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INHIBITION OF RAT ADRENAL STEROIDOGENESIS BY ADENINE CONTAINING COMPOUNDS

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SUMMARY

The succinate-supported synthesis of corticosterone from endogenous precursors in a cell-free system from rat adrenals is inhibited by ADP and ATP. The inhibition is observed at short incubation times followed by a recovery from the inhibition and it appears to be specific for adenine containing compounds. The inhibition by ADP(ATP) requires phosphate, is increased by the presence of glucose plus hexokinase and eliminated by the presence of atractyloside or phosphoenolpyruvate plus pyruvate kinase. The inhibition is also eliminated if ATP is added shortly after the start of the incubation or if succinate is replaced by isocitrate. The inhibition has been found to occur only at the 11β-hydroxylation of deoxycorticosterone and the inhibition of this reaction shares many of the properties of the inhibition of corticosterone synthesis from endogenous precursors. It is concluded that the inhibition may be attributed to a competition for reducing equivalents between the hydroxylase system and oxidative phosphorylation as well as a direct effect on the hydroxylase system.

INTRODUCTION

The synthesis of corticosterone from cholesterol in the rat adrenal cortex consists of a series of reactions which occur in the mitochondria and the microsomes. The transformation of endogenous cholesterol to pregnenolone takes place in the mitochondria [1]; the formation of progesterone from pregnenolone and the 21-hydroxylation of progesterone to yield deoxycorticosterone occur in the microsomes [2, 3]; and the final 11β-hydroxylation of deoxycorticosterone to give corticosterone takes place in the mitochondria [2]. There is thus present an interaction between two organelles to accomplish the synthesis of the final product. There is evidence that there are regulatory factors involved in this complex series of reactions in addition to the primary regulation by ACTH at the transformation of cholesterol to pregnenolone [4, 5]. Thus, it has been found that 11β-hydroxylation of deoxycorticosterone inhibits the conversion of cholesterol to pregnenolone, but the reverse situation does not occur [6]. Pregnenolone can inhibit its own formation from cholesterol [7]. Ca^{2+} stimulates pregnenolone synthesis [8] and 11β-hydroxylation of deoxycorticosterone [9]. Reduced NAD^+ inhibits the formation of progesterone from pregnenolone and this inhibition can be reversed by ascorbic acid among other substances [10]. Both NAD^+ and NADH stimulate the 5-ene-3-keto steroid isomerase [11]. It is not known what physiological functions these modifications of the steroidogenic enzyme reactions may have, but the existence of such a number suggests the possibility that this sequence of reactions may well be functionally a highly integrated system. In addition, a number of investigations have been carried out on the relationship between steroid 11β-hydroxylation and oxidative phosphorylation [12–16].

In this communication we describe an inhibition by ADP(ATP) of corticosterone synthesis from endogenous precursors. Several of the properties of this inhibition have been investigated and it has been found to take place only in the 11β-hydroxylation of deoxycorticosterone.

MATERIALS AND METHODS

Rat adrenal glands were removed, homogenized and the homogenate fractionated according to the procedures described previously [17] except that the homogenization medium consisted of 0.25 M sucrose which contained 1 mM EDTA and 12 mM Tris buffer at pH 7.5. The preparation of mitochondria contained, on the average, about 2.0–2.5 mg protein per ml and the microsomes about 1.8–2.1 mg protein per ml.

The incubation conditions in cell-free systems for the synthesis of corticosterone from endogenous precursors (mitochondria plua microsome) and from deoxycorticosterone (mitochondria), the synthesis of deoxycorticosterone from progesterone (microsomes), the formation of progesterone from pregnenolone (microsomes) and the synthesis of pregnenolone from endogenous precursors (mitochondria) have been given elsewhere [17]. All incubations were carried out at 20° in the presence of phosphate and succinate unless otherwise indicated. The analytical methods used have been described [17] except that pregnenolone was determined by a different procedure [18].

