ADENYL CYCLASE AND HORMONE ACTION, I. EFFECTS OF ADRENOCORTICOTROPIC HORMONE, GLUCAGON, AND EPINEPHRINE ON THE PLASMA MEMBRANE OF RAT FAT CELLS

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Abstract.—A large number of hormones, of diverse molecular structure, evoke characteristic responses in target cells via the intermediary 3′,5′-AMP, the specificity of hormone action upon cell type being achieved by selective stimulation of adenyl cyclase. In the fat cells of rat adipose tissue, adenyl cyclase is stimulated by a number of hormones of disparate molecular structure, posing the question whether this cell type posesses multiple cyclase systems with distinctive specificities for individual hormones, or a single cyclase with broad specificity to a variety of hormones.

Studies of the stimulatory effects of adenocorticotropin, glucagon, and epinephrine upon the adenyl cyclase of the rat fat cell "ghosts" (plasma membrane sacs) have shown that distinctive selectivity sites for each of these hormones can be differentiated. The β-adrenergic blocking agent Kö 592 abolished the stimulatory effect of epinephrine without influencing adenocorticotropin or glucagon; Ca was required for adenocorticotropin action, but not for glucagon or epinephrine. Dose-response curves show that the affinity of hormones to the cyclase system was in the order: glucagon > adenocorticotropin ≫ epinephrine; the magnitude of cyclase activation achieved by maximal doses of hormones had a reversed order. Combinations of maximal doses of hormones failed to produce additive stimulation. The results show that in the membrane of the fat cell a single catalytic unit of adenyl cyclase is coupled to distinctive selectivity sites for three lipolytic hormones.

The action of a hormone in a multicellular organism may be viewed as an informational transaction, where a specific chemical signal input evokes a specific adaptive response by the cell.[1] We should like to know the underlying principles and detailed molecular mechanisms involved in the selective *reception* and *readout* of hormonal signals.

The cyclic nucleotide 3′,5′-AMP has been strongly implicated as the critical intermediary in the readout of a set of peptide hormones and biogenic amines, including: glucagon, adenocorticotropin (ACTH), vasopressin, thyroid-stimulating hormone, luteinizing hormone, parathormone (perhaps gastrin), catecholamines, and serotonin.[1, 2] In characteristic target tissues and cells, these signals activate a membrane-associated enzyme, adenyl cyclase, that converts ATP to 3′,5′-AMP. The cyclic nucleotide generated in turn initiates a sequential set of reactions[3]—which differ in various specialized cell types—in each case leading to a specific cellular response characteristic of hormonal stimulation.

Adenyl cyclases in membrane preparations of certain target tissues have been shown to respond to the same set of added hormones that are effective in intact

cells or tissues.[2] The cyclases of some tissues exhibit high selectivity and respond to a single hormone (ACTH in adrenal cortex,[4] thyroid-stimulating hormone in thyroid,[5] catecholamines in nucleated erythrocytes[6]); other tissues respond to two or more hormones with an increased 3′,5′-AMP formation (e.g., epinephrine and glucagon in liver).[7] Rat adipose tissue responds to a multiplicity of hormones of disparate molecular structure (including epinephrine, ACTH, glucagon, and thyroid-stimulating hormone) with a single lipolytic response[8] presumably mediated via 3′,5′-AMP.[2, 9, 10] To elucidate the mechanisms of hormone selectivity and adenyl cyclase activation, plasma membrane sacs of rat fat cells which possess an adenyl cyclase moiety sensitive to ACTH,[11] glucagon,[12] and epinephrine[12] have been selected as a model system for detailed investigation.

Materials and Methods.—Male Sprague-Dawley rats (Rockland Farms), weighing 130–180 gm and subjected to a 3-day starving/3-day refeeding cycle, were used in these experiments. Plasma membrane sacs ("ghosts") were prepared by lysing fat cells isolated from rat epididymal fat pads as described by Rodbell.[11] The ghosts were suspended in 1 mM $KHCO_3$ (containing 0.1% bovine serum albumin) and assayed after being kept at 0° within 1–2 hr or after storage at −20°.

