ACTH and adrenal aerobic glycolysis. I: Effects of O-nitrophénylsulphinyl and other ACTH analogues, vasoactive intestinal peptide and human parathyroid hormone(1–34) on lactic acid, steroid and cyclic AMP production by mouse adrenocortical cells

J. Hinson* and M. K. Birmingham†

Departments of †Biochemistry and ‡Psychiatry, McGill University, ‡Allan Memorial Institute, 1033 Pine Avenue West, Montreal, Quebec H3A 1A1, Canada

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ABSTRACT

The structural requirements in the ACTH molecule for evocation of the glycolytic response in suspensions of mouse adrenal cells were investigated by examining the effects of analogues containing modifications at positions 8, 9 and 10 and of peptides containing homologies with the amino-terminal segment of ACTH. Introduction of a nitrophénylsulphinyl (NPS) group into the tryptophan moiety at position 9 of ACTH(1–24) greatly reduced both the potency and the capacity for maximal glycolytic response. It also virtually abolished cyclic AMP formation. In contrast, the capacity for a maximal steroidogenic response remained unimpaired in the NPS derivative, although steroidogenic potency was reduced to 0·4% of that of ACTH(1–24).

Replacement of the tryptophan moiety with phenyl-alanine had intermediate inhibitory effects on glycolysis and steroid output; replacement with alanine virtually abolished both these responses. Replacement of arginine in position 8 with lysine in the Phe9 analogue caused a fifty-fold increase in glycolytic potency, but rendered it steroidogenically inactive. Cyclic AMP production was abolished in the Ala9 analogue and greatly impaired in the Phe9 and Lys8,Phe9 analogues. Replacement of the glycine moiety in position 10 with l-alanine, d-alanine, β-alanine or α-aminoisobutyric acid had little or no effect on steroidogenic or glycolytic capacity, although potency was reduced with all substitutions excepting l-alanine.

Vasoactive intestinal peptide, which contains homologies with positions 3, 7, 15 and 16 of ACTH, proved completely inactive in dispersed mouse adrenal cells under our experimental conditions, in contrast to human parathyroid hormone(1–34) (hPTH (1–34)) which contains homologies with positions 3, 4, 5, 9 and 11 and was steroidogenic at the lowest concentration tested (0·1 nmol/l), eliciting an eleven-fold increase in steroid production, a response which might be physiologically relevant. It induced near maximal steroidogenesis at a concentration of 10 nmol/l, without affecting cyclic AMP production, and stimulated glycolysis at concentrations above 10 nmol/l, accompanied by a slight rise in cyclic AMP levels.

The examples of dissociation between glycolysis and steroidogenesis suggest that different receptors may mediate the two responses. The examples of increased steroidogenesis unaccompanied by a rise in cyclic AMP accord with the concept that cyclic AMP is not an obligatory second messenger for the steroidogenic response.


INTRODUCTION

The control and significance of the production of lactic acid under aerobic conditions are still debated. The phenomenon has been observed in, amongst others, the brain (McIlwain, Anguiano & Cheshire, 1951; Wollenberger, 1955; Dittmann & Herrmann, 1970; Benjamin & Verjee, 1980), tumour cells (Warburg, Posener & Negelein, 1924), vascular smooth muscle (Debellis, Mandl, MacLennan & Howes, 1954; Paul,
Bauer & Pease, 1979), erythrocytes (Rodbell, 1966) and endocrine tissues (Hamberger & Ahrén, 1967; Birmingham, Huberman & Riven, 1968). In the ovary the process is stimulated by luteinizing hormone and follicle-stimulating hormone, and in the adrenal gland by adrenocorticotrophin (ACTH). In earlier work from this laboratory, ACTH was found to cause a three- to sixfold increase in lactic acid output from the mouse adrenal, an effect which was also elicited by cyclic AMP (cAMP). The response to ACTH and cAMP were dose-dependent and usually paralleled steroid production by the gland (Bartova & Birmingham, 1971a). Exogenous calcium was required for the glycolytic response to ACTH, but not to cAMP or dibutyryl cAMP (dbcAMP) (Birmingham & Bartova, 1973). The glycolytic response to ACTH and dbcAMP could also be demonstrated in suspensions of mouse adrenal cells (Hinson & Birmingham, 1985). Although basal glycolysis was stimulated by a variety of substrates, and most effectively by fructose-1,6-diphosphate, the response to ACTH and dbcAMP proceeded only in the presence of added glucose.

