KINETICS AND DOSE–RESPONSE CHARACTERISTICS OF ADENOSINE 3′,5′-MONOPHOSPHATE PRODUCTION BY ISOLATED RAT ADRENAL CELLS STIMULATED WITH ADRENOCORTICOTROPHIC HORMONE

C. MACKIE, M.C. RICHARDSON and D. SCHULSTER
School of Biological Sciences, University of Sussex, Falmer, Brighton BN1 9QG, England

Received 17 April 1972

1. Introduction

Current evidence suggests that adenosine 3′,5′-monophosphate (cyclic AMP) is the intracellular mediator of adrenocorticotrophic hormone (ACTH) stimulated steroidogenesis in the adrenal gland. ACTH has been shown to increase cyclic AMP content in bovine adrenal cortical slices [1], perfused cat adrenal gland [2], and rat adrenal gland both *in vitro* and *in vivo* [3]. Moreover, cyclic AMP addition to adrenal tissue has been shown to stimulate steroidogenesis [4] and ACTH has been shown to bind to purified adenyl cyclase preparations from adrenal tumours [5].

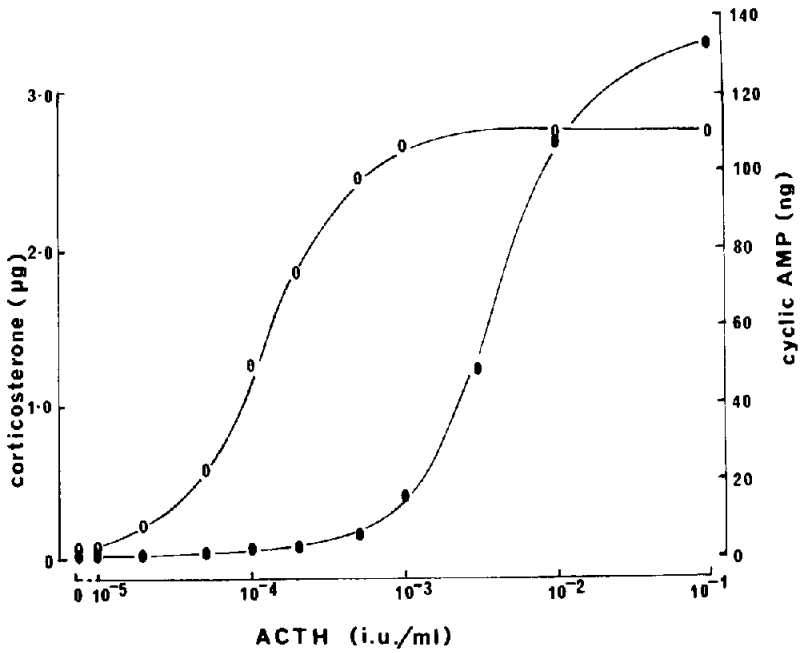

Fig. 1. The effect of ACTH on corticosterone production (o—o—o) and cyclic AMP levels (•—•—•) in isolated rat adrenal cells. Incubations were for 1 hr and values shown represent amounts found in cells isolated from the equivalent of 2 adrenals. Corticosterone was measured by fluorometric assay. The points plotted are averages of duplicate incubations.

We have measured the kinetics of the ACTH effect on cyclic AMP levels, using an isolated rat adrenal cell suspension that facilitates examination of the response during the first few minutes of hormonal stimulation. We found that cyclic AMP levels, measured by a highly sensitive saturation analysis assay, rose before any detectable increase in steroidogenesis. The sensitivity and magnitude of the steroidogenesis response to ACTH by this system has previously been reported [6]. We now present the dose–response characteristics between ACTH and adrenal cyclic AMP production. A linear relationship between log (cyclic AMP produced) and log (ACTH concentration) was observed over the range 10^{-5}–10^{-2} I.U. ACTH. Part of this work has been reported in preliminary form [7].

2. Materials and methods

[U-^{14}C] Cyclic AMP (322 mCi/mmole) was obtained from the Radiochemical Centre, Amersham, England, while [8-^{3}H] cyclic AMP (16.3 Ci/mmole) was obtained from Schwarz/Mann, Orangeburg, N.Y. Dowex AG 50W–X8 (100–200 mesh) was purchased from BioRad Laboratories, Richmond, Calif. ACTH (Acthar Corticotrophin) was a gift from Armour Pharm., Eastbourne, England.

Isolated adrenal cells were prepared by collagenase digestion of decapsulated rat adrenal glands [6]. Cell suspensions were incubated in 1 ml samples which contained cells isolated from the equivalent of one adrenal. An aliquot (0.2 ml) from each incubate was taken for corticosterone estimation; the remainder was frozen, and 6% (w/v) trichloroacetic acid and [^{14}C] cyclic AMP recovery indicator (0.71 nCi) added. Ultrasonication of the incubate was followed by a centrifugation step, when acid-precipitated protein was spun down. Supernatants were chromatographed on Dowex 50 [8], lyophilised, and then dissolved in Tris buffer (50 mM; pH 7.4). Samples were taken for estimation of loss of cyclic AMP during the purification procedure. Recoveries were usually about 60%. Cyclic AMP levels were measured in the extracts by saturation analysis, using a cyclic AMP binding protein isolated from bovine adrenal homogenates as described by Brown et al. [9]. The method was modified to operate over the range 0–500 pg (11.6 nCi [^{3}H] cyclic AMP were used per assay tube). Sensitivity of the assay was 20 pg; precision at 500 pg was 40 pg.

