Mediatory Role of Calcium and Guanosine 3′,5′-Monophosphate in Adrenocorticotropin-Induced Steroidogenesis by Adrenal Cells

Abstract. *When incubated in a calcium-free medium, isolated rat fasciculata cells showed neither an increase in the concentration of guanocine 3′,5′-monophosphate (cyclic GMP) nor an increase in corticosterone production in response to adrenocorticotropic hormone (ACTH). In response to submaximum and maximum steroidogenic concentrations of ACTH, corticosterone formation was directly proportional to increases in calcium concentration ranging from 0 to 2.5 m*M. *Higher concentrations of calcium, however, inhibited maximal ACTH-induced steroidogenesis. In the absence of ACTH, calcium did not stimulate cyclic GMP accumulation and corticosterone formation. ACTH-induced corticosterone synthesis, preceded by an increase in cyclic GMP, was restored when ACTH and calcium were both present in the medium. Cyclic GMP or dibutryl cyclic GMP-induced steroidogenesis was substantially reduced in the absence of calcium, but in contrast to the ACTH effect a significant amount of corticosterone formation occurred without calcium. It is proposed that at the physiological concentrations of the hormone, calcium regulates the transduction of information between hormone receptors and guanylate cyclase.*

Since the original observation of Birmingham *et al.* that calcium is required for stimulation of adrenal steroidogenesis by adrenocorticotropic hormone (ACTH), several investigators have confirmed this finding (*1*). The exact mechanism by which the ion exerts its action is still not clear. With the advancement of the proposal that adenosine 3′,5′-monophosphate (cyclic AMP) is the mediator of ACTH-induced steroidogenesis (*2*), workers at several laboratories have examined the effect of calcium on the cyclic AMP system and have attempted to correlate these results with the process of steroidogenesis. Many of their data indicate that calcium is not necessary for the interaction of ACTH with the adrenal receptor (*3*). Other investigators have concluded that the major effect of calcium on ACTH-induced steroidogenesis is on the step or steps preceding the formation of cyclic AMP (*4*), thus implying the action of calcium at sites between the binding of ACTH to the receptor and the activation of adenylate cyclase. However, evidence provided by Farese (*5*) has implicated calcium in the maintenance of optimal protein synthesis thought to be required for the steroidogenic effect of ACTH and cyclic AMP (*6*).

Studies at this laboratory have demonstrated the important mediatory role of guanosine 3′,5′-monophosphate (cyclic GMP) in ACTH-induced steroidogenesis in isolated rat adrenal cells (*7*). In the study described here, we investigated the role of calcium on the generation of cyclic GMP and corticosterone production when isolated adrenal cells were incubated with ACTH. We also have examined the effect of calcium on the stimulation of corticosterone production by exogenous cyclic GMP and dibutyryl cyclic GMP. On the basis of these studies, we propose a model for the role of calcium and cyclic GMP in the ACTH-propagated signal for steroidogenesis.

Figures 1A and 2 show that 100 μU of ACTH in the presence of 2.5 m*M* calcium induced maximum steroidogenesis, but no corticosterone production was observed in response to ACTH in concentrations of up to 50,000 μU when calcium was omitted from the incubation medium (Fig. 1A). This indicates that the requirement of calcium is absolute in ACTH-induced steroidogenesis. Corticosterone formation was directly proportional to calcium concentrations ranging from 0 to 2.5 m*M* in the presence of submaximum and maximum steroidogenic concentrations of ACTH. However, higher concentrations of calcium inhibited steroidogenesis. These results are in contrast to earlier findings (*4*), where it was shown that 10,000 to 50,000 μU of ACTH caused steroidogenesis in the absence of calcium. The discrepancy between these results is not clear, but the steroidogenesis in calcium-free media observed at other laboratories could have been due to residual calcium present in the cells. Also, other commercially available reagents added to the incubation media would have contained trace amounts of calcium. It is possible that calcium may not be titrated out completely with EDTA from cell suspensions originally prepared by proteolytic digestion.

