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The relation of adenyl cyclase to the activity of other ATP utilizing enzymes and phosphodiesterase in preparations of rat brain; mechanism of stimulation of cyclic AMP accumulation by NaF

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Summary

1. A method for measuring the rate of production of ^{14}C-labelled adenine nucleotides, including cyclic adenosine 3′,5′monophosphate (cyclic AMP), from [^{14}C]-adenosinetriphosphate (ATP) was developed and used to study the effects of ATP, adenosine diphosphate (ADP) and adenosine 5′-monophosphate (AMP) on the rate of accumulation of cyclic AMP in cell-free preparations of adenyl cyclase from rat brain.

2. The mechanism by which NaF increases cyclic AMP accumulation was studied by comparing its effect on adenine nucleotide metabolism with that of an ATP regenerating system.

3. ADP and ATP are potent inhibitors of phosphodiesterase (PDE) and it is the sum of the concentrations of these two nucleotides which controlled the rate of destruction of cyclic AMP. The effect of these nucleotides was significant even in the presence of 6·7 mM theophylline; theophylline itself inhibited PDE only 50–60%.

4. Fluoride ion had no direct effect on PDE but it inhibited the rate of hydrolysis of ADP and ATP and thus indirectly inhibited PDE. The effect of fluoride ion on cyclic AMP accumulation can be explained, at least in part, by this indirect inhibition of PDE.

5. Studies on a more purified preparation of adenyl cyclase clearly demonstrated a direct action of NaF on adenyl cyclase.

Introduction

The activation of adenyl cyclase by hormones and non-specific agents such as fluoride ion has been studied in cell-free preparations of many different tissues (Dousa & Rychlik, 1970; Rodbell, 1967; Pastan & Katzen, 1967; Weiss, 1969). An alteration in cyclic 3′,5′-adenosine monophosphate (cyclic AMP) in these preparations is usually interpreted as a direct effect of these agents on adenyl cyclase. However, these preparations of adenyl cyclase are invariably contaminated with other enzymes, particularly nucleotidases. It is obvious, therefore, that inhibition of enzymes which hydrolyse adenine nucleotides would lead to an increase in cyclic AMP accumulation.

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Fluoride ion increases the rate of accumulation of cyclic AMP in a variety of tissues but the mechanism of action is not known (Sutherland, Rall & Menon, 1962; Streeto & Reddy, 1967; Weiss, 1969). It is considered unlikely that fluoride ion increases cyclic AMP accumulation simply by preserving substrate because the effect of this ion can still be observed when an ATP regenerating system is used. However, in most studies in which an ATP regenerating system was used, it was not established that the ATP concentration was maintained throughout the duration of the experiment (Rabinowitz, Desalles, Meisler & Lorand, 1965; Streeto & Reddy, 1967; Dousa & Rychlik, 1968).

Cyclic nucleotide phosphodiesterase (PDE) activity has been found in almost all cell-free preparations of adenyl cyclase. It is generally believed that fluoride ion does not increase cyclic AMP accumulation by inhibiting PDE because cyclic AMP accumulation is increased by fluoride ion in the presence of theophylline and caffeine, both of which inhibit this enzyme. However, the methyl xanthines only inhibit PDE of brain by about 60% even when present in high concentrations (Cheung, 1966; Streeto & Reddy, 1967). Therefore, the possibility that fluoride ion increases cyclic AMP accumulation in these tissues by an effect on PDE cannot be entirely eliminated. Cheung (1966) showed that polyphosphates, including several nucleotides and inorganic pyrophosphate, are potent inhibitors of purified rat brain PDE; adenosine triphosphate (ATP) and inorganic pyrophosphate were more potent inhibitors than the methyl xanthines. Fluoride ion is known to inhibit a number of phosphatases and it therefore seemed possible that fluoride ion might affect the rate of accumulation of cyclic AMP in brain by allowing the accumulation of PDE inhibitors.

In this study the relation between adenyl cyclase, other membrane-bound ATP utilizing enzymes and PDE was investigated. This was done by the development of a method whereby the rates of production and destruction of ATP, adenosine diphosphate (ADP), adenosine-5'-monophosphate (5'-AMP) and cyclic AMP could be determined in a single experiment. This present work describes such a procedure and shows that fluoride ion increases cyclic AMP accumulation by a direct effect on adenyl cyclase and indirectly by inhibition of PDE activity.

