Biochimica et Biophysica Acta, 526 (1978) 605—612

BBA 68547

INHIBITION OF BOVINE ADRENOCORTICAL ADENYLATE CYCLASE ACTIVITY BY ADENOSINE

PAUL GLYNN * and DERMOT M.F. COOPER **

Biochemistry Laboratory, School of Biological Sciences, University of Sussex, Falmer, Brighton, Sussex BN1 90G (U.K.)

(Received March 3rd, 1978)

Summary

Incubation of bovine adrenocortical membranes with corticotropin and 5-guanylylimidodiphosphate produced a state of adenylate cyclase (ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1) with maximal catalytic activity and an increased sensitivity to inhibition by adenosine. Due to metabolism of adenine nucleotides during adenylate cyclase assays a quantitative assessment of the nature of this inhibition was not possible. However, when determined at 0.2—1.0 mM $MgATP^{2-}$, half-maximal inhibition of the basal and maximally active states of the enzyme was observed at adenosine concentrations of 210—330 and 70—90 μM, respectively. The inhibition appeared to be partially competitive, suggesting that the nucleoside may act as an allosteric negative effector which reduces the affinity of the active site for substrate.

Adenosine was 5—6 times more potent as an inhibitor of adrenal adenylate cyclase than 2-chloroadenosine. Adenosine deaminase abolished the inhibitory effect of the nucleoside, whilst theophylline had no effect on activity either in the absence or presence of adenosine.

Introduction

The effects of adenosine on adenylate cyclase (ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1) activity appear to be tissue specific. Thus, the nucleoside has been shown to enhance the activity of the enzyme in brain tissue and tumours derived therefrom [1—3]. By contrast, adenosine inhibits adenylate

* Present address: Department of Crystallography, Birkbeck College, Malet St., London WC1 7HX, U.K. U.K.

** Present address: Section on Membrane Regulation, Laboratory of Nutrition and Endocrinology, N.I.A.M.D.D., N.I.H., Bethesda, Md. 20014, U.S.A. (Author to whom correspondence should be addressed.)

cyclase activity in membrane preparations from rat adipocytes [4], guinea-pig lung [5] and rat liver [6].

Wolff and Cook [7] reported that adenosine stimulated steroidogenesis in Y-1 adrenal tumour cells and was a partial agonist of adenylate cyclase activity in membrane preparations from these cells. Evidence presented by these authors suggested that the nucleoside acted via an external receptor, in a fashion analogous to that proposed for its action on the enzyme from brain [3].

Recent studies in this laboratory [8] indicated that, in normal rat adrenal cells, adenosine potentiated corticotropin-stimulated, but not basal, steroidogenesis, by inhibiting cyclic AMP phosphodiesterase activity (cf. ref. 9).

To elucidate the early events in the action of adenosine on normal adrenal tissue, the effects of the nucleoside on adenylate cyclase activity in a bovine adrenocortical plasma membrane preparation were examined. We have demonstrated that, in contrast to its reported stimulatory effect in adrenal tumour cells, the nucleoside inhibits the enzyme from bovine adrenal cortex, both in the basal or maximally activated states.

Materials and Methods

Synachten (corticotropin-(1-24)-tetracosapeptide; $ACTH_{1-24}$) was a generous gift from CIBA, Horsham, Sussex, U.K. 5′-Guanylylimidodiphosphate (disodium salt; Gpp(NH)p) synthesized by I.C.N., was kindly provided by Dr. S. Howell, University of Sussex. Adenosine, 2-chloroadenosine, theophylline and adenosine deaminase were purchased from Sigma (London) Chemical Co., London, S.W.6. The adenosine deaminase preparation (calf intestinal mucosa; crystalline suspension in saturated $(NH_4)_2SO_4$) was dialysed for 16 h at 4°C against 10 mM Tris · HCl (pH 7.6).

Before use the activity of the dialysed preparation was checked spectrophotometrically [10] and, by this criterion, it appeared stable at 4°C for 1 week, maintaining a potency of approx. 200 units/mg protein. The sources of other reagents and the method for preparation of a plasma membrane fraction from bovine adrenal cortex have been documented previously [11]. Membrane protein concentration was determined by the method of Lowry et al. [12] using crystalline bovine serum albumin as standard.

