Isolated Adrenal Cells: Adrenocorticotropic Hormone, Calcium, Steroidogenesis, and Cyclic Adenosine Monophosphate

Abstract. *Corticosterone production by isolated adrenal cells in response to adrenocorticotropic hormone is reduced when the cells are incubated in a medium that contains no calcium. This reduction is associated with an equal reduction of accumulation of cyclic adenosine monophosphate. Production of corticosterone and accumulation of cyclic adenosine monophosphate are increased when the calcium concentration in the medium is increased (from zero to 7.65 millimolar). This is in contrast to the situation in "subcellular membrane fragments" of adrenal tissue where high calcium in the medium (> 1.0 millimolar) inhibits cyclic adenosine monophosphate accumulation. We propose that adenyl cyclase in the intact plasma membrane is located in a compartment wherein calcium concentration is low and remains unaffected by the concentration of calcium in the extracellular space. It is proposed that, as the concentration of calcium in the incubation medium is increased from zero to 7.65 millimolar, the strength of the signal generated by the interaction of adrenocorticotropic hormone with its receptor and transmitted to the adenyl cyclase compartment is proportionately increased.*

As is true for a number of tissues, calcium exerts stimulatory and inhibitory effects at various loci within the cells of the adrenal cortex. Birmingham and co-workers (*1*) showed that increased steroidogenesis by rat adrenal quarters, which normally occurs in response to adrenocorticotropic hormone (ACTH), was abolished when calcium was absent from the incubation medium. Furthermore, the ability of the perfused cat adrenal to secrete corticosteroids is reduced when calcium is omitted from the perfusate (*2*). Conversion of deoxycorticosterone to corticosterone (B) by mitochondrial preparations of bovine adrenal cortex increases as the concentration of calcium increases in the medium (*3*). However, according to Lefkowitz *et al.* (*4*) concentrations of calcium greater than 1.0 m*M* suppress binding of ACTH to receptor and inhibit activation of adenyl cyclase by ACTH when measured in "subcellular cell membrane particles" of mouse adrenal tumor tissues. In an attempt to further elucidate the regulatory role of calcium and to delineate its sites of action, we have measured corticosterone production and cyclic adenosine monophosphate (AMP) accumulation when ACTH is added to isolated adrenal cells (*5*) suspended in buffer containing zero, 2.55, or 7.65 m*M* calcium.

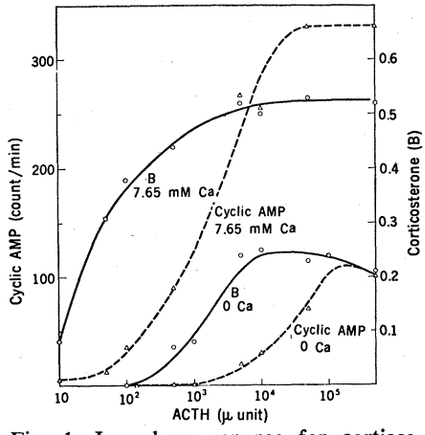

Fig. 1. Log dose response for corticosterone (B) production (μg/120 minutes) by portions (0.9 ml; 250,000 cells) of isolated adrenal cells incubated at 37°C for 120 minutes in an atmosphere of 95 percent O_2 and 5 percent CO_2 in response to ACTH (3rd International Standard) in microunits (*μ unit*). The pool of isolated adrenal cells was prepared by the method of Sayers *et al.* (*5*), divided into three portions, centrifuged, and suspended in buffer containing zero, 2.55, or 7.65 m*M* calcium. The points are averages of B analyses (*15*) on duplicate portions of cell suspension. Corticosterone production is expressed as B in portions to which ACTH was added (in 0.1 ml of vehicle) minus B in blanks (vehicle only). The blanks were incubated for 120 minutes. Calcium did not influence the quantity of B in cell suspensions to which no ACTH was added. Corticosterone in the blanks was 0.05 μg for zero, 0.08 μg for 2.55, and 0.09 μg for 7.65 m*M* calcium.

The results of three experiments were in excellent agreement; the curves of the log dose response (LDR) for one of them are presented in Fig. 1. Maximum B production (B_{max}) was the same for cells suspended in 2.55 and 7.65 m*M* calcium; B_{max} was reduced to approximately 50 percent of this value when the buffer had no calcium. The dose of ACTH which induces one-half B_{max} (A_{50}) was slightly but significantly smaller for the cells suspended in 7.65 m*M* calcium as compared to cells in 2.55 m*M*. When the cells were in zero calcium, A_{50} was increased. The results indicate that, over the range tested, the higher the concentration of calcium, the more responsive were the cells to ACTH as measured by corticosterone secretion.

