. 2). The thromboxanes are derived from endoperoxides, but the presence of an oxane ring makes them structurally different from other prostaglandin compounds, and they are extremely unstable with a half-life of 30-40 seconds (Hamberg et al.,



PROSTAGLANDIN BIOSYNTHESIS

1976). Thromboxanes are formed in numerous tissues including platelets, spleen, kidney, umbilical artery, and leukocytes (Samuelsson, 1976), where they appear to exert effects similar to prostaglandins, but due to the limitations of administering or accurately quantitating tissue levels of such unstable compounds, their physiological role is uncertain. Some tissues and plasma have the capacity for synthesizing prostaglandin A(PGA) and B(PGB) from PGE precursors via dehydration and PGA isomerase activity (Levine, 1973; Horton, 1972; McGiff et al., 1974).

An alternative synthetic route for the generation of $PGF_{2\alpha}$, other than through the endoperoxide reductase pathway, has been identified in several tissues as a PGE_2 conversion reaction catalyzed by 9-keto reductase (Lee et al., 1974). Homogenates of pigeon heart, brain, lung, liver, and of monkey brain, liver, spleen, kidney, lung, uterus, and heart, contain the 9-keto reductase which is most often found in the cytoplasmic cell fraction (Lee and Levine, 1974). Ordinarily, PGE and PGF compounds are not considered to be interconvertible, but Lee and co-workers have demonstrated that the 9-keto reductase reaction is reversible.

The direction of PGE and PGF interconversion via 9-keto reductase may depend upon the relative levels of oxidized and reduced pyridine nucleotides, with NADPH acting as the coenzyme facilitating the reduction of PGE. Studies on renal function suggest that the ratio of the levels of PGE to PGF in the canine kidney, and thus the activity of a PGE 9-keto reductase, could determine the physiological effects of prostaglandins in this tissue (McGiff et al., 1976a, b). Whether or not other factors, such as protein synthesis or cation dependency, alter the activity levels of PGE 9-keto reductase remains to be determined. It has been proposed, however, that estrogen alters prostaglandin levels in the uterus to favor the synthesis of PGF by inducing the formation of 9-keto reductase, in addition to inducing endoperoxide reductase, inhibiting endoperoxide isomerase, or changing tissue cofactor levels

(Kuehl et al., 1976).

The major pathways for the catabolism of prostaglandins involve a multiplicity of enzyme reactions. The initial step in the catabolic sequence is the formation of the 15-keto catabolite by 15-hydroxydehydrogenase, a pyridine nucleotide dependent enzyme (Pace-Asciak, 1976). The 15-keto catabolite provides substrate for Δ13-reductase, resulting in the 15-keto-13,14-dihydro derivative. A third enzyme, 9α-hydroxydehydrogenase, oxidizes the 9α-hydroxyl group of the 15-keto-13,14-dihydro-PGF_{α} compound to the corresponding 15-keto-13,14-dihydro-PGE compound (Pace-Asciak, 1975; 1976).

C. Inhibition of prostaglandin biosynthesis

The ubiquitous nature of the prostaglandin synthetase has rendered the classical endocrinological techniques, such as organ removal or ablation of a particular tissue, less than satisfactory as efficient methods of removing prostaglandins from physiological preparations. The pharmacological approach to inhibiting prostaglandin biosynthesis has therefore been adopted through the use of specific antagonists to prostaglandins or specific inhibitors of prostaglandin synthesis. Substrate analogues and certain fatty acid derivatives inhibit prostaglandin synthetase. An acetylenic analogue of arachidonic acid, eicosa 5, 8, 11, 14-tetraynoic acid, is reported to cause the irreversible inhibition of sheep vesicular gland prostaglandin synthetase (Ahern and Downing, 1970) and also blocks prostaglandin release from heart, spleen and seminal vesicle (Flower, 1974). It appears that acetylenic compounds inhibit prostaglandin synthetase by destruction of the catalytic site (Lands et al., 1973). A number of fatty acids also exhibit inhibitory activity against prostaglandin synthetase, and include the 12-trans analogues of di-homo-γ-linolenic acid and arachidonic acid, which behave as competitive inhibitors (Nugteren, 1970).

Attention has been mainly focused upon aspirin-like drugs, however, in pursuing efficient inhibitors of prostaglandin synthesis.

The aspirin-like drugs all share the antipyretic, analgesic and anti-inflammatory actions which are characteristic of aspirin, in addition to being potent inhibitors of prostaglandin synthetase. The relative potencies of the drugs vary and may be ranked in order of decreasing potency: meclofenamic acid > indomethacin > mefenamic acid > flufenamic acid > naproxen > phenylbutazone > aspirin or ibuprofen (Flower, 1974). The nature of the inhibition caused by these drugs is competitive and nonreversible, except for the newer anti-inflammatory compound designated SU-21524 and oxyphenbutazone (Ku and Wasvary, 1973). While the mechanism of the inhibitory action of the aspirin-like drugs includes more than one mode of action, the likely alternatives include competition at the substrate or cofactor site, irreversible inactivation of the enzyme, a chelating action, or a free radical destroying mechanism (Flower, 1974). Unfortunately, these diverse mechanisms of action of the aspirin-like drugs also contribute to their inhibitory effects at higher concentrations upon other cellular enzymes, including phosphodiesterase, oxidative phosphorylation enzymes, and prostaglandin dehydrogenase (Flower, 1974).

D. Prostaglandin receptors

The synthesis and release of prostaglandins from most cell types has contributed to the hypothesis that prostaglandins initiate or mediate many cellular phenomena induced by hormones and other stimulatory agents. Since prostaglandins are generally considered as local or intracellular hormones in view of their rapid inactivation by intravascular, lung and kidney enzyme systems (Golub et al., 1975), it is not unexpected that specific receptors for prostaglandins are found in many tissues including the plasma membrane of rat liver (Smigel and Fleischer, 1974) rat stomach, thyroid, luteal cell (Kuehl, 1974), and adrenal gland (Dazord et al., 1974).

The existence of prostaglandin receptors has been implied from the effects of structurally related prostaglandin antagonists, such as

7-oxa-13-prostynoic acid, which competitively block the stimulatory effect of prostaglandins on smooth muscle and other tissues (Kuehl, 1974). The rat liver plasma membrane contains receptors specific for PGE, and the prostaglandin agonists 16,16-dimethyl PGE_2 and 15s-15 methyl PGE_2, compete for the PGE_2 binding site (Smigel and Frölich, 1974). The study by Dazord et al. (1974) determined the binding characteristics of radiolabeled PGE_1 and PGE_2 to purified membranes of human and ovine adrenal glands. The binding of prostaglandins in these preparations was specific, and since the effects of maximum stimulatory concentrations of prostaglandins and ACTH were additive in promoting steroidogenesis, the prostaglandin receptors are believed to be distinct from ACTH receptors. In addition, as with ACTH receptor binding, calcium does not affect the prostaglandin binding in adrenal cell preparations. In contrast, high-affinity prostaglandin receptors in bovine corpus luteum cell membranes are dependent upon calcium (Rao, 1975).

E. Prostaglandins and cyclic nucleotides

As was stated earlier, the actions of polypeptide hormones are often mediated in the target tissue through the activation of adenylyl cyclase. The fact that prostaglandins have been demonstrated to increase adenylyl cyclase activity in several cell types (Kuehl, 1974) may imply that the effects of certain hormones on the adenylyl cyclase-cyclic AMP system may be expressed through the actions of one or another of the prostaglandins. Since prostaglandins are synthesized by most tissues, they may affect adenylyl cyclase activity by an endogenous biochemical interaction, or through their actions as local hormones stimulating specific receptors on cell membranes. In this regard, it has recently been proposed that prostaglandins may in fact be synthesized by the same cell whose membrane receptors they activate (Bito, 1975).

Support for an adenylyl cyclase-activating role of prostaglandins derives from the observation that purified solubilized myocardial adenylyl

cyclase can be stimulated by prostaglandins (Kuehl, 1973). In the mouse ovary, PGE_1 and PGE_2 mimic luteinizing hormone in augmenting cyclic AMP formation (Kuehl et al., 1972), and kinetic studies suggested that the activation of a prostaglandin receptor was essential to the action of luteinizing hormone in stimulating adenylyl cyclase in the ovary (Kuehl et al., 1970). In other systems, PGE increased cyclic AMP accumulation in broken cell preparations of human leukocytes and platelets, as well as in incubations of intact cell preparations of lung, spleen, diaphragm, kidney and testes (Butcher et al., 1968). Several prostaglandins have also been reported to stimulate pituitary adenylyl cyclase and increase cyclic AMP levels in the intact gland (Sundberg et al., 1975).

In human and ovine adrenal glands prostaglandins bind to plasma membrane receptors and increase the maximum velocity of adrenal adenylyl cyclase activity (Dazord et al., 1974). This adrenal preparation also demonstrated that prostaglandins are not obligatory for at least certain actions of ACTH, since inhibition of their synthesis by indomethacin did not inhibit adenylyl cyclase stimulation induced by ACTH. The thyroid gland is similar to the adrenal gland in that prostaglandins and the tropic hormone TSH increased adenylyl cyclase activity in thyroid slices, as well as in sheep and dog thyroid homogenates (Mashiter and Field, 1974). In addition, aspirin and indomethacin failed to inhibit the TSH-induced elevation of cyclic AMP, suggesting that prostaglandins are not obligatory intermediates in the action of TSH on the thyroid.

The stimulatory actions of prostaglandins are not always associated with enhanced adenylyl cyclase activity, however, since in rat adrenal gland preparations prostaglandins did not increase cyclic AMP levels in vitro (Halkerston, 1975), and in feline isolated adrenal cortical cell suspensions the PGE_2-induced increase in cyclic AMP was not statistically significant and small in relation to that found with equipotent steroidogenic ACTH concentrations (Warner and Rubin,

1975). The trypsinization procedure used to isolate the feline adrenal cortical cells may in part account for the depressed stimulation of membrane-associated adenylyl cyclase (Dazord et al., 1974). Studies employing indomethacin treated rats showed a depressed steroidogenic response to ACTH while the response to dibutyryl cyclic AMP was unaffected (Gallant and Brownie, 1973), suggesting that prostaglandins were regulating the action of ACTH either at the receptor site, the transmission of the signal from the ACTH receptor to the adenylyl cyclase moiety, or in the modification of enzymes involved with cyclic nucleotide metabolism (Halkerston, 1975). In fat cells a binding protein for prostaglandins has been isolated (Kuehl, 1974), but fat cells provide a classic example of prostaglandins antagonizing the activation of adenylyl cyclase. Ordinarily, epinephrine stimulates the accumulation of cyclic AMP in lipocytes, but PGE antagonizes this action (Butcher and Baird, 1968; Dalton and Hope, 1974). While no direct prostaglandin inhibition of adenylyl cyclase has been demonstrated, Gorman et al. (1976) reported that the endoperoxide, PGH_2, inhibits directly adenylyl cyclase in isolated adipocyte membranes. It was suggested that PGH_2 may be an endogenous feedback regulator of lipolysis in adipose tissue; the significance of endoperoxides in stimulating or inhibiting adenylyl cyclase and regulating hormone action in different cell types remains to be determined.

Prostaglandins possess the capacity to stimulate intracellular cyclic AMP production as well as to increase cyclic GMP levels in several tissues. This dual action has been viewed as an important means of regulating cell function. Generally, prostaglandins of the E series are concerned with the regulation of cyclic AMP levels, whereas those of the F series are responsible for alterations in cyclic GMP levels (Kuehl, 1973). In those tissues where the actions of PGE and PGF are antagonistic, the action of the PGF may be mediated by cyclic GMP which opposes the PGE activation of adenylyl cyclase. Such a

relationship between the activities of the prostaglandins is evident when considering prostaglandins as modulators of the autonomic nervous system, since PGE_1 dilates lobar arteries and veins by increasing cyclic AMP levels in smooth muscle cells, whereas $PGF_{2\alpha}$ increases intracellular cyclic GMP in isolated lobar veins (Brody and Kadowitz, 1974). Thus, augmented levels of cyclic GMP may play a role in the vasoconstrictor effect of $PGF_{2\alpha}$, whereas increases in the levels of cyclic AMP may be important in the vasodilator action of PGE. It has also been proposed that endoperoxides, which stimulate vascular smooth muscle and antagonize cyclic AMP regulatory functions, may act through the stimulation of cyclic GMP synthesis (Gorman, 1975).

In regard to the mediation of hormonal stimulation, evidence has been provided that cyclic GMP has the capacity to stimulate steroidogenesis in rat adrenal quarters although it is much weaker in this regard than cyclic AMP (Glinsman et al., 1969). As to the relationship of prostaglandins, cyclic nucleotides and ACTH stimulated steroidogenesis, it is conceivable that prostaglandins increase the levels of cyclic GMP prior to increases in cyclic AMP and increased steroid release. Although the studies of Warner and Rubin (1975) showed only marginal PGE_2-elicited cyclic AMP increases in the feline adrenal cortex, the time period chosen to measure such increases may not have been optimal. On the other hand, as noted earlier, in such preparations as the perfused feline adrenal cortex (Rubin et al., 1972) there is a sufficient lag between the administration of ACTH and the onset of maximal steroidogenesis that rapid increases in cyclic GMP levels (Kuehl, 1973), in response to ACTH or prostaglandins, could influence the onset of steroidogenesis in the adrenal gland. In the uterus, however, it has been demonstrated that prostaglandins do not mediate the estrogen-induced increases in cyclic GMP levels (Kuehl et al., 1976), thus suggesting that cyclic GMP generation in some tissues occurs prior to PGF formation, which in turn may influence adenylyl cyclase activity.

In addition to prostaglandin activation of cyclic nucleotide synthesis, there is evidence that the cyclic nucleotides stimulate prostaglandin formation. Treatment of isolated gonadal tissue with dibutyryl cyclic AMP increases synthesis of both E and F prostaglandins (Kuehl et al., 1973), and dibutyryl cyclic AMP increases the concentration of PGE_2 in adipocytes (Dalton and Hope, 1974). The finding that cyclic AMP increases prostaglandin levels in isolated rabbit Graafian follicles, as well as in mouse ovaries, rat testis, thyroid tissue, neuroblastoma, glioma, and fibroblast cell cultures (Marsh et al., 1974) led to the development of a model suggesting that the action of a trophic hormone is to increase the synthesis of cyclic AMP in certain target tissues, and the cyclic nucleotide is in turn responsible for prostaglandin synthesis (Bergstrom, 1967; Marsh et al., 1974). Cyclic nucleotide might exert its stimulatory effect on phospholipase, triglyceride lipase or cholesterol esterase, which would convert esterified fatty acids to free fatty acids; the free fatty acids would thereby provide the substrate for prostaglandin synthesis.

F. Prostaglandins and calcium

Calcium has been implicated in many aspects of the action of ACTH on the adrenal gland. If prostaglandins play a role in the tropic action of ACTH, their interrelationship with the distribution and actions of this important cation deserves to be assessed, especially in regard to their possible role in enzyme activation. In thyroid tissue, phospholipase activation and subsequent prostaglandin synthesis is calcium dependent, since EDTA diminishes TSH-stimulated phospholipase activity (Haye et al., 1976). In homogenates of bovine seminal vesicles, phospholipase A_2 activity is stimulated by calcium and inhibited by EDTA, with parallel effects on prostaglandin formation (Kunze et al., 1974). Thus, calcium is implicated as a modulator of prostaglandin synthesis in that it contributes to the availability of free fatty acids. The dependence of prostaglandin synthetase activity itself upon the presence

of calcium has yet to be evaluated. However, it may be inferred from experiments in which calcium deprivation enhances lipase activity and prostaglandin synthesis in the face of diminished phospholipase activity, that prostaglandin synthetase remains active in the absence of available calcium so long as substrate acids are provided by lipolytic enzymes (Kunze et al., 1974).

Prostaglandins appear to not only be regulated in their synthesis by calcium, but have been shown to affect cellular calcium distribution as well. For several years Hedqvist (1970a; 1974) has proposed that prostaglandins inhibit norepinephrine release from sympathetic nerves by inhibiting the calcium influx normally induced by depolarization (Hedqvist, 1970b). It was further demonstrated that prostaglandins affect calcium fluxes when $PGF_{2\alpha}$ enhanced the uptake of ^{45}Ca into helical strips of arterial and venous smooth muscle incubations (Greenberg et al., 1973). Evidence also suggests that prostaglandins affect calcium transport across platelet membranes resulting in alterations in certain aggregation phenomena (Vigdahl et al., 1969; MacIntyre and Gordon, 1975). Even the turkey erythrocyte, which is not known to synthesize prostaglandins, demonstrates a modified ^{45}Ca efflux in the presence of these unsaturated fatty acids (Shio et al., 1971). It is not unlikely that the secretion of substances such as adrenal corticosteroids - which is calcium dependent - might be altered by the calcium-mobilizing actions of prostaglandins.

Intracellularly, prostaglandins have membrane-associated actions similar to those that have been described for the plasma membranes of several tissues. The mitochondrial membranes seem to be especially affected by prostaglandins. Using configurational changes and resultant light-scattering alterations which occur when calcium ions bind to the inner mitochondrial membrane, Kirtland and Baum (1972) demonstrated the marked facilitation of non-energized calcium binding to rat liver

mitochondria in the presence of PGE_1. These results prompted the hypothesis that PGE_1 as a monobasic acid might act as an "ionophore" facilitating calcium exchange. Further studies on the interplay between prostaglandins and calcium at the mitochondrial level demonstrated that the cation stimulate prostaglandin binding by liver mitochondria, and the binding of the prostaglandins induce the efflux of calcium from the mitochondria (Malstrom and Carafoli, 1975). Interestingly enough, while the prostaglandin binding to mitochondria would be an effective way to sequester prostaglandins away from the cytosol, isotopic stoichiometry suggested that prostaglandins are probably transported into the mitochondria, where two molecules complex with a calcium ion resulting in the efflux of the cation from the organelle. The consequences of this action of prostaglandins is not only that calcium may redistribute intracellularly for participation in a multiplicity of reactions, but also that mitochondrial respiration is activated. This activation probably has profound effects upon biochemical syntheses occurring within this organelle.

G. Prostaglandins and gonadal steroidogenesis

In view of the purported effects of prostaglandins on mitochondria and calcium dynamics, cyclic AMP synthesis, and cholesterol availability, it might be expected that these unsaturated fatty acids would play a role in steroidogenesis since this process is dependent to some extent upon each of these parameters. Because the conversion of cholesterol esters to free cholesterol has been discussed as an initial event in steroidogenesis in several tissues including the adrenal gland, the regulation of cholesterol availability by prostaglandins is an important aspect in their mechanism of action. In the liver, the addition of prostaglandin E_1 or $F_{1\alpha}$ to liver microsomes inhibits the esterification of cholesterol with long-chain fatty acids (Schweppe and Jungman, 1970). Later studies revealed that prostaglandin E_1 can either enhance cholesterogenesis and fatty acid synthesis or inhibit these reactions, depending upon its

concentration in liver tissue (Calandra and Montaguti, 1973). These workers suggested that prostaglandin E_1 interferes with the formation of active acetyl, the precursor for the biosynthesis of fatty acids and sterols. In the rat ovary, a similar phenomenon was observed when prostaglandin $F_{2\alpha}$ depressed ovarian cholesterol ester turnover in vivo (Behrman et al., 1971). In vitro evidence, however, demonstrated that the incorporation of labeled acetate into ovarian cholesterol was not diminished by prostaglandin $F_{2\alpha}$ (Pharriss et al., 1968) and that steroidogenesis was stimulated in incubated slices of ovarian tissue (Behrman et al., 1971).

The conflicting effects of prostaglandins on cholesterol ester formation and steroidogenesis in vivo and in vitro might be resolved by an examination of the direct effects of prostaglandins on steroid synthesis, since the latter response is dependent upon changes in cholesterol metabolism. Steroid synthesis in several tissues has been studied in the presence of different species of prostaglandins (Shaw and Tillson, 1974). In the testes, initial studies found that the intra-arterial infusion of prostaglandin E_2 augmented testosterone secretion induced by human chorionic gonadotrophin in the dog (Eik-Nes, 1969), but in the rat $PGF_{2\alpha}$ reduced testicular testosterone secretion (Shaw and Tillson, 1974). The observed steroidogenic responses to these two prostaglandins, however, may depend in large part upon the evoked alterations in blood flow to the organ, rather than to the direct effect of these compounds on cellular secretion. Using minced rat testicular tissue, prostaglandin A_1 (PGA_1) diminished conversion of progesterone to androgens and PGE_1 reduced testosterone production compared with control samples (Shaw and Tillson, 1974). In the testes, therefore, some prostaglandins may be direct inhibitors of the steroidogenic process.

The ovary is another steroid secreting tissue which is responsive to prostaglandin stimulation. Pharriss and Wyngarden (1969) reported that $PGF_{2\alpha}$ increased progesterone synthesis in ovarian minces, and in

a similar tissue preparation PGE_2 increased the production of 20α-hydroxypregn-4-en-3-one (Bedwani and Horton, 1968). Studies on both human (Santos et al., 1973) and bovine (Speroff and Ramwell, 1970) corpora lutea demonstrated that $PGF_{2\alpha}$, PGE_2, PGE_1, and PGA_1 stimulate the synthesis of progesterone in vitro, thus supporting the view that prostaglandins are steroidogenic agents in luteal tissue. The PGE_2 induced increase in bovine corpus luteum progesterone in vitro was accompanied by an increased incorporation of acetate-1-^{14}C into steroid (Marsh, 1970), suggesting that cholesterol synthesis was not inhibited. On the other hand, contradictory evidence derives from in vivo studies, which describe $PGF_{2\alpha}$ as inhibiting corpus luteal progesterone secretion in several species (Behrman et al., 1971; O'Grady et al., 1972), presumably by inhibiting cholesterol ester synthetase activity and causing a decline in free cholesterol available for steroidogenesis. Although the disparity in these results has not been resolved, it appears that alterations in blood flow similar to those induced by prostaglandins in the testes, or an interference with in vivo LH and pituitary regulation of corpus luteum function, might be in part responsible for the in vivo luteolytic effects of prostaglandins (Kirton et al., 1976; Novy and Cook, 1973; Behrman et al., 1976). In the placenta, in vitro studies fail to demonstrate any effect of PGE_1 or $PGF_{2\alpha}$ on progesterone synthesis (Bedwani and Marley, 1971). Thus, not all reproductive steroidogenic tissues behave comparably to prostaglandin administration.

While prostaglandins influence the synthesis of steroids in several tissues including the gonads, the reciprocal effect of steroids upon prostaglandin synthesis and/or metabolism is also a factor in their relationship. In rat ovaries and homogenates of cultured Graafian follicles the suppression of steroidogenesis by aminoglutethimide did not impair the synthesis of prostaglandin in response to luteinizing hormone (Bauminger et al., 1975). Such results suggest that steroids play no mediator role in prostaglandin synthesis. In contrast, when guinea-pigs

were treated with estradiol and/or progesterone the result was a doubling in the $PGF_{2\alpha}$ synthesizing capacity of the uterus in response to estradiol (Poyser, 1976). Progesterone treatment alone did not increase prostaglandin synthetase activity. In vitro studies using uterine tissue showed an estradiol stimulation of prostaglandin synthesis, which was antagonized by progesterone (Naylor and Poyser, 1975). Thus, the products of steroid synthesis have the capacity for affecting and possibly regulating prostaglandin synthesis, although the mechanisms involved remain obscure. The observed inhibition of placental prostaglandin dehydrogenase by high concentrations of estrone and progesterone (Thaler-Dao et al., 1976) suggests that some steroids similar in dimension and shape to the prostaglandins might compete for prostaglandin synthesizing or metabolizing enzymes and regulate prostaglandin production accordingly.

