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The Mode of Adrenocorticotrophin Action in Stimulating Steroidogenesis: the Obligatory Role of Adenosine 3':5'-Cyclic Monophosphate and the Involvement of Rapidly-Turning-Over Protein

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Current concepts for the complicated mechanism whereby ACTH* stimulates corticosteroidogenesis involve the following aspects: ACTH binding at cell-surface receptors, activation of adenylate cyclase and increased production of intracellular cyclic AMP (adenosine 3':5'-cyclic monophosphate), activation of protein kinases with increased phosphorylation of (ribosomal?) protein, induction of labile protein(s) implicated in the rate-limiting step of steroidogenesis and intracellular translocation of steroid intermediates that influence cholesterol conversion into pregnenolone (3β -hydroxypregn-5-en-20-one) (Garren *et al.*, 1971). Two of these aspects, namely the role of cyclic AMP and the involvement of rapidly-turning-over protein, are the subject of this report.

The cyclic AMP content of collagenase-disaggregated rat adrenal cells (Richardson & Schulster, 1970) incubated with ACTH was assayed after Dowex chromatography (Montague & Cook, 1971) by a modification of the saturation-analysis procedure of Brown *et al.* (1971). A sigmoid log(dosage)-response curve was obtained (ED_{50} 5×10^{-3} i.u. of ACTH/ml) that was steepest over the range 5×10^{-4} – 10^{-2} i.u. of ACTH/ml; over this range a 13-fold increase in cyclic AMP (83% of maximum response) was apparent. By contrast, in the identical incubation samples, increasing ACTH concentrations from 10^{-5} to 10^{-3} i.u./ml led to the greatest change in corticosterone content (about 36-fold stimulation) where-

as higher ACTH concentrations elicited no greater corticosteroidogenic response. The ED_{50} for this sigmoid log(dosage)-response curve was 10^{-4} i.u. of ACTH/ml. Results from three experiments showed the relationship between log([ACTH]) and log([cyclic AMP]) to be linear over the entire range 10^{-5} – 10^{-2} i.u. of ACTH/ml; the lowest concentration that appreciably stimulated cyclic AMP concentrations (approx. 3-fold) was 5×10^{-5} i.u. of ACTH/ml. Since doses of ACTH that stimulated steroidogenesis were associated with increases in cyclic AMP and the two effects were not separable, the findings are consistent with an intermediary role for cyclic AMP in the ACTH-stimulated steroidogenic mechanism. However, there appears to be a vast adrenal reserve capacity for cyclic AMP production, and only a small fraction of the cells' potential to synthesize cyclic AMP need be activated in order to achieve maximum steroidogenesis. Thus at 10^{-3} i.u. of ACTH/ml (maximal for steroidogenesis) cyclic AMP concentrations were only 12% of maximum. This finding, obtained with cell suspensions, correlates well with that obtained *in vivo*, but differs markedly from that observed *in vitro* with adrenal quarters, where high concentrations of ACTH (0.5–1.0 i.u./ml) were found to be necessary to maximize steroidogenesis (Grahame-Smith *et al.*, 1967). This may be attributed to the relative inaccessibility of medium components to cells within the tissue of quartered glands, compared with isolated cells or perfused glands *in vivo*, where medium or blood components are readily available to each cell.

The effect of exogenously added cyclic AMP also implies an intermediary role for this nucleotide. Similar sigmoid log(dosage)-response characteristics were observed for corticosteroidogenesis in isolated cells stimulated by ACTH or cyclic AMP. Moreover graphs of log([ACTH]) against log([corticosterone]) and log([cyclic AMP added]) against log([corticosterone]) both exhibited linear relationships over the ranges 10^{-7} – 10^{-3} i.u. of ACTH/ml and 0.5–5 mM-cyclic AMP (ED_{50} 3 mM).