In a fourth experiment, corticosterone and cyclic [8-^{14}C]AMP accumulation were measured on portions from a single dispersate of cells incubated in buffer containing zero or 7.65 m*M* calcium. Log dose response curves are presented in Fig. 2. Accumulation of cyclic [8-^{14}C]AMP and B production are reduced when cells are suspended in zero rather than 7.65 m*M* calcium. Production of B reaches a maximum at much lower doses of ACTH than does cyclic AMP accumulation. For cells incubated in 7.65 m*M* calcium, the doses of ACTH producing one-half maximum steroidogenesis and one-half maximum accumulation of cyclic AMP were 33 and 2000 microunits, respectively. These observations confirm the work of Grahame-Smith *et al.* (*6*) and of Beall and Sayers (*7*) to the effect that cyclic AMP accumulation is not the limiting step in B production. For zero calcium, the A_{50} for B production was 1700 microunits and that for cyclic

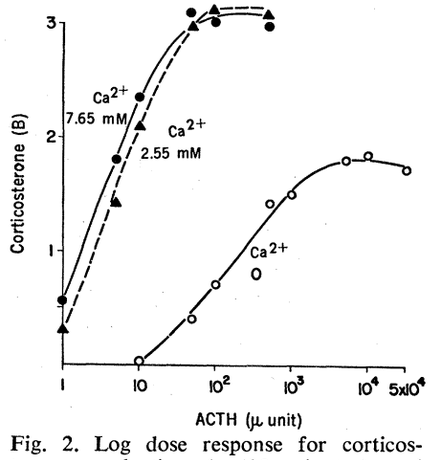

Fig. 2. Log dose response for corticosterone production (μg/30 minutes) and accumulation of cyclic [8-^{14}C]AMP (count/min) by isolated adrenal cells in response to various doses of ACTH in microunits. A single pool of rat-isolated adrenal cells was divided into two parts and centrifuged. One pellet was suspended in buffer which contained 7.65 m*M* calcium, the other in buffer which contained no calcium. Portions of cells (0.9 ml; 300,000 cells) plus ACTH in 0.1 ml of vehicle were incubated for 30 minutes at 37°C in 95 percent O_2 and 5 percent CO_2. The [8-^{14}C]adenine was added to the medium during the dispersion of the adrenal tissue and its incorporation into cyclic [8-^{14}C]AMP was measured by a modification of the method developed by Kuo and De Renzo (*16*). The values for B and cyclic [8-^{14}C]AMP are corrected for duplicate blanks to which no ACTH was added.

AMP production, 30,000 microunits. B_{max} was reduced by 50 percent when cells were suspended in zero calcium as compared to those in 7.65 m*M* calcium; maximum for cyclic AMP accumulation was reduced by 65 percent when calcium was omitted from the buffer. These observations indicate that, when the external medium has no calcium, cyclic AMP accumulation by isolated adrenal cells is reduced to about the same degree as B production. We conclude that the decrease in B production which follows removal of calcium from the buffer medium is in large measure a consequence of reduction of cyclic AMP accumulation.

Adenosine triphosphate (ATP), adenosine diphosphate (ADP), and 5′-AMP in deproteinized extracts of isolated adrenal cells were resolved by paper chromatography [95 percent ethanol and 1*M* ammonium acetate (7 : 3)]. Radioactivity in the [8-^{14}C]ATP fraction was the same for cells incubated 30 minutes with or without ACTH (5×10^5 microunits) in buffer containing zero calcium as for cells incubated in 7.65 m*M* calcium. The reduction in incorporation of [8-^{14}C]adenine into cyclic [8-^{14}C]AMP, when calcium is absent from the medium does not appear to be due to reduction in synthesis of [8-^{14}C]ATP (*8*).

The results of our investigation, together with various works of others, suggest a tentative working model for the steroidogenic action of ACTH. The sequence, from the interaction of ACTH with receptor to the increased rate of secretion of corticosterone, is set forth in Fig. 3. According to Lefkowitz *et al.* (*4*) the first event is ". . . the binding of ACTH to receptor . . ." (**1**, circled in Fig. 3) followed by the activation of adenyl cyclase **3**. These workers have shown that calcium in a concentration greater than 1.0 m*M* depresses the "binding" of [^{125}I]ACTH to the receptor on "subcellular cell membrane particles" from a mouse adrenal tumor. There is no convincing experimental evidence or theory that compels "depression of binding" to be equated with decrease in biological response. Actually, according to one theory proposed by Paton (*9*), occupancy of the receptor by hormone and anything favoring it produces antagonism. High efficacy of ACTH would be associated with high dissociation rate, low efficacy with low dissociation rate. In this connection, Rodbell *et al.* (*10*) have demonstrated that guanyl nucleotides play a specific and obligatory role in glucagon activation of adenyl cyclase located in rat liver plasma membrane fragments and decrease affinity of the binding sites for glucagon. Calcium may exert similar effects on the ACTH–receptor–adenyl cyclase complex on adrenal cell plasma membrane.