H. Prostaglandins and adrenal steroidogenesis

The adrenal gland represents another steroidogenic tissue responsive to prostaglandin administration. In response to PGA_1, there was a three-fold increase in the adrenal aldosterone secretion rate in man in vivo (Fichman and Horton, 1973), while PGE_1 inhibited aldosterone secretion from the autotransplanted sheep adrenal (Blair-West et al., 1971) and $PGF_{1\alpha}$ had a similar inhibitory effect on slices of beef adrenal tissue (Saruta and Kaplan, 1972). In the beef adrenal system, however, PGE_1 increased the synthesis of aldosterone, in contrast to the ovine response. One type of prostaglandin appears to be capable of affecting the synthesis of a particular steroid in different ways, depending upon the species studied or the experimental methods used.

The prostaglandin induced synthesis of adrenal corticosteroids, other than aldosterone, has received much attention. In vivo, the administration of PGE_2 to hypophysectomized rats (Gallant and Brownie, 1973) or of $PGF_{2\alpha}$ to dexamethasone treated humans (Wentz et al., 1973), had no direct stimulatory effect upon corticosterone or cortisol synthesis, respectively. In the absence of dexamethasone, $PGF_{2\alpha}$ elicited a three

fold increase in cortisol secretion, suggesting that prostaglandins were dependent upon the secretion of pituitary ACTH for an effect upon adrenal steroidogenesis. In vitro studies found this not to be the case, however, since PGE_1 and PGE_2 stimulated the synthesis of corticosterone and cortisol in slices of beef adrenal tissue (Saruta and Kaplan, 1972) and PGE, PGE_2 and $PGF_{2\alpha}$ enhanced steroidogenesis in superfused rat adrenal glands (Flack et al., 1969, 1972). Recent evidence demonstrated that PGE_1, PGE_2, and to a lesser extent, $PGF_{1\alpha}$ were steroidogenic when incubated with isolated feline adrenal cortical cells (Warner and Rubin, 1975). In addition, prostaglandin stimulated steroidogenesis was at least partially dependent upon calcium (Saruta and Kaplan, 1972; Warner and Rubin, 1975) and required protein synthesis (Flack et al., 1969). Thus, the possibility that prostaglandins are intracellular mediators of the action of ACTH arises from the fact that both agents require similar cofactors and synthetic pathways in promoting steroidogenesis.

IV. Summary and methods of approach

The foregoing introduction was meant to provide an in-depth perspective of the mechanism of action of ACTH on adrenal cortical cellular metabolism and steroidogenesis. In tracing the action of ACTH from its capacity to interact with membrane receptors, to stimulate the synthesis of second-messengers, to regulate protein synthesis, and finally to enhance steroid synthesis and secretion, it is hoped that an appreciation of the complexity of the mechanisms involved was gained. In this light, the role of the ubiquitous prostaglandins as possible mediators of the steroidogenic mechanism of ACTH was reviewed. Actually, very little is known about the physiological role of prostaglandins in cortical steroidogenesis, except that in pharmacological concentrations they are able to enhance steroidogenesis. However, the

ability of prostaglandins to alter cyclic AMP synthesis, redistribute calcium, and affect steroid synthesis in various other cell types contributes to an understanding of the ways in which prostaglandins might be expected to mediate the action of ACTH on the adrenal cortex. Often, conflicting evidence regarding the interrelationship of prostaglandins and other mediators of hormone action was reported in this overview. These data were delineated not to confuse, but rather to emphasize the options available for explaining a possible role of prostaglandins in the tropic action of ACTH on the adrenal cortex.

Conflicting data not withstanding, evidence supporting any physiological role for prostaglandins in ACTH-induced steroidogenesis must involve the demonstration of prostaglandins within cortical cells and an alteration of prostaglandin metabolism during stimulation of steroid production and release by ACTH. Hence, the primary aim of this investigation is to characterize the prostaglandins synthesized in adrenal cortical cells by the use of radioactive precursors and radioimmunoassay techniques and to attempt to correlate prostaglandin levels and steroid release. In addition, since cyclic AMP and calcium as well as prostaglandins are implicated in cortical steroidogenesis, the possible interaction of these steroidogenic mediators will be considered. Such an approach may help to unravel the complexities associated with ACTH action.

The preparation chosen to study the physiological and biochemical events associated with PG metabolism was the feline adrenal cortical cell suspension system (Warner and Rubin, 1975). This preparation maintains the integrity of cells, unlike cell homogenates, and although the basic architecture of the gland is destroyed, the advantages of the suspension allowing rapid distribution and elimination of chemical compounds remain. Adrenal cortical cell suspensions respond to physiological concentrations of ACTH, unlike adrenal sections which are one thousand times less sensitive and lack a homogenous

cell population. While cell suspensions show variability among preparations, the cells in each preparation are completely randomized and a number of different experiments can be performed on cells allocated to small separate aliquots. Since PGs are ubiquitous compounds synthesized by many cell types, it is of particular importance that the cell suspensions are relatively free of fat cells and cell fragments removed during centrifugation steps of the isolation procedure.

The adrenocortical cell suspension system therefore appears well suited to a study of prostaglandin metabolism. Not only are diffusion barriers, exclusive of the plasma membrane, removed, permitting unhindered access of ACTH and other pharmacological agents to the cells; but tritiated compounds used in biochemical synthesis studies also have ready access to target cells with a minimum of isotope dilution due to the uptake by non-adrenocortical tissue. Likewise, the release of chemical compounds and prostaglandins synthesized by the cells is not diffusion limited and these substances are contained within a specified volume of incubation media making them particularly amenable to qualitation and quantitation procedures. In addition, following centrifugation of the suspension the cell pellet is available for homogenization and extraction of prostaglandins for comparison with releasable prostaglandin species. The radioimmunoassay technique used to identify prostaglandins in this investigation relies upon antibodies specific for a particular prostaglandin, and provides a highly sensitive means of estimating endogenous prostaglandins, which compete against labeled antigen for binding to a specific antibody (Berson et al., 1956).

METHODS

I. Preparation of cortical cell suspensions

Cats (2-3 kg) were anesthetized with an intraperitoneal injection of pentobarbital sodium (30 mg/kg). The adrenal glands were quickly removed and placed in Modified Eagle's Medium (MEM) plus glutamine (2 mM), which was equilibrated with a gas mixture of 95% oxygen and 5% carbon dioxide and had a pH of 7.4. The glands were cut into 1 mm cubes, placed in siliconized trypsinizing flasks containing 10 ml MEM plus trypsin (0.15%) and bovine serum albumin (BSA) (1%) and incubated at room temperature in a metabolic shaker. After 90 minutes, the temperature was raised to 37° C and the rate of shaking increased from 120 to 300 rpm. After 1 hour the undigested material was removed and the suspension incubated for an additional 40 minutes at 120 rpm with sufficient trypsin inhibitor to block further proteolytic activity. The cell suspension was centrifuged at 3000 rpm for 10 minutes at 4° C and washed once with MEM and re-centrifuged. The cell pellet was re-suspended in MEM supplemented with 0.2% BSA and 0.04% trypsin inhibitor when prostaglandin analysis was performed and 0.6% BSA and 0.1% trypsin inhibitor when optimum responsiveness to ACTH was desired for measurement of corticc steroid release.

Contamination of the cortical cell preparation by other tissue components was minimized since noncortical tissue was either digested by the trypsin or separated from the cortical cells during centrifugation by remaining suspended while the cortical cells formed a pellet. The cortical cells were counted with a hemocytometer and the cell concentration adjusted so that an equal number of cells (circa 2.5×10^5) could be added to each beaker in a final incubation volume of 1 ml.

In the experiments concerned with calcium deprivation the basic medium was Kreb's bicarbonate solution fortified with vitamins plus amino acids in concentrations identical to those in MEM. Calcium was

excluded from the Kreb's solution and EGTA (0.4 mM) was added to chelate residual calcium.

II. Adrenal perfusion

The left cat adrenal gland was perfused in situ according to the method of Douglas and Rubin (1961). Perfusion was carried out at room temperature with Locke's solution which had the following composition (mM); NaCl 154; KCl 5.6; $CaCl_2$ 2.0; $MgCl_2$ 0.5, $NaHCO_3$ 12; dextrose 10. The perfusion medium was equilibrated with 95% oxygen and 5% carbon dioxide, and had a pH of 7.0. The rate of flow was maintained between 0.8-1.2 ml/min by regulation of the perfusion pressure and the addition of ACTH to the medium did not alter flow rate through the gland. The perfusate was collected in aliquots every 8 or 10 minutes from a cannula placed in the adrenolumbar vein.

III. Steroid analysis

Methylene chloride (5 ml) was used to extract 1 ml of medium. The steroid (hydrocortisone) concentration of the organic phase was determined by competitive protein binding using human transcortin as the binding agent (Jaanus et al., 1972). Each experimental procedure for prostaglandin determinations was accompanied by steroid analysis in order to assure the viability and responsiveness of the preparation.

IV. Prostaglandin synthetase activity: Intact cells

The cells were incubated for 75 minutes at 37° C with 1.0 ml MEM containing ^{3}H-arachidonic acid (4 μg/ml), in the presence or absence of ACTH. Control and stimulated cells were pooled separately, centrifuged at 3000 rpm for 10 minutes at 4° C and the cell pellets washed and re-centrifuged in 0.5 ml MEM. The supernatants were decanted, acidified to pH 3 with 1 N HCL, and extracted twice each with 20 ml ethyl acetate. The organic phase was evaporated in vacuo. The

cell pellets were homogenized in 4 ml incubation medium (titrated to pH 3 with formic acid) and centrifuged for one hour at 20,000 rpm at 4° C. The resulting supernatant was decanted into extraction tubes and the pellet was washed with 1.0 ml MEM (pH 3) and centrifuged for 10 minutes at 15,000 rpm. The combined supernatants were extracted twice with 15 ml of ethyl acetate:cyclohexane (2:1) and the pooled organic phases evaporated to dryness in vacuo.

Chromatographic separation of the prostaglandins was performed using silicic acid chromatography by a modification of the method of Jaffe et al. (1973). The prostaglandin fractions were obtained by developing glass columns (0.5 × 30 cm) serially with 7 ml solvent 3 (benzene: ethyl acetate, 60:40) (PGAB fraction), 18 ml solvent 1 (benzene: ethyl acetate: methanol, 60:40:2) (PGE fraction), and 9 ml solvent 4 (benzene: ethyl acetate: methanol, 60:40:20) (PGF fraction). The fractions were evaporated in vacuo at 42° C., and recovered from the evaporation flasks by re-suspending them in 0.5 ml distilled absolute ethanol and quantitatively transferring them to glass tubes and drying them under N_2 at 37° C. The separation procedure was validated by adding tritiated PGE_1 and $PGF_{1\alpha}$ to incubating cortical cells and extracting them in the same manner as the experimental samples. Recovery calculations on the extracts estimated that 82% of the ^3H-PGE_1 was eluted in the PGE fraction and less than 4% was recovered in either of the other two fractions. Sixty-six per cent of the ^3H-$PGF_{1\alpha}$ was recovered in the PGF fraction, 15% in the PGE fraction, and 5% in the PGAB fraction. In other experiments, ^3H-PGE was quantitatively converted to ^3H-PGB (Zusman, 1972), with resultant recoveries after silicic acid chromatography of 65% in the PGAB fraction and 4% in the PGE fraction. Arachidonic acid residues were largely removed in the PGAB fraction.

In preparation for thin layer chromatography (TLC), the sample residues were re-suspended in absolute ethanol and applied on silica gel G (250 microns) coated glass plates and developed in benzene:

chloroform: acetone: methanol: acetic acid (20:20:5:5:1 by volume). Standards were run in parallel and visualized with phosphomolybdic acid (Willis, 1970). A radioactive profile was obtained by scraping the plates into 1 cm zones, dissolving them into 10 ml Bray's scintillation solution and counting them by scintillation spectrometry.

V. Prostaglandin synthetase activity: Cell homogenates

Tritiated arachidonic acid was purified using TLC, and eluted from the silica gel with 3.5 ml methanol; the eluate was dried under N_2 at 37° C. and stored -4° C. The purified acid was re-suspended in ethanol for use in the incubation procedure.

The right and left adrenal glands were removed from a male cat, rinsed in Ringer's solution and the capsule and fat were trimmed away. The glands were hemisected and the medulla was removed as completely as possible using forceps; the remaining cortex was sliced into 4 mm cubes in a solution of calcium supplemented MEM (2.5mM). The cubes of tissue were suspended in 2.5 mM EDTA, 0.2 mM reduced glutathione, and 20 μg/ml L-norepinephrine bitartrate (Bauminger et al., 1973; Pace-Asciak, 1975). The tissue was kept on ice and homogenized using a Polytron at maximum speed for 10 seconds. The homogenate was centrifuged at 7500 rpm for 10 minutes at 4° C., and the supernatant was decanted and incubated with ^{3}H-arachidonic acid, for 30 minutes at 37° C.; ^{3}H-arachidonic acid was also incubated in the absence of homogenate to ascertain the spontaneous conversion of the labeled acid. The incubation was terminated by acidification to pH 3 with 1 M citric acid and the incubates were then extracted twice with 20 ml ethyl acetate: cyclohexane (2:1); ^{3}H-PGF_1 and ^{3}H-PGE_1 were extracted separately for calculation of recovery. The organic phase was dried in vacuo and re-suspended samples were applied to silicic acid columns. The E and F fractions were subject to TLC analysis and a radioactive profile was obtained as described above.

VI. Prostaglandin analysis by radioimmunoassay: Extraction and chromatographic separation of prostaglandins

Aliquots (1.5-3.0 ml) of MEM supernatant were acidified to pH 3 with 0.5 N HCL, and subjected to XAD-2 column chromatography using a modified procedure of Kierse and Turnbull (1973). Following application of the samples and 15 ml deionized water, the prostaglandins were eluted with 3 ml distilled ethanol. The prostaglandin fraction was taken to dryness by evaporation in N_2 at 55° C , and the dried prostaglandins stored at -20° C until radioimmunoassay (RIA) was carried out.

In certain other experiments, in order to eliminate the resin eluate contribution to non-specific binding during RIA, acidified aliquots (1.5 ml) of incubation media were extracted with diethyl ether according to the method of Levine et al. (1972). A small amount of ^{3}H-PGF_1 (circa 2000 cpm) was added to each sample to calculate the percent recovery. The combined ether layers were evaporated under N_2 at room temperature, and the dried extracts re-suspended in 50 µl methanol and applied to TLC plates (Silica Gel G) which were subsequently developed in the solvent system dioxane: benzene: acetic acid (10:20:1) (Jubiz et al., 1972). The developed plates were air dried, and those zones corresponding to PGE and PGF standards were scraped into glass tubes and the respective prostaglandins eluted with 1 ml methanol (2×) by vortexing. After centrifuging the tubes at 4° C, the methanolic supernatant was removed and dried under N_2 at 37° C, and stored at -20° C prior to RIA.

For intracellular estimation of prostaglandins, isolated cortical cells which had been incubated were homogenized in 4 ml acidified MEM (pH 3) and centrifuged for 15 minutes at 27,000 × g at 4° C. The resulting supernatant was extracted twice using either ethyl acetate: cyclohexane (2:1) or freshly distilled diethyl ether; extractions with either solvent system gave similar results. The extracts were dried in vacuo or under

N_2 and subjected to silicic acid chromatography, or in the case of the ether extracts, to thin layer chromatography. Those thin layer zones corresponding to PGF and PGE were scraped and the prostaglandins eluted with methanol. The eluates were dried under N_2 and re-suspended in sodium phosphate buffer (pH 7.4) for prostaglandin analysis by RIA.

VII. Antiprostaglandin antibodies

Antibodies to $PGF_{1\alpha}$ and $PGF_{2\alpha}$ were elicited by immunizing rabbits to the corresponding prostaglandin protein conjugate prepared in the manner described by Caldwell et al. (1971). The animals were injected once a week for 4 weeks, with monthly booster injections, thereafter. Following the sixth booster, the antibody titer was satisfactory for use in RIA, and the cross-reactivity of each antibody was measured by the percent binding of a variety of prostaglandins and prostaglandin metabolites in the presence of a tritiated prostaglandin binding marker specific for the antibody. $PGF_{1\alpha}$ and $PGF_{2\alpha}$ antisera were stored in 0.2 M $NaPO_4$-buffer (pH 7.4) prior to use in RIA, when dilutions of 1:7000 and 1:3000, respectively, were used.

PGE_1 and PGE_2 antibodies were generated in response to prostaglandin-ethyl chloroformate conjugates of the respective prostaglandins (Jaffe et al., 1971). The injection schedule and analysis of the cross-reactivity properties were carried out as described above. The PGE antisera were stored frozen in $NaPO_4$-buffer (pH 7.4) supplemented with 0.1% BSA, at a dilution of 1:10. The PGE_2 antiserum was diluted 1:500 with $NaPO_4$-buffer for use in RIA.

VIII. Radioimmunoassays

At least 2 ml of cells were pooled and centrifuged at 6000 rpm for 10 minutes at 4° C, and the supernatant decanted and frozen at -20° C. Aliquots of this medium were either assayed directly for PGF or PGE or the prostaglandins were extracted for subsequent assays.

All dilutions of haptens and antisera were made in 0.2 M $NaPO_4$-buffer (pH 7.4). For direct assay of prostaglandins, the following constituents were incubated together for 1 hour at room temperature: unlabeled prostaglandins (60-900 pg) or 300-400 µl of the unknown sample when the assay was for PGF and 100-200 µl when the assay was for PGE; tritiated prostaglandin (circa 7000 cpm); and 50 µl of $PGF_{2\alpha}$ or $PGF_{1\alpha}$ or PGE_2 antibody. All reaction mixtures were made to contain identical amounts of protein by adding appropriate volumes of MEM, and the final reaction volume was adjusted to 0.5 ml for the PGF assay and 0.35 ml for the PGE assay with $NaPO_4$ buffer. In both assay systems, MEM samples incubated in the absence of cells were processed as "blank" values for RIA. Following incubation, bound ^3H-prostaglandin was separated from unbound by nitrocellulose filtration; the filter was dissolved in Bray's solution and counted by liquid scintillation spectrometry. Prostaglandin concentrations were calculated by logit analysis on a Hewlett-Packard (9810) programmable calculator.

RIA of PGE_1 and PGE_2 was also performed following the reduction of samples with sodium borohydride ($NaBH_4$) (Levine, 1973). Although this procedure reduces PGE to approximately equal concentrations of PGF_α and PGF_β, the concomitant reduction of PGE standards takes into account the incomplete conversion to PGF_α in experimental samples during quantitation of prostaglandins. Aliquots of extracted samples (100-150 µl) and PGE standards were suspended in buffer in a total volume of 235 µl; 5 µl $NaBH_4$ (20 mg/ml) was added, and the mixture allowed to react for 30 minutes at room temperature. The reaction was terminated by the addition of 5 µl citric acid (0.65 mM), followed 15 minutes later by the addition of 5 µl NaOH (2 M) to neutralize the solution; the samples were stored at 4° C for 48 hours prior to assay. RIA of aliquots of the $NaBH_4$ reaction mixture was carried out using the $PGF_{2\alpha}$ or $PGF_{1\alpha}$ antiserum. Logit analysis of the displacement of tritiated PGF caused by reduced PGE in experimental samples was monitored by determining the recovery

of nanogram quantities of PGE from MEM. In those samples processed through XAD-2 columns but not subject to TLC, the amount of PGF present in the RIA aliquot, as previously determined, were subtracted in order to gain a more valid estimation of PGE values. In addition, MEM with appropriate ACTH and/or other pharmacological agent additions was extracted and chromatographed and subjected to $NaBH_4$ reduction; this provided a blank value which was subtracted from the experimental value.

IX. Characteristics of prostaglandin antisera

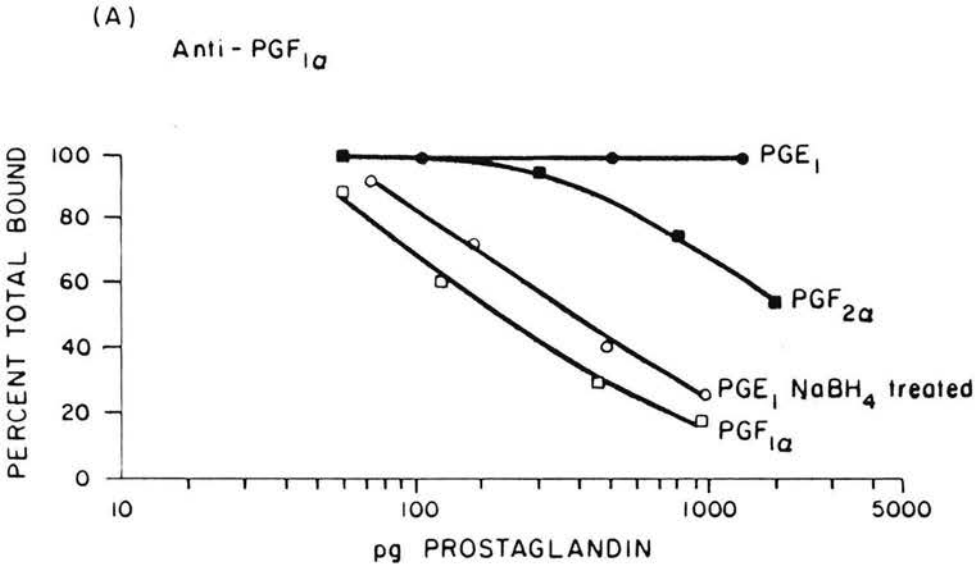
A. PGF antisera

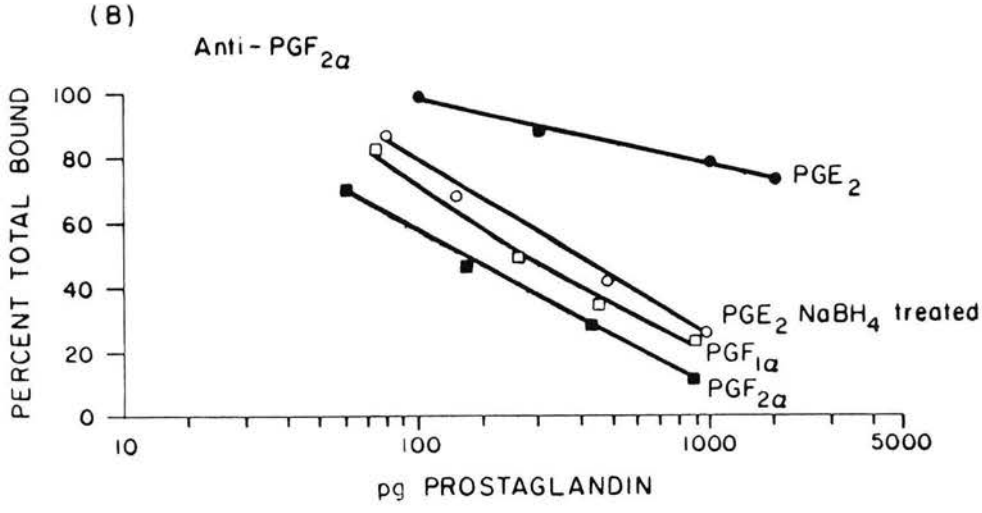
Fig. 3 depicts standard curves showing displacement of radioactive $PGF_{1\alpha}$ and $PGF_{2\alpha}$ from their corresponding antiserum. The calibration curves were linear over a range of 19-600 pg PGF when the percent binding was plotted against the logarithm of unlabeled prostaglandin added (Fig. 3). By computer analysis using a logit-log plot, the $PGF_{2\alpha}$ antibody standard curve was found to have a standard deviation in the slope of 0.08 for an unweighted regression analysis, and 0.06 for a weighted regression analysis. The linearity of the curve was further supported by the results of linear and parabolic regression tests which showed no significant nonlinearity in the curve ($p < .05$). The correlation coefficient of -0.99 indicates a strict linear relationship between the amount of prostaglandin added and the degree of displacement of radiolabeled prostaglandin from the antiserum.