Methods

Preparations of adenyl cyclase

Rat cerebral cortex preparation. Rat cerebral cortex was removed and transferred immediately to the homogenizing medium (2 mM Tris, pH 7·4 and 1·0 mM $MgSO_4$) at 4° C; all subsequent steps in the preparation of adenyl cyclase were done at 4° C. The tissue was minced and homogenized in 4·5 volumes of homogenizing medium and the homogenate was centrifuged at 2,000 *g* for 10 minutes. The supernatant was discarded, the pellet was resuspended in 10 volumes of homogenizing medium and again centrifuged at 2,000 *g* for 10 minutes. This final pellet was suspended in homogenizing medium to give approximately 30 mg protein/ml.

Rat synaptic membrane preparation of adenyl cyclase. A modification of the method of De Robertis, Rodriguez de Lores Arnaiz, Alberici, Butcher & Sutherland (1967) was used. Wistar rats (200 g) were guillotined and the brains removed and placed in homogenizing medium (0·32 M sucrose, 1 mM $MgSO_4$ and 0·05 mM Tris

pH 7·2). The cerebral cortex was dissected away from the remainder of the brain, minced and homogenized in 4·5 volumes of the same medium. The homogenate was diluted with 2 volumes of the same medium and centrifuged at 900 *g* for 10 minutes. The pellet was washed twice by suspending it in 30 ml of the same medium and the suspension was centrifuged at 900 *g* for 10 minutes. The supernatants were pooled and centrifuged at 11,500 *g* for 20 minutes. The pellet obtained was washed once in the same medium and the suspension centrifuged again. This pellet was homogenized in 9 volumes of a hypotonic medium consisting of 1 mM $MgSO_4$, pH 7·2 and the homogenate was centrifuged at 20,000 *g*. The pellet was resuspended in the preparation medium and layered on a discontinuous density gradient consisting of 6 ml of 1·2 M sucrose (density 1·15) and 5 ml each of 1·0 M (density 1·13), 0·9 M (density 1·11), and 0·8 M (density 1·10) sucrose. The gradient was centrifuged at 100,000 *g* for 1 h in a Beckman ultracentrifuge using an s.w. 27 rotor. The material at the top of the 1·2 M sucrose layer was collected and suspended in 30 volumes of a medium containing 1 mM $MgSO_4$ and 2 mM Tris pH 7·4 and centrifuged at 35,000 *g* for 20 minutes. The sediment was washed once by suspension in the same medium and centrifuged again. The resulting sediment was suspended in 40 mM Tris pH 7·4 to give a concentration of approximately 9 mg protein/ml. Electron microscope studies showed this preparation to consist mainly of synaptic membranes and to be free of mitochondria as was previously described by De Robertis *et al.* (1967).

Assay of adenyl cyclase and other membrane-bound ATP utilizing enzymes

In the standard assay the adenyl cyclase preparation was incubated in 2 ml incubation medium containing: 40 mM Tris, pH 7·4; 3·5 mM $MgSO_4$; 6·67 mM theophylline and 2·1 mM [^{14}C]-ATP (0·250 μCi/μmol). When an ATP regenerating system was used it consisted of 10 mM phosphoenol pyruvate and 25 μg/ml pyruvate kinase. In the assay of adenyl cyclase in brain synaptic membrane preparation, 5·0 mM $MgSO_4$ was used instead of 3·5 mM $MgSO_4$. After 5 min preincubation for temperature equilibration, the reaction was started by adding the [^{14}C]-ATP. Unless otherwise stated, the time of incubation was 10 minutes. The incubation was terminated by adding cold 45% trichloroacetic acid (final concentration 7·5%). Tracer amounts of ^{3}H-labelled 5′-AMP, ADP, ATP and cyclic AMP were added to each sample and the samples centrifuged at 4,000 *g*. The supernatant was extracted three times with five volumes of water saturated ether to remove trichloroacetic acid and the aqueous solution was then evaporated to dryness in a Buchler Rotary Evapo-Mix. The residue was dissolved in 0·2 ml of 1·0 M ammonium acetate, pH 4·4 and the adenine nucleotides determined as described below.