ATP-α-^{32}P (over 500 millicurie/millimole) was obtained from International Chemical and Nuclear Corp. Bovine serum albumin (Armour fraction V) was purified by chemical treatment.[15] Collagenase was obtained from Worthington, sodium phosphoenol pyruvate and pyruvate kinase (type III) from Sigma. Stock solutions of L-epinephrine (Sigma) (1 mg/ml in 0.01 *N* HCl) were stored in the dark, frozen, and then discarded after 1 week. Synthetic ACTH ("Synacthen," β^{1-24}-corticotropin, activity 104 IU/mg) was a gift of Dr. W. Rittl, Ciba, Basel, Switzerland. Synthetic glucagon (activity equivalent to natural porcine hormone) was a gift of Dr. E. Jaeger, Max-Planck-Institute, München, Germany. Kö 592 [1-(3-methylphenoxy)-3-isopropylaminopropanol-2] was a gift of Dr. A. Engelhardt, Boehringer-Sohn, Ingelheim, Germany.

Adenyl cyclase was assayed by a method we recently developed in this laboratory,[13] using ATP-α-^{32}P as substrate and trapping the radioactive 3′,5′-AMP formed with cold 3′,5′-AMP, under conditions where a constant level of ATP was maintained by a regenerating system (sodium phosphoenol pyruvate and pyruvate kinase). The formation of 3′,5′-AMP by fat cell membranes was found to be maximal at a pH of about 8.0–8.2 in the absence or presence of high doses of either ACTH, glucagon, or epinephrine. Adenyl cyclase activity in ghosts was found to decline (about 50%) on standing at 0° for 2–3 hr.[14] To correct for decay of cyclase activity during assay of a large number of tubes, control samples were included at intermittent intervals so that an interpolated value of basal cyclase activity for each group of tubes could be obtained. The basal rate of 3′,5′-AMP formation by ghosts was found to be linear for about 30 min and then declined slowly; the rates in the presence of NaF and hormones were linear for 60 min, suggesting that neither the enzyme nor the added hormones suffered detectable inactivation in the course of the standard 30-min incubation assay procedure. In the standard assay the components (in a total volume of 0.05 ml) were: 40 mM Tris-HCl (pH 8); 5 mM $MgCl_2$; 0.1% bovine serum albumin; 7 mM sodium phosphoenol pyruvate; 0.05 mg/ml pyruvate kinase; 1 mM unlabeled 3′,5′-AMP; 0.1–0.2 mg/ml ghost protein (determined by the Lowry method); 0.2 mM $KHCO_3$ (resulting from the addition of ghosts); and 0.1 mM ATP-α-^{32}P (300–500 millicurie/millimole). The reaction was started by addition of either ghosts or ATP, and incubations were carried out at 37° generally for 30 min. Reactions were stopped by boiling for 3 min, after addition of 0.05 ml 5 mM 3′,5′-AMP and 5 mM ATP. 3′,5′-AMP was separated by chromatography on precoated polyethyleneimine-impregnated cellulose thin-layer sheets (Polygram-PEI, Macherey-Nagel/Brinkman), developed with 0.3 *M* LiCl; 3′,5′-AMP has an R_f of about 0.4, while ATP and ADP remain almost entirely at the starting line. The 3′,5′-AMP and ATP spots were marked under ultraviolet light, cut out, and counted by a scintillation

technique. The per cent conversion of ATP to 3′,5′-AMP was determined and absolute rates of 3′,5′-AMP formation calculated.