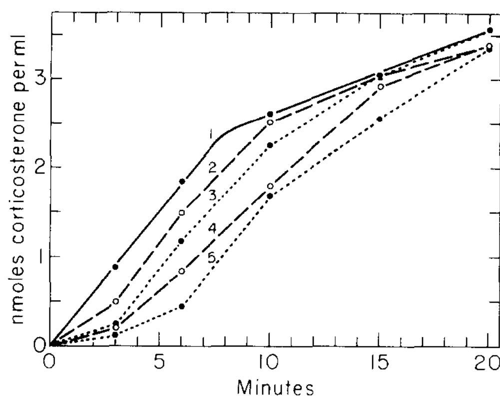

Fig. 1. The time-course of inhibition of corticosterone synthesis by ADP and ATP. Curve 1, no ADP or ATP present; curve 2, 0.1 mM ATP; curve 3, 0.1 mM ADP; curve 4, 1.0 mM ATP; curve 5, 1.0 mM ADP.

In most experiments the tissue was obtained from rats treated with ACTH [17] and unless otherwise indicated in the data corticosterone synthesis from endogenous precursor is presented.

RESULTS

The effects of ADP and ATP on the time course of synthesis of corticosterone from endogenous precursor in a cell-free system are shown in Fig. 1. The usual biphasic rate curve is obtained in the absence of ADP or ATP [17]. In the presence of these adenine nucleotides an initial inhibition of corticosterone synthesis is observed, followed by a recovery from this inhibition so that essentially no effect is found after 20 min of incubation. The extent and duration of the inhibition depends on the nucleotide and its concentration, ADP being more effective than ATP.

The inhibitory effects of various nucleosides and nucleotides are given in Table 1. It is seen that in the adenine series ADP is most effective followed by ATP and that AMP and adenosine are about equally effective. The nucleosides and nucleotides of guanine, uracil and cytosine are approximately equally effective with no inhibition by the nucleosides and with considerably less inhibition than ADP by the various nucleotides. In all cases the inhibitory effects decrease with an increase in time of incubation. Since ADP and ATP are metabolically readily interconvertible in this incubation system they have been used interchangeably in many of the following experiments.

A number of characteristics of the inhibition of corticosterone synthesis by ADP(ATP) have been determined. It is seen from the data in Table 2 that while phosphate has only a slight stimulatory effect on corticosterone synthesis from endogenous precursors in a cell-free system, its presence is required for the inhibition to take place. The decrease in the inhibition with an increase in incubation time occurs at all phosphate concentrations. It was found that a decrease in succinate concentration from the 12 mM routinely used to 5 mM, results only in a slight decrease (10%) in corticosterone synthesis but an increase in the inhibition by ATP from 40% to 62% after 3 min of incubation. The inhibition is prevented by the presence of 0.5 mM potassium atractyloside. The inhibition is also eliminated if the ATP is added to the reaction mixture 3 min after the start of the incubation (Table 3). The use of adrenal mitochondria and microsomes from rats given ACTH *in vivo* 15 min before killing has little effect on the inhibition, although there is a suggestion that the ACTH administration results in a somewhat greater and more protracted inhibition.

Several of these observations, particularly the elimination of the inhibition by atractyloside and the increase in the inhibition brought about by a decrease in succinate concentration suggest that a reaction

Table 1. The inhibition of corticosterone synthesis by various nucleosides and nucleotides

Experiment Addition		1 Adenine		2 Guanine		3 Uracil		4 Cytosine	
Incubation time		3 min	9 min	3 min	9 min	3 min	9 min	3 min	9 min
	mM	Percent inhibition of corticosterone synthesis							
Nucleoside	0.1	22	5	0	0	0	0	0	0
,,	2.0	78	13	0	0	0	0	0	0
Nucleoside-3′-P	0.1	0	0						
,,	2.0	11	0						
Nucleoside-5′-P	0.1	28	5	0	0	0	0	0	0
,,	2.0	72	15	15	0	12	8	13	10
Nucleoside-5′-diP	0.1	84	24	21	9	12	10	25	10
,,	2.0	100	44	26	9	29	11	25	13
Nucleoside-5′-triP	0.1	66	13	15	0	12	8	6	3
,,	2.0	100	34	21	6	24	8	25	15
ADP	0.1			64	15	53	13	69	15
,,	2.0			91	29	86	31	92	38

When derivatives of bases other than adenine were tested ADP was included in the experiment to provide a standard for the inhibitions caused by the other substances. The values, in nmoles of corticosterone per ml, for incubations with no nucleosides or nucleotides present for 3 min and 9 min incubation times are for experiment 1, 1.02 and 2.67; experiment 2, 0.87 and 2.05; experiment 3, 1.09 and 2.89; experiment 4, 1.11 and 2.77.