Adenylate cyclase. Adrenal membranes (2—3 mg/ml protein) were incubated (30°C; 0.5 ml) in 25 mM Tris · HCl (pH 7.6)/1 mM dithiothreitol/0.2% human serum albumin/5 mM $MgCl_2$, in the absence or presence of 0.1 mM Gpp(NH)p and 1 μM $ACTH_{1-24}$. The preincubation was terminated after 10 min by addition of 10 ml medium A (10 mM Tris · HCl (pH 7.6)/1 mM dithiothreitol at 4°C) and centrifugation at 80 000 × *g* for 10 min at 4°C. The pellets were washed by resuspension and centrifugation as above.

The adenylate cyclase activity of 20-μl aliquots of the washed membranes was determined at 30°C in a 5 or 10 min assay. The 50 μl reaction mixture contained: [α-^{32}P]ATP ($1 \cdot 10^6$—$2 \cdot 10^6$ cpm), cyclic[^{3}H]AMP (approx. 10 000 cpm), 0.1 mM cyclic AMP, 5 mM phosphocreatine, 25 units/ml creatine kinase, 0.2% human serum albumin and 1 mM dithiothreitol in 25 mM Tris · HCl (pH 7.6). The concentrations of ATP, $MgCl_2$ and other agents are listed in the appropriate figure legends. The reaction was terminated by addition of 100

μl 'stopping solution' (2% sodium dodecyl sulfate (SDS)/40 mM ATP/12.5 mM cyclic AMP at pH 7.5). Cyclic [^{32}P]AMP was isolated by the method of Salomon et al. [13] with the modifications described previously [11].

Unless stated otherwise, data represent the means of duplicate determinations which agreed to within less than 5%. All experiments have been repeated at least twice with essentially identical results.

Results and Discussion

Thin-layer and paper chromatography of adenylate cyclase reaction mixtures, in which either tritiated ATP or cyclic AMP were substituted for the ^{32}P-labelled substrate, revealed that [^{3}H]adenosine was formed from both [^{3}H]ATP and cyclic [^{3}H]AMP. Using 0.1 mM ATP and 1 mM cyclic AMP up to 30 μM adenosine could be formed during a 15 min assay. In addition, following such incubations, a significant fraction of the radioactivity from cyclic [^{3}H]AMP chromatographed with ATP. It was calculated that, using 0.1 mM [α-^{32}P]ATP and 1 mM cyclic AMP, the specific activity of the substrate would be diluted by approx. 10% within 15 min (unpublished data).

In an attempt to reduce the potential for distortion of data by these effects the present studies used 0.1 mM cyclic AMP, [α-^{32}P]ATP at concentrations not less than 0.2 mM and a maximum assay time of 10 min. Under these conditions, the final concentration of adenosine formed during the assay would not be expected to exceed 5 μM and the dilution of the specific activity of the substrate would be <1%.

Similar to findings in several other adenylate cyclase systems [14—16], preincubation of adrenal membranes with Gpp(NH)p and $ACTH_{1\text{-}24}$ leads to the rapid production of a maximally active state of the enzyme that cannot be

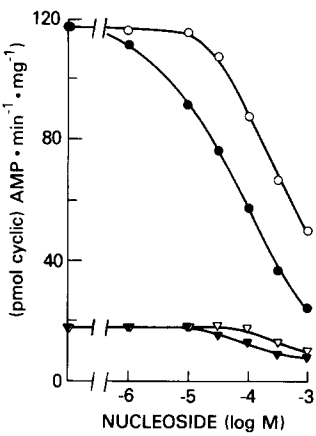

Fig. 1. Adrenal adenylate cyclase activity as a function of adenosine and chloroadenosine concentrations. Adrenal membranes (approx. 2 mg/ml protein) were incubated (0.5 ml, 30°C, 10 min) in 25 mM Tris · HCl (pH 7.6)/1 mM dithiothreitol/5 mM $MgCl_2$/0.2% human serum albumin in the presence (○, ●) or absence (▽, ▼) of 1 μM $ACTH_{1\text{-}24}$ and 0.1 mM Gpp(NH)p. The membranes were subsequently washed and their adenylate cyclase activities determined Assay conditions were: 1 mM ATP, 5 mM $MgCl_2$, and the concentrations of adenosine (●, ▼) and chloroadenosine (○, ▽) indicated in the figure. Membrane protein was 16.5 μg (●, ○) and 22.5 μg (▽, ▼) per assay.

reversed by washing the membranes [17]. In the present studies such preincubations were used so that the effects of adenosine on the maximally active and basal states of the adrenal enzyme could be compared.