To elucidate further the mechanism by which ACTH induces adrenal aerobic glycolysis, structure–function studies have been carried out to test the effects of various ACTH analogues on adrenal cell suspensions. Earlier investigations of synthetic ACTH have established that the amino-terminal segment of Met-Glu-His-Phe-Arg-Trp-Gly of ACTH is responsible for eliciting the hormonal response (Li, Meienhofer, Schnabel et al., 1960; Schwyzer, Rittel, Kappeler & Iselin, 1960; Hofmann, Yajima, Yanaihara et al., 1961; Selig, Lindley & Sayers, 1975). The present study, therefore, focuses on the effect of structural modification of this sequence on the ability of ACTH to stimulate aerobic glycolysis and compares it with the steroidogenic effect. In addition, vasoactive intestinal peptide (VIP) and human parathyroid hormone (hPTH(1–34)) were examined in view of noted structural similarities with the amino-terminal segment of ACTH.

MATERIALS AND METHODS

Chemicals

Corticotrophin(1–24) tetracosapeptide (Synacthen, lot Ba 50495; donated by the Ciba Pharmaceutical Company, Summit, NJ, U.S.A.) was used as a reference standard. Nitrophenylsulphenyl (NPS)-ACTH was a gift from Dr J. Ramachandran, University of California Hormone Research Lab., San Francisco, CA, U.S.A., [Gln5, Ala3]-ACTH(1–24), [Gln5, Phe3]-ACTH(1–24) and [Gln5, Lys8, Phe3]-ACTH(1–24) were donated by Dr K. Hofmann, Protein Research Laboratory, University of Pittsburgh, Pittsburgh, PA, U.S.A. The analogues ACTH(1–18)-NH2, [L-Ala10]-ACTH(1–18)-NH2, [D-Ala10]-ACTH(1–18)-NH2, [β-Ala10]-ACTH(1–18)-NH2 and [α-aminoisobutyric acid10]-ACTH(1–18)-NH2 were gifts from Drs M. Nakamura and A. Inuoe, Shinogi Research Laboratories, Osaka, Japan. Vasoactive intestinal peptide and hPTH were obtained from Bachem Inc., La Jolla, CA, U.S.A.

Preparation of adrenal cell suspensions

Twenty-four-day-old Swiss mice (CD-1), purchased from Canadian Breeders Ltd, La Prairie, Quebec, Canada, were accustomed to a noise-free environment under controlled lighting for at least 1 week. The animals were decapitated between 13.00 and 14.00 h. The adrenals from 100–200 mice were collected into ice-cold Krebs–Ringer bicarbonate buffer containing glucose (0·01 mol/l) and with the Ca2+ concentration increased to 7·6 mmol/l (KRBG). The adrenals were freed of fat and cut into small pieces with scissors. The washed adrenal pieces were preincubated in a 100 ml Teflon beaker for 1 h at 37 °C under an atmosphere of 95% O2–5% CO2 in KRBG solution (20 ml) containing collagenase (0·3%), DNase (0·025%) and 4% (w/v) bovine serum albumin (BSA). The enzyme solution was then carefully decanted, the adrenal pieces were washed twice with KRBG–BSA solution and the tissue was dispersed by gentle pipetting (five strokes) of the adrenal pieces, allowing the fragments to settle, removing 2 ml of the cell suspension and repeating the process until all pieces were dispersed.

Purification and preincubation of cells

The crude cell suspension was filtered on a nylon gauze into two polypropylene centrifugation tubes. The cells were washed twice by centrifugation at 25 g for 2 min at room temperature. The final pellet was further purified by layering onto 5 ml 5% (w/v) KRBG–BSA and allowing sedimentation under gravity for 30 min at 4 °C. The purified cells (0·5 ml) were preincubated in a 10 ml Teflon beaker under 95% O2–5% CO2 for 1 h at 37 °C with gentle oscillation. The preincubated cells were again washed twice with KRBG–BSA by centrifugation at 25 g for 2 min. The loose cell pellets were diluted with KRBG so that they contained 0·3% BSA (final volume 8–10 ml). Trypan blue (0·5%) was added to a small sample and the cells were counted in a haemocytometer. A yield of 300 000–500 000 cells/ml was normally obtained.