Figure 1B shows that concentrations of up to 50,000 μU of ACTH did not stimulate an increase in the cyclic GMP concentration in calcium-free medium. This indicates that the requirement of calcium is absolute for the activation of guanylate cyclase. Calcium (0.25 to 5 m*M*) alone neither increased the cyclic GMP level (data not shown) nor activated the process of steroidogenesis (Fig. 2). These data indicate that the presence of calcium is obligatory in the ACTH-activated formation of cyclic GMP, but cal-

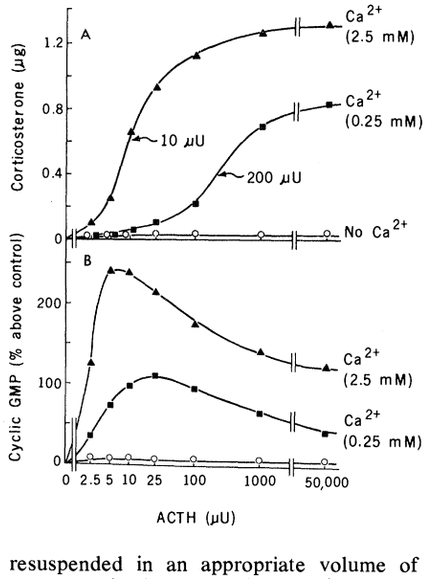

Fig. 1. Concentration response curves for the production of (A) corticosterone and (B) cyclic GMP in isolated rat adrenal cells incubated in the presence of 0 to 50,000 μU of ACTH at varying concentrations of calcium. Isolated adrenal cells were prepared as previously described (*8*), except that reagents used in the cell preparation were completely free of calcium. For cell preparation by the standard method, bovine serum albumin is used that has been dialyzed in Krebs-Ringer bicarbonate buffer containing calcium. In the present studies, the dialysis buffer did not contain any calcium. The cells were trypsin-digested as usual (*8*), resuspended in calcium-free Krebs-Ringer bicarbonate buffer, *p*H 7.4, containing 4 percent albumin, 0.2 percent glucose (KRB-GA), lima bean trypsin inhibitor, and 0.25 m*M* EGTA to ensure the removal of any residual calcium. The cells were incubated for 15 minutes and centrifuged at 100*g* for 45 minutes at 4°C. The cell pellet was suspended in 5 ml of calcium-free KRB-GA, pelleted by centrifugation as above, then resuspended in an appropriate volume of calcium-free KRB-GA. Each incubation mixture contained 2×10^6 isolated adrenal cells suspended in 0.8 ml; the reagents were dissolved in 0.2 ml of calcium-free KRB-GA. Exogenous calcium, when added, was in the form of calcium chloride. Experiments were conducted in quadruplicates; two of the samples were used for determination of corticosterone and two for the measurement of cyclic GMP (*11*). The samples used for cyclic GMP assays were incubated for 10 minutes, and those used for corticosterone assays were incubated for 2 hours. Results are expressed as the mean values of six separate determinations from three different experiments. Basal values (0.03 μg of corticosterone; and 2.0 pmole of cyclic GMP) have been subtracted from the experimental results. Arrows indicate the half-maximal ACTH concentration required for a specific response.

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cium alone cannot stimulate either guanylate cyclase or corticosterone formation.

Exogenous cyclic GMP and dibutyryl cyclic GMP were able to activate steroidogenesis in the absence of calcium (Fig. 3), although the maximal steroidogenic responses obtained with the cyclic nucleotides were significantly lower than those observed in the presence of calcium. This indicates that the presence of calcium is not obligatory to propagate the cyclic GMP steroidogenic response. These data also indicate that steroidogenic steps that occur after the formation of cyclic GMP are potentiated by calcium.

Previous studies with isolated fasciculata cells (*8*) have demonstrated that physiological concentrations of ACTH stimulate the formation of cyclic GMP (*9*), but not cyclic AMP, with corresponding increases in the protein kinase activity and corticosterone synthesis (*10, 11*). Excellent temporal correlation was observed between cyclic GMP formation, phosphorylation, and corticosterone synthesis in response to as little as 5 μU of ACTH (*11*). These observations indicate that the hormonal response is mediated by cyclic GMP through the cyclic GMP-dependent protein kinase. The presence, characterization, and specificity of cyclic GMP-dependent protein kinase from bovine adrenal cortex has been described (*12*). In contrast to the cyclic AMP-dependent protein kinase (*13*), the bovine adrenal cyclic GMP-dependent protein kinase is not dissociated into regulatory and catalytic subunits (*12*). Furthermore, the direct stimulatory effect of ACTH and cyclic GMP on the transformation of cholesterol to corticosterone has been demonstrated (*14*). Indirect evidence has been provided that the cycloheximide-sensitive translational control of the hormone mediated by cyclic GMP is at the entry of cytoplasmic cholesterol into the mitochondria (*14*). It has been further demonstrated that the cycloheximide-sensitive step is after the activation of protein kinase (*10*). Considering these factors, our previously proposed hypothesis (*14*) on the mechanism of the control of ACTH, as mediated by cyclic GMP, may be further extended.