Results.—Figure 1 shows typical dose response curves for ACTH, glucagon, and epinephrine tested in a single membrane preparation. Half-maximal activation (K_a) of cyclase was obtained with concentrations of approximately 7×10^{-8} *M* glucagon, 7×10^{-7} *M* ACTH, and 1.5×10^{-5} *M* epinephrine. Maximal effects on 3′,5′-AMP formation were achieved with concentrations of about 5×10^{-7} *M* glucagon, 4×10^{-6} *M* ACTH, and 1.5×10^{-4} epinephrine. The affinity of hormones for the adenyl cyclase system thus had the order: glucagon > ACTH ≫ epinephrine; however, the magnitude of hormonal stimulation of the cyclase had a reverse order. In other cyclase preparations prepared during various seasons of the year, ACTH was found in some assays to be equipotent to (or more potent than) epinephrine in terms of the magnitude of cyclase response. Figure 1 should not, therefore, be regarded as representing the absolute potencies of these hormones in fat cell membranes. The K_a for epinephrine and ACTH upon 3′,5′-AMP levels in intact fat cells can be estimated to be about 3–5 $\times 10^{-8}$ *M* [10, 16] and 5×10^{-10} *M*,[10] respectively. The sensitivity of cyclase to epinephrine and ACTH in the isolated ghost preparation is thus decreased relative to the intact cell by about 3 orders of magnitude. A similar decrease of hormone effectiveness upon the cyclase activity in broken cell preparations relative to intact cells has been noted in several other systems.[5]

The β-adrenergic blocking agent Kö 592 competitively inhibits the lipolytic effect of norepinephrine in the fat pad, half-maximal inhibition (K_i) being achieved at 2×10^{-7} *M;* Kö 592 also inhibits ACTH noncompetitively, the K_i being 5.2×10^{-4} *M*, presumably by acting at sites other than adenyl cyclase.[17] In ghosts, Kö 592 produced detectable inhibition of epinephrine (10^{-4} *M*) action at 0.001 mM, half-maximal inhibition at about 0.05 mM, and complete inhibition above 1 mM. Kö 592 at doses 50–100 times greater than the K_i value for epinephrine did not influence ACTH or glucagon activity. The experiments with Kö 592 on ghosts, coupled with the fact that this agent acts as a competitive inhibitor for catecholamines in intact fat cells,[17] establish that epinephrine occupies a specific site in the adenyl cyclase system of ghosts.

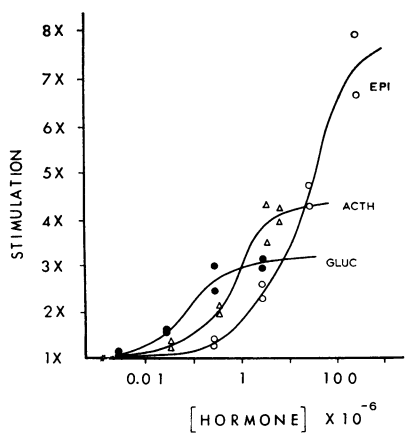

FIG. 1.—Dose response curves for ACTH, glucagon (Gluc), and epinephrine (EPI). Standard assays were conducted as described in the text. The basal activity in this preparation was 0.053 mμmole × mg^{-1} × min^{-1}. Individual points of duplicate determinations are shown.

Ca is specifically required for the lipolytic effect of ACTH (but not epinephrine).[18] The Ca requirement for ACTH appears to involve a step prior to 3′,5′-AMP action.[19] To determine whether ACTH requires Ca for the activation of fat cell adenyl cyclase, we have assayed the effects of hormones upon ghost preparations in the presence of the Ca-complexing agent, ethyleneglycol-bis(β-aminoethylether)-*N*,*N*′-tetraacetic acid complex constants[20] for Ca and Mg are 10^{11} and 10^{5} M^{-1}, respectively; thus, Ca will be complexed efficiently by EGTA in the presence of 5 mM Mg used for cyclase assay. Typical experiments are shown in Figure 2. It will be seen that EGTA (1.0 mM) selectively abolished the stimulatory effect of ACTH and that ACTH activity was restored by addition of 1 mM Ca to the incubation medium containing EGTA (1 mM). The minimal Ca concentration required for ACTH action was estimated to be about 10^{-7} *M*. Whether Ca is required for the initial binding of ACTH to its selective interaction site, or is involved at some secondary stage in the sequence of events which lead to activation of catalytic activity cannot presently be evaluated.

Having established distinctive sites for ACTH, epinephrine, and glucagon, maximal doses of these hormones were assayed separately, and in combination. The results of these experiments, shown in Figure 3, demonstrate that the maximal doses employed for combination were indeed maximal (since doubling the dose of each hormone produced no further increase in 3′,5′-AMP formation), and that additive effects were not obtained when maximal doses of ACTH, glucagon, and epinephrine were assayed in combination. Although these hormones exhibit a differential potency to activate cyclase activity, when combined in pairs, only the level of the more potent hormone of the pair was reached.

