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The Effect of Adenosine and Adenine Nucleotides on the Cyclic Adenosine 3′,5′-Phosphate Content of Guinea Pig Cerebral Cortex Slices

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SUMMARY

The content of cyclic adenosine 3′,5′-phosphate in guinea pig cerebral cortex slices increases 20–30-fold after a 5-min exposure to a medium containing 0.05 mM adenosine. A similar increase was observed upon exposure to adenine nucleotides. The effect appeared to be specific for adenine ribose monomers. Methylxanthines (0.5 mM) blocked the effect of adenosine, but the blockade could be surmounted by increasing the adenosine concentration. Mutual potentiation of effects was observed when norepinephrine or histamine was added together with adenosine. Nucleotidase activity was observed in slices and homogenates. While this may be related to the mechanism of the adenosine effect, a direct effect of adenosine on adenyl cyclase or cyclic 3′,5′-nucleotide phosphodiesterase could not be implicated in homogenates. Changes in the tissue compartmentation of adenine nucleotides probably play a major role in producing the previously observed increase of cyclic adenosine 3′,5′-phosphate during electrical stimulation of slices.

INTRODUCTION

In recent years evidence has accumulated that cyclic adenosine 3′,5′-phosphate is implicated in the hormonal regulation of many tissues. These hormones include catecholamines, ACTH, glucagon, antidiuretic hormone, and thyroid-stimulating hormone. This evidence has been recently reviewed (1, 2). Cyclic 3′,5′-AMP, together with enzymes for its synthesis and destruction, is widely distributed in almost all mammalian tissues. Sutherland, Rall, Butcher, and associates found that brain contains the highest activities of adenyl cyclase and cyclic 3′,5′-nucleotide phosphodiesterase (3, 4). Kakiuchi and Rall (5, 6) found that rabbit brain slices responded to both norepinephrine and histamine with large increases in cyclic 3′,5′-AMP content. Electrical pulses applied to slices of guinea pig cerebral cortex also resulted in large increases in cyclic 3′,5′-AMP content (7). Although specific regulatory actions of cyclic 3′,5′-AMP have not yet been established, it is presumed, by analogy with other tissues, that the nucleotide is involved in the regulation of function and metabolism of the central nervous system. The present paper is a further study of factors regulating the cyclic 3′,5′-AMP content of central nervous tissue.

n an effort to find endogenous substances that might mediate the effect of electrical pulses, Sattin and Rall (8) found that per-

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chloric acid or boiled 0.9% NaCl extracts of guinea pig cerebral cortex increased the cyclic 3′,5′-AMP content of guinea pig cortex slices by a factor of 20–30. In the course of that investigation, the ability of adenosine and adenine nucleotides to reproduce the effects of tissue extracts was noted. Exploration of the effects of adenosine and its derivatives on the cyclic 3′,5′-AMP content of brain slices is the subject of this paper.

METHODS AND MATERIALS

Male guinea pigs weighing 300–500 g were decapitated, the forebrain was removed, and the cortex was separated from adjacent striatal tissue. Procedures used for the preparation, incubation, and fixation of the tissue have been described (5), except that all preparation of tissue was carried out at room temperature instead of 3°. The composition of the medium was as follows: NaCl, 124 mM; KCl, 5 mM; $NaHCO_3$, 26 mM; KH_2PO_4, 1.24 mM; $CaCl_2$, 0.8 mM; $MgSO_4$, 1.3 mM; glucose, 10 mM; gas phase, 95% O_2–5% CO_2. After 50 min of preliminary incubation at 37°, the amounts of cyclic 3′,5′-AMP in the slices reached stable low levels. Slices were then transferred to fresh medium containing the substance(s) to be tested. Theophylline was not included in the preliminary incubation medium and was added to the incubation medium only when indicated. The time required for preparation of tissue from one to three animals was 40 min or less. Except as otherwise stated, each sample contained 100–200 mg of slices, wet weight.

Extraction of frozen slices, isolation and assay of cyclic 3′,5′-AMP in tissues and medium, and assay of protein have been described (5). In the Dowex 50 step of the isolation procedure, the chromatographic fraction preceding cyclic 3′,5′-AMP contained ATP and ADP. In the radiochemical incorporation experiments summarized in Table 6, the specific activity of slice ATP + ADP was estimated by measuring the ultraviolet absorption and the radioactivity of this fraction. In these experiments the specific activity of cyclic 3′,5′-AMP was determined after isolation on Dowex 50, followed by reisolation by descending chromatography on Whatman No. 41 paper, using 1-butanol–formic acid–water (77:10:13, by volume). This paper chromatography system was also used to separate adenosine from nucleotides present in incubation media after prior adsorption and elution of the purine derivatives from a charcoal-Celite mixture (9). The paper system also resolved adenosine and adenine, the latter having a higher R_F. The identity of the adenosine spot was confirmed with adenosine deaminase after elution from the paper.