Assay of phosphodiesterase activity

The adenyl cyclase preparation was incubated for 1 min at 37° C in 1 ml of a medium containing 40 mM Tris pH 7·5; 3·5 mM $MgSO_4$ and 1 μM [^{14}C]-cyclic AMP (specific activity 0·250 mCi/μmol). After 5 min preincubation the reaction was started by adding the [^{14}C]-cyclic AMP. The reaction was terminated by adding cold 45% trichloroacetic acid (final concentration 7·5%). [^{3}H]-AMP (0·310 μCi) and [^{3}H]-cyclic AMP (0·210 μCi) were then added to the samples, and the samples were mixed, centrifuged at 4,000 *g* and the supernatants extracted with ether and evaporated to dryness as described previously. The PDE activity was determined by measuring [^{14}C]-AMP and [^{14}C]-cyclic AMP present in the sample by the ion-exchange column chromatography method to be described.

Determination of adenine nucleotides

The procedure was based on the separation of each of the adenine nucleotides in pure form by anion-exchange column chromatography with AG 1 X8 resin in the acetate form in a 24 cm×0·5 cm column. The columns were eluted at room temperature at an initial flow rate of 15–18 ml/hour. The nucleotides were eluted by increasing the molar concentration of the eluant, ammonium acetate, at constant pH (4·4). The recovery of the ^{14}C-labelled nucleotides was determined by measuring the recovery of the ^{3}H-labelled nucleotides added to each incubation vessel immediately after the reaction had been terminated. The ratio of $^{3}H/^{14}C$ in the column eluates was a measure of the purity of each nucleotide removed from the column, and only samples with a constant ratio (i.e. less than 5% variation) were used in the final quantitation of each nucleotide.

Preliminary experiments determined the order of elution of ATP, ADP, AMP and cyclic AMP; samples of authentic nucleotides were placed on the column, effluent was collected at 8 min intervals and the radioactivity in 200 μl portions was determined. In Fig. 1, the sequence, molar concentration, and volume of each buffer used are illustrated along with the elution pattern of the nucleotides. The nucleotides were eluted in the following order: 5′-AMP, cyclic AMP, ADP, and ATP. Adenosine was not retained by the column under the conditions used and could be quantitatively eluted, free of the other adenine compounds, with 1·0 M ammonium acetate. This elution pattern was very consistent and the time of appearance of each peak could be accurately predicted. In practice, the column eluates were not collected continuously but 8–10 samples of not greater than 0·35 ml were collected directly into scintillation vials at the time that each nucleotide was expected. Radioactivity in each sample was determined in a Picker 220 liquid scintillation counter by standard methods for double isotope counting with external standardization. The counting data were processed using a PDP 8L computer (Digital Equipment Corp.) which was programmed to compute d/min for both isotopes and

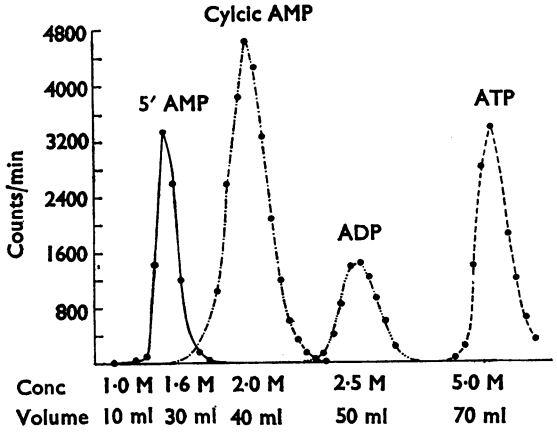

FIG. 1. Elution pattern of AMP, ADP, ATP and cyclic AMP from AG 1-X8 anion-exchange columns using a stepwise concentration gradient of ammonium acetate. Samples of ^{3}H-labelled nucleotides (0·18 μCi–0·32 μCi) were applied to AG 1-X8 columns (prepared as described in **Methods**) and eluted using a stepwise concentration gradient of ammonium acetate (pH 4·4). Effluent was collected at 8 min intervals and the radioactivity of 200 μl portions determined by liquid scintillation counting. The sequence of ammonium acetate concentrations and the volume of each used is shown at the bottom of the elution pattern.

the $^{3}H/^{14}C$ ratio for each sample. The recovery of each nucleotide was corrected to 100% by calculating the recovery of the corresponding ^{3}H-labelled nucleotide, and the quantity of each nucleotide present in each sample was calculated in μmol using the computer.