Incubation with peptides

The peptide diluent used was 0·05% (w/v) BSA in isotonic saline (pH 3·5) adjusted with HCl (1 mol/l). Aliquots of diluted cell suspension (0·5 ml) were added to 10 ml Teflon beakers containing 0·05 ml peptide solution in diluent. The cells were incubated in a
Dubnoff metabolic incubator at 37°C under an atmosphere of 95% O₂-5% CO₂. Incubation was terminated after 3 h by rapidly freezing the cell suspension at −80°C until analysis. For analysis of lactic acid and cAMP, aliquots of cell suspensions were boiled in polypropylene microcentrifuge tubes for 10 min before assay.

**Assays**

Lactic acid was determined, after deproteinization of samples, by the fluorescence of NADH, formed upon enzymatic conversion of lactate to pyruvate, using the Sigma Lactic Acid Kit (St Louis, MO, U.S.A.). For the estimation of corticosterone, the samples were washed with petroleum ether followed by extraction with dichloromethane. The phases of the emulsion were separated as described previously (Hinson & Birmingham, 1985). The extracts were washed with NaOH (0-1 mol/l), and corticosterone, the major steroid produced by the mouse adrenal, was measured by fluorimetry (Zenker & Bernstein, 1958).

**RESULTS**

**Nitrophenylsulphenyl-ACTH**

The analogue NPS-ACTH containing an O-nitrophenylsulphenyl substitution in the Trp⁹ residue of ACTH(1-24) was added to adrenocortical cells at concentrations ranging from 1 nmol/l to 10 μmol/l. Unmodified ACTH(1-24) was tested at concentrations of 0-1-10 nmol/l. The results revealed a marked disparity between the glycolytic and steroidogenic effects of the analogue (Fig. 1). The analogue produced the same maximal steroidogenic response as ACTH(1-24) at high concentrations, but was not capable of maximal glycolytic stimulation at any concentration tested. The maximal glycolytic activity obtained with NPS-ACTH was only about one-third that elicited by ACTH(1-24). The dose–response curves for both steroidogenesis and glycolysis were shifted to the right. The pooled log potency ratio for six assays from these experiments, calculated as described by Saffran & Schally, (1955), was −2.39±0.118 for the steroidogenic response, denoting a potency of 0.41% with fiducial limits of 0.23 and 0.71%. The glycolytic response, when it was not absent or too shallow to fulfill the bioassay requirements for parallelism, gave a log potency ratio of −3.33±0.27, corresponding to a potency of 0.04% and fiducial limits of 0.01 and 0.17%.

The formation of cAMP in the experiment depicted in Fig. 1 is shown in Fig. 2b, and that of a second experiment, in which the concentration of NPS-ACTH ranged from 0-1 to 100 nmol/l, in Fig. 2a. The

**Ala⁹, Phe⁹ and Lys⁸,Phe⁹ analogues**

The effects of substitution in positions 8 and 9 of ACTH (1-24) were tested at concentrations of 0-1-10 nmol/l. Replacement of Trp⁹ with alanine abolished steroidogenic activity at all concentrations tested and permitted a minimal glycolytic response only at 10 nmol/l, amounting to 10% of the lactic acid produced by a corresponding concentration of ACTH(1-24) (Fig. 3; analogue I). Replacement of Trp⁹ by phenylalanine (analogue II) improved the response over that obtained with the aliphatic amino acid in position 9. At 10 nmol/l, corticosterone production was 45% and lactic acid production 35% of that elicited by the same concentration of the unmodified peptide. Replacement of both Trp⁹ with phenylalanine and Arg⁸ with lysine (analogue III) completely abolished steroidogenesis but
had only a moderate inhibitory effect on the glycolytic capacity; thus, lactic acid production in response to the Lys$^8$,Phe$^9$ analogue at 10 nmol/l was 75% of that obtained with a corresponding concentration of ACTH(1–24) and twice that obtained with the Phe$^9$ analogue containing the natural amino acid in position 8. The glycolytic log potency ratios, with respect to ACTH(1–24), for compounds I, II and III were $-4.14 \pm 0.87$, $-2.61 \pm 0.26$ and $-0.86 \pm 0.17$ respectively, denoting potency ratios of 0.007, 0.25 and 14%. The steroidogenic log potency ratio for compound II, the only steroidogenically active analogue, was $-3.06 \pm 0.23$, indicating a potency ratio of 0.09%.