The $PGF_{1\alpha}$ antiserum had a high degree of specificity in that 220 pg of $PGF_{1\alpha}$ caused 50% inhibition of ^3H-$PGF_{1\alpha}$ binding, compared to 50% inhibition achieved by 2100 pg $PGF_{2\alpha}$ (Fig. 3a). Nanogram quantities of PGE_1 failed to displace a discernible amount of labeled PGF from the $PGF_{1\alpha}$ antibody, resulting in less than 2 percent cross-reactivity (Fig. 3a; Table 1). However, when PGE_1 was reduced to $PGF_{1\alpha}$ by treatment with sodium borohydride ($NaBH_4$), a 50% displacement of bound tritium was attained with less than 400 pg (Fig. 3a). Prostaglandins of

Figure 3. Standard curves for $PGF_{1\alpha}$ and $PGF_{2}\alpha$ antisera.

Using equimolar concentrations of the prostaglandins indicated, the percent of total binding of radiolabeled PGF is shown as a function of the displacement produced by the competing prostaglandin for A) $PGF_{1\alpha}$ or B) $PGF_{2\alpha}$-binding sites.


(A)
Anti - PGF1α
PERCENT TOTAL BOUND
100
80
60
40
20
0
10
100
1000
5000
pg PROSTAGLANDIN
PGE1
PGF2α
PGE1 NaBH4 treated
PGF1α


(B)
Anti - PGF2α
PERCENT TOTAL BOUND
100
80
60
40
20
0
10
100
1000
5000
pg PROSTAGLANDIN
PGE2
PGE2 NaBH4 treated
PGF1α
PGF2α

the B series showed a cross-reactivity with the $PGF_{1\alpha}$ antibody of 20-30% (Table 1). On the other hand prostaglandins of the A series were unable to effect a 50% displacement even in nanogram concentrations (Table 1). Likewise, several PGF and PGE metabolites displayed little affinity for the $PGF_{1\alpha}$ antibody as demonstrated by a less than 2% cross-reactivity with the antiserum (Table 1). The antibody generated against $PGF_{2\alpha}$ had a greater than two-fold higher specificity for $PGF_{2\alpha}$ than $PGF_{1\alpha}$. Whereas 120 pg $PGF_{2\alpha}$ produced a 50% inhibition of ^{3}H-$PGF_{2\alpha}$ binding to the $PGF_{2\alpha}$ antibody, 280 pg $PGF_{1\alpha}$ was required for a comparable degree of inhibition (Fig. 3b; Table 2). Again, PGE_2 effected a significant binding with the $PGF_{2\alpha}$ antiserum only after PGE reduction by $NaBH_4$, which resulted in a 50% displacement with 360 pg PGE_2 (Fig. 3b). Prostaglandins of the A and B series, as well as PGF metabolites, had little or no affinity for the $PGF_{2\alpha}$ antibody, with the exception of 13,14-dihydro $PGF_{2\alpha}$, which demonstrated a 7% cross-reactivity with the $PGF_{2\alpha}$ antibody (Table 2).

B. PGE antiserum

Analysis of the PGE antibody generated in our laboratory revealed that the standard curve was linear over the range of 20-200 pg PGE_2 (Fig. 4). Although PGE_1 was more than twice as effective as PGE_2 in displacing ^{3}H-PGE_2 from the PGE antibody, PGA_1, PGA_2, PGB_1, PGB_2, $PGF_{2\alpha}$, and 13,14-dihydro PGE_1 showed negligible cross-reactivity (4% or less) (Table 3). While the antiserum is not specific for one or another of the prostaglandins of the E series, differential assays using $PGF_{1\alpha}$ and $PGF_{2\alpha}$ antisera after $NaBH_4$ reduction established that PGE_2, as opposed to PGE_1, is the predominant PGE released by feline cortical cells; thus all PGE values are expressed as PGE_2 equivalents.

X. Drugs and reagents used

β1-25 Adrenocorticotropin (ACTH) (Ciba); bovine serum albumin (fatty acid free) (Sigma); cycloheximide (Sigma); Modified Eagle's

Table 1. $PGF_{1\alpha}$ antiserum specificity.

Prostaglandin	pg required to displace 50% of bound ^{3}H-$PGF_{1\alpha}$	Relative cross-reactivity (%)
$PGF_{1\alpha}$	220	100
$PGF_{2\alpha}$	2100	10
PGE_1	>10,000	<2
PGE_2	>10,000	<2
PGA_1	>10,000	<2
PGA_2	>10,000	<2
PGB_1	720	31
PGB_2	1075	20
15-keto-$F_{2\alpha}$	>10,000	<2
13,14-dihydro-15-keto$F_{2\alpha}$	>10,000	<2
13,14-dihydro-$F_{2\alpha}$	>10,000	<2
13,14-dihydro-PGE_1	>10,000	<2

Table 2. $PGF_{2\alpha}$ antiserum specificity.

Prostaglandin	pg required to displace 50% of bound ^3H-$PGF_{2\alpha}$	Relative cross-reactivity (%)
$PGF_{2\alpha}$	120	100
$PGF_{1\alpha}$	280	43
PGE_1	7100	2
PGE_2	7700	2
PGA_1	>10,000	<1
PGA_2	>10,000	<1
PGB_1	5000	2
PGB_2	>10,000	<1
15-keto-$F_{2\alpha}$	3000	4
13,14-dihydro-15-keto$F_{2\alpha}$	4300	3
13,14-dihydro-$F_{2\alpha}$	1700	7
13,14-dihydro-PGE_1	9550	1

Figure 4. Standard curve for PGE_2 antiserum.

Using increasing concentrations of unlabeled PGE_2, the percent total binding of radiolabeled PGE_2 is shown as a function of the displacement produced by the competing prostaglandin for PGE_2-binding sites.

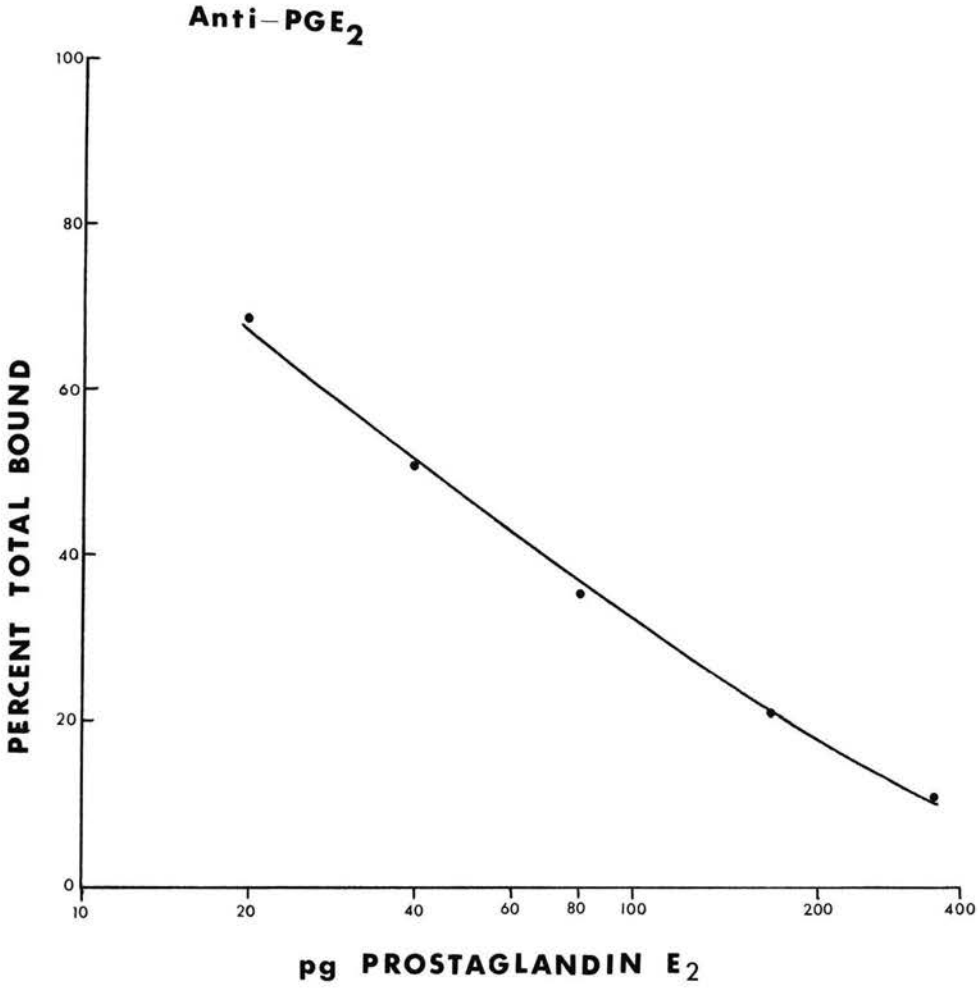

Anti-PGE2
PERCENT TOTAL BOUND
100
80
60
40
20
0
10
20
40
60
80
100
200
400
pg PROSTAGLANDIN E2

Table 3. PGE_2 antiserum specificity.

Prostaglandin	pg required to displace 50% of bound ^3H-PGE_2	Relative cross-reactivity (%)
PGE_2	42	100
PGE_1	21	200
PGA_1	>10,000	<1
PGA_2	>10,000	<1
PGB_1	3,400	1
PGB_2	7,800	<1
$PGF_{2\alpha}$	>10,000	<1
13,14-dihydro-PGE_1	1,200	4

Medium (GIBCO); 6-monobutyryl cyclic 3',5'-AMP (Sigma); NPS-ACTH from Dr. J. Ramachandran, University of California (San Francisco); pregnenolone (Sigma); non-radioactive prostaglandins (Upjohn); trypsin and lima bean inhibitor (Worthington); [^{3}H]-arachidonic acid (72 Ci/mM) (New England Nuclear); [^{3}H]-corticosterone (42 Ci/mM) (New England Nuclear); [^{3}H]-PGE_1 (87 Ci/mM) (New England Nuclear); [^{3}H]- PGE_2 (117 Ci/mM) (New England Nuclear); [^{3}H]- $PGF_{1\alpha}$ (100 Ci/mM) (New England Nuclear); [^{3}H]- $PGF_{2\alpha}$ (175 Ci/mM) (New England Nuclear). Indomethacin (Merck, Sharp & Dohme) and 5,8,11,14-eicosatetraynoic acid (RO 3-1428) (Hoffman-La Roche) 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.

RESULTS

I. Prostaglandin synthetase activity in adrenal cortex

A. Cell homogenates

The use of cell homogenates to assess the prostaglandin synthesizing activity of selected tissues has been proven successful in numerous studies. A preliminary investigation to determine if the feline adrenal cortex was active in synthesizing prostaglandins involved preparative procedures developed for similar studies in the rat kidney (Pace-Asciak, 1975) and sheep seminal vesicles (Bauminger et al., 1973). The cortex was initially separated from medulla as well as external fat and capsular tissue in order to minimize the contribution of extraneous cell types to the assessment of prostaglandin synthetase activity. In this trial study, the entire cortical region of the gland was homogenized in a KH_2PO_4-NaOH buffer solution containing reduced glutathione for its antioxidant activity and norepinephrine bitartrate which preserved the activity of prostaglandin synthetase (Pace-Asciak, 1973).

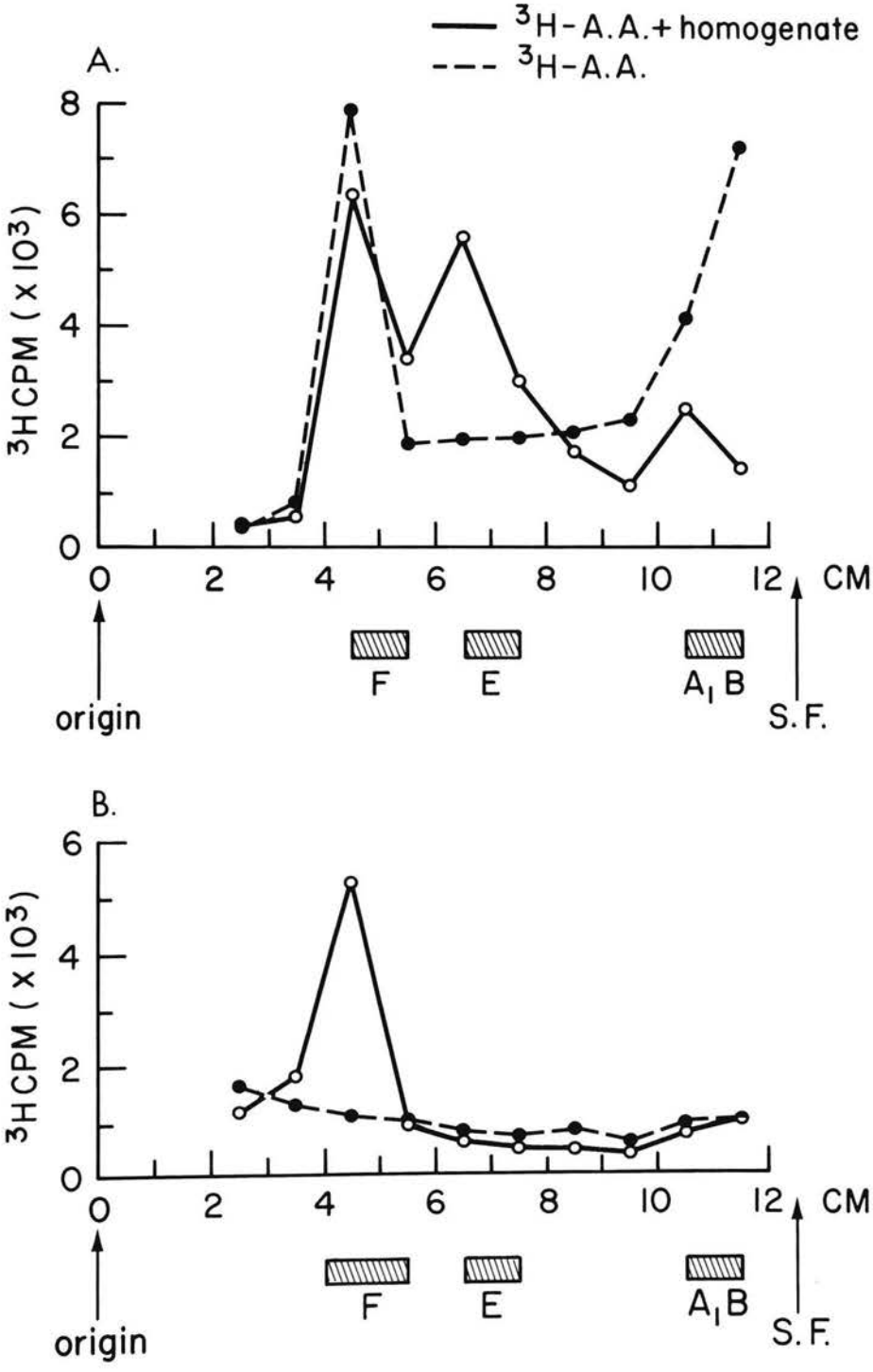
Fig. 5 shows the TLC profile resulting from the conversion of ^3H-arachidonic acid to prostaglandin-like compounds in adrenal cortical homogenates. Two peaks of radioactivity corresponding with the rf values for PGF and PGE are evident in the E-fraction from silicic acid chromatography (Fig. 5a). The spontaneous conversion of arachidonic acid to prostaglandin-like compounds is limited to a peak of radioactivity in the PGF zone of the migration profile (Fig. 5a), whereas radiolabeled PGE-like compounds appear only in homogenate extracts. Thus, cortical homogenates actively synthesize the PGE product, while the PGF occurring in the E-fraction arises at least in part as an artifact of arachidonate autoconversion. Unconverted arachidonic acid also appears as a radioactive peak in the A,B zone on TLC (Fig. 5a). In contrast, Fig. 5b depicts a single peak of radioactivity from the F-fraction migrating in the PGF zone on TLC. There is no spontaneous arachidonic acid

Figure 5. Conversion of ^{3}H-arachidonic acid into prostaglandin-like compounds in adrenal cortical homogenate.

Tritiated arachidonic acid (^{3}H-A.A.) (4μCi) was incubated in the presence and absence of cortical homogenate for 30 min at 37°C. The organic extract was dried and the prostaglandins of the resuspended samples separated by silicic acid and thin layer chromatography. A radioactive profile was obtained as described in Methods.

Fig. 1a represents two peaks of radioactivity corresponding to the rf values of PGF and PGE in the E fraction obtained from silicic acid chromatography. Fig. 1b depicts a single peak of radioactivity from the F fraction obtained by silicic acid chromatography. The dotted line in Fig. 1 represents the radioactive profile obtained after incubation of ^{3}H-arachidonic acid in the absence of homogenate.

Hatched bars indicate prostaglandin migration pattern on thin layer chromatography. The solvent front is represented as S.F.


A.
^{3}H-A.A.+ homogenate
^{3}H-A.A.
^{3}HCPM (X 10^3)
0
2
4
6
8
10
12
CM
origin
F
E
A$_1$B
S.F.
B.
^{3}HCPM (X 10^3)
0
2
4
6
8
10
12
CM
origin
F
E
A$_1$B
S.F.

conversion to a similarly migrating PGF substance (Fig. 5b). Thus, cortical cell homogenates synthesize a PGF-like compound(s) from a fatty acid precursor. These preliminary results point to the presence of an enzymatic synthesizing mechanism in the feline adrenal cortex, which is responsible for the synthesis of PGF and PGE-like compounds.

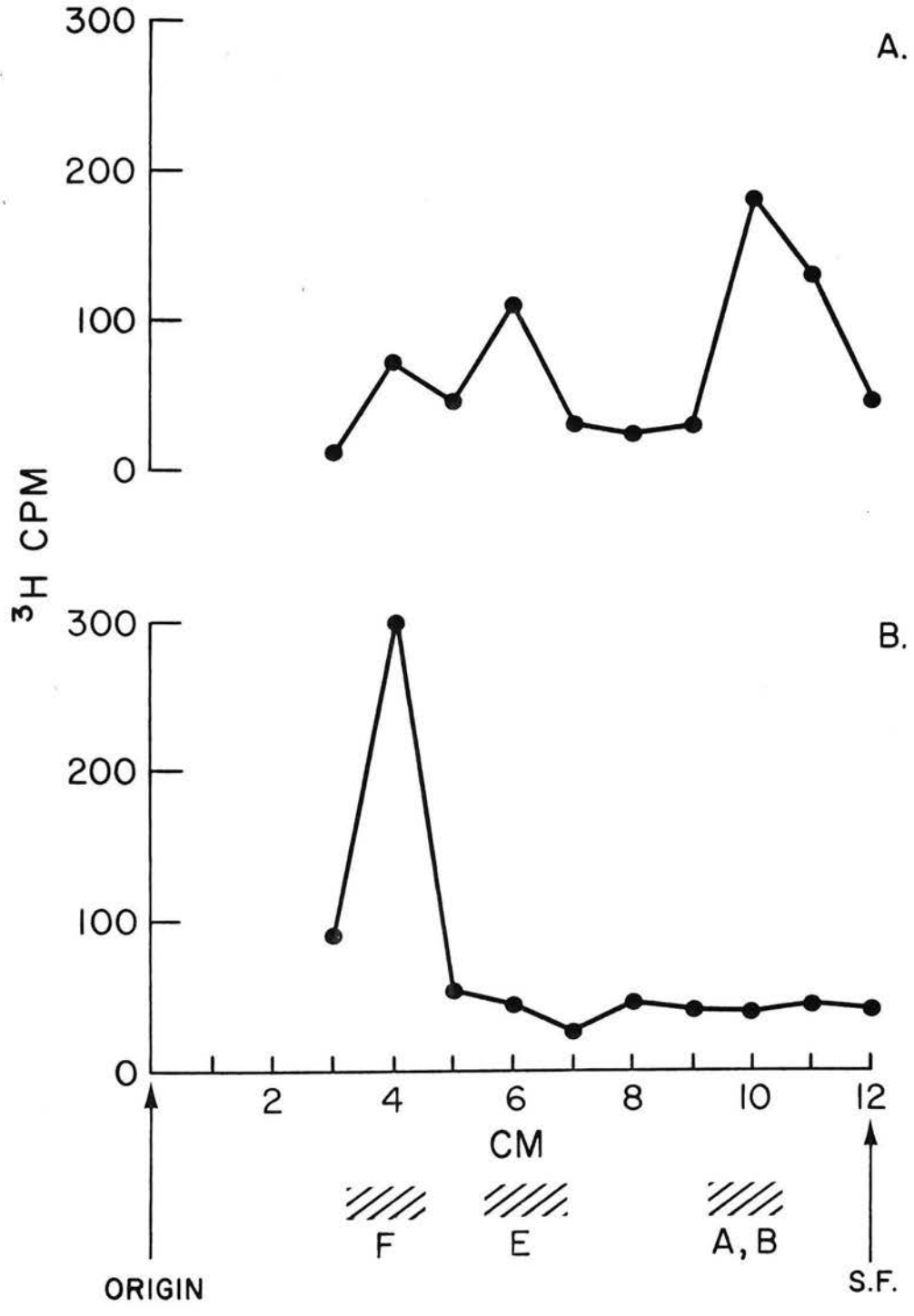
B. Cell suspensions

Since a cortical homogenate preparation was capable of converting arachidonic acid into prostaglandin-like compounds, the trypsin dispersed feline cortical cell preparation was used to demonstrate whether intact cells could perform a similar conversion. In addition, the effect of ACTH stimulation of steroidogenesis upon prostaglandin synthesis could be investigated in this whole-cell suspension and a comparison made between intracellular products of arachidonate metabolism and those compounds released from cortical cells. Following the incubation of isolated cortical cells in MEM containing ^{3}H-arachidonic acid, in the absence or presence of ACTH, the cells and their corresponding suspension media were separated by centrifugation for isolation and identification of intracellular and extracellular prostaglandins, respectively.