According to the current proposal, ACTH binds to the hormone receptor located on the adrenal cell plasma membrane (*15*). At physiological concentrations of the hormone, calcium regulates the transduction of information between hormone receptors and guanylate cyclase. This leads to the increase (*9*) in cyclic GMP concentration, which in turn activates the cyclic GMP-dependent protein kinase (*10, 11*), leading to the translation of a hypothetical preexistent messenger RNA (*2, 16*). The new protein, thus synthesized, controls the entry of cytoplasmic cholesterol into the mitochondrial precursor pool of cholesterol. This model is depicted in Fig. 4. According to this proposal, calcium alone cannot substitute for ACTH or cyclic GMP in promoting adrenal steroidogenesis.

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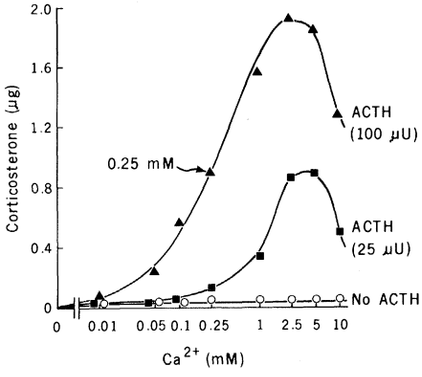

Fig. 2 (left). Concentration-response curves showing the production of corticosterone in isolated adrenal cells stimulated with 25 μU and 100 μU of ACTH and incubated for 120 minutes in the presence of 0 to 10 m*M* of calcium. The conditions of the experiment were identical to those in Fig. 1.

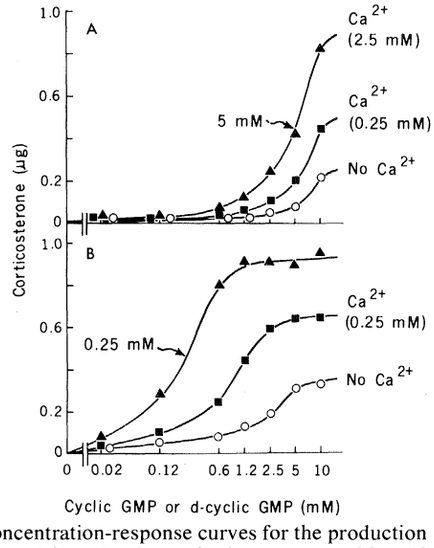

Fig. 3 (right). Concentration-response curves for the production of corticosterone in isolated adrenal cells incubated for 120 minutes in the presence of 0 to 10 m*M* of (A) cyclic GMP or (B) dibutyryl cyclic GMP at varying concentrations of calcium. The conditions of the experiment were identical to those in Fig. 1.

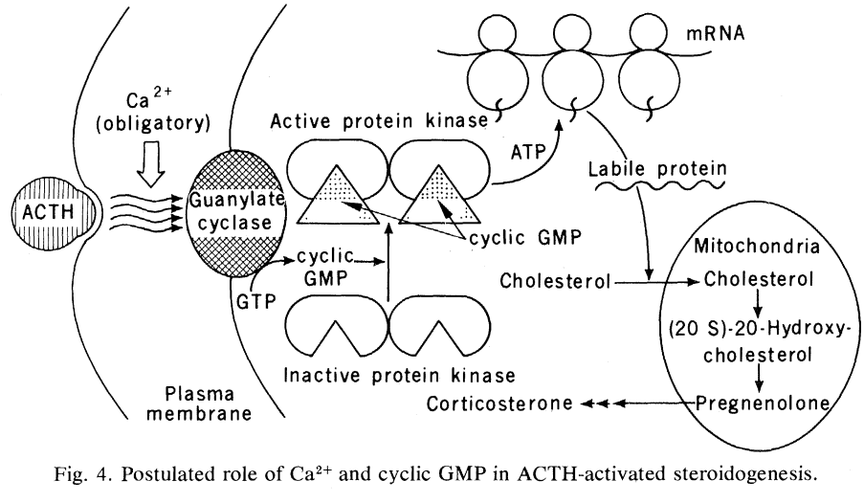

Fig. 4. Postulated role of Ca^{2+} and cyclic GMP in ACTH-activated steroidogenesis.