Table 2. The effect of phosphate on the inhibition by ADP and ATP

Incubation time Additions	3 minutes Corticosterone nmol/ml	Inhibition %	6 minutes Corticosterone nmol/ml	Inhibition %
No phosphate				
—	0.96		1.75	
ADP, 0.1 mM	0.90	6	1.75	0
ADP, 1.0 mM	0.72	25	1.69	0
ATP, 0.1 mM	0.90	6	1.80	0
ATP, 1.0 mM	0.81	16	1.81	0
1.7 mM phosphate				
—	0.96		1.98	
ADP, 0.1 mM	0.54	44	1.63	18
ADP, 1.0 mM	0.30	69	1.33	33
ATP, 0.1 mM	0.60	38	1.63	18
ATP, 1.0 mM	0.42	56	1.44	27
12 mM phosphate				
—	1.02		2.04	
ADP, 0.1 mM	0.12	88	1.27	38
ADP, 1.0 mM	0.12	88	0.54	74
ATP, 0.1 mM	0.36	65	1.51	26
ATP, 1.0 mM	0.12	88	0.75	63

Table 3. The effect of the late addition of ATP on corticosterone synthesis

ATP	Time after start of incubation of ATP addition	Incubation time 3 min	6 min	9 min	12 min
		nmol corticosterone/ml			
0		0.79	1.76	2.82	3.00
2 mM	0 min	0.18	0.57	1.41	2.20
2 mM	3 min		1.76	2.73	3.08
2 mM	6 min			2.82	3.08

located within the mitochondria is involved. A survey of the reactions of the major steps in the synthesis of corticosterone, i.e., the conversion of cholesterol to pregnenolone, the formation of progesterone from pregnenolone, the 21-hydroxylation of progesterone to give deoxycorticosterone and finally, the 11β-hydroxylation of deoxycorticosterone to yield corticosterone, showed that ATP inhibits only the conversion of deoxycorticosterone to corticosterone (Tables 4 and 5). The other mitochondrial reaction, the synthesis of pregnenolone, was not affected. It is also seen in Table 5 that the extent of 11β-hydroxylation decreases with increasing deoxycorticosterone concentration in confirmation of the observations made by Hayano and Dorfman[19]. The percentage of inhibition by ATP also decreases with increasing dioxycorticosterone concentrations so that at 100 μM virtually no inhibition is seen. It is to be noted, however, that the absolute amounts of corticosterone formed in the presence of ATP remains essentially constant at the shorter incubation times throughout the range of deoxycorticosterone concentrations.

Several additional lines of evidence also indicate that the inhibition by ADP(ATP) of corticosterone

Table 4. The effect of ATP on enzyme systems of steroidogenesis

Assay		ATP
	nmol product/min/mg protein	
Pregnenolone synthesis	0.33	0.33
Pregnenolone to progesterone	5.13	4.78
Progesterone to deoxycorticosterone	1.85	1.81

ATP when present was 2 mM.

Table 5. The effect of ATP on the conversion of 11-deoxycorticosterone to corticosterone

Deoxycorticosterone μM	ATP	Incubation time 3 min	6 min	10 min
		nmol corticosterone/ml		
12.5	—	1.98	5.92	10.0
	+	0.76	3.36	9.43
Inhibition		62%	43%	6%
25	—	1.52	4.72	10.0
	+	0.76	3.34	8.85
Inhibition		50%	29%	11%
50	—	1.22	3.34	7.18
	+	0.76	2.74	6.53
Inhibition		38%	18%	9%
100	—	0.91	3.04	6.53
	+	0.84	2.90	6.53
Inhibition		8%	0	0

ATP when present was 2 mM.