The cell pellets were homogenized and partitioned and the resulting extract was fractionated by silicic acid chromatography into three components corresponding to the prostaglandin AB, E, and F fractions. The AB fraction was discarded since it contained the major portion of the unmetabolized ^{3}H-arachidonic acid, which hampers any attempt to isolate and quantitate PGA or PGB using TLC methods. When the E fraction was subjected to TLC, three major peaks of radioactivity were found (Fig. 6a) corresponding to the reference markers PGF (rf 0.27-0.37) and PGE (rf 0.46-0.57) and PGA and/or PGB (rf 0.77-0.85). In contrast, the F fraction produced a single peak of radioactivity in the region of the plate where PGF migrates (Fig. 6b). The occurrence of a radioactive peak corresponding to PGF in the E fraction is ascribed to discernible amounts of standard PGF being recovered in this fraction following

Figure 6. Conversion of radioactive precursor into prostaglandins by isolated cat cortical cells.

Trypsin-dispersed cells (2.5×10^5 / beaker) were incubated in MEM for seventy-five min in the presence of ACTH (125μU/ml) plus ^{3}H-arachidonic acid (4μCi/ml). Contents of six beakers were pooled, and following centrifugation, the prostaglandins in the pellet were extracted. The E fraction (A) and F fraction (B) were separated by silicic acid chromatography and run separately on thin layer plates with the resulting radioactive profiles. Each sample was counted for 10 min and background subtracted from the total counts.


300
200
100
0
A.
^{3}H CPM
300
200
100
0
B.
2
4
6
8
10
12
CM
F
E
A, B
ORIGIN
S.F.

column chromatography (see Methods).

In order to ascertain the nature and extent of prostaglandin release from cortical cells, the incubation medium was extracted separately from cell homogenates and processed through silicic acid and thin layer chromatography. Total counts isolated from extracts of incubation media were some ten times higher than those isolated from cortical cell homogenates (compare Fig. 6 and 7), indicating that prostaglandins synthesized intracellularly are readily extruded into the extracellular fluid. Analysis of the E fraction revealed a peak of radioactivity which corresponded to that of PGE and a smaller peak with mobility properties similar to PGA and PGB (Fig. 7a). The F fraction taken from silicic acid chromatography of incubation media produced a single peak of radioactivity in the PGF zone (Fig 7b).

Although the prostaglandins contained within the first two radioactive peaks were indistinguishable from PGF and PGE, the prostaglandins represented by the third radioactive peak could not be positively identified. However, the possibility that the products in this zone were derived from biosynthesized PGE rather than arachiconic acid was entertained, since PGE is a labile substance which is convertible to other derivatives, including PGA and PGB (Levine, 1973). This supposition was tested by determining the fate of exogenous PGE after several extraction and elution procedures. $^{3}H\text{-}PGE_1$ was added to the incubation medium containing cortical cells and then extracted and isolated in the same manner as the experimental samples. Fig. 8 depicts the thin layer chromatogram of the resulting E fraction. A majority of the counts (57%) was localized in the E zone and only 4% was found in the F zone; however, 40% of the counts were recovered from the AB zone. By contrast, unprocessed $^{3}H\text{-}PGE_1$ gave a single radiolabeled peak in the E region of the thin layer chromatogram. This indicates that prior to TLC a portion of the PGE formed in the cell suspension is converted to a derivative with a polarity similar to PGA and PGB.

Figure 7. Release of biosynthesized prostaglandins from isolated cortical cells.

The experimental procedure for extracting and isolating prostaglandins in the incubation medium was essentially the same as described in fig.6 , except that following centrifugation the supernatant rather than the pellet was processed (see Methods).

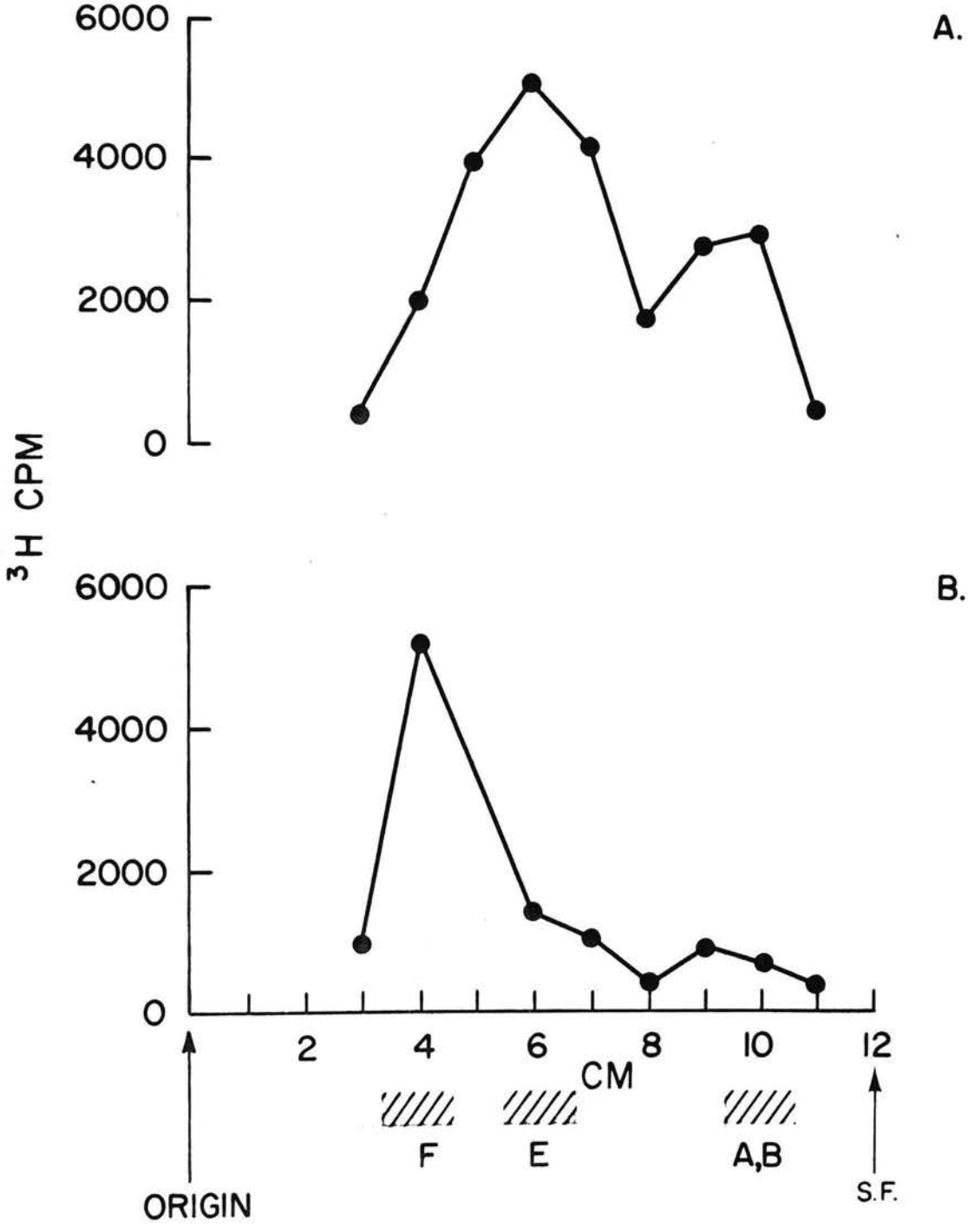
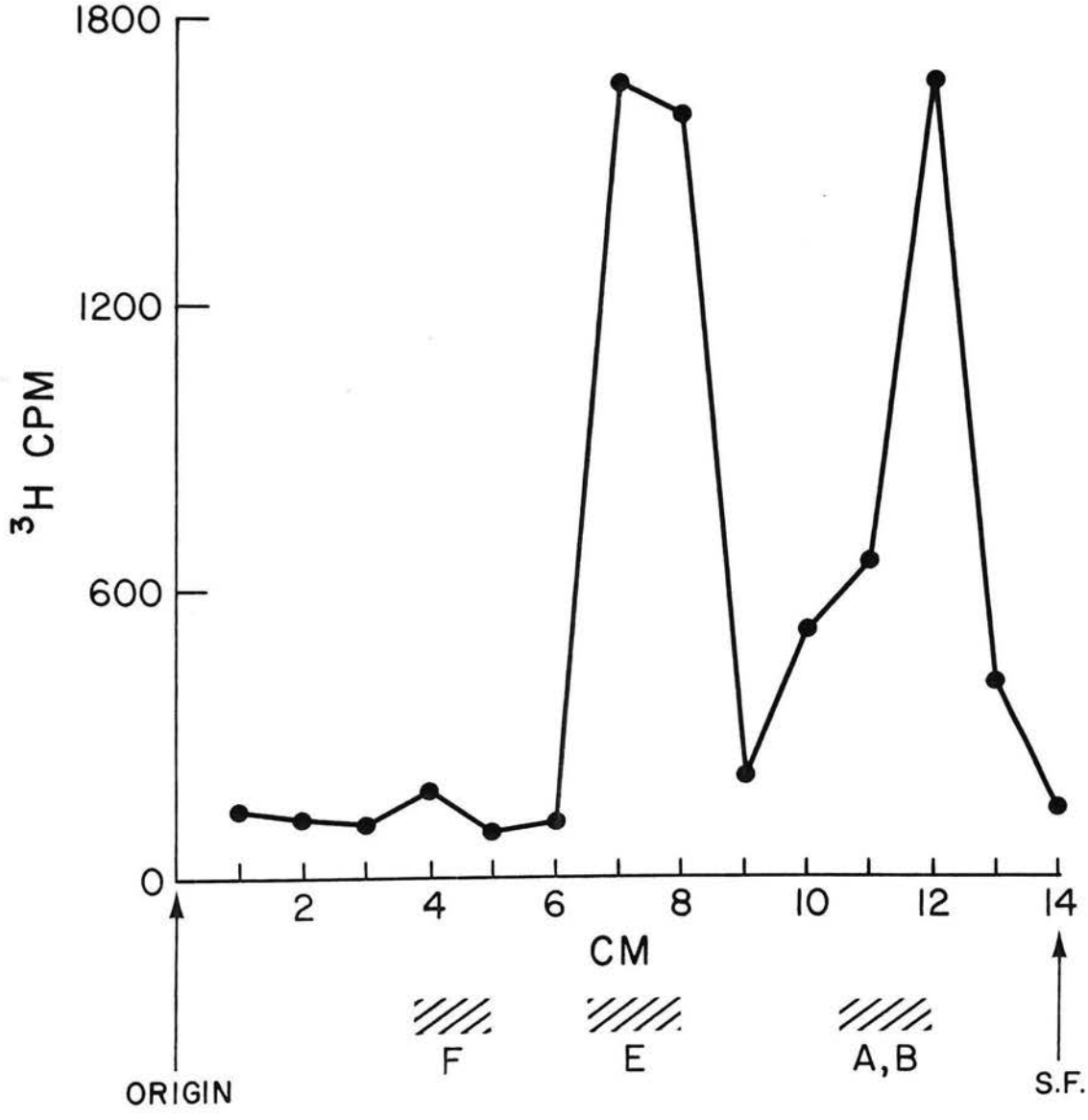

A.
B.
6000
4000
2000
0
^{3}H CPM
2
4
6
8
10
12
CM
F
E
A,B
ORIGIN
S.F.

Figure 8. Isolation of radioactive prostaglandins from medium bathing cortical cells following the addition of ^{3}H-PGE_1.

Cells were incubated with ^{3}H-PGE_1 (0.02μCi/ml) for seventy-five minutes; the medium obtained from beakers was then processed for thin layer chromatography, with the resulting radioactive profile.


1800
1200
600
0
^{3}H CPM
2
4
6
8
10
12
14
CM
F
E
A,B
ORIGIN
S.F.

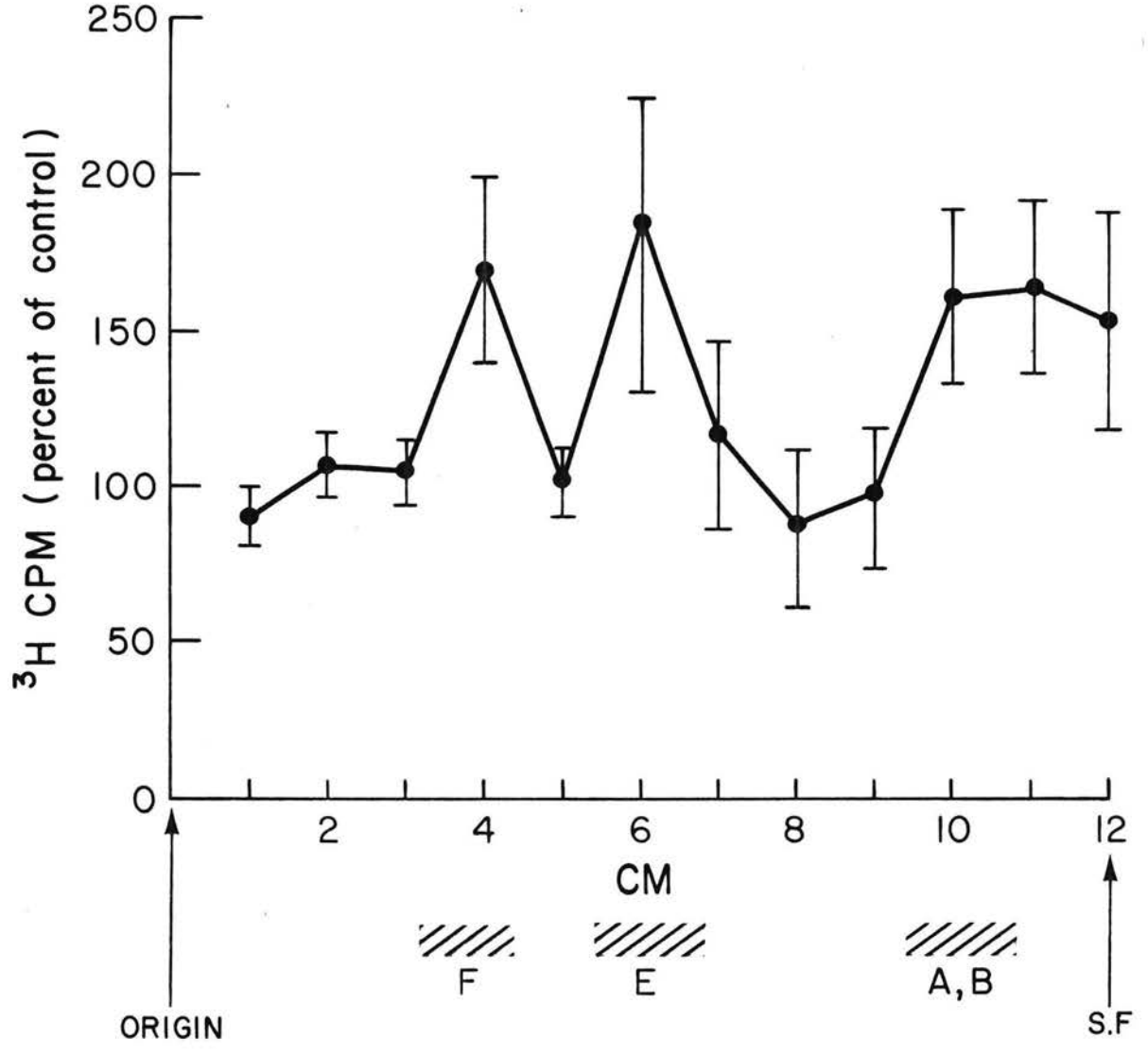
C. The effect of ACTH on ^{3}H-arachidonic acid incorporation

Having identified PGE, PGF, and a third component with a polarity similar to PGA or PGB in cortical cell homogenates and incubation medium, experiments were carried out to determine whether ACTH could influence the conversion of arachidonic acid into one or another of these prostaglandins. When fractions from unstimulated cells were compared by thin layer analysis with their respective fractions obtained from stimulated cells, submaximal steroidogenic concentrations of ACTH (125-250 μU) were found to augment the conversion of arachidonic acid into PGE and PGF (Fig. 9). The amount of label incorporated into PGF and PGE by cells exposed to ACTH was 158 and 193 percent respectively, of that incorporated by unstimulated cells. An ACTH-induced increase in the incorporation of label in the third zone was also observed (Fig. 9). Since this radioactive peak may represent degradation products of PGE, as discussed previously, the increase in PGE turnover induced by ACTH may be significantly greater than indicated by the radioactivity in the E zone alone.

TLC analysis of radiolabeled prostaglandins extracted from the incubation medium suggested a trend in incorporation similar to that described for the cell homogenates, although quantitatively the effects were less pronounced. The amount of labeled PGE and PGF released by ACTH-stimulated cells was 121 ($\pm$ 22) and 125 ($\pm$ 4) percent respectively, of the unstimulated cell basal release (n=4). The rather small effect of ACTH on prostaglandin release observed by analysis of the incubation medium suggests that a portion of the newly-synthesized prostaglandin may be metabolized intracellularly rather than released, and/or that the release of substantial amounts of unlabeled, as well as labeled, prostaglandin dilutes the detectable release of the radioactive product.

Figure 9. Radiochromatogram of ACTH-facilitated prostaglandin synthesis.

Cortical cells were incubated with ^{3}H-arachidonic acid with or without ACTH. The contents of six beakers containing unstimulated and stimulated cells were pooled separately into two aliquots, and the prostaglandins of the cell homogenates were extracted and separated by silicic acid and thin layer chromatography. Radioactivity (cpm) in each zone of ACTH-treated cells is expressed as a percent of the radioactivity in the corresponding zone of unstimulated cells. To simplify representation of the data, the chromatograms obtained from the E and F fractions were combined. Each mean value (± S.E.) was derived from four different cell preparations.


250
200
150
100
50
0
^{3}H CPM (percent of control)
2
4
6
8
10
12
CM
F
E
A, B
ORIGIN
S.F

II. Identification of adrenocortical prostaglandins by radioimmunoassay

A. Prostaglandin F

Having characterized the PGF antibodies regarding their specificity to bind $PGF_{1\alpha}$ or $PGF_{2\alpha}$, PGF determinations were carried out on samples of media incubating control and ACTH-stimulated cat cortical cells. The responsivity of these cells to ACTH was evidenced by its ability to enhance steroidogenesis from 21 ($\pm$ 4) to 110 ($\pm$ 24) ng/2.5×10^5 cells. Direct RIA using $PGF_{2\alpha}$ antiserum gave mean PGF values of 450 and 912 pg/2.5×10^5 cells for control and ACTH-stimulated cells, respectively (Table 4). When the more specific $PGF_{1\alpha}$ antiserum was used to assay the same samples of media, much lower PGF values were obtained (Table 4). Thus, the average prostaglandin value determined using the $PGF_{1\alpha}$ antiserum on samples treated with ACTH (132 $\pm$ 39 pg/2.5×10^5 cells) was only 14% of that obtained with the $PGF_{2\alpha}$ antiserum.

In order to assess the possibility that the higher PGF values obtained with the $PGF_{2\alpha}$ antiserum could be quantitatively accounted for by a $PGF_{1\alpha}$ cross-reaction, samples of media were analyzed with $PGF_{2\alpha}$ antibody and radioactive $PGF_{2\alpha}$ using the $PGF_{1\alpha}$ standards, in addition to orthodox analysis using the $PGF_{1\alpha}$ and $PGF_{2\alpha}$ antisera with homologous labeled and unlabeled prostaglandins. Using $PGF_{2\alpha}$ antiserum the average amount of $PGF_{2\alpha}$ released in response to ACTH was determined to be 676 ($\pm$ 120) pg/2.5×10^5 cells (n=3). Conducting parallel assays using $PGF_{1\alpha}$ standards established that 2276 pg of $PGF_{1\alpha}$ must be released to produce a comparable displacement of ^3H-$PGF_{2\alpha}$ antibody (Table 5). However, only 6% of this amount of $PGF_{1\alpha}$ (133 pg) was found using the specific $PGF_{1\alpha}$ antiserum. This indicates that, most, if not all of the PGF measured with $PGF_{2\alpha}$ antiserum represents $PGF_{2\alpha}$. Whether the prostaglandin measured with the $PGF_{1\alpha}$ antiserum is truly $PGF_{1\alpha}$ or due solely to the obviously larger amount of $PGF_{2\alpha}$ cannot be unqualifiedly ascertained, but it is clear from this differential analysis that $PGF_{2\alpha}$ is the predominant species.

Table 4. PGF release by isolated cortical cells in the presence and absence of ACTH.

Expt.	$PGF_{1\alpha}$		$PGF_{2\alpha}$	
	Control	ACTH	Control	ACTH
1	<2	110	1047	1666
2	<3	227	178	721
3	54	42	308	608
4	79	149	270	652
Mean	<35	132	450	912
±S.E.	±19	±39	±201	±252

Equal quantities of adrenocortical cells (2.5×10^5/ml) were incubated in MEM for 60 min in the presence and absence of ACTH (50-250 μU). The medium was analyzed for $PGF_{1\alpha}$ and $PGF_{2\alpha}$ using the corresponding antiserum. All values are expressed as pg/2.5×10^5 cells.

Table 5. Differential analysis of PGF release.

	$PGF_{2\alpha}$-Antiserum		$PGF_{1\alpha}$-Antiserum
	$PGF_{2\alpha}$ (pg)	$PGF_{1\alpha}$ (pg)	$PGF_{1\alpha}$ (pg)
Exp.			
1	652	2147	154
2	895	3033	150
3	482	1650	96
Mean	696	2276	133
± S.E.	±120	±404	±19

Samples of media incubating cortical cells exposed to ACTH (125 μU) for 60 min were assayed using $PGF_{1\alpha}$ and $PGF_{2\alpha}$ antisera with homologous radiolabeled and unlabeled prostaglandins. The same samples were also analyzed with $PGF_{2\alpha}$ antiserum and ^{3}H-$PGF_{2\alpha}$, but using $PGF_{1\alpha}$ standards to calculate the PGF equivalents. All values are given in pg/2.5×10^{5} cells.

In order to confirm the reliability of direct RIA of the samples of incubation media, the prostaglandins of certain samples were extracted and separated by TLC prior to RIA. The samples eluted from the F zone contained quantities of prostaglandins which were comparable to those measured by direct assay (compare Tables 4 and 6). While these experiments again illustrate the stimulating effects of ACTH, they also show that the enhanced PGF release is antagonized by indomethacin (Table 6). The ability of the assay method to demonstrate a depression of prostaglandin synthesis in the presence of the well-known inhibitor of prostaglandin synthetase provides additional confirmation of the reliability of techniques employed to measure PGF. The parallel displacement of labeled prostaglandin from $PGF_{2\alpha}$ antibody in response to different size sample aliquots (Table 7) also confirms the reliability of the radioimmunoassay technique for accurately determining prostaglandin release from adrenal cortical cells.