Substitution of Trp$^9$ with alanine abolished cAMP formation; with phenylalanine it permitted a minimal increase at 10 nmol/l. The Phe$^9$ and Lys$^8$,Phe$^9$ analogues evoked increases of 5–7 pmol/10$^6$ cells, compared with increases of 50 pmol/10$^6$ cells observed with the unmodified peptide (Fig. 4).

**Figure 2.** Effect of 0-nitrophenylsulphonyl-ACTH(1–24) (NPS-ACTH) on cyclic AMP formation in mouse adrenal cell suspensions. Arrows indicate basal production. Means ± S.E.M. for triplicate incubations are shown; S.E.M. values are not shown where they fall within the size of the symbols. The results of two experiments are presented.

**Figure 3.** Effects of substitutions at positions 8 and 9 of ACTH(1–24) on aerobic (a) glycolysis and (b) steroidogenesis by mouse adrenal cell suspensions. ACTH(1–24) (●), ACTH(1–24) analogue containing alanine instead of tryptophan in position 9 (▲; I), ACTH(1–24) analogue containing phenylalanine in position 9 (■; II) and ACTH(1–24) analogue containing phenylalanine in position 9 and lysine, instead of arginine, in position 8 (○; III) were used.

**Substitution of Gly$^{10}$**

Effects of replacement of the glycine residue in position 10 were examined utilizing analogues of ACTH(1–18)-NH$_2$, a fragment which has been found to be nearly as effective as ACTH(1–24) in stimulating lactic acid and steroid production at concentrations of 0.1–10 nmol/l (Hinson & Birmingham, 1987). The effects of L-alanine, d-alanine, ß-alanine and α-aminoisobutyric acid in position 10 are depicted in Fig. 5.

Replacement of glycine with L-alanine had no effect on glycolysis, although steroidogenic potency was reduced. Both responses were impaired with all other derivatives. The log potency ratios for L-alanine, d-alanine, ß-alanine and α-aminoisobutyric acid derivatives for glycolysis were $0.13 \pm 0.28$, $-1.40 \pm 0.20$, $-2.03 \pm 0.13$ and $-2.43 \pm 0.30$ respectively, denoting potencies of 136, 4, 1 and 0.4%. The corresponding log potency ratios for steroidogenesis were $-1.38 \pm 0.18$, $-2.50 \pm 0.23$, $-1.97 \pm 0.19$ and $-2.18 \pm 0.13$, respectively.
denoting potencies of 4, 0·3, 1 and 0·7%. It should be noted, however, that the substitutions had little or no effect on the ability to elicit maximal steroidogenesis or glycolysis at high concentrations, with the exception of substitution with ɑ-aminoisobutyric acid which affected the glycolytic capacity even at the highest concentration tested.

Vasoactive intestinal peptide and human parathyroid hormone(1-34)

Homologies of VIP and hPTH to the amino-terminal segment of ACTH, at positions 3, 7, 15 and 16, and 3, 4, 5, 9 and 11 respectively, have been considered responsible for steroidogenic effects, first noted by Kowal, Horst, Pensky & Alfonzo (1977) for VIP and by Rafferty, Zanelli, Rosenblatt & Schulster (1983) for hPTH.