Table 6. The effect of ATP dephosphorylation, ADP phosphorylation and phosphate on the ATP inhibition of corticosterone synthesis

Additions	A Corticosterone nmol/ml	Change %	B Corticosterone nmol/ml	Change %
—	1.64		2.90	
ATP	0.66	−60	1.92	−34
Glucose + hexokinase	1.58		2.42	
Glucose + hexokinase + ATP	0.40	−70	1.12	−54
PEP + pyruvate kinase	1.25		4.18	
PEP + pyruvate kinase + ATP	1.18	0	6.12	+ 46
P_i omitted			1.77	
P_i omitted + ATP			2.10	+ 18

When present, ATP was 2 mM; glucose 12 mM; PEP (phosphoenolpyruvate) 5 mM; crystalline hexokinase, 12 units per ml incubation medium; and crystalline pyruvate kinase, 5 units per ml incubation medium. Incubation time was 3 min. Corticosterone synthesis was determined in condition A from endogenous precursors in the presence of mitochondria and microsomes, and in condition B from added 11-deoxycorticosterone in the presence of mitochondria.

synthesis from endogenous precursors can be accounted for by the inhibition of 11β-hydroxylation. In both systems the inhibition decreases with time of incubation (Fig. 1 and Table 5). As is seen from the data in Table 6, the presence of glucose plus hexokinase increases the inhibition caused by ATP in both systems while the addition of PEP plus pyruvate kinase not only erases the inhibition but in the case of 11-hydroxylation results in a stimulation. The omission of phosphate also eliminates the ATP inhibition of 11-hydroxylation, as was found for corticosterone synthesis from endogenous precursors (Table 2). Since the omission of phosphate reduced the 11-hydroxylation of added deoxycorticosterone, the stimulation of this reaction by ATP in the absence of phosphate may be a consequence of phosphate derived from the ATP. A stimulation of 11β-hydroxylation by phosphate has been reported by others [15]. The addition of oligomycin, which would be expected to impair the rate of mitochondrial phosphorylation of ADP, results only in a partial reversal of the inhibition (Table 7). Higher concentrations of oligomycin inhibited corticosterone synthesis.

The above experiments were carried out with succinate as the source of electrons for the formation of NADPH required for steroid hydroxylation. This involves energy dependent processes such as reverse electron transport and tranhydrogenation. Steroid hydroxylation can be supported by isocitrate by an energy independent process [15] presumably by the NADP-linked isocitrate dehydrogenase. The effect of ADP of isocitrate supported corticosterone synthesis is given in Table 8. It is seen that little or no inhibition is observed. In the same experiment with succinate replacing isocitrate ADP caused a 67% inhibition in corticosterone synthesis from endogenous precursors (condition A) and a 42% inhibition in corticosterone synthesis from added corticosterone (condition B). Succinate was about 75% as effective as isocitrate in supporting corticosterone synthesis.

DISCUSSION

The succinate supported synthesis of corticosterone from endogenous precursors in a rat adrenal cell-free system consisting of mitochondria, microsomes and appropriate co-factors has been found to be inhibited by the addition of ADP or ATP. The evidence presented indicates that this inhibition takes place at the mitochondrial 11β-hydroxylation of deoxycorticosterone to form corticosterone. Of the four major steps in rat adrenal steroidogenesis only this reaction and not the formation of pregnenolone, progesterone or deoxycorticosterone is inhibited. In addition, many of the characteristics of the inhibition of the transformation of deoxycorticosterone to corticosterone are

Table 7. The effect of oligomycin on the inhibition of corticosterone synthesis by ADP

Additions	A Corticosterone nmol/ml	Change %	B Corticosterone nmol/ml	Change %
—	1.08		1.80	
Oligomycin	1.08	0	1.80	0
ADP	0.42	−61	0.71	−61
ADP + oligomycin	0.77	−29	1.16	−36

Conditions A and B are described in the legend to Table 6. Oligomycin was present at a concentration of 0.84 μg per ml incubation medium in A and 1.6 μg in B. The concentration of ADP was 2 mM. The incubation time was 3 min.