Increasing concentrations of adenosine over the range 1 μM to 1 mM caused progressive inhibition of adrenal adenylate cyclase activity (Fig. 1). Half-maximal inhibition of the basal and maximally active enzymes were observed at 0.3 and 0.1 mM adenosine, respectively. These concentrations of adenosine are of the same order as those reported to inhibit the enzyme in adipocyte ghosts [4], but are substantially greater than those required to inhibt cyclic AMP production by intact fat cells [18,19].

2-Chloroadenosine appeared to be 5—6 times less potent than the native nucleoside (Fig. 1). The reduced potency of the chloro derivative might result from either a reduced affinity for the adenosine-binding site in adenylate cyclase or from its resistance to metabolism to form an active product. In view of the metabolism of adenine nucleotides under the conditions of adrenal adenylate cyclase assays, the latter possibility merited consideration; however, the data in Table I suggest that adenosine itself, rather than one of its metabolites, is the most potent inhibitor of the adrenal enzyme. Thus, adenosine (0.1 mM) reduced the activity of maximally active adrenal adenylate cyclase by approx. 50% (cf. Fig. 1) but this inhibition was abolished in the presence of adenosine deaminase. This observation indicates that inosine has little, if any, inhibitory effect.

It is also noteworthy that adenosine deaminase had essentially no effect on the activity observed in the absence of exogenously added nucleoside, suggesting that production of adenosine from 1 mM ATP and 0.1 mM cyclic AMP during the assay was not sufficient to cause significant inhibition of adenylate cyclase. 0.1 mM 5′-AMP caused a small (approx. 13%) inhibition of activity, which was abolished in the presence of adenosine deaminase. 5′-AMP itself is a very poor substrate for adenosine deaminase and it seems likely that the apparent inhibition by 5′-AMP resulted from its hydrolysis to adenosine during the assay. It can be estimated that 5% conversion of 0.1 mM 5′-AMP to

TABLE I

EFFECTS OF ADENOSINE, AMP, AND ADENOSINE DEAMINASE ON ADRENAL ADENYLATE CYCLASE

Adrenal membranes (approx. 3 mg/ml) were preincubated with 1 μM $ACTH_{1-24}$ and 0.1 mM Gpp(NH)p and subsequently washed. The washed membranes were resuspended in 25 mM Tris · HCl (pH 7.6)/1 mM dithiothreitol/5 mM $MgCl_2$. After equilibrating to room temperature, 20 μl aliquots of the membranes were added to 30 μl adenylate cyclase assay medium to produce reaction conditions as follows: 1 mM ATP, 5 mM $MgCl_2$, membrane protein (20 μg per assay), adenosine and AMP (0 or 0.1 mM) and when present, adenosine deaminase (40 μg/ml = 8 units/ml). Assay time was 10 min.

Assay additions	Adenylate cyclase activity	
	Control	+ adenosine deaminase
None	122 ± 4	126 ± 6
+ adenosine (0.1 mM)	63 ± 3	116 ± 5
+ AMP (0.1 mM)	106 ± 3	116 ± 2

Results are expressed in pmol cyclic AMP · min^{-1} · mg^{-1} protein (mean ± S.E.; n = 3).