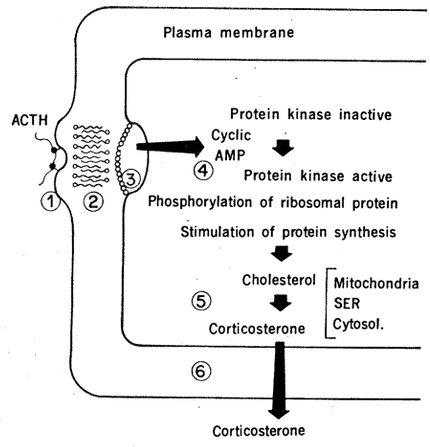

Fig. 3. Sequence of steps from interaction of ACTH with receptor on the plasma membrane to increased rate of secretion of corticosterone by a cell of the adrenal cortex of the rat. **1**, ACTH interacts with receptor. **2**, Signal is generated and transmitted through the plasma membrane. **3**, Adenyl cyclase is activated and synthesis of cyclic AMP increases. **4**, A protein kinase is activated. **5**, Cholesterol is converted to corticosterone. **6**, Corticosterone is secreted.

In our experiments, cyclic AMP production by intact cells increased as the concentration of calcium in the medium increased from zero to 7.65 m*M*. We propose that the strength of the signal generated at **1** and transmitted **2** (Fig. 3) through the membrane to the adenyl cyclase compartment is related to the concentration of calcium on the outside of the plasma membrane. On the other hand, activation by ACTH of adenyl cyclase of membrane fragments is optimum at a concentration of about $10^{-7}M$ calcium (*11*) and inhibited at calcium concentrations greater than $10^{-3}M$ (*4*, *11*). For this reason, we further propose that adenyl cyclase in the intact plasma membrane is confined to a compartment (**3**, Fig. 3) where the concentration of calcium approximates that of the cytosol (about $10^{-7}M$) and remains relatively fixed in the face of wide fluctuations in the concentration of the cation on the outside of the cell. There is evidence that adenyl cyclase itself is not the receptor for ACTH (*12*). If adenyl cyclase is located on the inner aspect of the plasma membrane as shown at **3**, then the enzyme of membrane fragments is exposed to abnormal ("outside") concentrations of calcium. This would explain why Lefkowitz *et al.* (*4*) observed inhibition of ACTH activation of adenyl cyclase of membrane fragments exposed to high concentrations of calcium (> 1 m*M*). The signal arising from the interaction of ACTH and its receptor was strong, but the abnormally high concentration of calcium inhibited adenyl cyclase. Furthermore, the model explains why Lefkowitz *et al.* (*4*) were obliged to use very high concentrations of ACTH ($10^{-6}M$) to activate adenyl cyclase when the membrane fragments were suspended in a medium of low concentration of calcium. In this instance, adenyl cyclase was responsive to the signal, but, in order to overcome the adverse effect of low calcium on signal strength, high concentrations of ACTH had to be used.

In analogy with the situation in skeletal muscle (*13*), we suggest a regulatory influence for calcium in the events concerned with activation of a kinase by cyclic AMP and the eventual synthesis of a protein (*14*) (**4**, Fig. 3) which is presumed to initiate steroid biosynthesis. Péron and McCarthy (*3*) have demonstrated that calcium influences the conversion of cholesterol to corticosterone **5**. That calcium does exert effects beyond cyclic AMP production is indicated by an experiment in which dibutyryl cyclic AMP was added to isolated adrenal cells suspended in buffer containing zero or 7.65 m*M* calcium. In response to dibutyryl cyclic AMP, B_{max} was reduced by 50 percent when calcium was absent from the medium. However, in contrast to the observations on ACTH, the dose of dibutyryl cyclic AMP which induced one-half B_{max} was not altered when calcium was absent from the medium. These results are interpreted to mean that the marked increase in the dose of ACTH required to induce one-half B_{max} and one-half maximum cyclic AMP accumulation is a result of the weakening of the signal at **2** in the absence of calcium; the reduction in B_{max} is primarily due to impairment of reactions subsequent to cyclic AMP production (**4** and **5**).

