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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 adrenaline, ouabain and Mn^{++}

S. KATZ* AND A. TENENHOUSE

Department of Pharmacology and Therapeutics, McGill University, Montreal, Quebec, Canada

Summary

1. The mechanism of stimulation of cyclic adenosine 3′,5′-monophosphate (cyclic AMP) accumulation by adrenaline and ouabain and the effect of Mn^{++} substitution for Mg^{++} as the metal ion requirement of this system was studied in cell-free preparations of adenyl cyclase from rat brain.

2. In the rat cerebral cortex preparation, substitution of Mn^{++} for Mg^{++} significantly increased cyclic AMP accumulation while significantly inhibiting adenosine triphosphate (ATP) and adenosine diphosphate (ADP) hydrolysis and adenosine 5′-monophosphate (AMP) accumulation. In the synaptic membrane preparation, in the absence of NaF, the highest amount of ATP hydrolysis was obtained in tissue prepared with Mn^{++} and incubated with Mg^{++}; under these conditions cyclic AMP accumulation was equal to that produced under any other condition and significantly higher than that observed in the presence of Mg^{++} prepared and Mg^{++} incubated tissue.

3. Preparation and/or incubation of tissue with Mn^{++} significantly reduced phosphodiesterase (PDE) activity compared to that observed in Mg^{++} prepared tissue.

4. Adrenaline and ouabain both significantly increased cyclic AMP accumulation in the rat cerebral cortex preparation but did not inhibit ATP or ADP hydrolysis. In the synaptic membrane preparation, in the presence of 0·01 mM Ca^{++}, adrenaline but not ouabain significantly increased cyclic AMP accumulation. Phenoxybenzamine (0·1 mM) and pronethalol (0·1 mM) significantly inhibited adrenaline-induced cyclic AMP accumulation in both these preparations.

5. Ouabain and adrenaline both failed to stimulate cyclic AMP accumulation in the presence of Mn^{++} prepared and/or incubated tissue.

6. Ouabain and adrenaline had no effect on PDE activity in either of these preparations.

7. It was concluded that Mn^{++} increased cyclic AMP accumulation in part by indirect inhibition of ATP and ADP hydrolysis which provides inhibitors

* Present address: Division of Haematology, Royal Victoria Hospital, Montreal, Canada.

of cyclic AMP destruction, by direct stimulation of adenyl cyclase and by inhibition of cyclic AMP destruction in a way unrelated to nucleotide inhibition of PDE. Adrenaline and ouabain appeared to stimulate cyclic AMP accumulation in a more direct manner.

Introduction

In previous studies (Katz & Tenenhouse, 1973), it was shown that in cell-free preparations of adenyl cyclase an effect on other enzyme systems can result in a net accumulation of cyclic adenosine 3′,5′-monophosphate (cyclic AMP). All these enzyme systems exist in the intact cell and it is not improbable that physiological stimulators of adenyl cyclase act through these systems in the same way as that described for NaF (Katz & Tenenhouse, 1973).

Adrenaline and ouabain have previously been shown to stimulate cyclic AMP accumulation in rat brain (Klainer, Chi, Friedberg, Rall & Sutherland, 1962; Shimizu, Creveling & Daly, 1970). The mechanism of action of these agents in stimulating cyclic AMP accumulation in these preparations is unknown.

It is considered that the substrate for adenyl cyclase is Mg-ATP (Birnbaumer, Pohl & Rodbell, 1969). Sutherland, Rall & Menon (1962) and Perkins (1970) had previously shown that Mn^{++} could substitute for Mg^{++} as the metal ion requirement for adenyl cyclase in brain particulate preparations. It has not been demonstrated, however, that agents that stimulate adenyl cyclase in the presence of Mg^{++} will do so in the presence of Mn^{++}, or whether any alteration in the activity of other ATP utilizing enzymes and cyclic 3′,5′-AMP phosphodiesterase (PDE) resulting from the change in divalent cation would lead to a change in cyclic AMP metabolism.

In this study, in which the method of adenine nucleotide determination previously described by Katz & Tenenhouse (1973) was used, an attempt was made to elucidate the nature of the stimulation of adenyl cyclase by adrenaline and ouabain in the presence of Mn^{++} and Mg^{++}.

Methods

Preparation of adenyl cyclase

The rat cerebral cortex preparation of adenyl cyclase was prepared as described previously (Katz & Tenenhouse, 1973).