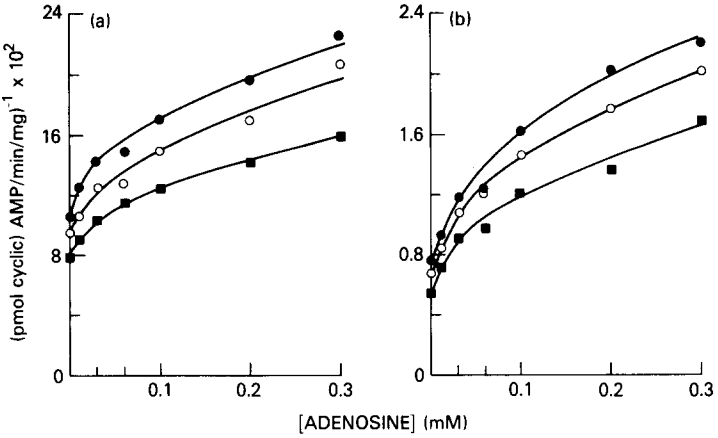

Fig. 2. Dixon plots for inhibition of adrenal adenylate cyclase activity by adenosine. Adrenal membranes (approx. 3 mg/ml) protein were incubated (1.0 ml, 30°C, 15 min) in 25 mM Tris · HCl (pH 7.6)/1 mM dithiothreitol/5 mM $MgCl_2$/0.2% human serum albumin, in the absence (a) or presence (b) of 1 μM $ACTH_{1-24}$ and 0.1 mM Gpp(NH)p. Preincubations were terminated and membranes washed. The amounts of ATP and $MgCl_2$ added to the adenylate cyclase assay were adjusted so as to maintain a constant concentration of 4 mM free Mg^{2+} whilst $MgATP^{2-}$ was varied (●, 0.2 mM; ○, 0.5 mM; ■, 1.0 mM). Assays contained either 29 μg (a) or 27.5 μg (b) membrane protein and the indicated concentrations of adenosine, with other conditions as in Materials and Methods. Data show reciprocal velocity (pmol cyclic AMP · min^{-1} · mg^{-1} protein)$^{-1}$ as a function of adenosine concentration for the basal (a) and Gpp(NH)p + $ACTH_{1-24}$)-activated (b) enzyme.

adenosine would be sufficient to cause the observed inhibition (cf. Fig. 1).

To examine the nature of the inhibition of basal and maximally-active adrenal adenylate cyclase, the effects of increasing adenosine concentrations on the enzyme activities were determined at various substrate concentrations whilst Mg^{2+} concentration was kept constant at 4.0 mM. Analysis of the data from this experiment by the method of Dixon [20] yielded a set of hyperbolae (Fig. 2). Hyperbolic Dixon plots indicate partial competitive inhibition. In both pure competitive and partial competitive inhibition the V of the reaction is unaffected by the inhibitor but, whilst in the former situation the K_m increases without bound with increasing inhibitor concentration, in the latter the K_m approaches an upper limit [21]. Partial competitive inhibition of several enzymes has been reported and has been interpreted by postulating that the inhibitor binds to an allosteric site and has been interpreted by postulating that the inhibitor binds to an allosteric site and consequently lowers indirectly the affinity of the active site for its substrate (e.g. ref. 22). Adenosine may inhibit adenylate cyclase in an analogous fashion.

In this experiment the maximally active form of adenylate cyclase was approximately three times more sensitive to inhibition by adenosine than the basal state. Basal activity was inhibited half-maximally by concentrations of adenosine of 210, 250, and 330 μM at substrate concentrations of 0.2, 0.5 and 1.0 mM, respectively. Half-maximal inhibition of the (Gpp(NH)p + corticotropin)-activated enzyme was observed at adenosine concentrations ranging from approx. 70 μM at 0.2 mM $MgATP^{2-}$ to approx. 90 μM at 1.0 mM $MgATP^{2-}$ (Fig. 2). McKenzie and Bär [23] showed that corticotropin-stimulated adenylate cyclase activity in rat adipocyte ghosts was considerably more

sensitive to inhibition by adenosine than basal activity. Weinryb and Michel [5] found that on increasing Mg^{2+} concentration from 1.8 to 11.8 mM, the concentration of adenosine required to inhibit half-maximally basal adenylate cyclase activity in guinea-pig lung decreased from 2 to 0.15 mM. Londos and Preston [6] have also shown that the sensitivity of the hepatic enzyme to inhibition was increased by Mn^{2+}, Mg^{2+}, and glucagon. Previously we have presented evidence to suggest that Mg^{2+} is an activator of adrenal adenylate cyclase, and that corticotropin, Gpp(NH)p and Mg^{2+} cooperate to produce a maximally active state of the enzyme [17]. Mg^{2+} may also be considered to be an activator of the rat hepatic and guinea-pig lung enzymes. Thus it might be inferred that in systems in which adenosine inhibits adenylate cyclase, effectors which activate the enzyme (i.e. hormones, guanine nucleotides and Mg^{2+}) also render it more sensitive to inhibition by adenosine.