As seen from the results depicted in Fig. 6, VIP, examined over a range of 10 nmol/l-0·1 mmol/l, increased neither corticosterone nor lactic acid production by dispersed mouse adrenal cells. In contrast, hPTH(1-34) was capable of inducing near maximal increases in both parameters, although the dose-response curve for corticosterone differed greatly from that for lactic acid (Fig. 7). Corticosterone production was stimulated 11-fold by a concentration of hPTH(1-34) as low as 0·1 nmol/l. At 10 nmol/l the response was 90% of that obtained with the same concentration of ACTH. Further increases in hPTH (1-34) caused a decline in response. Lactic acid formation was barely stimulated by hPTH at 10 nmol/l and was still increasing at 10 µmol/l, the highest concentration tested. The production of cAMP in this experiment is depicted in Fig. 8. An increase was noted with hPTH only at the highest concentration, 10 µmol/l.

DISCUSSION

Nitrophenylsulphonyl-ACTH

Nearly all the peptides examined in the present study were of greatly reduced potency which could
conceivably be ascribed to biologically active contaminants, even though reported purities were 95% or greater. That the potency of the NPS derivative is not due to any residual ACTH is suggested by the steroidogenic activity of the phenylalanine analogue produced by a synthetic method, as was pointed out by Moyle, Kong & Ramachandran (1973). They noted that the insertion of the orthonitrophenylsulphenyl group in the tryptophan moiety of ACTH abolished the lipolytic response in the rat and caused a dissociation between steroidogenic capacity, which was not impaired at high concentrations of the agonist, and ability to increase cAMP formation, which was greatly impaired (Ramachandran & Lee, 1970; Moyle et al. 1973). Our studies show that substitution with NPS-Trp⁹ also causes a dissociation between adrenal glycolysis, which is greatly impaired, and steroidogenesis, which may still be maximally induced in mouse adrenal cells albeit at a 240-fold higher concentration than that required for the unmodified peptide. Notably, this steroidogenic response was not accompanied by an increase in cAMP, indicating also a dissociation between these two components, in agreement with the observations of Moyle et al. (1973) on rat adrenal cell suspensions.

Insertion of the bulky NPS group at Trp⁹ would be expected to exert steric hindrance and impair the potential for hydrogen bonding at the indolyl-NH group. This could suggest that free access at this site may be essential for the activation of adenyl cyclase and the induction of glycolysis by a receptor or receptors not associated with steroidogenesis. Free access to the tryptophan moiety could also enhance the affinity for the steroidogenic receptor. The capacity for charge donation and hydrophobic interaction, on the other hand, is not impaired by NPS and could suffice for maximal excitation of the steroidogenic but not the other receptors, provided the agonist is added at a high concentration.

**Replacements in position 9**

The importance of Trp⁹ for lactic acid, steroid and, in particular, cAMP production is also apparent from...
the experiments in which ACTH(1–24) was compared with analogues containing alanine or phenylalanine in position 9. Replacement with the aliphatic amino acid virtually abolished all three responses. Substitution with Phe⁹, which imparts some capacity for charge donation (although far less than that of Trp⁹) but no hydrogen-binding capacity, improved steroidogenic and glycolytic activity somewhat, with equivocal effects on cAMP levels. The order of activity Trp⁹ > Phe⁹ > Ala⁹ was the same as that noted by Finn, Johns, Nishi & Hofmann (1976) for the steroidogenic effects of these compounds on bovine and rat adrenocortical cells.

Replacement in position 8

The results obtained with substitution of the Arg⁸ residue in the Phe⁹ derivative with lysine were unexpected. The Lys⁸,Phe⁹ analogue of ACTH(1–24) was fifty times more potent than the Arg⁸,Phe⁹ analogue in stimulating glycolysis but was steroidogenically inert, even though it caused the same minor increase in cAMP formation observed with a steroidogenic dose of Arg⁸,Phe⁹ analogue (10 nmol/l). This would suggest that the doubling in cAMP levels was not responsible for the steroidogenic effect of the Arg⁸,Phe⁹ analogue which should then have occurred also with the Lys⁸,Phe⁹ analogue. The striking dissociation between glycolysis and steroidogenesis observed with the Lys⁸ analogue points again to different receptors for the two responses. Basicity in position 8 appears essential for receptor activation leading to glycolysis, in contrast to the activation of steroidogenesis which would seem to require the characteristics of the guanidyl group.