Rat synaptic membrane preparation of adenyl cyclase was prepared by a modification of the method of De Robertis, Rodriguez de Lores Arnaiz, Alberici, Butcher & Sutherland (1967) as described previously (Katz & Tenenhouse, 1973) except in some experiments where equimolar amounts of Mn^{++} were substituted for Mg^{++} in the media used.

Cyclic adenosine 3′,5′-monophosphate phosphodiesterase, adenyl cyclase and other membrane-bound ATP utilizing enzymes were assayed as previously described (Katz & Tenenhouse, 1973).

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 Corporation and radioactive agents from Schwarz Bioresearch Inc.

Results

Effect of Mn^{++}

The effect of Mn^{++} on the metabolism of ATP in the rat cerebral cortex preparation of adenyl cyclase. Table 1 illustrates that when equimolar (3·5 mM) Mn^{++} was added to the incubation mixture of the rat cerebral cortex preparation of adenyl cyclase in place of Mg^{++} the amount of ATP hydrolysed during the 10 min incubation period was significantly reduced ($P<0·001$). There was also a significant reduction in AMP accumulation ($P<0·001$) and a significant increase in ADP accumulation ($P<0·001$) compared to the Mg^{++} incubated controls. The amount of cyclic AMP accumulated in the presence of Mn^{++} was also significantly increased ($P<0·001$). Incubation in the presence of both Mn^{++} and 10 mM NaF produced a slightly greater inhibition of ATP hydrolysis and AMP accumulation but these changes were not statistically different from the values obtained in the presence of Mn^{++} alone. Under these conditions, however, there was a significant increase in cyclic AMP accumulation ($P<0·001$). The amount of cyclic AMP accumulated was also significantly greater than that accumulated in the presence of Mg^{++} and NaF ($P<0·01$) although the amount of ATP hydrolysed and AMP accumulated in the presence of Mn^{++} and NaF was not significantly different from that observed in the presence of Mg^{++} and NaF.

TABLE 1. *The effect of Mn^{++} on ATP metabolism in the rat cerebral cortex preparation of adenyl cyclase*

		AMP (μmol)	Cyclic AMP (nmol)	ADP (μmol)	ATP (μmol)
Mg^{++}	(8)	3·88±0·06	5·80±0·86	0·16±0·03	0·05±0·01
Mg^{++}+NaF	(8)	0·88±0·21	57·30± 6·20	1·17±0·13	2·00±0·21
Mn^{++}	(4)	1·67±0·22[a]	30·10± 4·50[a]	0·98±0·17[a]	1·42±0·51[b]
Mn^{++}+NaF	(3)	0·84±0·45[a]	114·00±18·30[c, d, a]	0·57±0·28	2·58±0·61[a]

[a] $P<0·001$ compared to Mg^{++}; [b] $P<0·01$ compared to Mg^{++}; [c] $P<0·001$ compared to Mn^{++}; [d] $P<0·01$ compared to Mg^{++}+NaF. 2,000×*g* particulate preparation of rat cerebral cortex (450 mg wet weight tissue) was incubated at 37° C for 10 min in the presence of either 3·5 mM $MgSO_4$ or 3·5 mM $MnCl_2$. In addition, the incubation medium of 2 ml volume contained: 40 mM Tris, pH 7·4; 6·67 mM theophylline; 2·1 mM [^{14}C]-ATP (0·250 μCi/μmol); 10 mM phosphoenolpyruvate and 25 μg/ml pyruvate kinase and where indicated, 10 mM NaF. The adenine nucleotides were determined as described by Katz & Tenenhouse (1973). Numbers in parentheses indicate number of experiments per group. Results are expressed as mean±S.E.

The effect of Mn^{++} on the metabolism of ATP in the rat synaptic membrane preparation of adenyl cyclase. When tissue prepared in Mg^{++} was incubated in the presence of Mn^{++} (Table 2, Part A), the amount of ATP hydrolysed during the 10 minute incubation period was significantly reduced ($P<0·05$ compared to in-

cubation in Mg^{++}). The amount of AMP accumulated was also significantly reduced ($P<0.001$) and the accumulation of cyclic AMP was significantly increased ($P<0.01$). The addition of 10 mM NaF to the Mn^{++} incubated preparation did not significantly alter the hydrolysis of ATP or accumulation of cyclic AMP.