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Narcotic Analgesia: Fentanyl Reduces the Intensity but Not the Unpleasantness of Painful Tooth Pulp Sensations

Abstract. *Forty subjects rated the magnitude of painful electrical stimulation of tooth pulp before and after the intravenous administration of either fentanyl, a short-acting narcotic, or a saline placebo. The responses were choices of verbal descriptors from randomized lists of either sensory intensity (that is, weak, mild, intense) or unpleasantness (annoying, unpleasant, distressing) descriptors. The fentanyl significantly reduced the sensory intensity without reducing the unpleasantness of the tooth pulp stimuli, indicating that the mechanisms of narcotic analgesia may include a significant attenuation in pain sensation in addition to effects on pain reaction. These results stress the importance of using multiple measures of pain.*

Present beliefs (*1*) about the mechanisms of pain and narcotic analgesia remain virtually unchanged since the influential reports by Beecher (*2*). Beecher distinguished between relatively invariant "pain sensations" that relate to the intensity of noxious stimuli and varied "pain reactions" that reflect complex emotional and cognitive responses elicited by such stimuli. The latter are influenced by personality, past experience, and experiential context. He proposed that narcotics produce clinical analgesia primarily by altering the unpleasantness of pain reactions rather than by altering the intensity of pain sensations. This view, prevalent for the past 20 years, has de-emphasized the role of sensory processes in narcotic analgesia.

We now report evidence from human subjects that fentanyl, a short-acting narcotic, can reduce the intensity of electrically induced tooth pulp pain sensations independent of the reaction, or unpleasantness, that accompanies these sensations. We asked subjects to rate the magnitude of sensory intensity and unpleasantness by direct procedures, this having been done with other sensory modalities, such as taste, olfaction, and temperature (*3*). The subjects quantified painful electrical stimulation of tooth pulp by choosing words from randomized lists of 12 words that described either the sensory intensity or the unpleasantness associated with stimuli of varying magnitude (see Table 1). This method facilitates discrimination of a single dimension because it forces judgments based on word meaning rather than other cues in rating scales such as rank order or position in a rank-ordered list of verbal or nonverbal categories.

The relative magnitudes of the words within a dimension are quantified reliably by ratio scaling procedures (*4*). Standard testing criteria reveal a high degree of objectivity for both the sensory intensity and the unpleasantness scales, which indicates that an individual scale is predicted equally well by another scale from that individual or by a mean scale from another similar group (*5*). The validity of using quantified verbal descriptors to discriminate between sensory intensity and unpleasantness has been shown in an additional study. Subjects rated either the sensory intensity or unpleasantness of electrocutaneous stimuli before and after the administration of diazepam, a minor tranquilizer. Diazepam altered only the unpleasantness scale, consistent with evidence showing that diazepam attenuates the aversive aspect of stimuli but does not alter sensory magnitude (*6*).

Forty dental patients scheduled for an oral surgical procedure (third molar extractions) participated individually in two 1-hour sessions approximately 1 week apart. Each subject was randomly assigned to either a sensory intensity or an unpleasantness descriptor group. Relative magnitudes of the descriptors were determined in the first session by asking each subject to match the force of handgrip strength and the duration of a button press to their perceptions of (i) the lengths of seven lines and (ii) the magnitude of the sensory intensity or unpleasantness implied by 12 descriptors presented twice in randomized order for each response measure. With this method, common responses (handgrip force or time duration) are made to both test (words) and standard (line lengths) stimuli, and ultimately the test stimuli are expressed in terms of the standard stimuli. Patient estimates of line lengths produced mathematical power functions that were used to transform the mean response to each word from units of handgrip force or time duration to common units of line length, referred to as units of relative magnitude. This procedure [for details see (*7*)] reduces bias that occurs in direct scaling experiments and permits the comparison of different response measures within individuals and the comparison of responses across individuals (*8*). After the descriptor scaling in

Table 1. The words used to describe the relative magnitudes of sensory intensity and unpleasantness. Each magnitude was determined by cross-modality matching of perceived handgrip force or duration of a button press to the magnitude of the sensory intensity or unpleasantness implied by each descriptor. Additional cross-modality matches to physically measurable line-length stimuli produced calibration functions used to transform mean handgrip or time-duration responses to each descriptor from units of force or time to common units of line length, referred to as units of relative magnitude.

Sensory intensity		Unpleasantness	
Descriptor	Relative magnitude	Descriptor	Relative magnitude
Extremely intense	59.5	Very intolerable	44.8
Very intense	43.5	Intolerable	32.3
Intense	34.6	Very distressing	18.3
Strong	22.9	Slightly intolerable	13.6
Slightly intense	21.3	Very annoying	12.1
Barely strong	12.6	Distressing	11.4
Moderate	12.4	Very unpleasant	10.7
Mild	5.5	Slightly distressing	6.2
Very mild	3.9	Annoying	5.7
Weak	2.8	Unpleasant	5.6
Very weak	2.3	Slightly annoying	3.5
Faint	1.1	Slightly unpleasant	2.8

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