In another radiochemical incorporation experiment (Table 7), ATP was purified by adsorption and elution from charcoal-Celite, followed by adsorption onto a 20 × 7 mm column of Ecteola cellulose. AMP and ADP were eluted with 10 ml of 0.01 N HCl, and the ATP was then eluted with 3 ml of 0.1 N HCl. Nucleotidase activity was estimated by the liberation of P_i, which was then assayed colorimetrically (10).

Nucleotides, purines and their derivatives, and phosphocreatine were purchased from Nutritional Biochemicals Corporation, Sigma, Calbiochem, and Mann Research Laboratories. *N*-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid buffer was purchased from Calbiochem, and enzymes, from Sigma. 8-Bromoadenosine was purchased from Aldrich Chemical Company. Adenosine 5′-sulfate was purchased from Alfred Bader Chemicals as the Ba^+ salt. This was converted to the sodium salt and freed of any possible adenosine impurity by passing it through a column of Dowex 50-Na^+ at pH 2.4. DPN^+ was purified before use by chromatography on DEAE-cellulose. The carbocyclic analogue of adenosine in which the ring oxygen of the ribose moiety is replaced by a carbon atom was kindly supplied by Dr. Y. Fulmer Shealy, Southern Research Institute, Montgomery, Alabama. Adenosine-8-^{14}C was purchased from Calbiochem. Adenosine-5′-^{32}P and 8-^{14}C-5′-AMP were purchased from International Chemical and Nuclear Corporation. 8-^{14}C-Labeled 2′- and 3′-AMP were purchased from Schwarz BioResearch. The ^{14}C-nucleotides were repurified by adsorption and elution from Ecteola cellulose. Ion exchange resins and cellulose were obtained from Bio-Rad Lab-

oratories. The Norit SG Extra brand charcoal used in these experiments had been obtained in 1957; material obtained more recently was unsatisfactory because of poor recovery of nucleotides upon elution with aqueous pyridine.

RESULTS

The previously observed effects of brain extracts applied to slices of guinea pig cerebral cortex (8) were reproduced with similar extracts obtained from guinea pig liver, kidney, and skeletal muscle. Evidently acid-soluble constituents common to all tissues could produce a 20–30-fold increase in brain slice content of cyclic 3′,5′-AMP. The major portion of these effects could be accounted for by the content of adenosine and adenine nucleotides in the extracts. Further work with extracts was therefore suspended

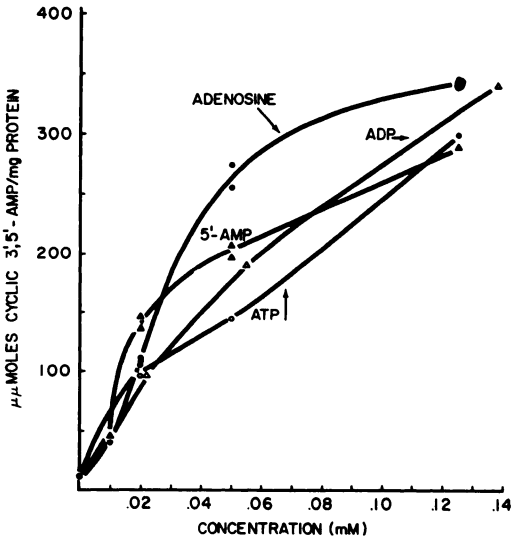

FIG. 1. *Cyclic 3′,5′-AMP content of cerebral cortex slices: dose-response curves for adenosine and adenosine 5′-mononucleotides*

Portions of slices (50–150 mg) held in quick transfer holders were incubated for 50 min at 37° in 40 ml of medium continuously gassed with 95% O_2–5% CO_2 in open 100-ml beakers, then transferred to similar beakers containing the same buffer (controls) or buffer plus added adenine ribose compound for an additional 5 min. Duplicate control values, shown as a single point on the vertical axis, were 9.0 and 9.3 μμmoles/mg of protein. The ADP used here contained 10% 5′-AMP as an impurity.

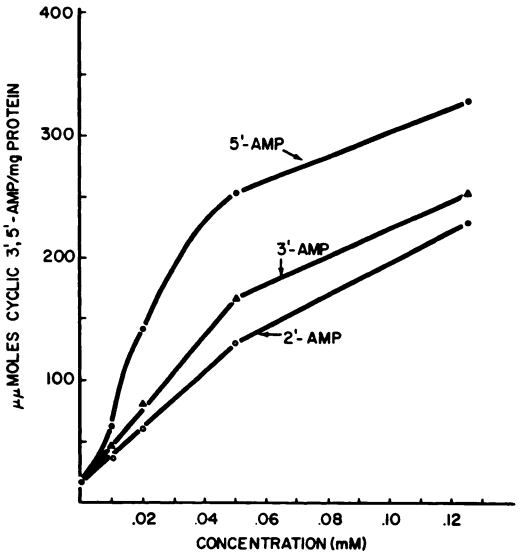

FIG. 2. *Cyclic 3′,5′-AMP content of cerebral cortex slices: dose-response curves for isomers of adenosine monophosphate*

The control value, shown as a point on the vertical axis, was 16.4 μμmoles/mg of protein. Conditions of incubation were the same as in Fig. 1.

in order to explore the effects of adenine derivatives on the content of cyclic 3′,5′-AMP in brain slices.