TABLE 2. *The effect of Mn^{++} on ATP metabolism in the synaptic membrane preparation of adenyl cyclase*

	(A) Tissue prepared in Mg^{++}				(B) Tissue prepared in Mn^{++}			
	Incubated in Mg		Incubated in Mn^{++}		Incubated in Mg^{++}		Incubated in Mn^{++}	
	Control	NaF	Control	NaF	Control	NaF	Control	NaF
AMP (μmol/mg protein)	0·45 ±0·03	0·14 ±0·01	0·04 ±0·01[b]	0·04 ±0·01	0·57 ±0·07[e]	0·18 ±0·03[e]	0·13 ±0·00	0·10 ±0·00
Cyclic AMP (nmol/mg protein)	1·60 ±0·26	5·60 ±1·30	4·40 ±0·60[c]	6·10 ±0·80	4·80 ±1·20[a]	12·00 ±2·10	4·80 ±1·00	8·80 ±1·10
ADP (μmol/mg protein)	0·69 ±0·11	0·49 ±0·10	0·53 ±0·07	0·35 ±0·05	0·88 ±0·15	0·58 ±0·06	0·75 ±0·06	0·61 ±0·05
ATP (μmol/mg protein)	0·90 ±0·17	1·40 ±0·16	1·47 ±0·14[a]	1·65 ±0·19	0·60 ±0·11[d,g]	1·27 ±0·02[f]	1·14 ±0·07	1·31 ±0·06

[a] $P<0.05$ compared to Mg^{++} prepared-Mg^{++} incubated control; [b] $P<0.001$ compared to Mg^{++} prepared-Mg^{++} incubated control; [c] $P<0.01$ compared to Mg^{++} prepared-Mg^{++} incubated control; [d] $P<0.01$ compared to Mn^{++} prepared-Mn^{++} incubated control; [e] $P<0.001$ compared to Mn^{++} prepared-Mn^{++} incubated control; [f] $P<0.01$ compared to Mn^{++} prepared-Mg incubated control; [g] $P<0.01$ compared to Mg^{++} prepared-Mn^{++} incubated control. Synaptic membrane fraction of rat cerebral cortex homogenates (2·0 mg protein) prepared in either 1 mM Mg^{++} (A), or 1 mM Mn^{++} (B), was incubated at 37° C for 10 min in either 5·0 mM $MgSO_4$ or 5·0 mM $MnCl_2$. The incubation medium of 2 ml volume also contained: 6·67 mM theophylline; 40 mM Tris, pH 7·4; 2·1 mM [^{14}C]-ATP (0·250 μCi/μmol) and where indicated, 10 mM NaF. The adenine nucleotides were determined as described by Katz & Tenenhouse (1973). Results are expressed as the mean of 5 experiments ± S.E.

Table 2, Part B shows the change in ATP metabolism produced when the synaptic membrane preparation of adenyl cyclase was prepared in a medium in which 1 mM Mn^{++} was substituted for 1·0 mM Mg^{++}. When tissue prepared in Mn^{++} was incubated in Mn^{++} the amount of ATP hydrolysed during the 10 min incubation period was significantly reduced compared to that measured when the Mn^{++} prepared tissues was incubated in Mg^{++} ($P<0.01$). Similarly, AMP accumulation was significantly lower in the Mn^{++} incubations ($P<0.001$). The cyclic AMP accumulation was the same under both conditions. When the tissue prepared in Mn^{++} was incubated in the presence of 10 mM NaF, ATP hydrolysis in the Mg^{++} incubations was significantly inhibited ($P<0.01$) but in the Mn^{++} incubations was virtually unchanged. The concentrations of ADP, ATP, and AMP were similar under these conditions whether Mg^{++} or Mn^{++} was present in the incubation media. Comparison of Parts A and B of Table 2 shows that the highest amount of ATP hydrolysis in the absence of NaF was obtained when the synaptic membranes were prepared in Mn^{++} and incubated in the presence of Mg^{++}. Conversely, the concentration of cyclic AMP accumulated under these conditions was equal to that produced under any other conditions and significantly higher than that seen in Mg^{++} prepared and Mg^{++} incubated tissue. In the presence of NaF, the amount of ATP hydrolysed was the same whether the tissue was prepared or incubated with Mn^{++} or Mg^{++}, but the amount of cyclic AMP accumulated in tissue prepared in Mn^{++} was considerably higher.

Effect of Mn^{++} on phosphodiesterase activity

Rat cerebral cortex preparation of adenyl cyclase. Table 3 illustrates the effect

of Mn^{++} on the destruction of cyclic AMP in the rat cerebral cortex adenyl cyclase preparation. Theophylline, 6·67 mM, was present in the incubation medium. Despite this, following the 5 min incubation period only 13·5% of the cyclic AMP added remained in the presence of Mg^{++}. If equimolar Mn^{++} was substituted for Mg^{++} the amount of cyclic AMP remaining following the incubation period was significantly increased ($P<0·05$).