The accuracy of this method in the determination of cyclic AMP is indicated by Table 1 in which the recovery of varying amounts of cyclic AMP in the presence of a constant excess of 5'-AMP is shown. The amount of [^{14}C]-5'-AMP applied to each column was 1 μmol (specific activity 0·240 μCi/μmol) and the amount of cyclic AMP (at constant specific activity, 0·250 μCi/μmol) was varied. There was little contamination of the cyclic AMP peak by the added AMP.

TABLE 1. *Recovery of known amounts of cyclic AMP in the presence of a large excess of AMP*

Cyclic AMP added (μmol)	Cyclic AMP recovered (μmol)	% Recovery
0·0050	0·0057	114
0·0100	0·0110	110
0·0250	0·0260	104
0·0500	0·0490	98

A known amount of [^{14}C]-cyclic AMP (specific activity: 0·250 μCi/μmol) was applied to the AG 1–X8 anion-exchange columns together with 1 μmol of [^{14}C]-AMP (specific activity: 0·240 μCi/μmol). Cyclic AMP recovery was determined as described in the text.

Protein determination

The biuret method (Kabat & Meyer, 1964) for protein determination was used.

Statistics

Student's *t* test for unpaired data was used to test the significance of the difference between results; two tailed tests were always used. Variation of results is expressed as ±standard error (S.E.).

Materials

AG 1 X8 resin (200–400 mesh) was purchased from Bio-Rad Laboratories; scintillation fluid components from Nuclear-Chicago Corp.; radioactive agents from Schwartz Bioresearch Inc.; ATP and cyclic AMP from Sigma Chemical Co.; theophylline from K and K Laboratories Inc.; phospho (enol) pyruvate kinase, type II from rabbit skeletal muscle, from Sigma Chemical Co.

Results

Time-course of the metabolic fate of ATP in the rat cerebral cortex

Preparation

Figures 2, 3 and 4 show the metabolic fate of ATP incubated with the rat cerebral cortex preparation of adenyl cyclase in the absence of either an ATP regenerating system or NaF (Fig. 2), in the presence of NaF (Fig. 3) and in the presence of the ATP regenerating system (Fig. 4).

In the absence of either NaF or an ATP regenerating system, ATP was rapidly hydrolysed; 95% was destroyed in the first min of incubation and more than 98% in the first 4 minutes. ADP accumulated during the first min of incubation, then

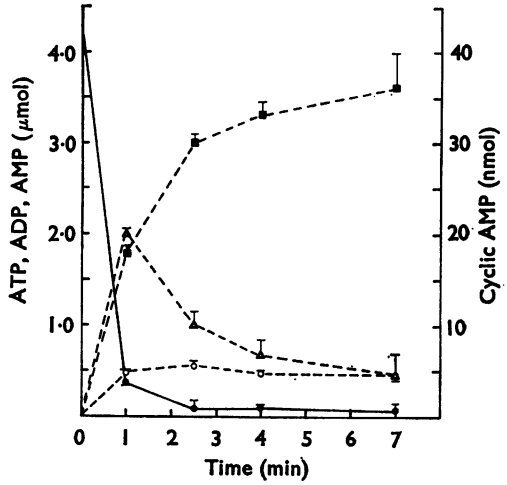

FIG. 2. The metabolic fate of ATP incubated with the rat cerebral cortex preparation of adenyl cyclase. 2,000 *g* particulate preparation of rat cerebral cortex (450 mg wet weight tissue) was incubated at 37° C in a total volume of 2 ml, in a medium containing: 40 mM Tris, pH 7·4; 3·5 mM $MgSO_4$; 6·67 mM theophylline and 2·1 mM [^{14}C]-ATP (0·250 μCi/μmol). The incubation was stopped at the times indicated and the adenine nucleotides determined as described in the text. Results are a mean of 3 observations and vertical bars represent ± S.E. AMP (■----■), ADP (△----△), and ATP (●——●) recovered expressed in μmol and cyclic AMP (○----○) recovered expressed in nmol.

declined rapidly to a concentration of 0·45 μmol after 7 min of incubation. AMP accumulated at a rapid rate; after 2·5 min incubation more than 70% of the added ATP could be accounted for as AMP. Cyclic AMP concentration reached a maximum after 2·5 min (5·0 nmol) and fell slightly through the rest of the incubation period.