Kinetic studies. Large increases in the content of cyclic 3′,5′-AMP were found in slices of guinea pig cerebral cortex following exposure to various concentrations of adenosine and adenine nucleotides (Figs. 1 and 2). The linear scale of concentration was used here to avoid compression of the data and to display the nearly proportionate responses to some of the dose increments. The existence of real differences in relative potency among the adenosine derivatives tested may not be concluded from the data of Fig. 1 because of variability within and between experiments. However, the data of Fig. 2 did suggest differences in relative potency among the three isomers of adenosine monophosphate. With higher medium concentrations of adenosine, the curve relating adenosine concentration to slice content of cyclic 3′,5′-AMP was bell-shaped (Fig. 3). The maximum content of cyclic 3′,5′-AMP was found with 0.1–1.0 mM adenosine; 0.03 mM adenosine gave approximately a half-maximal effect.

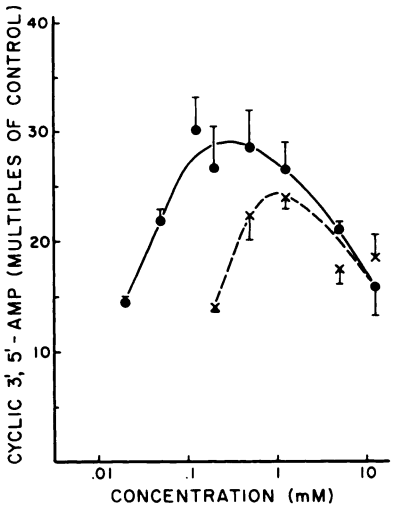

FIG. 3. *Cyclic 3',5'-AMP content of cerebral cortex slices: log dose-response curve to adenosine, showing surmountability of theophylline inhibition of effect of adenosine by higher concentrations of adenosine*

Cerebral cortex slices were incubated for 5 min with 0.02–12.5 mM adenosine (●——●) or adenosine + 0.5 mM theophylline (×- - -×). Points and bars represent means ± standard errors of data from three separate incubations. Control values ranged from 7.8 to 9.7 μμmoles of cyclic 3',5'-AMP per milligram of protein. Conditions of incubation were otherwise the same as in Fig. 1.

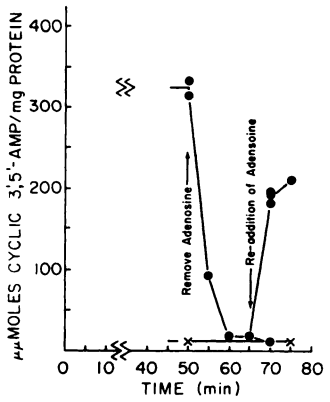

FIG. 4. *Cyclic 3',5'-AMP content of cerebral cortex slices: effect of removal and readdition of adenosine*

Without prior incubation, slices were incubated with 0.125 mM adenosine for 50 min, then rinsed and incubated in adenosine-free medium. At 65 min, 0.125 mM adenosine was added back to some samples. ×——× indicates control slices. Conditions of incubation were otherwise the same as in Fig. 1.

The 5-min incubation time used in most of these experiments was chosen to minimize metabolic alteration of the added compound. Later observations suggested that maximal levels of cyclic 3',5'-AMP may be found only after 10 min of incubation with adenosine or adenine nucleotides, and that the increment from 5 to 10 min (10–80%) varied with the compound added and its concentration. With 0.02 and 0.125 mM adenosine, maximal levels of cyclic 3',5'-AMP were maintained for 20–40 min. Slices incubated with adenosine for 50 min contained large amounts of the cyclic nucleotide. When adenosine was removed by rinsing following such incubation, the cyclic 3',5'-AMP content was reduced almost to the control levels in 10 min (Fig. 4). Subsequent addition of adenosine could again increase the level of cyclic 3',5'-AMP.

Structure-activity studies. The specificity of the response of slices to adenosine was further characterized by survey studies of structure-activity relationships and by blockade experiments. Structure-activity data are presented in Tables 1 and 2. Of the compounds tested, substitutions in either the adenine or the ribose moiety appeared to lower or nullify the response, and adenine itself and nucleosides other than adenosine appeared to be inactive. The two experiments of Table 1 suggested either that 2-deoxyadenosine was slightly active at higher doses or that the preparation used contained 1–2% adenosine as a contaminant. In separate experiments, ADP-ribose at 0.125 mM and DPN at 0.16 mM both appeared to increase levels of cyclic 3',5'-AMP by no more than 2–3-fold. In another experiment, adenosine 5'-sulfate at 0.125 mM produced changes of no more than 25%, but did not interfere with the effects of 0.02 mM adenosine or 5'-AMP. Poly A, the nucleic acid polymer of adenosine, was inactive (Table 2, experiment 3). In a separate experiment, 1.4 mg of yeast RNA per milliliter were also inactive.