TABLE 3. *Effect of Mn^{++} on the phosphodiesterase activity of rat cerebral cortex preparation of adenyl cyclase*

		% Cyclic AMP remaining
Mg^{++}	(3)	13·5±3·2
Mn^{++}	(3)	26·2±0·5[a]

[a] $P<0·05$ compared to Mg^{++}. 2,000×*g* particulate preparation of rat cerebral cortex, prepared as described by Katz & Tenenhouse (1973). 225 mg wet weight tissue was incubated for 5 min at 37° C in either 3·5 mM $MgSO_4$ or 3·5 mM $MnCl_2$. The incubation medium of 1 ml volume also contained: 40 mM Tris, pH 7·5; 1·0 μM [^{14}C]-cyclic AMP (0·250 mCi/μmol) and 6·67 mM theophylline. Cyclic AMP remaining was determined as described by Katz & Tenenhouse (1973). Numbers in parentheses indicate number of experiments per group. Results are expressed as mean±S.E.

Rat synaptic membrane preparation. Table 4 shows the PDE activity of the synaptic membrane preparation of adenyl cyclase prepared normally in 1 mM Mg^{++}, or prepared in 1 mM Mn^{++}. Theophylline (6·67 mM) was used throughout this study and the incubation time was 10 minutes. When this tissue was prepared in Mg^{++} and incubated in Mg^{++}, 51% of the cyclic AMP added was hydrolysed. If however, 5 mM Mn^{++} was substituted for Mg^{++} in the incubation medium, only 40% of the cyclic AMP was hydrolysed during the incubation period ($P<0·01$). If the adenyl cyclase was prepared with Mn^{++} instead of Mg^{++} the inhibition of PDE was even more pronounced. Preparation in Mn^{++} and incubation in Mg^{++} caused a significant inhibition of PDE compared with that observed in Mg^{++} prepared-Mg^{++} incubated adenyl cyclase preparations ($P<0·02$). If however, the Mn^{++} prepared tissue was incubated in Mn^{++}, only 29% of the cyclic AMP was hydrolysed in the 10 min incubation period compared to 44% cyclic AMP hydrolysed in the presence of Mn^{++} prepared-Mg^{++} incubated tissue ($P<0·01$) and over 50% cyclic AMP hydrolysed in the presence of Mg^{++} prepared-Mg^{++} incubated tissue ($P<0·001$).

TABLE 4. *Effect of Mn^{++} on phosphodiesterase activity of the synaptic membrane preparation of adenyl cyclase from rat cerebral cortex*

	% Cyclic AMP remaining			
Incubation media	Tissue prepared in Mg^{++}		Tissue prepared in Mn^{++}	
Mg^{++}	48·7±0·8	(6)	55·7±2·3[b]	(3)
Mg^{++}+NaF	52·9±2·1	(5)	57·0±1·7	(3)
Mn^{++}	59·3±3·1[a]	(4)	70·8±2·1[c,d,e]	(5)
Mn^{++}+NaF	56·7±4·7	(3)	74·1±2·4[c,e,d]	(5)

[a] $P<0·01$ compared to Mg^{++} prepared-Mg^{++} incubated; [b] $P<0·02$ compared to Mg^{++} prepared-Mg incubated; [c] $P<0·001$ compared to Mg^{++} prepared-Mg^{++} incubated; [d] $P<0·02$ compared to Mg^{++} prepared-Mn^{++} incubated; [e] $P<0·01$ compared to Mn^{++} prepared-Mg^{++} incubated. Synaptic membrane fraction of rat cerebral cortex homogenates prepared in either 1 mM $MnCl_2$ or 1 mM $MgSO_4$ (3·0 mg protein) was incubated at 37° C for 10 min in either 5·0 mM $MgSO_4$ or 5·0 mM $MnCl_2$. The incubation medium of 2 ml volume also contained: 40 mM Tris, pH 7·5; 6·67 mM theophylline; 0·1 μM [^{14}C]-cyclic AMP (2·50 mCi/μmol) and where indicated, 10 mM NaF. Cyclic AMP remaining was determined as described by Katz & Tenenhouse (1973). Numbers in parentheses indicate number of experiments in each group. Results are expressed as mean±S.E.

Effects of adrenaline and ouabain

The effects of adrenaline and ouabain on cyclic AMP accumulation and ATP metabolism in the cerebral cortex preparation of adenyl cyclase. Table 5 illustrates that following incubation in the presence of Mg^{++} there was no difference in the amount of ATP, ADP and AMP recovered in the presence of adrenaline (0·1 mM) or ouabain (0·1 mM) as compared to the controls. There was a significant difference, however, in the amount of cyclic AMP accumulated in the presence of adrenaline or ouabain compared to the controls ($P<0·02$ in both cases).