B. Prostaglandin E

Information regarding the relative amounts of PGE_1 and PGE_2 was initially obtained by the differential assay of media using the $PGF_{1\alpha}$ and $PGF_{2\alpha}$ antibodies, after the conversion of PGEs to their corresponding PGF equivalents by $NaBH_4$ (Table 8). The average PGE_2 content of media incubating stimulated cells (2802 pg) was significantly higher than that obtained following ether extraction, separation by TLC, and RIA measurement using PGE antiserum (428 pg) (Table 9). In order to discern the basis for the quantitative differences obtained using these two methods for assaying PGE, $NaBH_4$ reduction and subsequent assay using $PGF_{2\alpha}$ antiserum were carried out on samples which were processed through TLC; the mean PGE content in media with cells exposed to ACTH (50-175 uU) for 60 minutes was 409 ($\pm$ 140) pg/2.5×10^5 cells (n=3). This indicates that some assayable factor was removed from the PGE fraction during TLC. Despite the quantitative differences found with these two methods, both assays showed that ACTH was able to elicit

Table 6. ACTH-induced $PGF_{2\alpha}$ release and its inhibition by indomethacin.

Expt.		Control	$PGF_{2\alpha}$ ACTH	ACTH + Indomethacin
1		108	342	<2
2		243	439	107
3		1153	1431	799
4		116	281	-
5		115	131	-
	Mean	347	525	
	± S.E.	±203	±232	

Adrenocortical cells were incubated in MEM for 60 min in the presence or absence of ACTH (50-250 μU) and/or indomethacin (3×10^{-5} M). The samples were extracted with ether and processed through TLC; the PGF was eluted with methanol and assayed using $PGF_{2\alpha}$ antiserum. Values are expressed as pg/2.5×10^5 cells.

Table 7. Parallel displacement of labeled prostaglandin from antisera by increasing amounts of sample.

$PGF_{2\alpha}$ Antibody

Sample	Aliquot (µl)	% binding	$PGF_{2\alpha}$ (pg)
1	50	59%	66
	100	38%	150
2	50	55%	79
	100	29%	219
3	50	55%	78
	100	38%	155

PGE_2 Antibody

Sample	Aliquot (µl)	% binding	PGE_2 (pg)
1	25	18%	437
	50	10%	900
2	25	19%	418
	50	11%	817
3	25	50%	83
	50	34%	169

Varying aliquots of adrenal cell incubation media or samples of ether-extracted media subjected to TLC were assayed for prostaglandin content using the $PGF_{2\alpha}$ or PGE_2 antibody, respectively. The percent binding of labeled prostaglandin in the presence of different sample sizes is indicated. Picogram (pg) values for each sample have been corrected for the 'blank' contribution of varying size aliquots in the assay.

Table 8. PGE release by cortical cells in the presence and absence of ACTH

Expt.		PGE_1 (pg) Control	PGE_1 (pg) ACTH	PGE_2 (pg) Control	PGE_2 (pg) ACTH
1		77	46	1324	2711
2		166	<6	1466	2463
3		158	395	2339	3234
	Mean	133	149	1710	2802
	± S.E.	±28	±23	±317	±227

Following incubation of equal numbers of cells for 60 min in the presence or absence of ACTH (50-250 μU), the incubation medium was processed through XAD-columns; the ethanolic eluate was dried, resuspended in buffer and the PGE equivalents reduced to their corresponding PGFs with $NaBH_4$ (see Methods) and assayed using $PGF_{1\alpha}$ and $PGF_{2\alpha}$ antisera. All values were expressed as pg/2.5×10^5 cells.

Table 9. ACTH-induced PGE_2 release and its inhibition by indomethacin.

	PGE_2 (pg)		
Expt.	Control	ACTH	ACTH + Indomethacin
1	733	811	259
2	88	165	<2
3	298	309	-
Mean	373	428	-
± S.E.	±190	±196	-

Equal numbers of cells were incubated in MEM for 60 min in the presence or absence of ACTH (50-250μU) and/or indomethacin (3×10^{-5}M). After the prostaglandins were extracted with ether, the E prostaglandins were separated on TLC and assayed using anti-PGE antibody. Values are expressed in pg/2.5×10^5 cells.

a modest enhancement of PGE release (Tables 8 and 9), and indomethacin blunted this enhancement (Table 9).

The development of a suitable PGE antibody during the course of these studies eventually allowed PGE analysis by direct assay of the incubation medium (see Methods). With this antibody, a parallel displacement of labeled PGE_2 was observed with graded increases in sample volume (Table 7). The validity of this method is further attested to by the fact that the values obtained by direct assay of the incubation medium (299 ± 97 pg/2.5×10^5 cells) compared favorably with the RIA quantitation of these same samples after ether extraction and separation of the PGEs by thin layer chromatography (209 ± 106 pg) (n=4). This indicates that the prostaglandin values obtained using this PGE antibody for direct RIA of incubation media are largely, if not entirely, PGE species.

C. Intracellular prostaglandin levels

Although previous studies have clearly established that prostaglandin release is the result of de novo prostaglandin synthesis (Piper and Vane, 1971), a few experiments were carried out to substantiate this fact in feline cortical cells by comparing the amount of prostaglandin remaining in cortical cells to the amount released to the medium following a 60 minute exposure to ACTH (250 μU). The average amounts of $PGF_{2\alpha}$ (44 pg) and PGE_2 (41 pg) contained intracellularly was only about 15% of the amount of $PGF_{2\alpha}$ (309 pg) and PGE_2 (278 pg) released (Table 10). Comparably low amounts of $PGF_{2\alpha}$ (36 pg) and PGE_2 (55 pg) were measured intracellularly in the absence of ACTH (Table 10).

III. Temporal relation between prostaglandin and steroid release

Preliminary experiments using adrenocortical cell suspensions demonstrated that measurable increases in prostaglandin release occurred during the first 15-30 minutes of exposure to ACTH. However, earlier studies using this same preparation (Warner and Rubin, unpublished) had shown that increases in steroid release also occurred during these

Table 10. Comparison of intra- and extracellular prostaglandin levels.

Expt.	Control				ACTH			
	$PGF_{2\alpha}$ (pg)		PGE_2 (pg)		$PGF_{2\alpha}$ (pg)		PGE_2 (pg)	
	Pellet	Supernatant	Pellet	Supernatant	Pellet	Supernatant	Pellet	Supernatant
1	24	338	40	268	62	501	<10	257
2	10	324	79	300	6	310	7	424
3	76	125	85	384	65	116	107	152
Mean ± S.E.	36 ±20	262 ±69	55 ±27	317 ±35	44 ±19	309 ±11	41 ±32	278 ±79

Values for each experiment were obtained from medium incubating cortical cells or from ether-extracts of cells incubated for 90 min at 37° C in the presence or absence of ACTH (250 μU). Values are expressed as pg/2.5×10^5 cells.

same time intervals; thus, isolated cortical cells were deemed not to be the optimal system for studying the temporal sequence of prostaglandin and steroid release. An alternative approach to this problem was to study the dynamics of prostaglandin and steroid release in the isolated intact perfused cat adrenal gland.

The perfused feline adrenal displays a protracted pattern of steroid release, with maximum steroidogenesis occurring some 30-40 minutes following exposure to ACTH (Fig. 10). By contrast, $PGF_{2\alpha}$ release rapidly increased during perfusion with ACTH. After the stimulus was removed, $PGF_{2\alpha}$ release remained elevated over the next 10 minutes and then declined to basal levels (Fig. 10). In some experiments, corticosteroid secretion rates were still markedly increased some 90 minutes after the removal of ACTH, despite the fact that prostaglandin release had reverted to basal levels one hour before. Although these experiments elucidate the temporal sequence of PGF release, the barely measurable amounts of prostaglandin which were detected in the perfusate from these same experiments by direct assay using PGE antiserum made it impossible to quantitate the temporal sequence of PGE release.

IV. The effects of inhibitors of prostaglandin synthesis

A. Indomethacin

1. Basal steroid levels. Since one purpose of this investigation was to explore the relationship between steroidogenesis and prostaglandin synthesis, it was important to determine the effect of prostaglandin synthesis inhibitors upon steroid release. Indomethacin in the concentration range of 3×10^{-9} to 3×10^{-5} M was capable of augmenting basal steroid release (Table 11). The stimulant effect was small and inconsistent at the lowest concentration tested (3×10^{-9} M), but increased approximately two-fold with 3×10^{-7} and 3×10^{-5} M indomethacin, respectively (Table 11). This facilitatory action of indomethacin was not demonstrable to the same degree in every experiment, but was

Figure 10. Time course of $PGF_{2\alpha}$ and corticosteroid release from the perfused cat adrenal gland.

Left adrenal glands were perfused *in situ* with Locke's solution for an additional 50 min. The perfusate was collected during the 8 min perfusion with ACTH and at 10 min intervals thereafter. Corticosteroid (solid line) was extracted from 1 ml aliquots and assayed by competitive protein binding. $PGF_{2\alpha}$ (broken line) determinations were made by direct radioimmunoassay of 400 µl aliquots of perfusate. All values are expressed as percent of basal values obtained from perfusate collected during a 10 min interval immediately prior to exposure to ACTH. Each point represents the average rate of release (± S. E.) during the 8 or 10 min collection period from 4 different preparations.

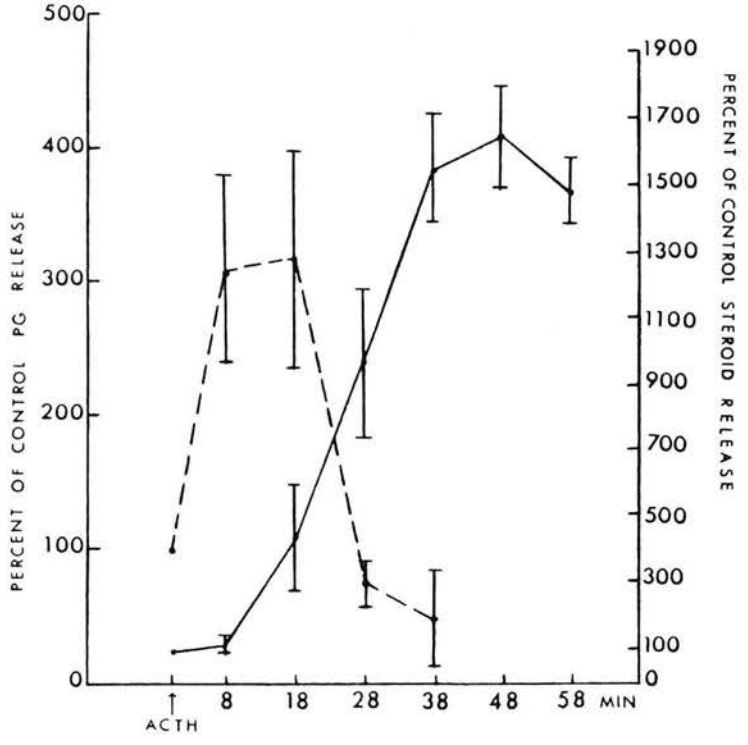

500
400
300
200
100
0
PERCENT OF CONTROL PG RELEASE
1900
1700
1500
1300
1100
900
700
500
300
100
0
PERCENT OF CONTROL STEROID RELEASE
ACTH
8
18
28
38
48
58
MIN

Table 11. The effect of indomethacin on basal corticosteroid and prostaglandin release.

Indomethacin (x3M)	Steroid	PGF	PGE
	(percent of basal)	(percent 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.) are expressed as percent increase over values obtained in the absence of drug.
Number of observations are indicated by number in parenthesis; each value was obtained from a different preparation.

generally manifest in those preparations showing a greater responsivity to ACTH.

2. ACTH-induced steroid production. If prostaglandins act as modulators of ACTH action, then interference with their synthesis should be reflected in changes in the physiological response to ACTH. Accordingly, strikingly low concentrations of indomethacin caused marked effects on ACTH-induced steroidogenesis in isolated cortical cells. Results from a single experiment employing a low ACTH concentration (25 μU) are depicted in Figure 11a; they show a marked facilitation of steroid release in the presence of 3×10^{-8} M indomethacin. More detailed analysis of this potentiation using near maximal ACTH concentrations (75-250 μU) demonstrated that 3×10^{-9} M indomethacin augmented ACTH-induced steroid release by almost two-fold (Fig. 12a). This facilitatory action of the drug was transformed into an inhibitory action as its concentration was increased (Fig. 11b; 12a). ACTH-evoked steroid release in the presence of indomethacin (3×10^{-7} M) was less than in its absence and in the presence of 3×10^{-5} M indomethacin, a slightly greater inhibition was detected (Fig. 12a). Despite the fact that low and high concentrations of indomethacin caused marked alterations in the steroidogenic response to ACTH, these same indomethacin concentrations did not elicit similar alterations in the steroidogenic response to exogenous PGE_2 (Fig. 11a and 11b).

3. Basal and ACTH-induced prostaglandin release. Steroidogenic concentrations of ACTH elicited dose-related increases in PGF and PGE release (Table 12, expt. 1). An indomethacin concentration (3×10^{-9} M) which facilitated ACTH-induced steroid release, likewise potentiated the effects of ACTH on PGF and PGE release (Table 12, expt. 2); in the same experiment this facilitatory effect of indomethacin on prostaglandin release was converted to inhibition by a high concentration of indomethacin (3×10^{-5} M) (Table 12, expt. 2). Experiment 3 of Table 12 illustrates the potent inhibitory action of indomethacin even in the

Figure 11. Comparison of the effects of indomethacin on ACTH and PGE_2-induced steroid release from isolated adrenal cortical 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 (25μU) or PGE_2 (2×10^{-4}M), (b) ACTH (125 μU) or PGE_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 PGE_2. These results were obtained from 2 different preparations.

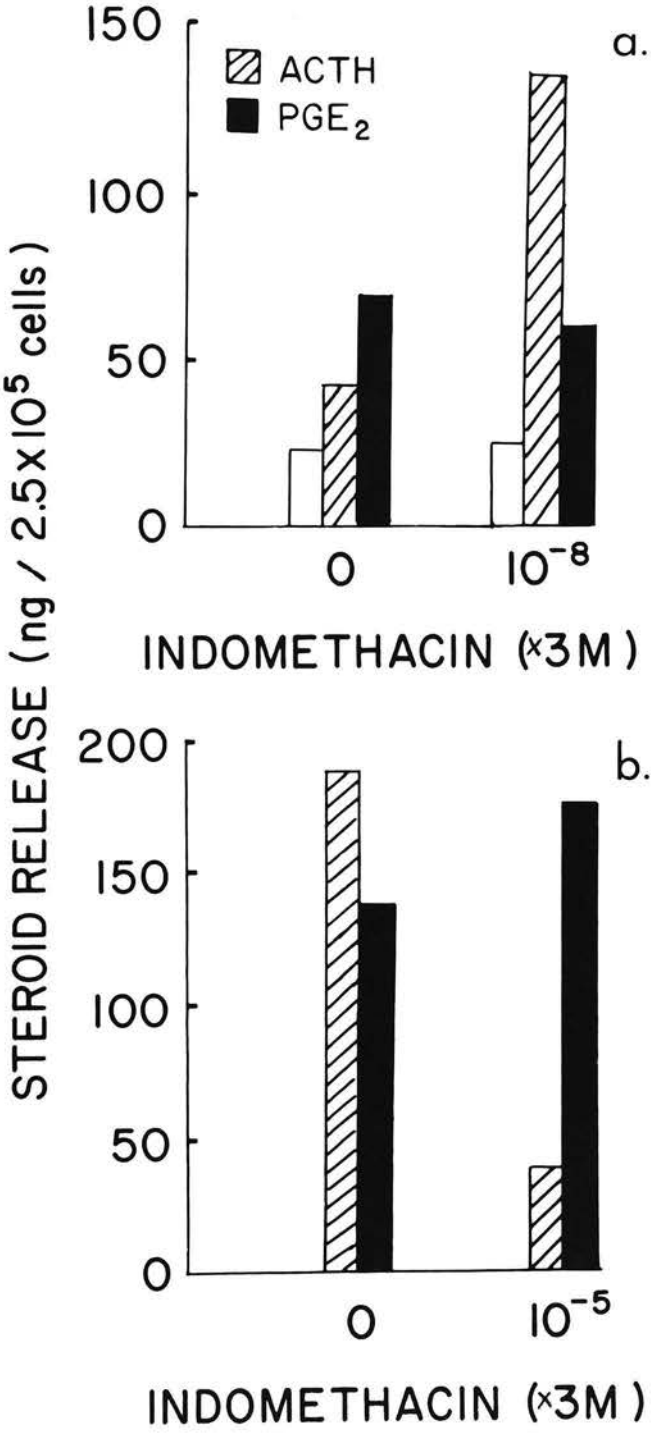

a.
ACTH
PGE2
150
100
50
0
0
10-8
INDOMETHACIN (×3M)
b.
200
150
100
50
0
0
10-5
INDOMETHACIN (×3M)
STEROID RELEASE (ng / 2.5x10^5 cells)

Figure 12. The effects of indomethacin on 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.

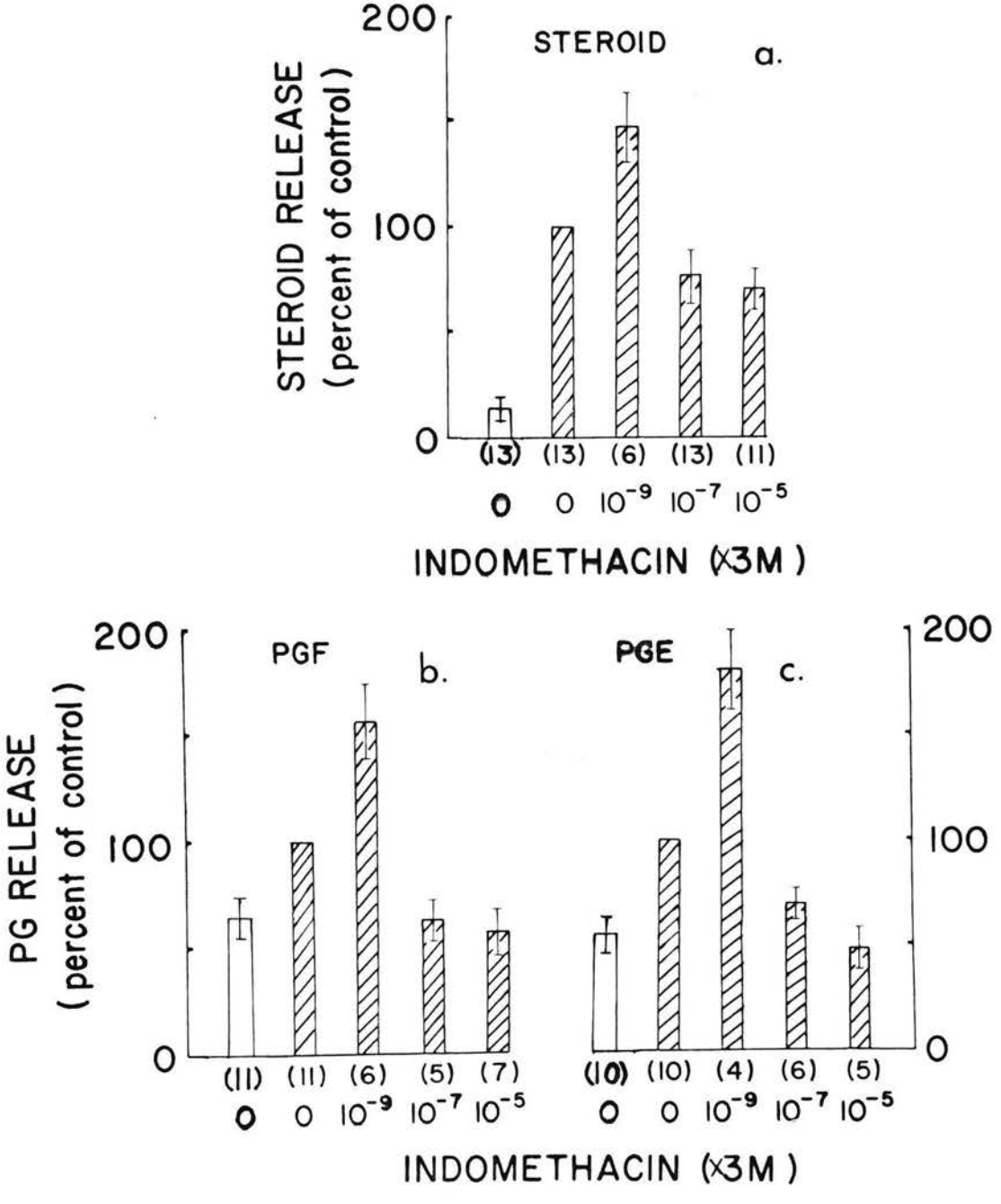

STEROID
a.
STEROID RELEASE
(percent of control)
200
100
0
(13) (13) (6) (13) (11)
0 0 10^{-9} 10^{-7} 10^{-5}
INDOMETHACIN (×3M)
PGF
b.
PGE
c.
PG RELEASE
(percent of control)
200
100
0
(11) (11) (6) (5) (7)
(10) (10) (4) (6) (5)
0 0 10^{-9} 10^{-7} 10^{-5}
INDOMETHACIN (×3M)

Table 12. Prostaglandin release from unstimulated cortical cells, and from cells exposed to ACTH with or without indomethacin.

Expt.	ACTH (μU)	Indomethacin (x3M)	PGF (pg)	PGE (pg)
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}[a]	<6	<6

Values for each experiment were obtained from medium incubating equal numbers of cells for 1 hour at 37°C and are expressed as pg/2.5x10^5 cells.

[a]Indomethacin present for 1 hour prior to addition of ACTH and then removed from the medium.

concentration of 3×10^{-7} M. The irreversible nature of this inhibition is evidenced by the finding that after a 1 hour exposure to indomethacin, cells re-incubated in an inhibitor-free medium were unable to release measureable amounts of PGE or PGF in response to ACTH (Table 12, expt. 4), despite large increases in steroid release.

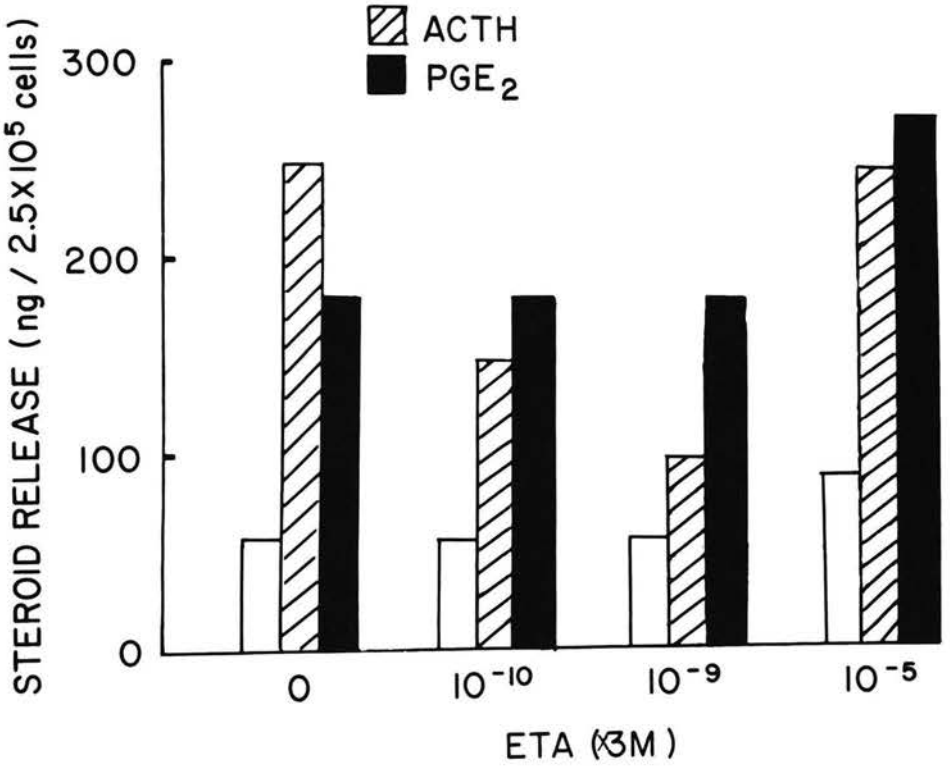
Figures 12b and c summarize the results of all experiments, including those presented in Table 12. Indomethacin at a concentration of 3×10^{-9} M potentiated ACTH-evoked release of prostaglandins by almost two-fold. Higher indomethacin concentrations (3×10^{-7} and 3×10^{-5} M) depressed ACTH-evoked PGE and PGF values to below basal levels; with these same indomethacin concentrations, ACTH still increased steroid release by six-fold or more above basal levels (Fig. 12a). Despite the fact that indomethacin in the concentrations of 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 PGE or PGF release (Table 11).