Effects of methylxanthines. In the initial studies with rabbit brain slices prepared in the cold, methylxanthines potentiated hor-

TABLE 1

Effect of nucleosides on cyclic 3′,5′-AMP content of cerebral cortex slices

Groups of three quick transfer holders, each containing 50–150-mg portions of slices, were incubated for 50 min at 37° in 25 ml of medium and then transferred to individual vessels containing the substance to be tested in 4 ml and incubated for an additional 5 min. In experiment 2 the preliminary incubation and incubation volumes were 40 ml for each portion. All media were continuously gassed with 95% O_2–5% CO_2 in an open system.

Addition	Concentration	Cyclic 3′,5′-AMP
	mM	*μμmoles/mg protein*
Experiment 1		
None		11
Adenosine	0.05	300
Adenine	0.3	11
Inosine	0.1	11
Guanosine	0.1	10
Cytidine	0.1	14
Uridine	0.1	14
TPN	0.1	14
2-Deoxyadenosine	0.1	13
Experiment 2		
None		6
Adenosine	0.05	129
2-Deoxyadenosine	0.5	25

mone-induced accumulation of cyclic 3′,5′-AMP (5, 6), presumably by inhibition of cyclic 3′,5′-nucleotide phosphodiesterase (4). However, when slices of guinea pig cerebral cortex prepared at room temperature were used, the content of cyclic 3′,5′-AMP was found not to be altered by methylxanthines in either the presence or absence of the hormonal agents (7). The latter conditions correspond to those used here. Methylxanthines were tested for their effect on the adenosine-induced accumulation of cyclic 3′,5′-AMP (Table 3 and legend to Table 6). Not only was there no potentiation of the adenosine effect, but the effect of adenosine was blocked. Theophylline appeared to be more effective than caffeine (Table 3). The inhibition of the adenosine response by 0.5 mM theophylline could be surmounted by increasing the concentration of adenosine (Fig. 3). In other experiments, adenine, inosine, hypoxanthine, and uric acid at 0.5

TABLE 2

Effect of nucleoside derivatives on cyclic 3′,5′-AMP content of cerebral cortex slices

Conditions of incubation were the same as in Table 1, experiment 2.

Addition	Concentration	Cyclic 3′,5′-AMP
	mM	*μμmoles/mg protein*
Experiment 1		
None		5
Adenosine	0.05	83
Adenosine 5′-sulfate	0.05	11
Adenosine 5′-sulfate	0.125	10
DPN	0.0835	15
8-Bromoadenosine	0.5	18
Experiment 2		
None		10
Adenosine	0.05	230
C-Ado[a]	0.05	17
Experiment 3		
None		10
Adenosine	0.125	244
Adenosine N^1-oxide	0.125	127
Poly A	0.125 (in P)	7

[a] Carbocyclic analogue of adenosine in which the ring oxygen of the ribose moiety is replaced by a carbon atom.

TABLE 3

Blockade of adenosine effect by methylxanthines

Conditions of incubation were the same as in Table 1, experiment 1. In a separate experiment, 0.5 mM theophylline by itself had no effect on the cyclic 3′,5′-AMP level.

Additions	Cyclic 3′,5′-AMP
	μμmoles/mg protein
None	12
Adenosine, 0.05 mM	177
Adenosine, 0.05 mM, + theophylline, 0.05 mM	120
Adenosine, 0.05 mM, + theophylline, 0.1 mM	44
Adenosine, 0.05 mM, + caffeine, 0.05 mM	146
Adenosine, 0.05 mM, + caffeine, 0.1 mM	109
Adenosine, 0.05 mM, + caffeine, 0.5 mM	34

TABLE 4
Effect of combined addition of hormone and adenosine

Conditions of incubation were the same as in Table 1, experiment 1.

Adenosine	Hormone	Cyclic 3′,5′-AMP
mM		*μμmoles/mg protein*
0	None	11
0.01	None	50
0.05	None	300
0.125	None	296
0	Histamine, 0.1 mM	80
0.01	Histamine, 0.1 mM	375
0.125	Histamine, 0.1 mM	806
0	Norepinephrine, 0.1 mM	18
0.01	Norepinephrine, 0.1 mM	148
0.125	Norepinephrine, 0.1 mM	603

mM appeared unable to reduce the response of slices to 0.05 mM adenosine.

Effects of biogenic amines. The combined addition of histamine or norepinephrine with adenosine increased cyclic 3′,5′-AMP levels to a greater degree than the sum of the increases observed when these agents were applied singly (Table 4). The potentiation was apparent in relative as well as absolute terms when 0.01 mM adenosine was added together with 0.1 mM norepinephrine. While norepinephrine alone produced less than a 2-fold increase over the control and adenosine alone produced less than a 5-fold increase, their combined addition produced an increase greater than 13-fold. The doses of histamine and norepinephrine used were considered to be maximal, and the levels of cyclic 3′,5′-AMP achieved were consistent with those previously observed in guinea pig cerebral cortex (7).