TABLE 5. *The effect of adrenaline and ouabain on ATP metabolism in the rat cerebral cortex preparation of adenyl cyclase*

	AMP (μmol)	Cyclic AMP (nmol)	ADP (μmol)	(ATP (μmol)
Control (9)	3·94±0·06	5·80±0·86	0·16±0·03	0·08±0·01
Ouabain, 0·1 mM (7)	3·73±0·26	12·30±2·20[a]	0·29±0·08	0·08±0·02
Adrenaline, 0·1 mM (6)	3·81±0·33	10·90±1·76[a]	0·19±0·05	0·10±0·03

[a] $P<0·02$ compared to control. 2,000×*g* particulate preparation of rat cerebral cortex (450 mg wet weight tissue) was incubated at 37° C for 10 min in the presence or the absence of adrenaline (0·1 mM) or ouabain (0·1 mM). The incubation medium of 2 ml volume also contained: 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 phosphoenolpyruvate and 25 μg/ml pyruvate kinase. The adenine nucleotides were determined as described by Katz & Tenenhouse (1973). Numbers in parentheses indicate number of experiments per group. Results are expressed as mean±S.E.

The effects of adrenaline and ouabain on cyclic AMP accumulation and ATP metabolism in the synaptic membrane preparation of adenyl cyclase. Table 6 illustrates that there was no increase in cyclic AMP accumulation with these agents when the synaptic membranes were prepared in the usual manner ($MgSO_4$, 1 mM, and Tris, 2 mM, pH 7·2) and little difference in the concentrations of ATP, ADP and AMP was found. When the synaptic membranes were prepared with 0·01 mM Ca^{++} present throughout the preparation procedure, however, adrenaline but not ouabain stimulated cyclic AMP accumulation ($P<0·02$). The addition of Ca^{++}

TABLE 6. *The effects of adrenaline and ouabain on ATP metabolism in the synaptic membrane preparation of adenyl cyclase*

	Prepation in Mg^{++}			Preparation in $Mg^{++}+Ca^{++}$		
	Control (5)	Adrenaline (3)	Ouabain (3)	Control (7)	Adrenaline (7)	Ouabain (3)
AMP (μmol/mg protein)	0·05±0·02	0·04±0·01	0·08±0·04	0·07±0·02	0·09±0·1 [a]	0·05±0·02
Cyclic AMP (nmol/mg protein)	2·90±0·30	3·20±0·60	2·70±0·20	2·60±0·30	3·90±0·30 [b]	3·00±0·50
ADP (μmol/mg) protein)	0·04±0·01	0·04±0·00	0·02±0·01	0·41±0·14[d]	0·65±0·09[c,e]	0·07±0·05
ATP (μmol/mg protein)	2·01±0·24	2·01±0·12	1·98±0·18	1·52±0·15	1·26±0·21 [c]	1·95±0·27

[a] $P<0·01$ compared to adrenaline prepared in Mg^{++}; [b] $P<0·02$ compared to control prepared in $Mg^{++}+Ca^{++}$; [c] $P<0·05$ compared to adrenaline prepared in Mg^{++}; [d] $P<0·05$ compared to control prepared in Mg^{++}; [e] $P<0·005$ compared to ouabain prepared in $Mg^{++}+Ca^{++}$. Synaptic membrane fraction of rat cerebral cortex homogenate (2·0 mg protein) prepared as described by Katz & Tenenhouse (1973) with and without the addition of 0·01 mM calcium ion to the prepartion media was incubated at 37° C for 6 min in the presence or the absence of adrenaline (0·1 mM) or ouabain (0·1 mM). The incubation medium of 2 ml volume also contained: 40 mM Tris, pH 7·4; 5·0 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 phosphoenolpyruvate and 25 μg/ml pyruvate kinase. The adenine nucleotides were determined as described by Katz & Tenenhouse (1973). Numbers in parentheses indicate number of experiments per group. Results are expressed as mean±S.E.

to the preparation media significantly increased ADP accumulation in the controls and the amount of ATP hydrolysed and of ADP and AMP accumulated in the presence of adrenaline ($P<0.05$ in all cases). The addition of Ca^{++} to the preparation media did not affect the amount of ATP hydrolysed in the presence of ouabain.

In both preparations of adenyl cyclase used in this study phenoxybenzamine or pronethalol in concentrations of 0·1 mM inhibited significantly the adrenaline-induced cyclic AMP accumulation. These agents did not affect the control concentrations of cyclic AMP.