Studies *in vitro*, with adrenal fragments to ascertain the time-lag between ACTH addition and onset of stimulated steroidogenesis (Grahame-Smith *et al.*, 1967), were complicated by the time required for ACTH to penetrate the tissue mass and for corticoid to diffuse out. Use of isolated cell suspensions eliminates these problems, allowing immediate access of added stimulants to each cell. However, despite these conditions increased corticoid production was undetectable until 3 min after ACTH addition (10^{-3} i.u./ml). A similar time-lag after ACTH infusion has been observed during perfusions *in vivo* (Urquhart & Li, 1968; Beaven *et al.*, 1964). Further, a 2–3 min time-lag followed exogenous cyclic AMP (5 mM) addition before steroidogenic stimulation was detectable, thus making it improbable that this is the time

* Abbreviation: ACTH, adrenocorticotrophin.

required for accumulation within the cell of sufficient cyclic AMP to actuate the steroidogenic machinery. This was further militated against by kinetic measurements of cyclic AMP production by isolated cells, which showed that the cyclic AMP concentration had more than doubled within 1 min of the addition of ACTH (10^{-2} i.u./ml) and within 3 min had increased 5-fold above basal value. It is suggested that a considerable portion of this 3 min period is required for the accomplishment of processes [e.g. synthesis of specific protein(s)] initiated by cyclic AMP. Since the average transit time for translation of mRNA in some eukaryotic cells has been assessed at 1–2 min (Fan & Penman, 1970; Vaughan *et al.*, 1971), this suggestion is consistent with current concepts of ACTH acting via translational control of protein synthesis (Garren *et al.*, 1971).

Further studies on the kinetics of the steroidogenic response utilized cycloheximide, which at high concentration inhibits protein synthesis by stopping movement of ribosomes along the mRNA strand. At low concentrations the antibiotic results in a decreased rate of translation. In the presence of 10^{-2} i.u. of ACTH/ml, cycloheximide (0.1, 1, 10 and 100 μ g/ml) inhibited protein synthesis by 23, 74, 88 and 94% respectively, and also inhibited steroidogenesis by 23, 66, 89 and 96% respectively. This result showed an ED_{50} of about 0.28 μ g of cycloheximide/ml (i.e. 1 μ M), and this dose was used to investigate further the time-lag before onset of ACTH-stimulated steroidogenesis. It was found that cycloheximide, at a concentration that halved the protein synthesis rate, concomitantly doubled the time-lag before manifestation of the ACTH response. It is concluded that, if indeed there is a role for induced protein in the steroidogenic effect of ACTH, then it plays a direct or obligatory role. No such prolongation of the time-lag would be occasioned if some species of rapidly-turning-over protein had simply a permissive involvement. Further support for this conclusion derives from dynamic superfusion studies with adrenal bisects, where infusion of cycloheximide simultaneous with ACTH infusion allowed no discernible stimulation of steroidogenesis (Schulster *et al.*, 1970).

Steroidogenesis was observed to reach its maximum rate 6 min after ACTH addition to isolated adrenal cells, and this rate remained constant for at least 1 h. When cycloheximide (1 μ g/ml) was added 6 min after the ACTH addition, the capacity for steroidogenesis rapidly decayed, and the decay half-life for the labile protein, invoked as an intermediary in ACTH steroidogenic action, was calculated (Hartwell & Margasanik, 1963) as 2–4 min. This compares with the decay half-life of 7–10 min reported by Garren *et al.* (1965) with the perfused rat adrenal *in vivo* and 45–49 min for the superfused adrenal bisect *in vitro* (Schulster *et al.*, 1970).

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The Effect of Adrenocorticotrophin *in vivo* on Pregnenolone Synthesis by Adrenal Mitochondria

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Administration of 5×10^{-3} i.u. of ACTH* intravenously to 3 h-hypophysectomized female rats promoted a fourfold increase in the rate of pregnenolone (3β -hydroxypregn-5-en-20-one) synthesis from endogenous substrates. The rate of pregnenolone synthesis in mitochondria from the ACTH-treated animals was 0.4 nmol/min per mg of protein with 5 mM-malate as electron donor. When 1% bovine serum albumin (fatty acid-free) was included in the mitochondrial incubation solution, 75% of the pregnenolone was found in the supernatant from mitochondrial preparations from both the control and the ACTH-treated animals. This finding suggests that ACTH did not alter the percentage of pregnenolone passing through the mitochondrial membrane. The stimulating effect of ACTH on pregnenolone synthesis was also observed when 5 mM- β -hydroxybutyrate, 5 mM- α -oxoglutarate and 5 mM-isocitrate were used as electron donors. The generation of reducing equivalents for pregnenolone synthesis from endogenous cholesterol was not affected by the respiratory-chain blockers cyanide and rotenone. The acute ACTH effect seen in these studies appears to be stimulation of a component of the hydroxylation pathway, as it occurs with

* Abbreviation: ACTH, adrenocorticotrophin.