Analysis of adenosine derivatives in medium. Since medium adherent to slices might have affected the analytical results, it was necessary to exclude the possibility of appreciable variation in the cyclic 3′,5′-AMP content of the incubation medium. After 10 min of exposure to 4 ml of medium without and with 0.05 mM adenosine, the respective amounts of cyclic 3′,5′-AMP in the medium were 3 and 6 μμmoles/mg of slice protein. Incubations with 5′-AMP and ATP yielded similar results. These observations were consistent with data obtained with other tissues, which suggested that adenyl cyclase is associated with a fraction containing membranes and that synthesis of cyclic 3′,5′-AMP is entirely intracellular (11, 3).

Following incubation of slices with adenine nucleotides, adenosine could be detected in the medium. In the experiment summarized

TABLE 5
Accumulation of adenosine in incubation media

After 50 min of preliminary incubation, four portions of slices were transferred to fresh media and incubated for 5 min. The slices were then transferred to fresh media containing the indicated nucleotides at 0.125 mM, and 5 μmoles of the indicated nucleotides were added to the previous incubation media. In all cases the incubation volume was 40 ml. After 5 min of additional incubation, the slices were removed and homogenized, and all media were acidified and passed through charcoal-Celite columns. The columns were eluted with aqueous pyridine, and the pyridine was removed by repeated evaporation in the presence of NH_3. The solutions were passed through columns of Dowex 1-chloride at neutral pH. The columns were washed with water, and the ultraviolet spectra of the combined eluates were measured. The optical densities of samples derived from media to which nucleotides had not been added were subtracted, and concentrations were calculated by using 15.4 as the millimolar extinction coefficient at 260 mμ. The protein content of the homogenized slices was determined, and the weight of tissue incubated was calculated on the basis of 120 mg of protein per gram.

Conditions	Weight of slices	Net adenosine recovered	
		Per ml of medium	Per g of tissue
	g	*mμmoles*	
Incubated after removal of slices			
2′-AMP	0.18	0.53	115
3′-AMP	0.21	1.1	206
5′-AMP	0.17	0.91[a]	210
Incubated with slices			
2′-AMP	0.18	0.89	193
3′-AMP	0.21	1.2	234
5′-AMP	0.17	5.2[b]	1210

[a] The A_{250}:A_{260} and A_{280}:A_{260} of this sample were 0.78 and 0.16, respectively.

[b] The A_{250}:A_{260} and A_{280}:A_{760} were 0.80 and 0.15, respectively.

in Table 5, approximately 5 times as much adenosine was recovered from the medium in which slices had been incubated with 5′-AMP as from media containing either 2′-AMP or 3′-AMP. As judged from the ultraviolet spectra, neither inosine nor hypoxanthine was recovered in appreciable quantities. Paper chromatography confirmed the presence of adenosine and failed to reveal the presence of adenine. Although other experiments indicated that the recovery of adenosine by these procedures was in excess of 90%, the recovery of adenine has not been investigated. In this experiment, the adenosine concentration in the medium did not appear to reach much more than 0.005 mM after incubation of slices for 5 min with 0.125 mM 5′-AMP. It is of interest that the rate of adenosine formation from 2′-AMP and 3′-AMP after removal of the slices was nearly as great as in the presence of slices and was presumably due to enzymes that had leached out of the damaged cells.

The greater rate of 5′-AMP metabolism compared to that of 2′-AMP and 3′-AMP was also seen in homogenates of guinea pig cerebral cortex. In an experiment measuring the appearance of inorganic phosphate, 0.2 ml of a 10% (w/v) homogenate was incubated with 2.5 μmoles of nucleotide, 2.7 μmoles of $MgSO_4$, and 11 μmoles of *N*-tris(hydroxymethyl)methyl - 2 - amino - ethanesulfonic acid buffer at pH 7.4 in a final volume of 1.1 ml. After 15 min of incubation at 37°, the level of inorganic phosphate found was as follows (micromoles per gram of tissue): 2′-AMP, 3.4; 3′-AMP, 1.7; 5′-AMP, 34.7. A requirement for added Mg^{++} was noted with all three substrates.

Mechanisms of action. Two types of studies were carried out in an attempt to define the mechanism of action of the adenine ribose compounds: studies of penetration and incorporation into slice nucleotides and studies of the effect of adenosine on enzymes in cerebral cortex homogenates.