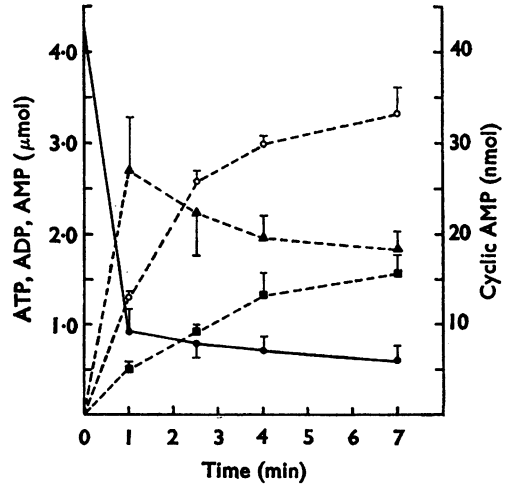

FIG. 3. The metabolic fate of ATP incubated with the rat cerebral cortex preparation of adenyl cyclase in the presence of NaF. 2,000 *g* particulate preparation of rat cerebral cortex (450 mg wet weight tissue) was incubated at 37° C in a total volume of 2 ml, in a medium containing: 40 mM Tris, pH 7·4; 3·5 mM $MgSO_4$; 6·67 mM theophylline; 2·1 mM [^{14}C]-ATP (0·250 μCi/μmol) and 10 mM NaF. The incubation was stopped at the times indicated and the adenine nucleotides determined as described in the text. Results are a mean of 3 observations and vertical bars represent ± S.E. AMP (■----■), ADP (△----△) and ATP (●——●) recovered expressed in μmol and cyclic AMP (○----○) recovered expressed in nmol.

In the presence of NaF (Fig. 3) in the first min of incubation the ATP concentration decreased by 77% to 0·94 μmol. Thereafter the concentration of this nucleotide declined slowly, to 0·72 μmol at 4 min and 0·61 μmol after 7 min of incubation. ADP accumulated rapidly in the first min of incubation and accounted for more than 80% of the ATP that disappeared during this time. The ADP concentration fell slowly for the rest of the incubation period to a concentration of 1·83 μmol at 7 minutes. There was a net accumulation of cyclic AMP throughout the entire incubation period to a maximum of 34·0 nmol after 7 min of incubation. However, the rate of accumulation fell after 2·5 min incubation as illustrated in Figure 5. This reduction in the rate of cyclic AMP accumulation occurred when the ATP concentration fell from 0·80 μmol to 0·60 μmol.

In the presence of an ATP regenerating system (Fig. 4), the rate of disappearance of ATP was clearly slower than it was in the presence of NaF. The concentration of ATP fell by only 25% in the first minute of incubation. From this time, the ATP concentration fell rapidly so that by 4 min it was at a lower concentration than it was in the presence of NaF (0·59 μmol compared to 0·72 μmol) and after 7 min the ATP concentration was similar to that present in the controls (0·07 μmol compared to 0·09 μmol). ADP concentration increased during the first 2·5 min of incubation at a rate that approximately paralleled the rate of disappearance of ATP, it then continued to rise for the next 1·5 min and fell rapidly over the final 3 min of incubation to 1·16 μmol. There was a net accumulation of 11·0 nmol of cyclic AMP during the first 2·5 min of incubation. The rate of accumulation of cyclic AMP fell significantly between 2·5 and 4 min and this occurred when the ATP concentration fell from 1·45 μmol to 0·59 μmol. During the last 3 min of incubation there was a net destruction of cyclic AMP so that its final concentration was 7·0 nmol as compared to 11·7 nmol at the peak of its accumulation. The des-