Corticosterone was estimated in cells plus medium both by a fluorometric assay [10] and by a more sensitive, specific radioimmunoassay using an antibody raised against corticosterone-21-hemisuccinate–albumin conjugate. The sensitivity of the latter assay was 30 pg; precision at 1 ng was 70 pg.

3. Results and discussion

Addition of increasing doses of ACTH to the isolated cells (fig. 1) resulted in a sigmoid log-dosage response curve for corticosterone production (ED_{50}:10^{-4} I.U. ACTH/ml). Similar characteristics have already been reported by several workers using the isolated adrenal cell suspension system [10–12]. Measurements of cyclic AMP in cells plus medium from the same incubation samples also gave a sigmoid

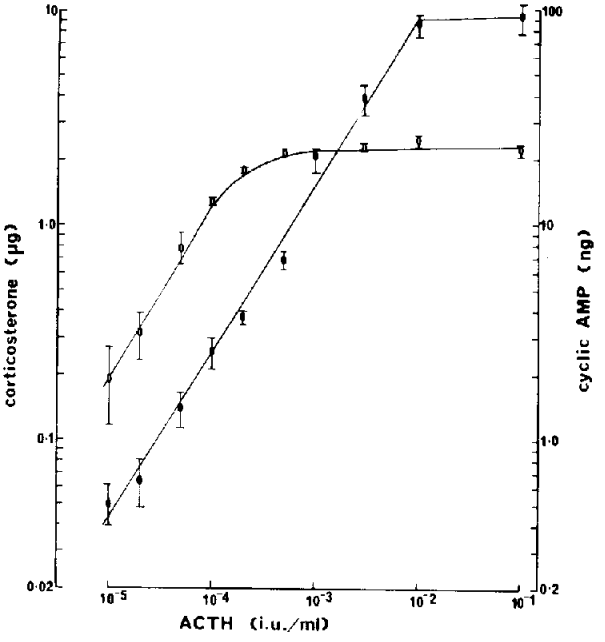

Fig. 2. Logarithmic plot showing the effect of ACTH on corticosterone production (o—o—o) and cyclic AMP levels (•—•—•) in isolated adrenal cells. Conditions of incubation were the same as in fig. 1. Combined data is given from 3 experiments, in each of which incubations were in duplicate; vertical bars represent S.E.M. Corticosterone was estimated by a fluorometric assay. Control incubations without ACTH gave values of: 0.22 ± 0.10 μg corticosterone and 0.56 ± 0.13 ng cyclic AMP.

log-dosage response curve, with estimated ED_{50} of 5×10^{-3} I.U. ACTH/ml. The data from several experiments is shown in fig. 2, where log (cyclic AMP levels) and log (corticosterone production) are plotted against log (ACTH concentration). Here, stimulation of cyclic AMP levels by low doses of ACTH can be clearly seen. A linear relationship between log (ACTH concentration) and log (cyclic AMP levels) is apparent over a considerable dose range; the lowest dose of ACTH which elicited an appreciable increase in cyclic AMP was 5×10^{-5} I.U. ACTH/ml. Only at the dose 2×10^{-5} I.U. ACTH/ml was increased steroid production (7% of maximum) seen where there was no clear increase in cyclic AMP levels. The data shown is consistent with an intermediary role for cyclic AMP in the ACTH effect on steroidogenesis, as we achieved no appreciable separation of the effect of ACTH on steroidogenesis from its effect on cyclic AMP production. However, only relatively little cyclic AMP needs to be made inside the cell to achieve maximum steroidogenesis. Thus at a concentration of ACTH (10^{-3} I.U./ml) which just elicits maximum steroidogenesis, cyclic AMP levels were only 12% of maximum. This correlates well with data obtained *in vivo* by Grahame-Smith et al. [3]. It should be noted that this relationship cannot be shown in adrenal quarters, where high levels of ACTH (0.5 I.U., approx.) are needed to produce maximum steroidogenesis [3]. This may be because cells within the quartered gland are not readily accessible to medium components.