The effect of Mg^{++} concentration on cyclic AMP accumulation and ATP metabolism

Figure 1 summarizes the effect of varying the Mg^{++} concentration in the incubation medium on ATP metabolism and cyclic AMP accumulation in the synaptic membrane preparation of adenyl cyclase. A concentration of 0·01 mM Ca^{++} was present throughout the preparation procedure and the time of incubation was 6 minutes. When the concentration of Mg^{++} was reduced (from 5·0 mM to 2·0 mM) the amount of ATP hydrolysed in the controls was unchanged while in the presence of adrenaline ATP hydrolysis was significantly increased ($P<0.02$). When the concentration of Mg^{++} was increased (from 5·0 mM to 10·0 mM) the amount of ATP hydrolysed in the controls changed little whereas in the presence of adrenaline

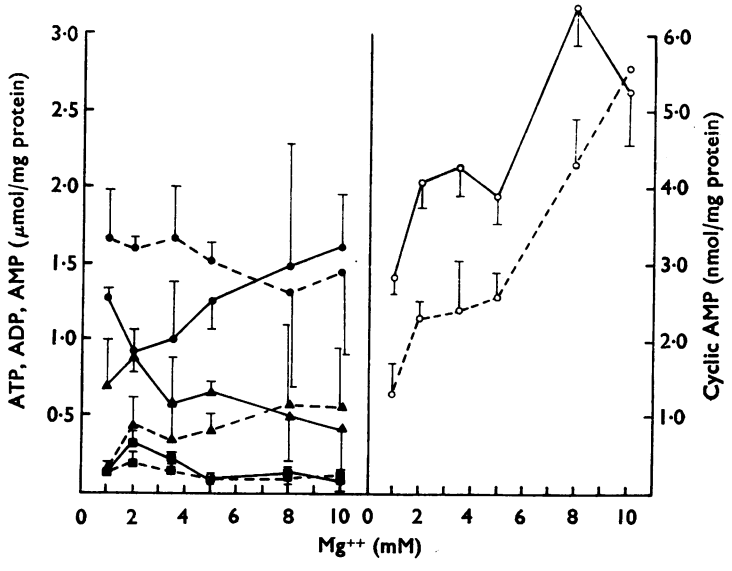

FIG. 1. The effect of the Mg^{++} concentration of the incubation medium on ATP metabolism in the presence and absence of adrenaline in a synaptic membrane preparation of adenyl cyclase. Synaptic membrane fraction of rat cerebral cortex homogenate prepared as described by Katz & Tenenhouse (1973) (2·0 mg protein) with 0·01 mM Ca^{++} present throughout the preparation procedure was incubated for 6 min in the presence and absence of 0·1 mM adrenaline at different Mg^{++} concentrations (from 1·0 mM–10·0 mM). The incubation medium (2 ml vol.) also contained: 40 mM Tris, pH 7·4; 6·67 mM theophylline; 2·1 mM [^{14}C]-ATP (0·250 μCl/μmol) and the ATP regenerating system consisting of 10 mM phosphoenolpyruvate and 25 μg/ml pyruvate kinase. The adenine nucleotides were determined as described by Katz & Tenenhouse (1973). Results are a mean of at least 3 observations per group. Vertical bars represent ± S.E., AMP (■), ADP (▲) and ATP (●) recovered in μmol/mg protein; cyclic AMP (○) recovered in nmol/mg protein. (- - - -)=Controls, (——)=adrenaline.

it decreased by 22%. There was little change in the amount of ADP accumulated in the controls when the Mg^{++} concentration was altered. The amount of ADP accumulated in the presence of adrenaline was highest at 2·0 mM and lowest at 10·0 mM Mg^{++}. AMP accumulation was highest at low Mg^{++} concentrations (from 1·0 mM to 5·0 mM Mg^{++}) in both the controls and in the presence of adrenaline.

Cyclic AMP accumulation increased steadily in the controls with increasing Mg^{++} concentration. The accumulation in the presence of adrenaline increased to a peak value at 8·0 mM Mg^{++}. At all concentrations of Mg^{++} the amount of cyclic AMP accumulated in the presence of adrenaline was higher than that accumulated in the controls ($P<0·01$ at 2·0 mM Mg^{++}, $P<0·02$ at 5·0 mM Mg^{++}) except at the highest Mg^{++} concentration when the amount of cyclic AMP accumulated under both conditions was the same.