B. 5,8,11,14-Eicosatetraynoic acid

In addition to experimentation probing the actions of indomethacin, which inhibits prostaglandin dehydrogenase and thus the conversion of prostaglandin to a keto derivative (Flower, 1974), a few studies were carried out using the acetylenic analogue of arachidonic acid, 5,8,11,14-eicosatetraynoic acid (ETA); this analogue inhibits the conversion of arachidonic acid to PGE and PGF (Ahern and Downing, 1970). ETA in concentrations as low as 3×10^{-10} M impaired the steroidogenic response to ACTH (Fig. 13). The inhibition was concentration dependent over the range of 3×10^{-10} to 3×10^{-7} M. However, at the highest ETA concentration tested (3×10^{-5} M), the inhibitory action was reversed (Fig. 13). The stimulant action of exogenous PGE was not markedly affected by an ETA concentration as high as 3×10^{-7} M, but its action, like that of ACTH, was potentiated by 3×10^{-5} M ETA (Fig. 13). Basal steroid release was increased by ETA concentrations as low as

Figure 13. Comparison of the effects of 5,8,11,14-eicosatetraynoic acid (ETA) on ACTH and 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 ETA concentrations, in the presence of either ACTH (50μU, hatched columns) or PGE_2 (2×10^{-4}M, solid columns), or in their absence (open columns).


ACTH
PGE2
300
200
100
0
STEROID RELEASE (ng / 2.5X10^5 cells)
0
10^-10
10^-9
10^-5
ETA (M)

3×10^{-7} M, with the maximum effect observed at 3×10^{-6} M (Table 13). Although ETA 3×10^{-5} M also augmented basal steroid release (Table 13) this same concentration failed to enhance basal PGF release, since prostaglandin values remained within 67% of control values in the presence of inhibitor (Table 14). In the presence of ETA ACTH-induced $PGF_{2\alpha}$ release was only 66% of that observed in the absence of inhibitor (Table 14). In fact, ACTH-evoked $PGF_{2\alpha}$ release was reduced to below basal values by ETA (3×10^{-5} M) in some experiments.

V. Comparison of the effects of pregnenolone and ACTH on steroid and prostaglandin release

The use of pregnenolone to stimulate steroid production could help to elucidate further the juxtaposition of prostaglandin synthesis in the train of events which culminates in steroidogenesis, since this corticoid precursor circumvents the membrane events triggered by ACTH. Isolated cortical cells incubated with pregnenolone (3 μM) responded with a more than 30-fold rise in steroid release (Fig. 14). By contrast, the potently steroidogenic concentration of pregnenolone failed to augment $PGF_{2\alpha}$ and PGE_2 release. This lack of effect of pregnenolone was manifest whether the data were expressed in terms of absolute prostaglandin concentrations or in terms of prostaglandin released from pregnenolone-treated cells as a percent of unstimulated controls (Table 15).

In the same experiments, ACTH (125 μU) augmented steroid release by 16-fold and also evoked an increase in PGE_2 and $PGF_{2\alpha}$ release (Table 15). Variability in the prostaglandin values from preparation to preparation hindered statistical analysis of the data when they were represented in absolute concentrations; however, when the ACTH-stimulated prostaglandin value of each experiment was expressed as a percent of its unstimulated control, ACTH elicited a 122% increase in PGE_2 release and a 173% increase in $PGF_{2\alpha}$ release (Table 15).

Table 13. Basal steroid release in the presence of ETA.

ETA (x3M)	Percent of Control	
10^{-9}	104 ± 7	(3)
10^{-7}	121 ± 7	(3)
10^{-6}	381 ± 111	(3)
10^{-5}	339 ± 145	(6)

Cells were incubated with varying ETA concentrations under conditions previously described. After 90 min, the incubation medium was extracted with methylene chloride and assayed for corticosteroids. Numbers in parentheses indicate number of experiments. Values represent means ± S. E.

Table 14. Effect of ETA on basal and ACTH-evoked $PGF_{2\alpha}$ release.

	$PGF_{2\alpha}$ (pg)			$PGF_{2\alpha}$ (pg)		
Expt.	Basal(A)	Basal + ETA(B)	B/A	ACTH(C)	ACTH + ETA(D)	D/C
1	107	53	50	375	387	103
2	1043	814	78	1660	578	55
3	68	49	72	92	37	40
Mean ± S.E.			67 ±9			66 ±19

Each experimental value was obtained by RIA after incubating equal numbers of cells in MEM for 60 min in the presence or absence of ACTH (125-250 μU) and/or ETA (3×10^{-5} M). Values are expressed as pg/2.5×10^5 cells.

Figure 14. The effect of cycloheximide on steroid release from isolated adrenal cortical cells.

Cells were incubated for 90 min in the presence or absence of ACTH (125μU) or pregnenolone (3μM) and/or cycloheximide (CX) (36 μM). Each vertical bar represents mean steroid release (± SEM) derived from 5 different preparations.

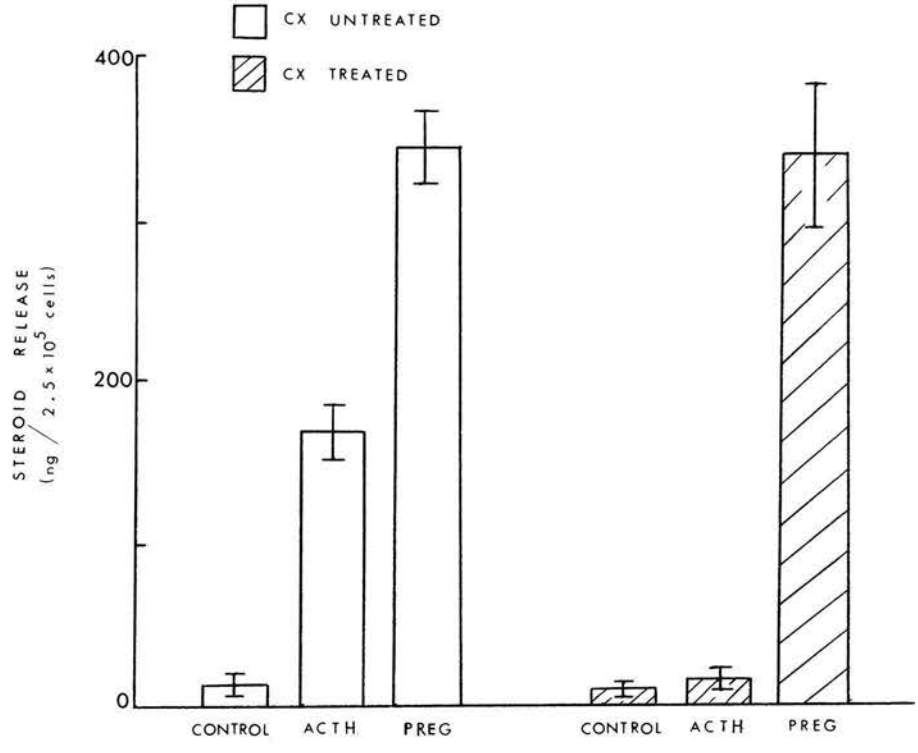

CX UNTREATED
CX TREATED
400
200
0
STEROID RELEASE
(ng / 2.5 x 10^5 cells)
CONTROL
ACTH
PREG
CONTROL
ACTH
PREG

Table 15. Comparative effects of pregnenolone and ACTH on prostaglandin formation.

Expt.		PGE		PGF	
		pg	% of basal	pg	% of basal
1	Basal	205	---	280	---
	ACTH	225	110	401	143
	Pregnenolone	122	60	152	54
2	Basal	218	---	714	---
	ACTH	266	122	772	108
	Pregnenolone	222	102	603	84
3	Basal	92	---	113	---
	ACTH	117	127	232	205
	Pregnenolone	97	105	114	101
4	Basal	269	---	431	---
	ACTH	344	128	1014	235
	Pregnenolone	259	96	232	54
Mean ± S.E.	Basal	196± 37	---	384± 128	---
	ACTH	238± 47	122±4	605± 177	173± 29
	Pregnenolone	175±39	91±10	275± 112	73±12

Values were obtained by RIA of incubation media bathing cells in the presence or absence of ACTH (250μU) or pregnenolone (3μ M). Incubations were for 90 min at 37°C. Values are expressed in pg/2.5 x 10^5 cells.

VI. The effects of cycloheximide

A. ACTH and pregnenolone-induced steroid release

Cycloheximide is a potent inhibitor of steroidogenesis since protein synthesis is a required event in the processes which control steroid biosynthesis (Garren et al., 1971). Incubation of cortical cells with cycloheximide completely blocked the steroidogenic effects of ACTH (Fig. 14). By contrast, the steroidogenic response to pregnenolone was unaffected by the same concentration of cycloheximide (Fig. 14), indicating that the inhibitor was not impairing the general viability of these cells.

B. Basal prostaglandin levels

Cells incubated with cycloheximide tended to release less PGE_2 (155 pg) than in its absence (232 pg) (Table 16), although due to variability from experiment to experiment this difference was not statistically significant ($p > 0.1$). When PGE_2 values for the cycloheximide-treated samples in each experiment were expressed as a percent of those obtained in the absence of inhibitor the average amount of PGE_2 released in the presence of cycloheximide was $73 \pm 12\%$ of control. Treatment with cycloheximide resulted in a small decrease in basal $PGF_{2\alpha}$ levels (Table 16). The difference in the average amount of $PGF_{2\alpha}$ released in the presence (348 pg) and absence (459 pg) of cycloheximide was not significantly different ($p > 0.4$); but when in each experiment the values obtained in the presence of cycloheximide were expressed as a percent of the values obtained in the absence of cycloheximide, the average amount of $PGF_{2\alpha}$ released in the presence of cycloheximide was $78 \pm 6\%$ of control ($p < .01$).

C. ACTH-stimulated prostaglandin release

ACTH-enhanced PGE_2 release was unimpaired by cycloheximide; in fact, in the presence of cycloheximide ACTH produced a slightly greater facilitation of PGE_2 release over its corresponding control value (234%), than in the absence of inhibitor (174%) (Table 16). When the response

Table 16. Effect of cycloheximide on prostaglandin release.

Expt.	pg PGE_2 Control	pg PGE_2 ACTH	Percent of Control	pg $PGF_{2\alpha}$ Control	pg $PGF_{2\alpha}$ ACTH	Percent of Control
1	205	225	110	280	401	143
2	218	266	122	714	772	108
3	92	117	127	113	232	205
4	269	344	128	431	1014	235
5	375	469	384	759	934	123
Mean	232	284	174	459	670	163
±S.E.	±46	±59	±53	±124	±152	±24

	Cyclohex	ACTH +Cyclohex	Percent of Control	Cyclohex	ACTH +Cyclohex	Percent of Control
1	133	154	115	165	184	111
2	184	179	97	530	488	92
3	93	172	185	106	288	272
4	241	399	166	369	551	149
5	122	743	609	570	695	122
Mean	155	329	234	348	441	149
±S.E.	±26	±113	±95	±94	±92	±32

Values represent the number of pg prostaglandin released by 2.5×10^5 cortical cells in the presence or absence of ACTH (250μU), cycloheximide (0.04 mM), or both agents. Incubations were for 90 min at 37°C.

to ACTH in the presence and absence of cycloheximide were compared in each experiment, they also were found to be comparable (111 ± 19%). In the presence of cycloheximide ACTH augmented $PGF_{2\alpha}$ release by an average of 149% (Table 16). This small increase was manifest only if the ACTH-induced $PGF_{2\alpha}$ release in the presence of inhibitor was expressed as a percent of basal cycloheximide-treated samples, since the average amount of $PGF_{2\alpha}$ released by ACTH in the presence of inhibitor (441 pg) approximated basal $PGF_{2\alpha}$ levels in the absence of inhibitor (459 pg) (Table 16). Moreover, the mean $PGF_{2\alpha}$ release induced by ACTH in the presence of cycloheximide (441 pg) was significantly less than that obtained in the absence of cycloheximide (670 pg), when the data from each experiment were analyzed by a paired observation students' t test ($p=.05$) (Table 16). When the inhibitory effects of cycloheximide on ACTH-facilitated $PGF_{2\alpha}$ release were expressed as a percent of ACTH-facilitated $PGF_{2\alpha}$ release in the absence of cycloheximide, ACTH-induced $PGF_{2\alpha}$ release in the presence of inhibitor was 72 ± 14% of control.

VII. Cyclic AMP and prostaglandin synthesis

A. Effect of exogenous cyclic AMP

A number of investigations have implicated cyclic AMP as a mediator of ACTH-induced steroidogenesis (Robison et al., 1971). In this regard, previous studies have shown that cyclic AMP and its monobutyryl analogue augment steroid production and release from isolated feline cortical cells in a dose-related manner (Rubin et al., 1975). Hence, experiments were designed to elucidate the relationship between cyclic nucleotide stimulation of steroidogenesis and prostaglandin synthesis. Table 17 illustrates conclusively that the monobutyryl analogue of cyclic AMP (BCAMP) (0.5 mM) dramatically facilitates the cortical release of $PGF_{2\alpha}$ and PGE_2 into incubation media assayed directly using the appropriate antisera. In fact, BCAMP was more effective in stimulating prostaglandin release than an ACTH concentration which had

Table 17. Cyclic AMP and prostaglandin synthesis.

		PGF		PGE	
		pg	% basal	pg	% basal
Expt. 1	Control	209	---	169	---
	BCAMP	3855	1844	694	411
Expt. 2	Control	309	---	283	---
	BCAMP	704	228	489	173
Expt. 3	Control	193	---	324	---
	BCAMP	1399	725	955	295
Expt. 4	Control	113	---	92	---
	BCAMP	239	212	1410	1532
Mean ± S.E.	Control	206 ± 40	---	217 ± 53	---
	BCAMP	1549 ± 805	752 ± 383	887 ± 199	602 ± 313

Cells were incubated in the presence or absence of monobutyryl cyclic AMP (BCAMP) (0.5mM) for 90 min. RIA for PGF, using anti-$PGF_{2\alpha}$ antiserum, and PGE, using anti-PGE_2 antiserum, was carried out on 400 and 100 µl aliquots, respectively, of incubation medium as described in Methods. Amounts of prostaglandins are expressed as pg/2.5×10^5 cells.

markedly greater steroidogenic activity (Fig. 15).

B. Effect of NPS-ACTH

The steroidogenic action of ACTH on the adrenal cortex appears to be mediated not only by activation of adenylyl cyclase and a resultant increase in cyclic AMP (Robison et al., 1971), but as the preceding results illustrate, also by an enhanced biosynthesis and release of endogenous prostaglandins. The question thus arises as to the interdependence of increases in cyclic AMP and prostaglandin synthesis during augmented steroidogenesis. Information pertaining to this problem may be gained with the aid of the o-nitrophenyl sulfenyl derivative of ACTH (NPS-ACTH). Although the ACTH analogue is approximately one six-hundredth as potent as ACTH on feline cortical cells, it elicits a dose-related facilitation of steroid release; however, unlike ACTH, NPS-ACTH fails to induce a measurable increase in cortical cyclic AMP (Rubin, 1975). Since NPS-ACTH appears to dissociate the early event of elevated cyclic AMP levels from steroid biosynthesis, it was of great interest to ascertain whether it, like ACTH, is still capable of altering prostaglandin metabolism. Figure 15 illustrates that both ACTH and NPS-ACTH enhanced the release of PGE_2 and $PGF_{2\alpha}$. Although the enhanced prostaglandin release elicited by both stimulants was completely suppressed by indomethacin, ACTH and NPS-ACTH still markedly stimulated steroid release (Fig. 16).

VIII. Calcium deprivation and prostaglandin release

Incubation of isolated feline cortical cells in a calcium-deprived medium containing EGTA completely suppresses the steroidogenic response to even high ACTH concentrations (Warner and Rubin, 1975). This finding was confirmed in the present investigation (Fig. 15), which also elaborated the calcium dependency of ACTH stimulation to include prostaglandin release. The ACTH-facilitated $PGF_{2\alpha}$ and PGE_2 release were reduced to below basal levels in calcium-deprived cells (Fig. 15).

Figure 15. The effects of calcium deprivation on prostaglandin and steroid release induced by ACTH, NPS-ACTH, and BCAMP.

Isolated cortical cells were incubated for 60 min in normal or calcium-free MEM containing EGTA (0.4mM), in the presence or absence of ACTH (250 μU), NPS-ACTH (3.2 μM), or BCAMP (0.1 mM). Aliquots of incubation medium were assayed for steroid, PGE_2, and $PGF_{2\alpha}$. Each vertical bar represents a mean value (± S.E.), expressed as percent of basal values, from 3 or more preparations.

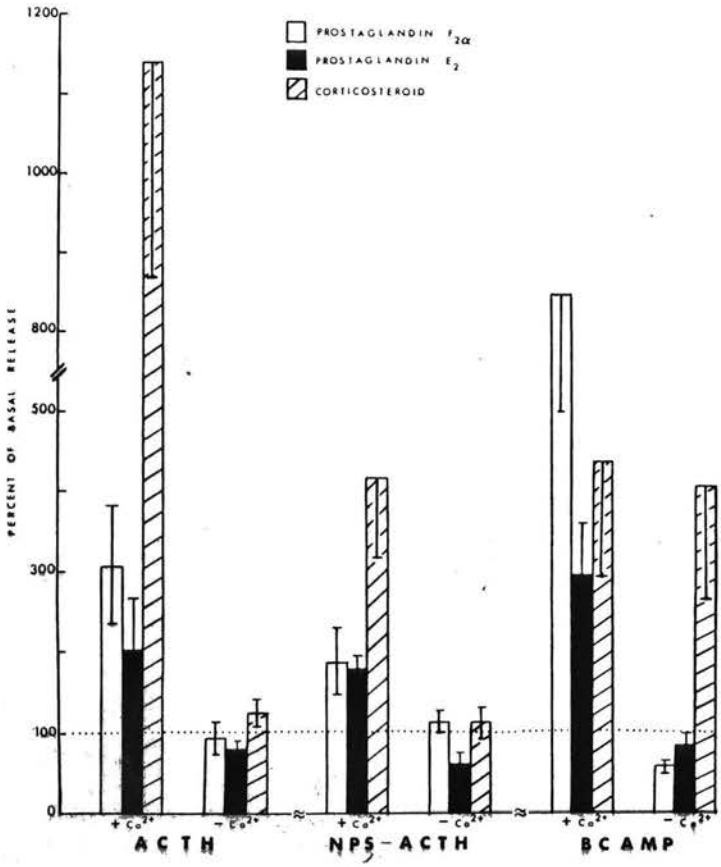
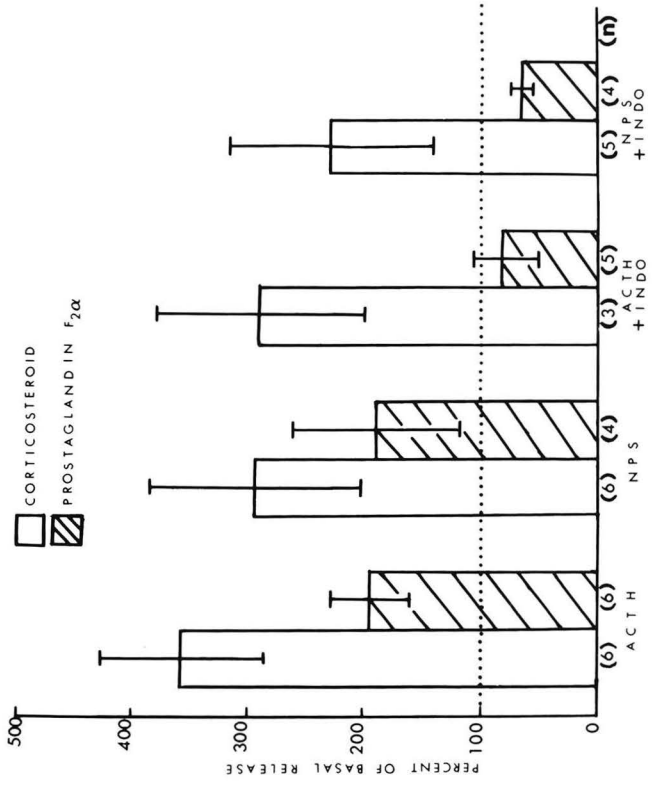

PROSTAGLANDIN $F_{2\alpha}$
PROSTAGLANDIN E_2
CORTICOSTEROID
PERCENT OF BASAL RELEASE
1200
1000
800
500
300
100
0
+ Ca^{2+}
- Ca^{2+}
ACTH
NPS-ACTH
BCAMP

Figure 16. The effects of indomethacin on ACTH- and NPS-ACTH-induced corticosteroid and $PGF_{2\alpha}$ release.

Equal numbers of cells were incubated for 90 min in MEM. ACTH (0.3 nM) or NPS-ACTH (3200 nM) was added as indicated in the presence or absence of indomethacin (3×10^{-5}M). $PGF_{2\alpha}$ determinations were made by direct radioimmunoassay of 400 µl aliquots of incubation medium, using $PGF_{2\alpha}$ antiserum. Values normalized per 2.5×10^5 cells are expressed as percent of unstimulated control samples. Each vertical bar represents the mean (± S.E.) of the number of independent experiments indicated by the number in parentheses at the base of the bar.


CORTICOSTEROID
PROSTAGLANDIN $F_{2\alpha}$
PERCENT OF BASAL RELEASE
500
400
300
200
100
0
(6) (6) ACTH
(6) (4) NPS
(3) (5) ACTH +INDO
(5) (4) NPS +INDO
(n)

Likewise, calcium lack blunted the enhancement of both steroid and prostaglandin release elicited by NPS-ACTH (Fig. 15). By contrast, the steroidogenic action of the monobutyryl analogue of cyclic AMP (BCAMP) was not significantly diminished by calcium deprivation, although calcium lack blocked BCAMP facilitated PGE_2 and $PGF_{2\alpha}$ release (Fig. 15).