Fig. 3 shows a time-course of production of cyclic AMP in response to ACTH, compared with that of corticosterone. The levels of cyclic AMP had increased within 1 min, whereas corticosterone production showed a distinct lag of about 3 min. Three minutes after the addition of ACTH, corticosterone levels were the same as those at time zero, whereas cyclic AMP levels were 5-fold above basal. This sequence of events agrees with the data obtained by Grahame-Smith et al. [3] using *in vitro* incubations of quartered glands. In the present study, however, diffusion problems have been eliminated, and directly ACTH is added, it is available to all cells in the suspension. Thus steps between cyclic AMP release and final steroid production require at least 2–3 min. If cyclic AMP is involved in ACTH action, this lag could represent the time

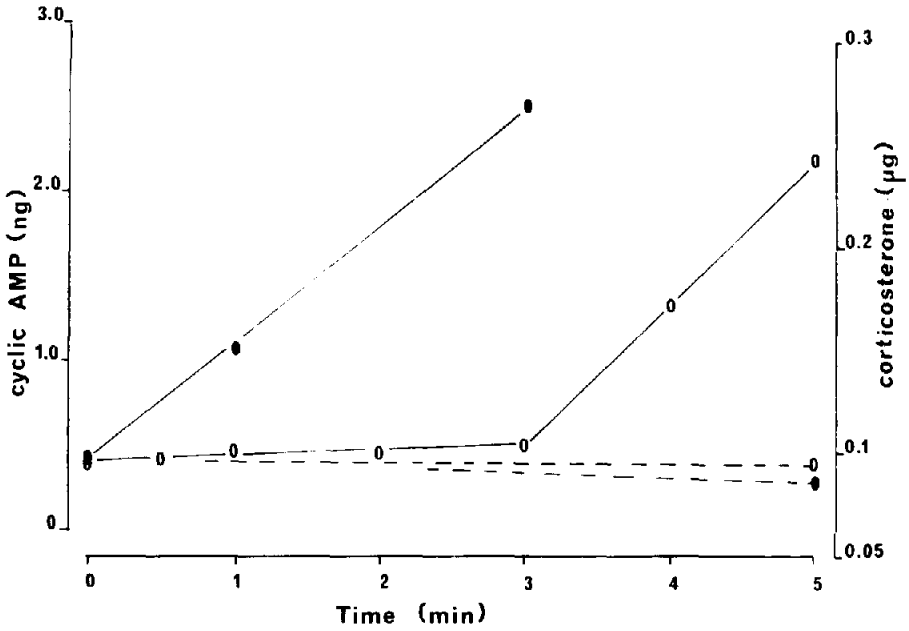

Fig. 3. Time-course study of the effect of ACTH on corticosterone production (o—o—o) and cyclic AMP levels (•—•—•) in isolated adrenal cells. After a preincubation time of 10 min, ACTH (10^{-2} I.U.) was added at zero time. Corticosterone was measured by radioimmunoassay. Values shown are the averages of duplicates and represent the amounts found in the cells isolated from the equivalent of 2 adrenals.

needed for accumulation of sufficient cyclic AMP to stimulate steroidogenesis. Alternatively, this time may be necessary for the operation of processes initiated by cyclic AMP, such as synthesis of the labile protein postulated by Garren [13]. The latter possibility is more likely, as addition of 5 mM cyclic AMP to cells results in a time lag of about 3 min before corticosterone is produced [14]. Our measurements of cyclic AMP levels in cells after stimulation by ATCH are thus consistent with the hypothesis that cyclic AMP is the intracellular mediator of ACTH stimulated steroidogenesis.

Acknowledgements

The authors are grateful to the Medical Research Council for a maintenance grant (to C.M.) and for financial support (to D.S.). We thank the Science Research Council for a maintenance grant (to M.C.R.), and Prof. and Mrs. J.F. Tait for corticosterone antiserum. We are grateful to Dr. W. Montague for helpful advice.

References

[1] R.C. Haynes, Jr., J. Biol. Chem. 233 (1958) 1220.
[2] R.A. Carchman, S.D. Jaanus and R.P. Rubin, Mol. Pharmacol. 7 (1971) 491.
[3] D.G. Grahame-Smith, R.W. Butcher, R.L. Ney and E.W. Sutherland, J. Biol. Chem. 242 (1967) 5535.
[4] R.C. Haynes, Jr., S.B. Koritz and F.G. Peron, J. Biol. Chem. 234 (1959) 1421.
[5] R.J. Lefkowitz, J. Roth, W. Pricer and I. Pastan, Proc. Natl. Acad. Sci. U.S. 65 (1970) 745.
[6] M.C. Richardson and D. Schulster, Biochem. J. 120 (1970) 25P.
[7] C. Mackie, M.C. Richardson and D. Schulster, J. Endocrinol. 52 (1972) xxiii.
[8] W. Montague and J.R. Cook, Biochem. J. 122 (1971) 115.
[9] B.L. Brown, J.D.M. Albano, R.P. Ekins and A.M. Sgherzi, Biochem. J. 121 (1971) 561.
[10] G. Sayers, R.L. Swallow and N.D. Giordano, Endocrinology 88 (1971) 1063.
[11] R. Haning, S.A.S. Tait and J.F. Tait, Endocrinology 87 (1970) 1147.
[12] A.E. Kitabchi and R.K. Sharma, Endocrinology 88 (1971) 1109.
[13] L.D. Garren, R.L. Ney and W.W. Davis, Proc. Natl. Acad. Sci. U.S. 53 (1965) 1443.
[14] M.C. Richardson and D. Schulster, submitted for publication.