It should be noted that a dissociation of the glycolytic from the steroidogenic response was achieved many years ago by Kowal (1969) with the steroidogenic inhibitor aminoglutethimide added to mouse adrenal cell cultures which did not synthesize corticosterone, but 20α-dihydropregosterone derivatives. In intact incubated mouse adrenal glands, aminoglutethimide inhibits both glycolysis and corticosterone production (Bartova & Birmingham, 1971a).

Replacements in position 10

The glycine residue in position 10 is considered to be a conformationally and functionally sensitive locus, as it joins the N-terminal segment of message, ACTH (1–9), with the binding address segment, ACTH(11–18), and, furthermore, connects the two message sequences Met¹–Glu⁵–His⁶–Phe⁷–Arg⁸–Trp⁹ and Lys¹⁰–Pro¹²–Val¹³ (Schwyzer, 1982). The substitutions (L- α- and β-alanine and α-aminoisobutyric acid) reduced potency without affecting the capacity for a maximal steroidogenic or glycolytic response, with the exception of L-

alanine, which reduced only steroidogenic potency, and of α-aminoisobutyric acid, which impaired both glycolytic potency and capacity. Nevertheless, at the highest concentration tested, well over 60% of the maximal response was obtained with α-aminoisobutyric acid which, with two bulky groups attached to the α-carbon, would be expected to hinder both free rotation and α-helix formation. Even the α-helix breaker D-alanine only impaired potency. Any functional or conformational disadvantages introduced by replacement of Gly¹⁰ would, therefore, seem to be overcome by increasing the concentration of the agonist.

Vasoactive intestinal peptide and human parathyroid hormone(1–34)

The 28-amino acid peptide hormone VIP, containing homologies to sites, 3, 7, 15 and 16 of ACTH, has been found in chromaffin cells of the rat and human adrenal and has been reported to stimulate steroidogenesis in mammals, albeit at a much higher dose than that required for ACTH, and in amphibians (Kowal et al. 1977; Morera, Cathiard, Laburthe & Saez, 1979; Rotsztejn, Besson, Briand et al. 1980; Lebouleguer, Perroteau, Netchitaiole et al. 1984). Kowal et al. (1977) noted that the potency of VIP resembled that of NPS-ACTH. Although we found that dispersed mouse adrenal cells responded with a maximal steroid output to NPS-ACTH concentrations of 0-1 µmol/l and higher, VIP proved to be completely inactive over a concentration range of 10 nmol/l to 0-1 nmol/l for unknown reasons. In contrast, hPTH(1–34), which contains homologies to sites 3, 4, 5, 9 and 11 of ACTH, demonstrated both steroidogenic and, at high concentrations, glycolytic activity. The steroidogenic response to hPTH(1–34) was optimal at a concentration of 10 nmol/l and then declined, in contrast to the response to ACTH. Parallelism in the slope of the two steroidogenic responses occurred between 1 and 10 nmol/l, permitting an estimate of potency of about one-fifth that of ACTH within this range (log potency ratio = −0.70 ± 0.44). The fact that hPTH (1–34) was steroidogenically active even at the lowest concentration tested (0-1 nmol/l) suggests that a steroidogenic response to hPTH(1–34) might be physiologically relevant. Parallelism between the two glycolytic response curves occurred at ACTH concentrations of 1 and 10 nmol/l and hPTH concentrations of 1 and 10 µmol/l, with a glycolytic potency ratio as low as 0-06% (log potency ratio = −3.24 ± 0.24).

Steroidogenesis was stimulated nearly maximally by hPTH(1–34) in the presence of basal cAMP levels. This provided the third example in the present work of non-involvement of cAMP in steroidogenesis, corroborating similar evidence in the literature (for review see Bristow, Gleed, Fauchère et al. 1980).
Bristow et al. (1980) concluded from their own studies with ACTH analogues on rat adrenocortical cells that native ACTH may evoke steroidogenesis through two receptors, only one of which involves cAMP. A hypothesis invoking different receptors responsible for glycolysis and steroidogenesis would, therefore, seem plausible. This does not exclude contributions to the glycolytic response by corticosterone as well as cAMP, as both these agents are capable of stimulating adrenal lactic acid production directly (Bartova & Birmingham, 1971a,b; Hum, Barta-Bartova & Birmingham, 1981).

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