The effect of Mn^{++} on adrenaline- and ouabain-stimulated cyclic AMP accumulation. When equimolar Mn^{++} was substituted for Mg^{++} in the preparation and/or incubation of the adenyl cyclase preparations, adrenaline and ouabain were ineffective in stimulating cyclic AMP accumulation.

Effect of adrenaline and ouabain on phosphodiesterase activity of the adenyl cyclase preparations. These agents, in the concentration that stimulated cyclic AMP accumulation in the rat cerebral cortex preparation of adenyl cyclase, did not alter the PDE activity of this preparation.

Discussion

In experiments with a partially purified preparation of PDE from rat brain we have found that ATP and ADP were potent inhibitors of cyclic AMP destruction. This finding indicated that substances which inhibited the ATP utilizing enzymes might act not only to preserve substrate for adenyl cyclase but might also indirectly inhibit cyclic AMP destruction. In the present experiments, Mn^{++} was shown to increase cyclic AMP accumulation. In studies with the cerebral cortex preparation of adenyl cyclase it was shown that when equimolar Mn^{++} was added to the incubation medium in the place of Mg^{++} there was a significant increase in cyclic AMP accumulation. There was also a significant reduction in the amount of ATP hydrolysed and a significant increase in ADP accumulation. Mn^{++} thus increased cyclic AMP accumulation while inhibiting ATP and ADP hydrolysis. It appeared that Mn^{++} could be increasing cyclic AMP accumulation by preserving substrate for adenyl cyclase and inhibiting PDE activity by maintaining high concentrations of ADP and ATP. In the synaptic membrane preparation, a clear dissociation was found between the stimulating effect of Mn^{++} on cyclic AMP accumulation and its effect on ATP hydrolysis. Mn^{++} in this preparation appeared to stimulate cyclic AMP accumulation in a way not related to inhibition of other ATP utilizing enzymes.

It was proposed that in both these preparations PDE activity might be altered in the presence of Mn^{++} in a way unrelated to nucleotide inhibition of PDE, thus resulting in the difference in the amount of cyclic AMP accumulated in the presence of Mn^{++} and Mg^{++}. When the effect of Mn^{++} on PDE activity was studied it was shown that PDE activity was significantly reduced in the presence of Mn^{++} in both the cerebral cortex preparation of adenyl cyclase and the synaptic membrane

preparation of adenyl cyclase. This finding explains at least part of the significant amount of cyclic AMP accumulation found in the presence of Mn^{++}.

These experiments show that the mechanisms by which Mn^{++} increases cyclic AMP accumulation in preparations of adenyl cyclase are: (1) inhibition of ATP and ADP hydrolysis, which provides inhibitors of cyclic AMP destruction; (2) direct stimulation of adenyl cyclase; (3) inhibition of cyclic AMP destruction in a way unrelated to nucleotide inhibition of PDE; and, (4) maintenance of substrate for adenyl cyclase by inhibition of ATP hydrolysis.

In the rat cerebral cortex preparation of adenyl cyclase, Mn^{++} appeared to increase cyclic AMP accumulation by inhibition of ADP and ATP hydrolysis and thus by indirect inhibition of PDE and also by inhibition of cyclic AMP destruction in a manner unrelated to nucleotide inhibition. In the rat synaptic membrane preparation of adenyl cyclase where PDE activity was much lower, these factors appeared to be secondary to a more direct stimulation of adenyl cyclase. A direct stimulation of adenyl cyclase is difficult to prove conclusively in preparations of adenyl cyclase that are contaminated with so many other enzyme systems. However, the large quantity of cyclic AMP accumulated in the presence of Mn^{++} in the synaptic membrane preparation, a system where ATP and ADP hydrolysis were much reduced and PDE activity was minimal, strongly suggests a direct effect of Mn^{++} on adenyl cyclase. The maintenance of substrate for adenyl cyclase by Mn^{++} probably becomes important at very low concentrations of ATP and cannot be excluded as an important factor in promoting cyclic AMP accumulation in systems where ATP hydrolysis is extensive.

The experiments with adrenaline and ouabain indicated that their ability to stimulate cyclic AMP accumulation was not due to an effect on other membrane-bound ATP utilizing enzymes or on PDE activity. In the rat cerebral cortex preparation of adenyl cyclase even though there was no difference in the amount of AMP, ADP and ATP recovered in the presence and absence of these agents, cyclic AMP accumulation was significantly increased. In the synaptic membrane preparation of adenyl cyclase, although ATP hydrolysis was significantly greater than in the controls, at low Mg^{++} concentration in the presence of adrenaline, cyclic AMP accumulation was significantly increased. In the control experiments, variation in the Mg^{++} concentration did not affect ATP hydrolysis but cyclic AMP accumulation was significantly increased at high Mg^{++} concentrations. Furthermore, adrenaline and ouabain did not alter the PDE activity of these preparations. It was concluded that ouabain and adrenaline were acting in a more direct manner to stimulate cyclic AMP accumulation in these preparations of adenyl cyclase.