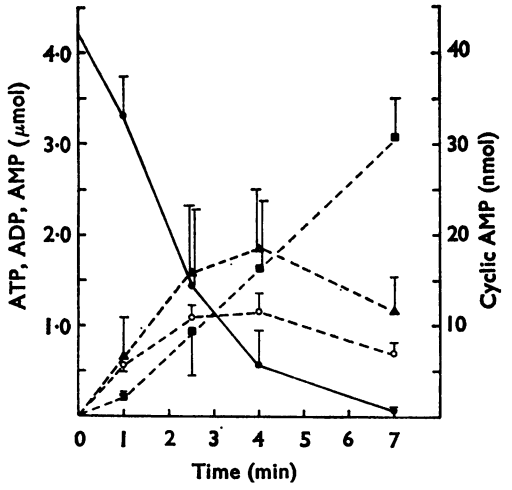

FIG. 4. The metabolic fate of ATP incubated with the rat cerebral cortex preparation of adenyl cyclase in the presence of the ATP regenerating system. 2,000 *g* particulate preparation of rat cerebral cortex (450 mg wet weight tissue) was incubated at 37° C in a total volume of 2 ml, in a medium containing: 40 mM Tris, pH 7·4; 3·5 mM $MgSO_4$; 6·67 mM theophylline; 2·1 mM [^{14}C]-ATP (0·250 μCi/μmol) and the ATP regenerating system consisting of 10 mM phosphoenol pyruvate and 25 μg/ml pyruvate kinase. The incubation was stopped at the times indicated and the adenine nucleotides determined as described in the text. Results are a mean of 3 observations and vertical bars represent ±S.E. AMP (■----■), ADP (△----△) and ATP (●——●) recovered expressed in μmol and cyclic AMP (○----○) recovered expressed in nmol.

truction of this nucleotide coincided with the disappearance of ADP. The rate of accumulation of AMP was slow for the initial minute and then linear for the remainder of the incubation period.

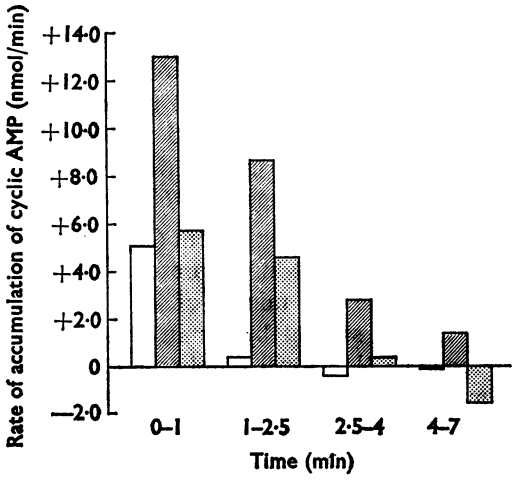

FIG. 5. The effect of NaF and the ATP regenerating system on the rate of accumulation of cyclic AMP in the standard cerebral cortex adenyl cyclase assay. The accumulation of cyclic AMP under the incubation conditions described in the controls (Fig. 2), in the presence of NaF (Fig. 3) and in the presence of the ATP regenerating system (Fig. 4) expressed in nmol/minute. Open columns, controls; hatched columns, NaF; stippled columns, ATP regenerating system.

Phosphodiesterase activity of the rat cerebral cortex preparation of adenyl cyclase

In previous experiments (Katz & Tenenhouse, unpublished observations) we had shown that ATP and ADP were potent inhibitors of a soluble PDE preparation from rat brain and AMP a weak inhibitor. In the present experiments the effects of ADP and ATP in the presence and absence of theophylline were determined in the rat cerebral cortex preparation of adenyl cyclase (Table 2). The concentrations of ADP, ATP and cyclic AMP were chosen to approximate those measured in the adenyl cyclase assay after 30 s incubation in the presence of NaF (in μmol/ml incubation media). The cyclic AMP concentration was 1·0 μM, the ADP concen-

TABLE 2. *Phosphodiesterase (PDE) activity of rat cerebral cortex preparation of adenyl cyclase*

Additions	% cyclic AMP remaining	% Inhibition of PDE
None	14·6±2·3	—
NaF	14·3±2·2	—
ADP+ATP	34·4±3·7*	19·4±4·4
ADP+ATP+NaF	44·2±6·7*	28·5±5·0
Theophylline	58·7±5·9	52·1±7·2 (—)
Theophylline+ADP+ATP	71·2±5·9	67·8±7·2 (30·1)
Theophylline+ADP+ATP+NaF	81·6±3·4†	77·7±2·2‡ (48·9)