In view of the high potency of adenosine and the low extracellular accumulation of cyclic 3′,5′-AMP, it seems unlikely that adenine nucleotides added to incubation media were directly converted to the cyclic nucleotide. This conclusion is supported by data showing that 5′-AM^{32}P in the medium was poorly incorporated into either cyclic 3′,5′-AMP or the (ATP + ADP) fraction of slices (Table 6, experiment 2). By contrast, radioactivity from adenosine-8-^{14}C in in the medium was readily incorporated into these substances (Table 6, experiment 1). Theophylline did not lessen this incorporation, although it severely reduced the effect of adenosine on the accumulation of cyclic 3′,5′-AMP. The observation that the specific activity of the cyclic nucleotide is at least twice that of ATP has been confirmed repeatedly in experiments using both adenine-8-^{14}C and adenine-^{3}H, even under conditions in which the pool size of cyclic 3′,5′-AMP was not changing. This suggests

TABLE 6

Incorporation of labeled adenine ribose compounds into ATP + ADP and cyclic 3′,5′-AMP of cerebral cortex slices

Conditions were the same as in Table 1, experiment 1. Incubation time was 5 min. Final concentrations of substances added to the medium were: adenosine, 0.05 mM; theophylline, 0.5 mM; 5′-AMP, 0.125 mM. Where indicated, theophylline was added to the medium. Nucleotides were isolated from slice extracts on Dowex 50, followed by paper chromatography. Further isolation of ^{32}P-nucleotides was required to remove impurities. Samples of slices in experiment 1, incubated in parallel with those shown but using unlabeled adenosine, gave the following values for cyclic 3′,5′-AMP (μμmoles per milligram of protein): control, 9; theophylline, 6; adenosine, 153; adenosine + theophylline, 12. All values are the averages of duplicate portions of slices, except for the one shown in parentheses.

Components analyzed	Specific activity
	cpm/mμmole
Experiment 1	
Medium adenosine-8-^{14}C	2796
Slice ATP + ADP	159
Slice ATP + ADP (+ theophylline)	218
Slice cyclic 3′,5′-AMP	457
Slice cyclic 3′,5′-AMP (+ theophylline)	(590)
Experiment 2	
Medium 5′-AM^{32}P	4897
Slice ATP + ADP	6
Slice cyclic 3′,5′-AMP	31

TABLE 7

Incorporation of labeled adenine ribose compounds into ATP of cerebral cortex slices

Conditions were the same as in Table 1, experiment 1, except for the additional incubation time, which was 10 min. In previous incubations, the chosen concentrations of adenine-8-^{14}C ribose compounds gave tissue levels of 40–60 $\mu\mu$moles of cyclic 3′,5′-AMP per milligram of protein.

^{14}C-Compound added	Concentration	Specific activity		B/A
		Medium (A)	Slice ATP (B)	
	mM	*cpm/mμmole*		
Adenosine	0.01	27,470	768	0.028
5′-AMP	0.01	38,165	700	0.018
3′-AMP	0.02	40,287	957	0.024
2′-AMP	0.02	43,100	1,198	0.028

that the cyclic nucleotide is derived from a more highly labeled pool of ATP.

In the light of the foregoing considerations, the adenine moiety of nucleotides would be expected to enter the intracellular space only after removal of any phosphate groups. Thus the rate of incorporation of ^{14}C-labeled nucleotides into ATP might be determined by the rate of dephosphorylation in the vicinity of the cell membrane. Table 7 summarizes the data from an experiment comparing the specific activity of ATP extracted from slices incubated for 10 min with 8-^{14}C-labeled adenosine, 2′-AMP, 3′-AMP, and 5′-AMP. The concentration of each compound used was that which usually produced about a 5-fold increase in cyclic 3′,5′-AMP content. The specific activities achieved in each case were remarkably similar, especially in view of the large differences in the the ability of slices or homogenates to dephosphorylate the various adenosine monophosphate compounds noted above.

The potentiation of hormone-induced accumulation of cyclic 3′,5′-AMP by adenosine suggested inhibition of the cyclic 3′,5′-nucleotide phosphodiesterase as one possible explanation for this effect. This enzyme has been found in both supernatant and particulate fractions of brain tissue (4). Such fractions were prepared and were incubated with low concentrations of cyclic 3′,5′-AMP in order to simulate levels in slices (Table 8). The effect of adenosine was compared with that of theophylline, a known inhibitor of this phosphodiesterase (4). Adenosine appeared to inhibit the activity of both preparations, but to a lesser degree than theophylline.

A stimulatory effect of adenosine on adenyl cylase was another possible explanation of the adenosine-induced accumulation of cyclic 3′,5′-AMP. This possibility was explored with a particulate fraction of guinea pig cerebral cortex tissue (3), employing phosphocreatine and creatine phosphokinase to maintain the concentration of low substrate levels of ATP (Table 9). However, the addition of 0.05 mM adenosine produced no consistent alteration in the accumulation of cyclic 3′,5′-AMP. In the same experiment, the addition of 0.1 mM histamine was also without effect (values not shown). ATP estimations revealed that substrate levels of 0.01 mM ATP were not significantly altered after 6 min by 1.0 ml of enzyme preparation, with other conditions as described in

TABLE 8

Cyclic 3′,5′-nucleotide phosphodiesterase activity of subcellular fractions of guinea pig cerebral cortex

The fractions were obtained at 0–4° from a 10% homogenate in 0.32 M sucrose. After a nuclear fraction had been removed by centrifugation at 6500 × *g* for 15 min, the particulate fraction was deposited between 10,000 × *g* for 15 min and 27,000 × *g* for 60 min. Incubations were conducted for 15 min at 30°, using Tris buffer (pH 7.8), 2 mM Mg^{++}, and 3–5 mg of original tissue equivalent in a final volume of 2.0 ml. The reaction was stopped by boiling the tubes, and cyclic 3′,5′-AMP was assayed directly after removal of a precipitate. The initial concentration of cyclic 3′,5′-AMP averaged $5.47 \pm 0.07 \times 10^{-6}$ M.