In order to ascertain that calcium deprivation was not irreversibly altering the response of cortical cells to ACTH, in 2 different experiments cells were incubated for 60 minutes in calcium-deprived medium plus ACTH and then re-suspended for the same time interval in calcium containing medium plus ACTH. The average amount of $PGF_{2\alpha}$ and PGE_2 released by ACTH (250 μU) in the absence of calcium was 212 and 105 pg, respectively, and subsequently, in the presence of calcium was 417 and 234 pg, respectively. A similar reversibility of the effects of calcium lack on steroid production was also demonstrable in these same experiments. Thus, the average amount of steroid released during and following calcium deprivation was 15 and 182 ng, respectively.

Although variability from preparation to preparation somewhat limits statistical analysis of the data in terms of absolute concentrations, Table 18 gives the $PGF_{2\alpha}$, PGE_2, and steroid concentrations when data from different experiments were combined. Certain general conclusions can be drawn from these results which are not readily apparent from the data expressed as percent of control. Although basal $PGF_{2\alpha}$ and PGE_2 levels were generally comparable, $PGF_{2\alpha}$ release was augmented to a greater extent by ACTH and BCAMP than by NPS-ACTH. NPS-ACTH appeared to enhance $PGF_{2\alpha}$ and PGE_2 release to a similar degree. Although basal prostaglandin levels appeared slightly depressed by calcium deprivation, the addition of stimulating agents generally failed to augment prostaglandin release above these resting levels, with the exception of a two-fold increase in BCAMP-induced PGE_2 release; however, even this increase is within the limits of variability imposed by the standard error of control and stimulated values. It should be noted that the

Table 18. Effect of calcium deprivation on prostaglandin and steroid release

Experiment	$PGF_{2\alpha}$ pg		PGE_2 pg		Steroid ng	
	+Ca	-Ca	+Ca	-Ca	+Ca	-Ca
Control	206 ±55	182 ±50	170 ±30	125 ±30	14 ±2	14 ±2
ACTH	561 ±145	172 ±33	284 ±65	152 ±22	140 ±23	15 ±3
Control	204 ±51	134 ±44	190 ±53	98 ±29	26 ±12	12 ±2
NPS-ACTH	313 ±59	152 ±60	311 ±117	94 ±38	75 ±17	<12 ±2
Control	237 ±36	192 ±89	258 ±46	155 ±16	15 ±3	<15 ±3
BCAMP	1986 ±955	140 ±31	712 ±134	215 ±69	54 ±14	46 ±13

All mean values (± S.E.) were derived from at least 3 experiments described in Figure 15 and are expressed as pg or ng/2.5×10^5 cells.

amount of PGE_2 released after BCAMP in the absence of calcium was less than the amount released under basal conditions in the presence of calcium.

DISCUSSION

The foregoing investigations were carried out to identify and quantitate prostaglandins which might be present in the adrenal cortex. A thorough knowledge of these proposed cellular mediators might help to elucidate the mechanism of action of ACTH and the role of prostaglandins in the steroidogenic response to the tropic hormone. The present studies have demonstrated that: 1) the adrenal cortex possesses the enzymatic machinery to synthesize prostaglandins; 2) at least two species of prostaglandins (PGE_2 and $PGF_{2\alpha}$) are synthesized and released by cortical cells; and 3) ACTH enhances the synthesis and release of cortical prostaglandins. This discussion will attempt a comprehensive analysis of these basic findings in the hope of elaborating the functional role which prostaglandins may play in the tropic action of ACTH.

I. Prostaglandin synthetase activity in the adrenal cortex

Implicating a potential intermediate in the action of a given hormone dictates that the substance under question be synthesized in the responsive tissue, and that some changes in its metabolism be demonstrable during the action of the hormone. In exploring the putative role of prostaglandins in ACTH-induced corticosteroidogenesis, studies have been carried out utilizing adrenal cortical cell homogenates and suspensions to demonstrate that cortical cells possess an active prostaglandin synthetase responsible for converting radiolabeled arachidonic acid into prostaglandins. This is the first demonstration of synthetase activity in the adrenal cortex, although it has been identified in a number of other tissue types (Pace-Asciak and Wolfe, 1970; Takeguchi and Sih, 1972). In general, the synthetases found in different tissues exhibit a common requirement for the reducing cofactor glutathione (Lands et al., 1971; Samuelsson, 1973), the protective dihydroxybenzene group of L-norepinephrine bitartrate (Pace-Asciak, 1973), and the chelating agent EDTA (Pace-Asciak and Wolfe, 1970). The prostaglandin synthetase of

adrenal cortical cells appears to share the chemical properties of synthetase from other tissues since cortical homogenates successfully synthesized prostaglandins from arachidonic acid in the presence of these agents.

The experiments employing ^{3}H-arachidonic acid metabolism as a measure of prostaglandin synthesis and release indicated that PGE_2 and $PGF_{2\alpha}$, rather than PGE_1 and $PGF_{1\alpha}$, were prominent species of prostaglandin in the adrenal cortex, since arachidonic acid is the precursor of the former two prostaglandins (Christ and van Dorp, 1972). Precedent for the use of ^{3}H-arachidonic acid to demonstrate the prostaglandin synthesizing activity of tissues was established by Bergström and co-workers (1964) using sheep vesicular gland homogenates, and Pace-Asciak (1973, 1975) using homogenates of rat stomach fundus and kidney papilla. Both groups of investigators have identified radiolabeled PGE_2 and $PGF_{2\alpha}$ after thin layer chromatographic analysis of tissue homogenate extracts.

While the spontaneous conversion of arachidonic acid to PGF-like compounds does occur to a limited extent in the adrenal cortical homogenate extracts examined on thin layer chromatography, the thin layer radioactive profiles indicate that the cell homogenate contained an active principle responsible for the synthesis of PGE and PGF in amounts 3- and 6-fold, respectively, above the levels of arachidonic acid recovered. The peak of radioactivity occurring in the PGA/B region of the thin layer chromatographic profile might be described as a mixture of unconverted arachidonic acid, PGA and/or PGB, and prostaglandin metabolites such as the 15-keto-dihydroxy derivatives (Pace-Asciak, 1975). Nevertheless, these initial results signified that the feline adrenal cortex contained an enzymatic mechanism for the synthesis of prostaglandins.

Those experiments in which intact adrenal cortical cells were allowed to incorporate ^{3}H-arachidonic acid further demonstrated the presence of a functional prostaglandin synthetase in these cells. In addition, these studies showed that prostaglandins E_2 and $F_{2\alpha}$ were

synthesized intracellularly from arachidonic acid and released into the incubation medium. In regard to the total radioactivity recovered in the PGF and PGE zones of prostaglandins extracted from adrenal cells and incubation medium, the two-fold higher level of radiolabeled prostaglandins in the medium supports the proposal that these unsaturated fatty acids are released rather than sequestered by the cells (Piper and Vane, 1971). These findings suggestive of de novo synthesis and release are corroborated by the quantitation of intracellular versus released adrenal cortical prostaglandins using radioimmunoassay, since only 15% or less of the PGE_2 and $PGF_{2\alpha}$ released into the incubation medium was contained within the cortical cells.

A portion of the radiolabeled product found in cells and medium with mobility properties similar to PGA or PGB on thin layer chromatography was derived from PGE oxidation products, as evidenced by the fact that a portion of the [^{3}H-PGE] added to incubating cortical cells was converted to a substance with mobility properties indistinguishable from the unidentified prostaglandin in the A/B zone. Therefore, the synthesis of PGE_2 may be even more pronounced than is evidenced by the amount of tritiated product found in the E zone on thin layer chromatography. However, this does not rule out the possibility that prostaglandin metabolites contribute to the radioactivity found in the PGA/B zone.

Subsequent to substantiating the capacity of isolated cortical cells to synthesize and release prostaglandins, the influence of ACTH on this synthetic process was sought in order to determine a role for prostaglandins in the mechanism of action of ACTH. The results of ^{3}H-arachidonic acid conversion to prostaglandins by cells exposed to ACTH showed that prostaglandin synthesis was augmented by as much as twice that occurring in control cells. The fact that the percent increase in released prostaglandins in response to ACTH was less than the increase observed for intracellular prostaglandins may be explained in several ways. The release of substantial amounts of both labeled and unlabeled prostaglandins

would tend to dilute the detectable release of radioactive product. The release of unlabeled as well as labeled prostaglandin in response to ACTH is likely since the adrenal cortex is rich in arachidonic acid. In addition, some of the radiolabeled prostaglandins may be metabolized intracellularly rather than released, thus contributing to a diminished percent rise in radiolabeled prostaglandin release. The fact, however, that the same species of prostaglandins increase intracellularly and extracellularly in response to ACTH, supports the concept that these unsaturated fatty acids may mediate ACTH-induced steroidogenesis.

II. Qualitative and quantitative analysis of prostaglandins

Correlative and more quantitative evidence for PGE and PGF synthesis obtained by the studies concerned with the conversion of radiolabeled arachidonic acid was sought using RIA techniques. The serologic procedure of prostaglandin analysis offers several advantages over other analytical methods in that it can be highly specific and permit estimation of the prostaglandins in fluids without chemical fractionation, and it can detect small amounts of antigens in small samples of biological fluids. The RIA analyses of prostaglandin release from isolated cortical cells confirm the ACTH-inducible synthesis of PGE_2 and $PGF_{2\alpha}$. The reliability and specificity of these prostaglandin assays were confirmed in a number of ways. The PGF and PGE values of several hundred picograms per ml obtained in the present study are in agreement with values obtained by assay of other biological fluids (Jaffe and Parker, 1973); moreover, the consistent increase in prostaglandin synthesis caused by ACTH was blocked by indomethacin, a prototype inhibitor of prostaglandin synthetase. Also, direct analysis of cellular prostaglandins gave much lower PGF and PGE values than obtained by assay of released antigen - a finding consistent with data from other systems which demonstrate that the newly synthesized prostaglandin is immediately released by the cell (Ramwell and Rabinowitz, 1972). In addition, the results of

the RIA technique are reproducible as indicated by the parallel increase in antigen binding with increasing sample size, and the comparable levels of prostaglandin found in media assayed directly or after extraction with organic solvents.

The usefulness of immunological methods for assay of prostaglandins is limited by the specificities of the antibodies employed. The antibodies used to measure cortical prostaglandins have been described as binding one class of prostaglandin more strongly than heterologous prostaglandins of similar structure, since a correlation of chemical structure and serologic activity of antibodies is mandatory before any absolute identification of a prostaglandin in a biological fluid can be made.

With the relative affinities of heterologous prostaglandins to the PGF antibodies known, it was possible to assay the amounts of $PGF_{1\alpha}$ and $PGF_{2\alpha}$ released by the cortical cells. The direct assay of samples of incubation medium using the $PGF_{2\alpha}$ antibody gave much higher values than determinations using $PGF_{1\alpha}$ antiserum. These higher values cannot be ascribed to cross-reactivity by heterologous PGA and PGB which have little affinity for the $PGF_{2\alpha}$ binding site; moreover, separation of PGF from the heterologous prostaglandins by TLC prior to RIA confirmed that PGF equivalents in samples of media were mainly responsible for the displacement of $^{3}H\text{-}PGF_{2\alpha}$ from $PGF_{2\alpha}$ binding sites.

It could still be argued that the determination of PGF equivalents using $PGF_{2\alpha}$ antiserum gave higher values than determinations using $PGF_{1\alpha}$ antiserum, because $PGF_{2\alpha}$ antiserum is capable of detecting both $PGF_{1\alpha}$ and $PGF_{2\alpha}$. However, the fact that the quantity of $PGF_{1\alpha}$ as detected with the $PGF_{1\alpha}$ antibody was not sufficient to cross-react discernibly with the $PGF_{2\alpha}$ antibody makes it clear that $PGF_{2\alpha}$ is the predominant species and that the amount of $PGF_{1\alpha}$ released must be extremely low, if indeed, any is released at all. Although these experiments have established that $PGF_{2\alpha}$, rather than $PGF_{1\alpha}$ is the predominant

PGF produced by feline cortical cells, they do not exclude the possibility that PGF metabolites are also concomitantly released.

The relative specificities of the $PGF_{1\alpha}$ and $PGF_{2\alpha}$ antisera also made it possible to identify the released PGE as PGE_2, after reducing the PGE equivalents to their corresponding PGF derivatives. Although the estimated quantity of endogenous PGF was subtracted from each sample, the PGE values derived by this method were higher than those obtained by RIA using PGE antiserum following PGE separation by TLC. There is reason to believe that the higher values obtained using the sodium borohydride ($NaBH_4$) reduction of PGE without prior separation from the other prostaglandins are due to the presence of some interfering substance(s) which is removed by ether extraction and TLC. This conclusion is based upon the fact that $NaBH_4$ reduction and RIA of PGE samples which were chromatographically separated rendered values which closely approximated those obtained using the PGE antiserum. In any event, the development of a PGE_2 antibody which allowed direct assay of the incubation medium, proved to be a reliable method for the determination of PGE. Although this antibody does not cross-react with prostaglandins of the A, B, or F series, it does cross-react with PGE_1; however, since the amount of PGE_1 in the feline cortex is probably negligible in comparison to PGE_2, the inability of the antibody to distinguish between PGE_1 and PGE_2 does not mitigate its usefulness.

III. The temporal relation of prostaglandin and steroid release

Our initial effort to correlate the synthesis of prostaglandins and ACTH-induced steroidogenesis involved an examination of the temporal pattern of prostaglandin and corticosteroid release from the perfused adrenal gland. Since it has been shown that steroid synthesis increases shortly after the administration of ACTH, any ACTH-induced alteration in the adrenal that is to be considered part of the biological machinery by which ACTH stimulates steroidogenesis must occur within the first

several minutes following ACTH administration. Otherwise, the event in question follows, rather than precedes, the enhanced synthesis of steroid hormones and does not constitute a necessary link in the chain of events culminating in increased steroidogenesis. The release of $PGF_{2\alpha}$ from the in situ perfused adrenal gland occurred within 10 minutes following exposure of the gland to ACTH, and at least 20 minutes prior to maximum steroid release. This finding not only confirms the release of prostaglandins from isolated cortical cells in vitro in response to ACTH, but also suggests that prostaglandins are participating in the steroidogenic mechanism induced by ACTH.

The in situ release of prostaglandins raises several questions regarding the physiological role of these compounds. Prostaglandins may exert biochemical effects related to steroidogenesis prior to their release from the gland and their catabolism in the circulation (Golub et al., 1975). In this regard, it is interesting that prostaglandin release in response to ACTH precedes the maximum secretion of corticosteroid by the same time interval reported for the synthesis of adrenal cyclic AMP induced by ACTH (Carchman et al., 1971). Thus, the possibility arises that the formation and/or release of prostaglandins are somehow linked to the cyclic AMP mediation of steroidogenesis.

The question of the physiological importance of released prostaglandins in the regulation of steroidogenesis also deserves consideration since the presence of prostaglandin receptors in adrenal glands (Dazord et al., 1974) suggests that membrane receptor coupling is an integral part of their mechanism of action. According to Bito (1975), prostaglandins or their precursors are released into extracellular fluids of tissues to act on receptors located on the outer surface of cells. This interpretation of the functional role of prostaglandins considers them to be local hormones (autocoids). The close association between prostaglandin receptors and adenylyl cyclase (Kuehl, 1973) further indicates that a functional locus of action of prostaglandins may be at

the membrane level in adrenal cortical cells where the prostaglandins could modulate the action of ACTH by modifying adenylyl cyclase activity.

Prostaglandins released from adrenal cortical cells in response to ACTH might also couple with receptors on cells other than steroid secreting cells. While the adjacent glomerulosa cells appear unresponsive to PGE and PGF (Blair-West et al., 1971; Fichman and Horton, 1973), the circulating prostaglandins may influence the release of catecholamines from the adrenal medulla (Brody and Kadowitz, 1974; Boonyaviroj and Gutman, 1975). Perhaps more importantly, since vascular smooth muscle receptors might also be influenced by circulating prostaglandins (see Introduction), the functional role of releasable adrenal cortical prostaglandins includes the regulation of adrenal blood flow. In the rat adrenal, blood flow and steroid release increase following the intravenous administration of ACTH (Maier and Staehelin, 1968). The reported increase in blood flow is maximum 10 minutes after ACTH and then gradually declines to control levels; whereas steroid secretion reaches a maximum at 60 minutes and then declines. This temporal relationship between blood flow and steroidogenesis in the rat adrenal in vivo mirrors the prostaglandin-steroid release profile of the perfused cat adrenal. Thus, adrenal cortical prostaglandins may be vasoactive and cause an increase in adrenal blood flow in vivo which could activate metabolism and facilitate steroidogenesis. On the other hand, the steroidogenic effect of exogenous prostaglandins on isolated cortical cells indicates that a vascular effect of the prostaglandins cannot be its sole mechanism of action.

IV. Prostaglandin inhibitors and steroidogenesis

In attempting to elucidate further the role of prostaglandins in the mechanism of action of ACTH, the use of prostaglandin inhibitors was invoked. This approach assumes that if endogenous prostaglandins are an integral component of steroidogenesis then not only should ACTH

be capable of stimulating prostaglandin biosynthesis but inhibitors of prostaglandin synthesis should modify the response to ACTH stimulation. The competitive and irreversible prostaglandin synthetase inhibitors used were indomethacin and the tetraacetylenic acid, ETA.

High concentrations of indomethacin caused an irreversible inhibition of ACTH-induced PGE and PGF release, accompanied by a more modest impairment of ACTH-evoked steroid release; thus, steroid release was still augmented approximately 6-fold despite prostaglandin release being at or near basal levels. This finding suggests that enhanced prostaglandin synthesis may not be a sine qua non for the steroidogenic action of ACTH, just as indomethacin-inhibited prostaglandin synthesis was found not to be essential to the stimulatory effect of LH on gonadal steroidogenesis (Kuehl et al., 1974), the action of human chorionic gonadotrophin (HCG) in evoking corpus luteal progesterone synthesis (Santos et al., 1973), or the ability of TSH to increase adenylyl cyclase activity and promote thyroid hormone release (Mashiter and Field, 1974). In these latter tissues as well as in the adrenal cortex, however, exogenous prostaglandins can mimic the physiological effects of the tropic hormone, which suggests a modulatory role, albeit not an essential one, for the unsaturated fatty acids in hormone action. In this regard, it is possible that decreased prostaglandin synthesis in response to 10^{-5} M indomethacin might be responsible for the modest depression of the steroidogenic response to ACTH under these conditions.

In contrast to the effects of high concentrations of indomethacin on cortical cells, indomethacin in low concentrations produced a parallel potentiation of steroid and prostaglandin release - providing strong support for the concept that prostaglandins play a key physiological role in ACTH-induced steroidogenesis. The likelihood that prostaglandins contribute to the increase in steroid release in the presence of indomethacin derives from the evidence that exogenous prostaglandins stimulate steroidogenesis, not only in the feline adrenal, but in the bovine

(Saruta and Kaplan, 1972) and rat (Flack et al., 1972) adrenal glands as well. The converse relationship of steroid synthesis resulting in increased prostaglandin synthesis is not supported by the finding that pregnenolone-induced steroidogenesis fails to increase $PGF_{2\alpha}$ or PGE_2 in adrenal cortical cells. In addition, the inhibition of adrenal steroidogenesis by cycloheximide did not remarkably impair the synthesis of prostaglandins in response to ACTH; this latter finding is reminiscent of the lack of an effect of aminoglutethimide in suppressing prostaglandin synthesis while inhibiting steroidogenesis in rat ovaries and Graafian follicles (Bauminger et al., 1975).

The unexpected finding that indomethacin, a prototype inhibitor of prostaglandin synthesis, enhances prostaglandin release in low concentrations may relate to an ability of the drug to impair the catabolism of endogenous prostaglandins. If prostaglandin dehydrogenase were inhibited by indomethacin (Flower, 1974; Beatty et al., 1976) then the intracellular levels of the prostaglandin might be expected to increase and explain the potentiated effect of ACTH on steroidogenesis.

Another action of low indomethacin concentrations, not related to prostaglandin catabolism but to the catabolism of cyclic AMP, might account for the potentiating effect of the drug on steroid and prostaglandin release. Since an increase in adrenal cyclic AMP occurs in response to ACTH (Carchman et al., 1971; Jaanus et al., 1972) and is believed to mediate the steroidogenic action of ACTH (see Introduction), the potent phosphodiesterase inhibitory capacity of indomethacin (Kuehl, 1974; Beatty et al., 1976) might be expected to augment intracellular levels of the cyclic nucleotide and thereby enhance steroidogenesis. Indeed, the fact that basal steroid values increase after treatment of adrenal cells with indomethacin even though prostaglandin levels are depressed to below basal values, argues that the potentiation of basal steroidogenesis and possibly ACTH-induced steroidogenesis may be at least partly due to the inhibition of phosphodiesterase activity by

indomethacin. Moreover, during calcium deprivation cyclic AMP can enhance steroidogenesis despite any stimulation of prostaglandin synthesis, suggesting that the steroidogenic activity of supra-physiological levels of cyclic AMP does not require the participation of prostaglandins.

ETA, the other prostaglandin synthetase inhibitor studied, depressed ACTH-evoked steroid release from cortical cells, except at high concentrations when ACTH-stimulated steroidogenesis returned to normal levels and basal steroid release was increased. These effects of ETA on steroid release occur even though prostaglandin release is depressed to less than control levels. Therefore, the evidence once again substantiates the expendability of these unsaturated fatty acids in the steroidogenic process. The reversal of the steroid inhibition seen with a high ETA concentration cannot be explained at present; it may be another consequence of certain membrane perturbations (Wolfe et al., 1976) induced by the drug or nonspecific effects on enzymes such as lipoxidases (Ahern and Downing, 1970).

V. Prostaglandins, steroidogenesis and protein synthesis

Protein synthesis has been demonstrated to be essential for the steroidogenic action of ACTH (see Introduction), while the exact role of protein synthesis in controlling steroidogenesis has not been precisely defined. Studies using the protein synthesis inhibitor, cycloheximide, were conducted to determine the nature of the role of protein synthesis in the chain of events linking prostaglandin and steroid synthesis. An inhibition of protein synthesis would also answer the question of whether the activity of prostaglandin synthetase in response to ACTH depends upon de novo enzyme synthesis.

The data demonstrating that cycloheximide effectively inhibited ACTH-induced steroidogenesis while the steroidogenic response to pregnenolone remained unaltered, is consistent with the hypothesis that the protein-dependent rate-limiting reaction in steroidogenesis

occurs prior to the synthesis of pregnenolone (see Introduction). In addition, potently steroidogenic concentrations of pregnenolone failed to increase prostaglandin release and may even have obtunded the release of PGF; an occurrence reminiscent of the lack of an effect of progesterone on uterine prostaglandin levels (Poyser, 1976).

The pregnenolone studies, which dissociated steroid release from prostaglandin formation, indicate that the biosynthesis of steroid end-products is not an obligatory precedent event for prostaglandin formation. These data are a biochemical confirmation of that obtained from the perfused gland, where maximal prostaglandin release preceded maximal steroid release induced by ACTH.

Another implication of the experiments with cycloheximide relates to the finding that PGE and PGF release were not significantly altered by protein synthesis inhibition. Thus, the increase in prostaglandin release elicited by ACTH is due to the activation of a preformed synthetase complex rather than to the transcription of new enzyme protein. It is also apparent from these results that prostaglandin formation per se is not sufficient for stimulating the conversion of cholesterol to corticosteroid, since cycloheximide inhibits steroidogenesis in the face of increased prostaglandin production. In guinea-pig placental slices, prostaglandins also have no effect on the conversion of pregnenolone to progesterone (Bedwani and Marley, 1971).