*$P<0·01$ compared to controls; †$P<0·05$ compared to theophylline; ‡$P<0·05$ compared to theophylline. 2,000*g* particulate preparation of rat cerebral cortex, prepared as described in Methods (225 mg wet weight tissue) was incubated at 37° C for 1 min in 1 ml medium containing 40 mM Tris, pH 7·5; 1·0 μM [^{14}C]-cyclic AMP (0·250 mCi/μmol) and 3·5 mM $MgSO_4$. The concentrations of the various substances added were: theophylline, 6·7 mM; ADP, 0·5 mM; ATP, 1·0 mM and NaF, 10 mM. Cyclic AMP remaining was determined as described in the text. The numbers in parentheses represent the percentage inhibition of PDE relative to the activity of the enzyme in the presence of theophylline. Results are a mean of 3 experiments±S.E.

tration 0·5 mM, and the ATP concentration 1·0 mM. In the absence of any added ATP, ADP or NaF, 85% of the cyclic AMP was destroyed in the first minute of incubation. The addition of 0·5 mM ADP and 1·0 mM ATP significantly inhibited the destruction of cyclic AMP ($P<0·01$). NaF inhibited cyclic AMP destruction even further, so that only 55% of the cyclic AMP was now destroyed. In the absence of ADP and ATP, NaF had no effect on the rate of cyclic AMP destruction. The standard concentration of theophylline used in the studies of cyclic AMP accumulation in this preparation, 6·7 mM, was shown to inhibit PDE activity approximately 59%. ADP and ATP added together with theophylline inhibited the enzyme by 71% ; the addition of NaF increased the inhibition to 82%. If the rate of destruction of cyclic AMP by the adenyl cyclase preparation in the presence of 6·7 mM theophylline is taken as the basal PDE activity, then the addition of ADP (0·5 mM) and ATP (1·0 mM) inhibited this activity by 30%. The presence of NaF increased this inhibition to 49%. This increase in inhibition is significant ($P<0·05$) compared to the inhibition obtained when theophylline alone was present.

The effect of NaF and an ATP regenerating system on the metabolism of ATP in the synaptic membrane preparation of adenyl cyclase

Table 3 illustrates that the amount of ATP hydrolysed in the controls of this preparation following 10 min incubation was much reduced compared to the rat cerebral cortex preparation. Approximately 43% of ATP remained following the 10 min incubation period with 33% of total nucleotide recovered as ADP and 23% as AMP. The addition of 10 mM NaF to the incubation mixture reduced the rate of ATP hydrolysis but this reduction was not statistically significant. NaF still produced a significant increase in the amount of cyclic AMP accumulated ($P<0·02$). Thus, in contrast with the cerebral cortex preparation, in the synaptic membrane preparation of adenyl cyclase, NaF stimulated cyclic AMP accumulation without having a significant effect on ATP hydrolysis. In the presence of 10 mM NaF and the ATP regenerating system the concentration of ATP had fallen only 2% following the 10 min incubation period. Under these conditions, the concentration of ATP was significantly higher than the other three conditions ($P<0·01$ compared

TABLE 3. *The effect of the ATP regenerating system on the metabolism of ATP in the presence and absence of NaF in the synaptic membrane preparation of adenyl cyclase*

	AMP (μmol/mg protein)	Cyclic AMP (nmol/mg protein)	ADP (μmol/mg protein)	ATP (μmol/mg protein)
Control (5)	0·45±0·04	1·60±0·26	0·69±0·11	0·90±0·17
Control+ATP regenerating system (7)	0·21±0·06[b]	2·10±0·34	0·61±0·14	1·16±0·19
NaF (5)	0·14±0·01[a]	5·60±1·30[c,g]	0·49±0·10	1·40±0·16
NaF+ATP regenerating system (5)	0·04±0·01[a,d,g]	6·60±0·84[c,f]	0·01±0·00[e,a,f]	1·97±0·02[a,e,f]

[a] $P<0·001$ compared to control; [b] $P<0·01$ compared to control; [c] $P<0·02$ compared to control; [d] $P<0·001$ compared to NaF; [e] $P<0·01$ compared to NaF; [f] $P<0·01$ compared to control + ATP regenerating system; [g] $P<0·02$ compared to control + ATP regenerating system. Synaptic membrane fraction of rat cerebral cortex homogenate prepared as described in **Methods** (2·0 mg protein) was incubated for 10 min at 37° C in the presence and absence of NaF, 10 mM, and an ATP regenerating system consisting of phosphoenol pyruvate, 10 mM and pyruvate kinase, 25 μg/ml. The incubation medium of 2 ml volume also contained: 40 mM Tris, pH 7·4; 5·0 mM $MgSO_4$; 6·67 mM theophylline; and 2·1 mM [^{14}C]-ATP (0·250 μCi/μmol). Numbers in parentheses indicate number of experiments per group. Results are expressed as mean ± S.E.