The possibility exists that the enhanced production of cyclic AMP induced by positive effectors could lead, via the actions of phosphodiesterase and 5'-nucleotidase, to increased levels of adenosine, which could provide an indirect feedback inhibition of the system in intact cells.

It is possible, although unlikely under the assay conditions used, that the non-linear Dixon plots (Fig. 2) might result from an inhibition of phosphodiesterase activity by high concentrations of adenosine (cf. ref. 9). However, theophylline had no effect on adrenal adenylate cyclase activity determined under the conditions described in Table II. In addition, theophylline did not affect the inhibition caused by adenosine (Table II) contrasting with its antogonistic effect on the activation by the nucleoside of adenylate cyclase in other systems [3,7].

A major point of interest of the present report is the striking contrast between the effects of adenosine on adenylate cyclase activity in normal adrenal tissue and adrenal tumour cells. Thus, Wolff and Cook [7] found that the enzyme in membrane preparations from Y-1 adrenal tumour cells was activated half-maximally by adenosine concentrations fo 30—50 μM, whereas such

TABLE II

INHIBITION OF ADRENAL ADENYLATE CYCLASE ACTIVITY BY ADENOSINE: EFFECT OF THEOPHYLLINE

Adrenal membranes (approx. 3 mg/ml protein) were preincubated with 1 μM $ACTH_{1-24}$ and Gpp(NH)p (0.1 mM) and subsequently washed. The washed membranes were incubated (30°C, 6 min, 40 μl) in Tris · HCl (pH 7.6), dithiothreitol, $MgCl_2$, human serum albumin and adenosine, at 1.25 times the concentrations noted in the table, and in the presence or absence of theophylline (1.0 mM). The adenylate cyclase assay was initiated by addition of 10 μl medium. The final composition of the reaction mixture was: 0.2 mM ATP, 5 mM $MgCl_2$, adenosine (0, 30 or 300 μM) and membrane protein (14 μg per assay). The assay was terminated after 10 min.

Adenosine (μM)	Adenylate cyclase activity	
	Control	+ theophylline
0	91.4 ± 4	91.4 ± 4
30	63.0 ± 2	63.6 ± 4
300	23.6 ± 0.6	23.6 ± 4

Results are expressed in pmol cyclic AMP · min^{-1} · mg^{-1} protein (mean ± S.E.; n = 3).

concentrations of the nucleoside cause substantial inhibition of the (GPP(NH)p + corticotropin)-activated enzyme in bovine adrenal membranes (Fig. 2). The entirely opposite effects of adenosine in the two adrenal cells types may reflect other differences in their adenylate cyclase systems. The K_m for adenylate cyclase from Y-1 cells is approx. 1.3 mM $MgATP^{2-}$ [24], which is almost an order of magnitude greater than K_m values determined for the enzymes in rat and bovine adrenal membrane preparations (0.15—0.25 mM; [26,25]). In addition it has been noted that the Y-1 enzyme is inhibited by Mg^{2+} concentrations greater than 2 mM [7]. The adenylate cyclase activity of bovine adrenal cortex, even in the presence of corticotropin guanine nucleotides, is much less sensitive to inhibition by the cation [25,17].

In a very recent report Londos and Wolff [27] reviewed evidence suggesting that two distinct adenosine-affected sites which differed in their chemical specificity and biochemical action might be present in many cell types.