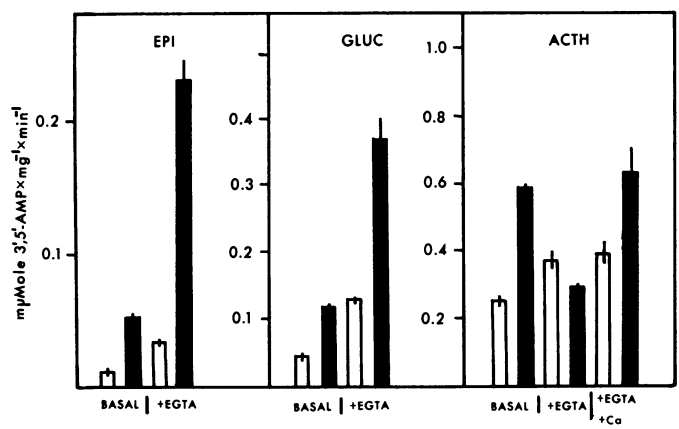

FIG. 2.—Hormonal stimulation in the absence and presence of EGTA. Ghosts were incubated ±1 mM EGTA in the absence (*open bars*) and presence (*solid bars*) of hormones. In the case of ACTH, an additional group, incubated with 1 mM EGTA plus 1 mM $CaCl_2$, was included. The concentrations of hormones were: 0.55×10^{-4} *M* epinephrine, 0.29×10^{-6} *M* glucagon, and 0.34×10^{-5} *M* ACTH. Standard errors of triplicate determinations are indicated. Different preparations of frozen-thawed ghosts were used with epinephrine and glucagon. A fresh preparation was used with ACTH (similar results have been obtained with frozen ghosts).

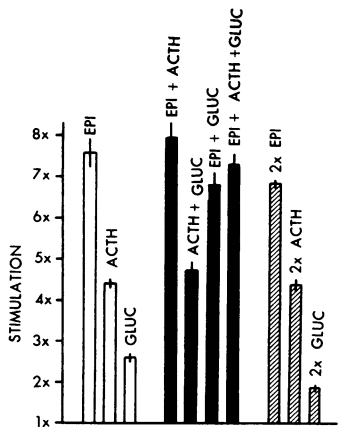

FIG. 3.—Additivity studies with maximal doses of epinephrine (EPI), glucagon (Gluc), and ACTH. Standard assays were conducted as described in the text. Concentrations of hormones were: 0.22×10^{-3} *M* epinephrine, 0.34×10^{-5} *M* ACTH, 0.29×10^{-5} *M* glucagon. These doses were doubled in the columns labeled "2× hormones." The same ghost preparation (frozen-thawed) was used as that employed in the dose-response studies shown in Fig. 1. Standard errors of triplicate determination are indicated.

Discussion.—Given cases where there are very exacting specificities of cyclase for ACTH,[4] thyroid-stimulating hormone,[5] and epinephrine,[6] it has been suggested that a hormone-responsive adenyl cyclase system consists of at least two types of molecular species coupled in the membrane of a target cell—one for hormone selectivity, the other for catalytic activity. Sutherland and his associates[2] consider that adenyl cyclase may be a *unitized receptor*, hormone selectivity being ascribed to a *regulatory subunit* bonded to a *catalytic subunit*. In an alternative formulation[1] the selectivity units have been designated as *hormonal discriminators* associated in the membrane with an *enzymic generator*, adenyl cyclase, the mode of coupling being left open. There are inadequate data to distinguish between these mechanistic alternatives;[21] both formulations, however, lead to the possibility that there may be multiple forms of hormone-sensitive adenyl cyclase, so that ACTH action in the adrenal might involve a cyclase specific for ACTH, thyroid-stimulating hormone, a thyroid-stimulating hormone-sensitive cyclase in thyroid, etc. The existence of multiple cyclase systems has been reported in rat liver[22]—one preferentially sensitive to glucagon, the other to epinephrine.