Table 8. The effect of ADP on isocitrate supported corticosterone synthesis

Additions	A Corticosterone nmol/ml	Change %	B Corticosterone nmol/ml	Change %
—	1.35		3.00	
ADP	1.13	−16	3.03	0
Antimycin A	1.28		2.89	
ADP + Antimycin A	1.13	−12	2.84	0

DL-Isocitrate was present at 24 mM, ADP at 2 mM, and antimycin A at 4.2 μg per ml incubation medium. The incubation time was 3 min. Conditions A and B are described in the legend to Table 6. In concomitant incubations with succinate in place of the isocitrate, ADP caused a 67% inhibition in A and a 42% inhibition in B.

similar to those found in the formation of corticosterone from the endogenous precursors. Thus, in both, the inhibition decreases with time of incubation, oligomycin causes a partial reversal of the inhibition, the presence of glucose plus hexokinase increases the inhibition while the presence of phosphoenolpyruvate plus pyruvate kinase reverses the inhibition, and the presence of phosphate is required for the inhibition to take place. In addition, in both cases the inhibition is greatly reduced or eliminated if succinate is replaced by isocitrate. These data support the conclusion that in the succinate supported steroidogenic sequence of reactions only 11β-hydroxylation is inhibited by ADP or ATP.

A possible explanation for an inhibition by ADP(ATP) of a mitochondrial hydroxylation reaction dependent upon the oxidation of a Krebs cycle acid would be a competition for reducing equivalents by the hydroxylating system and oxidative phosphorylation. A number of the observations can support this interpretation. Thus, the inhibition requires phosphate, is partially reversed by oligomycin and is increased when the succinate concentration is decreased. Furthermore, maintenance of high ADP levels by the addition of glucose plus hexokinase increases the inhibition and, in the reverse situation where ADP concentration is reduced by the addition of phosphoenolpyruvate plus pyruvate kinase, the inhibition is eliminated. Also in support of this interpretation is the essential elimination of the inhibition when succinate is replaced by isocitrate. In this case the generation of reducing equivalents required for steroid hydroxylation does not involve energy generating systems [15]. These observations would indicate that the inhibitor is ADP rather than ATP, a suggestion in keeping with the finding that, in general, ADP is more inhibitory than ATP. The inability of other investigators [14, 15] to find an inhibition of 11β-hydroxylation by ADP may be due to the high concentrations of deoxycorticosterone used or to the absence of phosphate.

However, an explanation of the inhibition solely on the basis of a competition for reducing equivalents does not appear to account for the reversibility of the inhibition with increasing time of incubation and for the absence of the inhibition if ATP is added shortly after the start of the incubation. These observations suggest an effect on steroidogenesis by a mechanism in addition to the postulated competition, and preliminary experiments have shown that ADP will inhibit NADPH supported 11β-hydroxylation in disrupted mitochondria.

The finding that the ADP(ATP) inhibition of 11β-hydroxylase decreases with increasing incubation times suggests the possible accumulation of a substance which either modifies or counteracts that inhibition. The extent of the accumulation of such a substance under the more dynamic conditions present during *in vivo* hydroxylation can now determine the magnitude of the ADP inhibition. This inhibition might then impose a regulatory process on steroidogenesis secondary to the ACTH sensitive step, the conversion of cholesterol to pregnenolone in the mitochondria. It is of interest that this latter reaction is not inhibited by ADP when supported by succinate. This would suggest that this reaction can more effectively compete with the cytochrome chain for reducing equivalents than can 11β-hydroxylation.

The data do not permit a single explanation for the characteristics of the inhibition produced by ADP(ATP). Both a competition for reducing equivalents by the hydroxylating system and oxidative phosphorylation, which appears to be a major factor, as well as a more direct effect of the adenine nucleotides on the hydroxylating system must be considered.

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