Addition	Disappearance of cyclic 3′,5′-AMP	Inhibition
	mμmoles/ml	%
Supernatant fraction		
Control	2.78	
Adenosine, 0.05 mM	2.72	2
Adenosine, 0.5 mM	2.06	26
Theophylline, 0.05 mM	2.08	25
Theophylline, 0.5 mM	1.41	49
Particulate fraction		
Control	1.91	
Adenosine, 0.05 mM	1.59	17
Theophylline, 0.05 mM	1.11	42

TABLE 9

Effect of adenosine on adenyl cyclase activity of guinea pig cerebral cortex

The enzyme was a freshly prepared particulate fraction (3). Incubation was carried out in 25 × 100 mm glass tubes in 10 ml containing 40 mM Tris (pH 7.4), 3 mM $MgSO_4$, 4 mM phosphocreatine, 0.16 mg of creatine phosphokinase, 0.012 mg of myokinase, and 0.4 mg of bovine plasma albumin. Variations in adenosine and ATP additions are shown. Sodium fluoride and caffeine were not used. The reaction was started by adding enzyme to tubes, which were then transferred from ice to a 30° bath for stated times. The reaction was stopped with 0.4 ml of 70% perchloric acid. Cyclic 3′,5′-AMP was determined (see METHODS AND MATERIALS) after the centrifuged extracts had been adsorbed and eluted from charcoal-Celite.

ATP	Enzyme	Adenosine, 0.05 mM	Cyclic 3′,5′-AMP	
			3 min	6 min
mM	*ml*		*μμmoles*	
0	1.0	−		20
0.01	0	−		8
0.01	0.5	−	173	269 ± 61
0.01	0.5	+	203	277 ± 24
0.01	1.0	−	450	478 ± 25
0.01	1.0	+	422	454 ± 41
0.01	2.0	−	676	879 ± 55
0.01	2.0	+	701	908 ± 3
0.1	1.0	−	1520	1640
0.1	1.0	+	810	1370
2.0	1.0	−	1810	2290
2.0	1.0	+	1530	2250

Table 9, in either the presence or absence of 0.05 mM adenosine. Therefore the preparation could not have contained enough adenosine kinase activity to alter the ATP concentration. The adenosine deaminase activity of a similar preparation of adenyl cyclase was estimated by adding 0.2 ml of enzyme preparation to 1 ml of a 0.05 mM adenosine solution. After 5 and 10 min of incubation at pH 7.4 and 25°, there was no progressive lowering of optical density at 260 mμ. Thus adenosine was probably not deaminated to any significant degree during the adenyl cyclase incubations.

DISCUSSION

Adenosine and the adenine mononucleotides may now be added to the list of substances capable of altering the cyclic 3′,5′-AMP content of tissues. At present, this action is established only for slices of guinea pig cerebral cortex. However, preliminary experiments have indicated that adenosine induces large increases in the cyclic 3′,5′-AMP content of guinea pig cerebellar slices. The application of adenosine *in vitro* to other tissues that respond to hormones by altering their content of cyclic 3′,5′-AMP has thus far given negative results. Adenosine at 0.1 mM, with or without added thyroid-stimulating hormone, produced no change in the cyclic nucleotide content of beef thyroid slices, and equivalent doses of 5′-AMP and ATP were also without effect (12). Adenosine with or without added epinephrine produced no change in the cyclic nucleotide content of intact rat diaphragm *in vitro*.[1] However, since adenosine and adenine nucleotides have been observed to mimic ACTH in producing steroidogenesis in cultured adrenal tumor cells (13), it is possible that adenine ribose compounds can cause increased accumulation of cyclic 3′,5′-AMP in such cells.

The present data do not permit definite conclusions regarding three related questions. First, what are the molecular species capable of producing increases in cyclic 3′,5′-AMP? Second, where is the site of action of active compounds (i.e., do they interact with extracellular or intracellular structures)? Third, what is the mechanism of action (e.g., is the rate of synthesis or the rate of metabolism of cyclic 3′,5′-AMP being influenced)? Although active substances were confined to a limited number of compounds containing the adenosine moiety, it cannot be decided as yet whether any of the phosphorylated compounds themselves possessed activity. The data of Table 7 suggest that activity of adenine nucleotides could be the result of conversion to adenosine in the vicinity of the cell membrane. Conversion to adenosine might also explain the activity of adenosine N^1-oxide (14). It is of considerable import-

[1] J. W. Craig and T. W. Rall, unpublished observations.