VI. Calcium, cyclic AMP, and prostaglandins

The assumption has heretofore been made that ACTH augments cortical prostaglandin release by stimulating de novo synthesis from fatty acid precursors. This assumption is supported by the very low intracellular cortical prostaglandin levels in the presence and absence of ACTH. The added finding that the intracellular prostaglandin values obtained after calcium-deprivation are also quite low, substantiates the idea that the low levels of prostaglandin in the incubation medium during

calcium deprivation is the result of an inhibition of synthesis rather than release. Thus, in elucidating the role of calcium in the regulation of prostaglandin formation, yet another major action of calcium in the mechanism of action of ACTH has been uncovered. The finding that 3 different agents, ACTH, NPS-ACTH, and BCAMP, each promote prostaglandin synthesis only in the presence of calcium, suggests a role for this cation which is intimately associated with the prostaglandin biosynthetic mechanism.

The data showing the calcium dependency of ACTH-stimulated steroidogenesis supports the findings of Rubin et al. (1972) and others (Halkerston, 1975) that the ACTH stimulation of adenylyl cyclase and steroid synthesis requires calcium. The calcium independent BCAMP-induced steroidogenesis suggests a major regulatory role for calcium in feline cortical cells prior to the formation of cyclic AMP. But, the additional requirement for calcium in other biosynthetic mechanisms is apparent from the studies using the ACTH analogue, NPS-ACTH. Since NPS-ACTH is believed to enhance steroidogenesis via a mechanism which circumvents significant increases in cyclic AMP (Moyle et al., 1973; Rubin, 1975), the severely obtunded steroidogenic response to NPS-ACTH in calcium-deprived media suggests a role for calcium in steroid synthesis which does not involve the adenylyl cyclase-cyclic AMP system.

Since both BCAMP and NPS-ACTH seemingly augment steroidogenesis by circumventing adenylyl cyclase, the ability of BCAMP to stimulate corticosterone production in the absence of calcium, while the action of NPS-ACTH is almost entirely inhibited under identical conditions, is difficult to interpret. High concentrations of BCAMP may bypass certain intermediate steps of steroidogenesis and so elicit steroid release much in the manner that extremely high ACTH concentrations stimulate the synthesis of rat adrenal corticosterone in the absence of calcium (Haksar et al., 1973).

This may suggest that the critical action of calcium is associated with specific membrane events not necessarily related to adenylyl cyclase. Alternatively, if calcium were to be mobilized from a cellular reserve by BCAMP, as suggested for ACTH action (Rubin et al., 1972), this might allow for steroid synthesis in calcium-deprived media; however, such a pool would appear to be unavailable to the prostaglandin biosynthetic pathway which remains inhibited in the absence of extracellular calcium and the presence of BCAMP.

The involvement of ACTH-stimulated cyclic AMP formation in prostaglandin synthesis has only been suggested heretofore due to similarities in their temporal release pattern and the circumstantial evidence suggesting that an inhibition of phosphodiesterase by indomethacin might enhance prostaglandin formation by preventing the breakdown of cyclic AMP. Although cautioned by the awareness that the steroidogenic action of exogenous cyclic nucleotide may not necessarily be a reflection of a physiological effect of endogenous cyclic AMP, the potent stimulatory effect of BCAMP upon cortical release of $PGF_{2\alpha}$ and PGE_2 point to a role for cyclic AMP in adrenal prostaglandin metabolism. Exogenous cyclic AMP also stimulates the synthesis and release of prostaglandins from isolated Graafian follicles, mouse ovaries, and rat testis (Marsh et al., 1974).

The question of whether or not cyclic AMP, as generated during the action of ACTH, is directly responsible for the activation of prostaglandin synthetase, or whether it acts via some intermediate in the synthesis of prostaglandins is answered, in part, by those experiments using NPS-ACTH as a steroidogenic agent. NPS-ACTH, while not eliciting detectable increases in feline adrenal cortical cyclic AMP (Rubin, 1975), elicited an increase in $PGF_{2\alpha}$ release equal to that induced by ACTH itself. Thus, while cyclic AMP may not be obligatory to the activation of prostaglandin synthetase, these results do not negate the important role cyclic AMP may play in the physiological

mechanism of ACTH and prostaglandin synthesis.

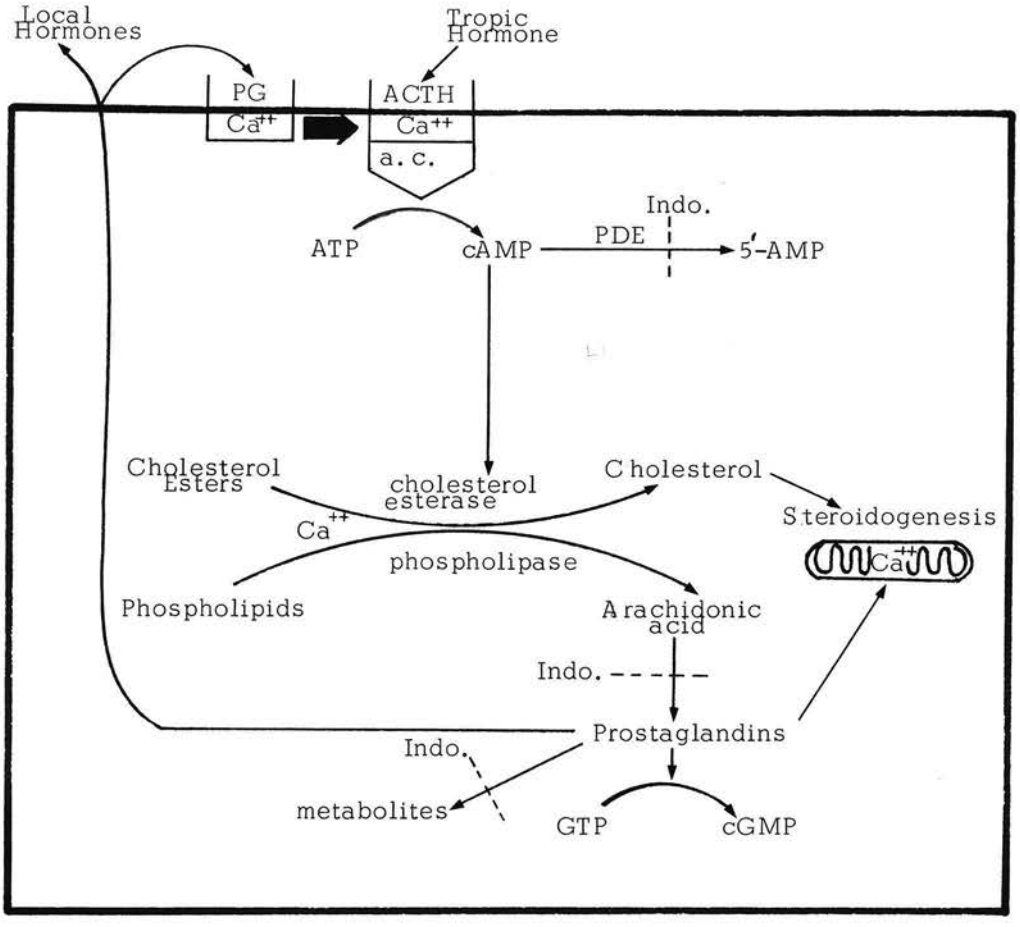
VII. Conclusions and model of prostaglandin interaction in the mechanism of ACTH

The ubiquitous prostaglandins, demonstrated to be present in almost all animal tissues, have also been shown to be synthesized de novo and released from isolated feline adrenal cortical cells. The ability of ACTH to stimulate the synthesis of prostaglandins suggests a possible interrelationship between prostaglandins and the other known mediators of ACTH action, cyclic AMP and calcium. The results support an interaction between the three modulators of steroidogenesis since: a) calcium is required not only for the ACTH-receptor stimulation of adenylyl cyclase (Rubin et al., 1972) and subsequent steroidogenesis, but also for the synthesis of prostaglandins; b) exogenous cyclic AMP can stimulate steroidogenesis as well as prostaglandin synthesis in the presence of calcium.

The model as depicted in Figure 17 is an attempt to summarize the data relating prostaglandins to steroidogenesis and the tropic action of ACTH. The model takes into account the presence of ACTH receptors in adrenal cortical cell membranes (Lefkowitz et al., 1971; Golder and Boyns, 1972) as well as the probability that prostaglandin receptors also reside in the membrane (Dazord et al., 1974). The translated form of the message transmitted as a result of ACTH-receptor activation, as typified by the synthesis of the second-messenger cyclic AMP, has been demonstrated not only for adrenal cells (Haynes, 1958; Rubin et al., 1972), but also for other steroidogenic cells (Shaw and Tillson, 1974) and is incorporated into Figure 17 as a facet of the action of ACTH. It must be kept in mind, however, that the experiments with NPS-ACTH indicate that all ACTH receptors may not activate adenylyl cyclase (Moyle et al., 1973). In addition, the figure depicts the role of cyclic AMP primarily as it has been hypothesized to affect prostaglandin and

Figure 17. Proposed model for the participation of prostaglandins in the mechanism of action of ACTH.

This scheme depicts the following proposed sequence of events in the adrenal cortical cell during ACTH stimulation. Interaction of ACTH with its cell membrane receptor results in membraneous calcium redistribution and causes an increase in adenylyl cyclase (a.c.) activity, with a consequent rise in tissue cyclic AMP (cAMP). The cyclic AMP can then either be converted enzymatically by phosphodiesterase (PDE) to 5'-AMP, or it can activate cholesterol esterase and a calcium-dependent phospholipase. Cholesterol esterase can cleave fatty acids from the cholesterol ester and thereby release free cholesterol for steroidogenesis and fatty acids (including arachidonic acid) for metabolism and prostaglandin synthesis. Phospholipase catalyzes the release of arachidonic acid from phospholipids, and arachidonate enters the prostaglandin synthesizing pathway. Prostaglandins synthesized can either stimulate steroidogenesis by: 1) affecting mitochondrial reactions associated with calcium availability by acting as an "ionophore"; 2) mediating the formation of cyclic GMP (cGMP); 3) leaving the cell to act as a local hormone either by interacting with the prostaglandin (PG) receptor of the cortical cell, or by affecting adjacent tissues, such as adrenal vasculature. PG receptor binding may influence the activity of the adjacent adenylyl cyclase. Indomethacin (Indo.) is indicated by dotted lines as inhibiting several enzymatic reactions which might alter steroid and prostaglandin synthesis.


Local Hormones
Tropic Hormone
PG
Ca++
ACTH
Ca++
a. c.
Indo.
ATP
cAMP
PDE
5'-AMP
Cholesterol Esters
cholesterol esterase
Cholesterol
Steroidogenesis
Ca++
phospholipase
Ca++
Phospholipids
Arachidonic acid
Indo.
Prostaglandins
Indo.
metabolites
GTP
cGMP

steroid production, without attempting to illustrate the many other possible cellular roles of this cyclic nucleotide (Robison et al., 1971).

Cholesterol esterase and phospholipase are enzymes likely to be influenced by cyclic AMP (Dalton and Hope, 1974; Kuehl, 1974) to stimulate steroidogenesis and prostaglandin synthesis. These enzymes liberate cholesterol and free fatty acids (including arachidonic acid), the substrates for cholesterol desmolase and prostaglandin synthetase, respectively. This enzymic stimulatory action of cyclic AMP may be mediated by the activation of a protein kinase (Schulster, 1974). As noted in the figure, calcium may modulate the activity of phospholipase (Kunze et al., 1974; Haye et al., 1976), thus, accounting for the inhibition of prostaglandin synthesis in calcium-deprived incubation media.

Indomethacin is illustrated in Figure 17 as inhibiting phosphodiesterase (Kuehl, 1974). Hence, potentiation of the ACTH-elicited release of steroid and prostaglandins in the presence of low indomethacin concentrations may be explained by an increase in cellular levels of cyclic AMP when the catabolism of the cyclic nucleotide is inhibited. The lipolytic activity of cyclic AMP results in an augmented supply of precursor fatty acids for prostaglandin formation as well as cholesterol for steroid synthesis. At low levels of indomethacin, therefore, increased amounts of substrate fatty acids could effectively compete with this synthetase inhibitor, resulting in an increased prostaglandin synthesis rather than the customary decrease.

Alternatively, indomethacin may be exerting an inhibitory effect upon prostaglandin dehydrogenase (Fig. 17). The diminished catabolism of the prostaglandins might increase their effective cellular concentration and account not only for their enhanced release in response to ACTH in the presence of low concentrations of indomethacin, but also account for the increased steroid release under these conditions if prostaglandins mediate the synthesis of steroids.

Once the phospholipase and esterase are activated and liberate fatty acids for prostaglandin synthesis, the role of prostaglandins in mediating steroidogenesis can be envisioned in several ways. Figure 17 shows newly synthesized prostaglandins being released at the cortical cell surface to either function as local hormones on adrenal vasculature, or to combine with a cortical cell prostaglandin receptor (Bito, 1975). The function of an activated prostaglandin receptor can only be theorized at present. In human and ovine adrenal glands the binding of prostaglandins stimulates adenylyl cyclase, but as in the indomethacin-treated feline adrenal, this binding is not obligatory to the action of ACTH (Shio et al., 1971; Dazord et al., 1974).

On the other hand, since prostaglandins elicit only minimal increases in feline adrenal cortical cyclic AMP (Warner and Rubin, 1975), the popular concept of a feedback regulatory role for prostaglandins (Bergstrom, 1967) deserves consideration. In the renal medulla, for example, prostaglandins synthesized in response to vasopressin actually decrease the hormone-receptor activation of adenylyl cyclase (Kalisker and Dyer, 1972). If, in the model of ACTH action (Fig. 17), prostaglandin assumes a modulatory role which is inhibitory, this might account for the decline in cyclic AMP within several minutes after the onset of ACTH stimulation (Jaanus et al., 1972). The decline in cyclic AMP would then also account for the temporal decline in prostaglandins due to diminished phospholipase and prostaglandin synthetase activity. The inhibition of the ACTH-receptor stimulation of adenylyl cyclase might reside in the ability of prostaglandins to redistribute calcium within the membrane (Kirtland and Baum, 1972; Carafoli and Crovetti, 1973) since the activity of adenylyl cyclase is extremely dependent upon calcium availability (Lefkowitz et al., 1970; Sayers et al., 1972; Rubin et al., 1972).

Other possible actions of prostaglandins synthesized by the adrenal cortical cell in response to ACTH, include the direct facilitation

of mitochondrial steroidogenesis via changes in calcium availability (Malmstrom and Carafoli, 1975) and the enhanced generation of cyclic GMP which has been found to increase steroidogenesis in the rat adrenal (Halkerston, 1975). Indomethacin in sufficiently high concentrations to inhibit prostaglandin synthesis would prevent the participation of prostaglandins in these potentially steroidogenic events. Thus, the inhibited mediator role of prostaglandins in the presence of high indomethacin concentrations might account for the depressed ACTH-induced steroid release.

The model of prostaglandin interaction in the tropic action of ACTH, as depicted in Figure 17, is by no means complete regarding the roles of cyclic AMP and cyclic GMP in protein synthesis (Schulster, 1974) or the role of prostaglandins in altering membrane permeability, cholesterogenesis, or other metabolic processes (Calandra and Montaguti, 1973). It should also be noted that different species of prostaglandins can manifest not only stimulatory, but inhibitory, effects on steroidogenesis (Shaw and Tillson, 1974). The PGE_2 synthesized by cortical cells may be concerned with cell membrane regulatory effects on steroidogenesis in light of its steroidogenic action in pharmacologic concentrations (Warner and Rubin, 1975); while the more-weakly steroidogenic $PGF_{2\alpha}$ may be involved with metabolic alterations in cyclic guanosine nucleotides (Kuehl, 1973). However, the cellular mechanisms outlined in Figure 17 account for most of the results reported in the studies probing the role of prostaglandins in the mechanism of action of ACTH and adrenal cortical steroidogenesis.

SUMMARY

1. Studies were carried out on the feline adrenal gland to ascertain the role of prostaglandins in the mechanism of action of ACTH.

2. Adrenal cortical homogenates contain an enzyme mechanism for converting ^{3}H-arachidonic acid to prostaglandin-like compounds. On TLC, the radiolabeled products are identified as PGE and PGF-like substances.

3. Prostaglandins synthesized from ^{3}H-arachidonic acid by trypsin-dispersed cat adrenocortical cells were isolated by silicic acid and thin layer chromatography. PGE, PGF, and a third component with mobility properties indistinguishable from either PGA or PGB were identified both in cortical cell homogenates and incubation medium.

4. Concentrations of ACTH (125-250 μU) which stimulate steroidogenesis enhanced the conversion of labeled arachidonic acid to PGE, PGF and the PGA/B products extracted from cortical cells and incubation media.

5. Prostaglandin biosynthesis by isolated cortical cells was studied by radioimmunoassay (RIA). The antisera generated against conjugates of PGE_2, $PGF_{1\alpha}$, and $PGF_{2\alpha}$ were characterized with respect to binding specificity and reliability in assay of cortical prostaglandins. Parallel assays of incubation media using $PGF_{2\alpha}$ and $PGF_{1\alpha}$ antisera established that $PGF_{2\alpha}$ is the primary PGF released by feline cortical cells. Following the reduction of PGE to PGF with $NaBH_4$ these same two antisera were also used to identify PGE_2 as the primary PGE released. RIA using a PGE antiserum confirmed the presence of PGE in the incubation medium.

6. Steroidogenic concentrations of ACTH (50-250 μU) enhanced in a dose-related manner the PGE and PGF release by isolated cells, as determined by RIA. Indomethacin suppressed the ACTH facilitated release. Evidence for ACTH-induced prostaglandin synthesis supports the hypothesis that prostaglandins play some role in the steroidogenic

action of ACTH.

7. The perfused adrenal gland shows maximum steroidogenesis occurring 30-40 minutes following exposure to ACTH, while $PGF_{2\alpha}$ release remained elevated during the 10 minutes following ACTH perfusion and then declined to basal levels.

8. The effects of prostaglandin synthesis inhibitors on steroid and PGE and PGF release from cortical cells were investigated. Low indomethacin concentrations potentiated ACTH-evoked prostaglandin and steroid release, whereas higher concentrations depressed both responses to ACTH. The steroidogenic response to exogenous PGE_2 was not markedly altered over a wide range of indomethacin concentrations.

9. Indomethacin enhanced basal steroid release but did not enhance basal PGE or PGF release.

10. 5,8,11,14-Eicosatetraynoic acid (ETA), another inhibitor of prostaglandin synthesis, elicited a concentration-dependent inhibition of ACTH-induced steroid release, but had little effect on PGE_2-induced steroid release. A high concentration of ETA inhibited PGE and PGF release.

11. Pregnenolone (3 μM) elicited a 30-fold rise in steroid release from isolated cortical cells but failed to augment $PGF_{2\alpha}$ and PGE_2 release. ACTH, on the other hand, increased steroid release by 16-fold and also evoked an increase in PGE_2 and $PGF_{2\alpha}$.

12. Cycloheximide completely blocked the steroidogenic effects of ACTH, while the steroidogenic response to pregnenolone was unaffected, attesting to the viability of the cells in the absence of protein synthesis. Cycloheximide depressed basal $PGF_{2\alpha}$ and PGE_2 release by 27% and 22%, respectively, while ACTH-facilitated prostaglandin release was not significantly impaired by cycloheximide.

13. ACTH, NPS-ACTH, and BCAMP increased $PGF_{2\alpha}$ and PGE_2 and steroid release by isolated cortical cells. Indomethacin completely blocked the prostaglandin effects of the polypeptides but failed to

markedly suppress steroid release. Calcium-deprivation blocked prostaglandin and steroid release evoked by ACTH and NPS-ACTH, but only inhibited prostaglandin release elicited by BCAMP without affecting steroid release.

14. These studies suggest a functional role for prostaglandins in mechanism of action of ACTH. Although the nature of this role remains to be elucidated, it appears to involve some complex interaction with calcium and cyclic nucleotides.

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ABSTRACT OF
PROSTAGLANDIN SYNTHESIS IN THE
FELINE ADRENAL CORTEX

by

Suzanne Gale Laychock
Department of Pharmacology
Medical College of Virginia
School of Basic Sciences
Virginia Commonwealth University
August, 1976

Studies were carried out on the feline adrenal gland to ascertain the role of prostaglandins in the mechanism of action of ACTH. Using tritiated arachidonic acid as a prostaglandin (PG) precursor, it was demonstrated by column and thin layer chromatography techniques that isolated trypsinized adrenocortical cells possess an active PG synthetase capable of synthesizing radiolabeled PGE, PGF, and PGA/B-like substances. Concentrations of ACTH (125-250 μU) which stimulate steroidogenesis enhanced the conversion of radiolabeled arachidonic acid to PGE, PGF and the PGA/B products extracted from cortical cells and incubation media.

PG biosynthesis by isolated cortical cells was studied by radioimmunoassay (RIA) using antisera generated against conjugates of PGE_2, $PGF_{1\alpha}$ and $PGF_{2\alpha}$. $PGF_{2\alpha}$ and PGE_2 were identified as the primary PGs released by feline cortical cells, and steroidogenic concentrations of ACTH (50-250 μU) enhanced their release in a dose-related manner. Indomethacin (10^{-5} M) inhibited PG and steroid release, whereas low indomethacin concentrations (10^{-9} M) potentiated ACTH-evoked PG and steroid release. The steroidogenic response to exogenous PGE_2 was not markedly altered by indomethacin. 5,8,11,14-Eicosatetraynoic acid (ETA) inhibited PGE and PGF release, and elicited a concentration-dependent inhibition of ACTH-induced steroid release. Therefore, there appears to be a functional relationship between PG and steroid release. Such a relationship was further supported by

studies on the perfused adrenal gland, which demonstrated that maximal $PGF_{2\alpha}$ release in response to ACTH preceded the maximal steroidogenic response. Moreover, pregnenolone (3 μM) elicited a 30-fold increase in steroid release from isolated cortical cells but failed to augment $PGF_{2\alpha}$ and PGE_2 release; this study further supports the concept that PG synthesis occurs prior to the steroidogenic response to ACTH.

Cycloheximide did not block the steroidogenic response to pregnenolone, but completely blocked the steroidogenic effects of ACTH. Cycloheximide also depressed basal $PGF_{2\alpha}$ and PGE_2 release, while ACTH-facilitated PG release was not significantly impaired. Thus, the enzymes responsible for increasing PG synthesis are activated rather than formed de novo in response to ACTH.

Three steroidogenic agents, ACTH, an ACTH analogue NPS-ACTH, and monobutyryl cyclic AMP (BCAMP), increased $PGF_{2\alpha}$ and PGE_2 release from isolated adrenocortical cells. Calcium deprivation blocked PG and steroid release evoked by ACTH and NPS-ACTH, but only inhibited PG release elicited by BCAMP without affecting steroid release. These studies suggest a functional role for PGs in the mechanism of action of ACTH. Although the nature of this role remains to be elucidated, it appears to involve some complex interaction with calcium and cyclic nucleotides.