Occupation of one ('R') site generally leads to stimulation of adenylate cyclase while occupation of the second ('P') site leads to inhibition of the enzyme. In the same report these authors intimated that adenosine not only stimulated but also inhibited Y-1 adrenal tumour adenylate cyclase activity. If this suggestion is confirmed then the lack of stimulation by adenosine of bovine adrenal adenylate cyclase requires explanation. It is possible that the complex pattern observed in the Dixon plot (this report: Fig. 2) may obscure two opposing actions of adenosine; inhibition at low (<0.05 mM) adenosine concentrations and both inhibition and stimulation at higher (>0.1 mM) concentrations (Fig. 2). However, the lack of effect of theophylline under these conditions (Table II) argues strongly against the presence of stimulatory ('R') sites [27]. The use of adenosine analogues which are specific for the activation site in other systems [27] may unambiguously determine whether stimulatory adenosine sites are present in bovine adrenal adenylate cyclase.

It is important to define clearly the possible actions of adenosine at the level of adenylate cyclase in adrenal plasma membranes in order to speculate on the regulation of steroidogenesis in the adrenal cortex by endogenous adenosine. The results presented herein make it possible that adenosine may act as a feedback regulator of hormonal stimulation in intact adrenal cells.

Acknowledgements

Tenure of an S.R.C. postgraduate studentship (for P.G.) is gratefully acknowledged. We wish to thank Drs. C. Londos and M. Rodbell for their helpful suggestions in the preparation of this manuscript.

References

1 Sattin, A. and Rall, T.W. (1970) Mol. Pharmacol. 6, 13—23
2 Clark, R.B., Gross, R., Su, Y.F. and Perkins, J.P. (1974) J. Biol. Chem. 249, 5296—5303
3 Perkins, J.P., Moore, M.M., Kaliskar, A. and Su, Y.F. (1975) Adv. Cyclic Nucleotide Res. 5, 641—660
4 Fain, J.N., Pointer, R.H. and Ward, W.F. (1972) J. Biol. Chem. 247, 6866—6872
5 Weinryb, I. and Michel, I.M. (1974) Biochim. Biophys. Acta 334, 218—225
6 Londos, C. and Preston, M.S. (1977) J. Biol. Chem. 252, 5951—5956
7 Wolff, J. and Cook, G.H. (1977) J. Biol. Chem. 252, 687—693
8 Cooper, D.M.F. and Gleed, C.D. (1978) J. Steroid Biochem., in the press

9 Amer, M.S. and Kreighbaum, W.E. (1975) J. Pharmacol. Sci. 64, 1—37
10 Kalckar, H.M. (1947) J. Biol. Chem. 167, 445—459
11 Glynn, P., Cooper, D.M.F. and Schulster, D. (1977) Biochem. J. 168, 277—282
12 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
13 Salomon, Y., Londos, C. and Rodbell, M. (1974) Anal. Biochem. 58, 541—548
14 Cuatrecasas, P., Jacobs, S. and Bennett, V. (1975) Proc. Natl. Acad. Sci. U.S. 72, 1739—1743
15 Schramm, M. and Rodbell, M. (1975) J. Biol. Chem. 250, 2232—2237
16 Spiegel, A.M., Brown, E.M., Fedak, S.A., Woodard, C.J. and Aurbach, G.D. (1976) J. Cyclic Nucleotide Res. 2, 47—56
17 Glynn, P., Cooper, D.M.F. and Schulster, D. (1978) Biochim. Biophys. Acta 524, 474—483
18 Fain, J.N. and Wieser, P.B. (1975) J. Biol. Chem. 250, 1027—1034
19 Schwabe, U., Ebert, R. and Erbler, H.C. (1975) Adv. Cyclic Nucleotide Res. 5, 569—584
20 Dixon, M. (1953) Biochem. J. 55, 170—171
21 Webb, J.L. (1963) in Enzyme and Metabolic Inhibitors, Vol. 1, Academic Press, New York
22 Burger, R.M. and Lowenstein, J.M. (1975) Biochemistry 14, 2362—2366
23 McKenzie, S.G. and Bär, H.P. (1973) Can. J. Physiol. Pharmacol. 51, 190—196
24 Schimmer, B.P. (1972) J. Biol. Chem. 247, 3134—3138
25 Glossmann, H. and Gips, H. (1975) Naunyn-Schmiedeberg's Arch. Pharmacol. 289, 77—97
26 Londos, C. and Rodbell, M. (1975) J. Biol. Chem. 250, 3459—3465
27 Londos, C. and Wolff, J. (1977) Proc. Natl. Acad. Sci. U.S. 74, 5482—5486