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References and Notes

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Rat Brain Binds Adrenal Steroid Hormone: Radioautography of Hippocampus with Corticosterone

Abstract. *Tritiated corticosterone injected subcutaneously into adrenalectomized male rats 1 hour before killing produced intense labeling of the hippocampus in radioautograms prepared by a method that reduced or prevented diffusion of the radioactive material. The pyramidal neurons of the cornu ammonis and the granule neurons of the gyrus dentatus contained more radioactivity than did other regions of the brain; however, the intensity of labeling varied among adjacent neurons. The nuclei of many neurons were clearly labeled but radioactivity was relatively sparse in the cytoplasm, in the axons where they branch from cell bodies, and in adjacent neuropil.*

Corticosterone, the principal adrenal glucocorticoid in the albino rat (*1*), enters the brain of this species from the blood and attaches to highly specific, limited-capacity binding sites (*2*). Biochemical studies of tissue obtained by gross brain dissection showed that the hippocampus contains the highest concentration of these binding sites (*3*). By subcellular fractionation of brain tissue, it was shown that substantial binding occurs in cell nuclei isolated from the hippocampus and other brain regions (*2*, *4*).

In this report we present evidence from a radioautographic study (*5*) that demonstrates binding of radioactive corticosterone in the hippocampus. We have confirmed and extended observations of sensitivity of the hippocampus to adrenocorticoids—observations made by the use of biochemical techniques (*2–4*), electrical recording of single-unit neuronal activity (*6*), and corticoid implantation (*7*). A radioautographic method that was designed to reduce or prevent artifacts from the translocation of labeled substances (*8*) permitted us to determine the distribution of radioactivity in the hippocampus with a high degree of spatial resolution (*9*). The resolution was adequate to detect a heterogeneity of labeling among adjacent cells of the hippocampus, to identify the labeled cells as neurons, and to observe heavy labeling of cell nuclei.

We employed a modification of the radioautographic technique described by Anderson and Greenwald (*8*), and analyzed radioautograms from four brains. To eliminate competition for binding sites in the brain by endogenous corticosterone released during the stress of injecting [^{3}H]corticosterone, we removed the adrenal glands of male rats of the Sprague-Dawley strain. The rats were given free access to standard rat chow and a solution of 0.8 percent sodium chloride. Between days 9 and 23 after adrenalectomy, each animal received a single subcutaneous injection of 200 to 300 μc of [1,2-^{3}H]corticosterone (*10*) dissolved in 0.1 ml of ethanol. The rats were decapitated 1 hour after the injections, the time at which binding reaches its peak in cell nuclei isolated from the hippocampus (*2*).

The brains were quickly removed and divided longitudinally at the midline, and the left hemisphere was used for sagittal sectioning and the right hemisphere for coronal sectioning. Each piece of brain was mounted onto a sandwich consisting of a thin copper disk between two wafers of fine stainless steel mesh. The mounted tissue was rapidly frozen by partial submersion in liquid nitrogen. Tissues were stored in a liquid nitrogen refrigerator until they were sectioned. Frozen sections 4 μm thick and spaced 100 μm apart were cut in a cryostat at −20°C. In darkness, individual sections were picked up from the cold knife by barely touching them with warmer glass slides, which had been coated with Kodak NTB-3 liquid emulsion (*11*) and desiccated before use. The radioautograms were exposed for 180 to 320 days at 4°C in sealed, double-depth, black plastic slide boxes (*12*) that were half-filled with Drierite and stored in a large box lined with lead. To control for artifacts due to negative chemography, some radioautograms from each brain were thoroughly air-dried and exposed uniformly to light before storage. The brain from an uninjected rat was also processed to control for artifacts due to positive chemography (*13*).

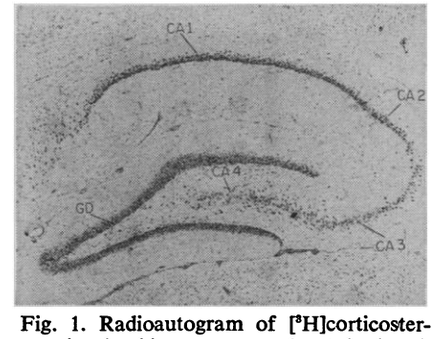

Fig. 1. Radioautogram of [^{3}H]corticosterone in the hippocampus of rat brain. A sagittal frozen section was exposed 180 days and stained lightly with Darrow red and light green. Shown are the longitudinal fields *CA1* to *CA4* of the pyramidal neuron layer in the cornu ammonis, and the granule neuron layer in the gyrus dentatus (*GD*) (×17).