ance to determine whether adenine nucleotides are active in themselves. If they were active, it could be concluded that interaction with extracellular structures was involved. This in turn would tend to eliminate a number of possible mechanisms of action, such as inhibition of the degradation of cyclic 3′,5′-AMP. Measurement of the disappearance (Table 8) and the accumulation of cyclic 3′,5′-AMP (Table 9) using broken cell preparations gave inconclusive results. In these experiments adenosine was less effective than theophylline in slowing the disappearance of the cyclic nucleotide. This finding is in agreement with a study of cyclic 3′,5′-nucleotide phosphodiesterase inhibition in adipose tissue (15). It is doubtful that the small degree of inhibition observed could have accounted for the large changes in tissue content of cyclic 3′,5′-AMP found. On the other hand, the failure to observe an adenosine-induced increment in cyclic 3′,5′-AMP accumulation in homogenates does not rule out a possible regulatory effect of adenosine on adenyl cyclase activity in slices. Inasmuch as histamine was also without effect, such regulatory influence might require a degree of preservation of cell membrane structure not found in the broken cell preparation used.

Methylxanthines have now been shown to possess a new pharmacological property. They are capable of blocking that action of adenosine which alters the cyclic 3′,5′-AMP content of cerebral cortex slices. Preliminary results with purines and other xanthines suggest that the effect may be specific for methylxanthines. On the other hand, the ability of methylxanthines to inhibit the cyclic 3′,5′-nucleotide phosphodiesterase does not appear to be relevant to the accumulation of cyclic 3′,5′-AMP in slices of guinea pig cerebral cortex, as evidenced by the inertness of methylxanthines in both the presence and absence of hormonal agents (7). Preliminary experiments with slices of rabbit cerebral cortex have indicated that chilling of the tissue prior to incubation reduces the response to histamine in the absence of theophylline, while in slices prepared at room temperature histamine produces the same large increment in cyclic 3′,5′-AMP in the absence or presence of theophylline. This might provide an explanation for earlier observations (5, 6). It is possible that in the brain *in situ* a methylxanthine-sensitive phosphodiesterase does not function in the disposition of cyclic 3′,5′-AMP. Current experiments involving the study of the turnover of cyclic 3′,5′-AMP under various conditions may provide more insight into this problem.

Theophylline has been shown to prevent most of the augmenting effect of 5–10 min of electrical stimulation on the level of cyclic 3′,5′-AMP in guinea pig cortex slices (7). The present observations suggest that the increase in cyclic 3′,5′-AMP during electrical stimulation of such slices is mediated by endogenous adenine ribose compounds. Large amounts of adenine nucleotides are present in incubated brain slices (16). In the present experiments, the tissue ATP content, 1 μmole/g,[2] appears to be 100 times the concentration needed to produce a 5-fold increase in cyclic 3′,5′-AMP when applied externally. Therefore, changes in the tissue compartmentation of adenine nucleotides probably play a role in the effects of electrical stimulation. Two possible means of translocating adenine ribose compounds can be visualized. The first possibility may be associated with metabolic alteration of ATP. For example, ATP is known to be dephosphorylated during electrical stimulation of slices *in vitro* (17) and *in vivo* following an electrically induced seizure (18) or decapitation (19). In the latter case, adenosine accumulation was shown to occur. Thus, dephosphorylation of ATP to adenosine would produce a substance capable of escaping from the cell by diffusion. The second possibility is that ATP itself might be released from certain nerve endings at synaptic sites. This would be analogous to the release of ATP from the adrenal medulla in proportion to the release of catecholamines (20). The same ratio of 4 catecholamine molecules to 1 of ATP has been found in the analysis of granules isolated from the adrenal medulla (21) or from the splenic nerve (22). If an analogous situation exists in norepinephrine-containing nerve endings in the central ner-

[2] A. Sattin and T. W. Rall, unpublished observations.

vous system, ATP might be released at synaptic sites in an effective concentration along with the catecholamine. It is also conceivable that ATP is released from nerve endings together with other biogenic amines, such as serotonin or histamine, or with as yet unidentified transmitter substances (23).

If this view proves to be correct, the potentiation of the effects of histamine and norepinephrine by adenine ribose compounds reported here could have considerable physiological significance. One possibility that arises is that the "neurotransmitter" at central adrenergic synapses is in fact several substances, and that the postjunctional events at such synapses represent a composite response. This added complexity could become crucial in deciphering central nervous system pharmacology. For example, the mild central stimulation produced by the methylxanthines might be due (as indicated by the antagonism reported here between theophylline or caffeine and adenosine) to the diminished effectiveness of any ATP being released at various synapses. This interpretation of methylxanthine action would seem to force the formulation of a hypothesis in which elevated levels of cyclic 3′,5′-AMP in some way cause "inhibition" or "depression" in the central nervous system. However, it would be most unfortunate to become trapped by an oversimplified view of the functional role of cyclic 3′,5′-AMP in the central nervous system at this early stage of investigation. In view of both the diverse functions of cyclic 3′,5′-AMP in various other tissues and the cellular inhomogeneity within the central nervous system, it might be a mistake to presume at the outset that elevation of intracellular cyclic 3′,5′-AMP will produce similar effects in all cells within the nervous system.

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