to NaF alone and the ATP regenerating system alone and $P<0{\cdot}001$ compared to the controls) and the amount of AMP recovered was significantly reduced compared to the amount recovered in the controls or in the presence of NaF alone ($P<0{\cdot}001$) and that recovered in the presence of the ATP regenerating system alone ($P<0{\cdot}02$). The amount of cyclic AMP accumulated, however, was not significantly greater than that accumulated in the presence of NaF alone.

Phosphodiesterase activity of the synaptic membrane preparation of adenyl cyclase

In this preparation PDE activity was much reduced; 50% of the cyclic AMP added remained following 10 min of incubation.

Discussion

Cheung (1966, 1967) proposed that ATP, which is of obvious importance as the substrate for adenyl cyclase, might have a second indirect effect on cyclic AMP metabolism. This suggestion was based on the observation that ATP and pyrophosphate, but not AMP, were potent inhibitors of PDE. This inhibition of cyclic AMP hydrolysis could lead to the accumulation of cyclic AMP in conditions where PDE was present and active. The rat cerebral cortex preparation of adenyl cyclase used in the studies reported here was contaminated with PDE and enzymes which rapidly hydrolysed ATP and ADP. The rate of destruction of cyclic AMP in this preparation was rapid even in the presence of 6·67 mM theophylline. ATP and ADP significantly inhibited this PDE activity and although fluoride ion itself did not directly affect the rate of cyclic AMP hydrolysis it augmented the inhibitory effect of the nucleotides. These results confirm the observation of Cheung (1966) that ATP inhibits PDE activity and illustrate that ADP is also a very effective inhibitor of this enzyme (about 80% as effective as ATP). They further illustrate that fluoride ion, presumably by retarding the rate of hydrolysis of ATP and ADP, can indirectly inhibit PDE activity. That NaF significantly reduces the rate of hydrolysis of ATP and ADP by rat cerebral cortex preparations is clearly demonstrated. It was also found that the concentration of ATP and, particularly, ADP is maintained only in the presence of NaF and only under these conditions is there a net accumulation of cyclic AMP after 4 min incubation. These results suggest that the maintenance of adenine nucleotide (ATP and/or ADP) concentration does contribute to the accumulation of cyclic AMP by inhibition of PDE and further suggest that, at least in part, the fluoride ion's effect on cyclic AMP accumulation results from the inhibition of ATP and ADP hydrolysis by this ion. It is impossible from this data to quantitate the importance of this effect of fluoride ion, however, it appears from our studies that the major action of this ion is on cyclic AMP generation. In comparing the effect of NaF and the ATP regenerating system during the first 2·5 min of incubation it was found that even though the concentration of ATP was higher in the presence of the regenerating system and the sum of the concentrations of ATP and ADP was the same in both conditions, the rate of accumulation of cyclic AMP was significantly higher in the presence of NaF. This is most easily explained by a NaF stimulation of cyclic AMP synthesis. Since this stimulation is obviously not the result of maintenance of substrate (ATP) concentration it is interpreted as a direct effect of fluoride ion on adenyl cyclase independent of ATP concentration. A similar conclusion with

respect to the effect of NaF on fat cell adenyl cyclase was reached by Birnbaumer, Pohl & Rodbell (1969). This direct effect of NaF on adenyl cyclase is more clearly illustrated when the synaptic membrane system is used. This system possesses considerably less PDE and phosphatase contamination but responds to NaF with a significant increase in cyclic AMP accumulation.

It is thus our contention that the primary effect of NaF is a direct one on brain adenyl cyclase. The indirect inhibition of PDE becomes important in maintaining cyclic AMP concentration only when ATP concentration is low; the important nucleotide being ADP.

Although there is currently no evidence to suggest that any physiological agent which stimulates cyclic AMP accumulation does so by acting on other ATP and ADP utilizing enzymes, it appears from our studies that under certain conditions such an action is possible. This has been investigated and results reported in the following paper.

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