The rat fat cell responsive to ACTH, thyroid-stimulating hormone, glucagon, and epinephrine might, on this basis, be expected to have multiple forms of adenyl cyclase with different specificities for hormones. The failure to produce additive effects on 3′,5′-AMP levels in fat cells, when various lipolytic hormones were tested in combination,[10] suggested that multiple forms of hormone-sensitive cyclase were unlikely. The present findings that maximal doses of ACTH, glucagon, and epinephrine failed to produce additive effects on the cyclase activity of fat cell membranes now provides definitive evidence that the fat cell membrane contains a single adenyl cyclase unit that can be stimulated by multiple hormones of diverse structure.

Although the sites of hormone interaction with ACTH, glucagon, and epinephrine can be differentiated in the fat cell membrane in terms of a specific Ca requirement for ACTH and specific inhibition of epinephrine action by a β-adrenergic blocking agent, the experimental evidence presently available does not permit us to differentiate between at least three alternative models. A single

catalytic unit of cyclase could be associated in the membrane with (1) a single discriminator unit, possessing distinctive sites for epinephrine, ACTH, and glucagon; (2) two discriminator units—one for peptide hormones, the other for catecholamines; and (3) multiple discriminator units, each of which interacts specifically with a particular hormone. The alternatives raised will only be solved when the molecular species postulated to exist in hormone-sensitive cyclase systems are isolated and chemically identified, in this membrane as well as in membranes from other responsive cell types.

If a set of discriminator units specific for ACTH, glucagon, catecholamines, thyroid-stimulating hormone, etc., actually exist, as indicated in (3), the question arises as to whether all cell types responsive to a particular hormone contain an identical discriminator. If this should be the case, the distinctive features of signal reception by specialized cells (like their distinctive pattern of macromolecules) would be the resultant of genetic instructions, expressed in selective transcription of the genome, during differentiation and development. The known differences in the response of adipose tissue from different species to various lipolytic hormones[8] would be explicable on the view that the genome possesses genetic information for a complete set of hormone discriminators and is differentially expressed in target cells. The finding that adenyl cyclase in the tadpole erythrocyte is hormone-insensitive but becomes epinephrine-sensitive following metamorphosis[23] provides strong evidence for the concept that hormonal discriminator units exist as entities separate from cyclase and are under genetic control.

We wish to thank Miss Betty Krohn for skillful technical assistance in this work.

Abbreviations used: ACTH, adenocorticotropin; EGTA, ethyleneglycol-bis(β-aminoethyl-ether)-*N,N'*-tetraacetic acid.

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[3] Perhaps by way of a 3',5'-AMP-dependent protein kinase (Walsh, D. A., J. P. Perkins, and E. G. Krebs, *J. Biol. Chem.*, **243**, 3763 (1968)), which promotes phosphoryl transfer from ATP to a variety of proteins including phosphorylase kinase, casein, and protamine. Similar phosphoryl transfer promoted by 3',5'-AMP has been observed with phosphatidylinositol kinase (Cunningham, E. B., *Biochim. Biophys. Acta*, **165**, 574 (1968)) and with the kinase for histone phosphorylation (Langan, T. A., *Science*, **162**, 579 (1968)).

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[14] Attempts to prevent decay of cyclase activity at 0° by addition of 3′,5′-AMP, Mg^{++}, Ca^{++}, ATP, phospholipid, and mercaptoethanol were unsuccessful. However, it was observed that Mg^{++}, Ca^{++}, and ATP, in the presence of EDTA, all reduced the rate of decay; ATP in the absence of EDTA was hydrolyzed by ghosts at 0°.

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[21] In either formulation, information conveyed to the cell by a hormonal signal would be relayed from the selectivity unit to the catalytic unit of the cyclase system before it is translated into the intracellular regulatory signal, 3′,5′-AMP. Given the real possibility that a biophysical (or chemical) signal(s) serves to couple the initial step of hormone reception to the final step of 3′,5′-AMP generation, the widely accepted designation of 3′,5′-AMP as a second messenger[2] would seem to be unjustified in terms of theoretical cybernetic considerations and is certainly premature on experimental grounds.

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