The requirement of Ca^{++} in the preparation media of the synaptic membrane preparation of adenyl cyclase, in order for cyclic AMP accumulation to be stimulated by adrenaline, further demonstrates the difference in the mechanism of stimulation of cyclic AMP accumulation by NaF or Mn^{++} and by adrenaline. This requirement of Ca^{++} is probably related to the preservation of the integrity of the membrane preparation (Kavanau, 1965; Cavallito, 1967; Abood & Gabel, 1965) as Ca^{++} does not appear to be an ion requirement *per se* of adenyl cyclase. In fact, in concentrations slightly greater than those used, Ca^{++} is a potent inhibitor of adenyl cyclase activity (Perkins, 1970; Bar & Hechter, 1969).

In the present studies, ouabain stimulated cyclic AMP accumulation in the rat cerebral cortex preparation but did not stimulate cyclic AMP accumulation in the

synaptic membrane preparation of adenyl cyclase. The lack of stimulation in this latter case probably suggests a loss of some property in the fractionation of this homogenate that is necessary for ouabain stimulation of adenyl cyclase. In this case, the addition of Ca^{++} to the preparation media did not restore the effect of ouabain on cyclic AMP accumulation. Ouabain has previously been shown to stimulate brain slice preparations of adenyl cyclase (Shimizu *et al.*, 1970) but this is the first report of its stimulation of cyclic AMP accumulation in any kind of broken cell preparation. In the brain slice preparation, ouabain appeared to be stimulating cyclic AMP accumulation in a manner different from that of the biogenic amines, noradrenaline and histamine ; it was postulated that ouabain stimulated cyclic AMP accumulation by a depolarizing effect requiring the presence of Ca^{++} (Shimizu *et al.*, 1970). The present studies do not support this explanation as ouabain was shown to stimulate cyclic AMP accumulation in a particulate preparation of adenyl cyclase in which, presumably, membrane depolization is not a factor.

The present experiments did not succeed in characterizing the effect of adrenaline on cyclic AMP accumulation as either an α- or β-adrenoceptor stimulating type of effect. Adrenaline-stimulated cyclic AMP accumulation in these preparations was inhibited by both phenoxybenzamine, an α-adrenoceptor blocking agent, and pronethalol, a β-adrenoceptor blocking agent. In studies by other workers (Weiss, 1969 ; Kakiuchi & Rall, 1968) in various brain preparations, β-adrenoceptor blocking drugs were found to inhibit noradrenaline-stimulated cyclic AMP accumulation but α-adrenoceptor blockers were without effect. It seemed possible that the concentration of phenoxybenzamine used in this study produced a 'non-specific' inhibition of these preparations. This concentration, though, did not affect the control values of cyclic AMP accumulation and was much lower than those used by other workers. It is thus possible that the different preparations of adenyl cyclase used could explain the differing results.

The results, obtained with various Mg^{++} concentrations in the synaptic membrane preparation of adenyl cyclase, are similar to those found by Birnbaumer *et al.* (1969) in studies on the effect of ACTH on cyclic AMP accumulation in rat fat cell ghosts. As in their studies, in the absence of hormones, Mg^{++} concentration, in excess of that required for maximal Mg-ATP concentrations (Walaas, 1958 ; Burton, 1959), enhanced the basal activity of adenyl cyclase. If, as suggested by Birnbaumer *et al.* (1969), Mg-ATP is the true substrate for adenyl cyclase, free ATP an inhibitor and free Mg^{++} an allosteric effector, it is possible that the observed accumulation of cyclic AMP in the presence of adrenaline is an action of the hormone in increasing the affinity of adenyl cyclase for Mg^{++} at a site distinct from the catalytic site. The lack of stimulation by adrenaline of cyclic AMP accumulation in the presence of Mn^{++} can be interpreted to mean that Mn^{++} binds to this allosteric site better than Mg^{++} and under basal conditions stimulated the catalytic activity of the enzyme and that adrenaline cannot further alter the binding of Mn^{++} at this site. This result is supported by the work of Birnbaumer *et al.* (1969) in the rat fat cell ghost preparation where stimulation of cyclic AMP accumulation by ACTH was not observed in the presence of Mn^{++}.

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