F⁻-INDUCED CHANGES AND ITS REVERSAL BY ITP IN MEMBRANE TURBIDITY AND ADENYLATE CYCLASE ACTIVITY OF CHICK BRAIN MICROSOMES

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Abstract—The activation of adenylate cyclase by NaF was dependent on the previous incubation time and the concentration of F⁻. The activation by F⁻ was irreversible and Mg²⁺ was required for the maximum effect. Turbidity of microsome suspension was also greatly increased by F⁻ plus Mg²⁺. These effects on adenylate cyclase and membrane turbidity were specific for F⁻ and F⁻-saturation curves for both were similar, though Mg²⁺-saturation curves for both were dissimilar. The increase in turbidity induced by F⁻ plus Mg²⁺ was rapidly reversed by ATP, GTP, ITP, UTP and CTP. However, ITP only, among all the triphospho-nucleotides tested, reversed the activity of adenylate cyclase previously activated by NaF plus MgCl₂. The activity of the enzyme reversed by ITP was not, however, re-enhanced by the presence of NaF in the assay medium. These results suggest the possibility that F⁻ induces a change in the membrane structure itself, and this change can be reversed by incubation with ITP. Consequently, adenylate cyclase may be conformed either to an activated or an un-activated state.

It is acknowledged that cyclic AMP is an important metabolic regulator and a mediator of hormone actions in a wide variety of tissues. Adenylate cyclase locates in a particulate fraction and is stimulated by NaF and various hormones in many tissues. Stimulation of this enzyme by NaF was originally reported by Rall and Sutherland (1) and Sutherland et al (2). Although the mechanism of stimulation of adenylate cyclase by NaF is unknown, recently some properties of stimulating effects of NaF have been reported (3, 4). Stimulation of adenylate cyclase by NaF is apparently nonspecific and differs from that by hormones. NaF stimulates adenylate cyclase activity only in a broken cell preparation, and does not do so in intact cells of many tissues (5) including central nervous system (6). The stimulating effect of hormones is reversible (7), whereas the stimulating effect of NaF is not reversed on the enzyme from parotid glands (8), adrenal cortex (9, 10), rat cerebral cortex (11) and skeletal muscles (12). However, the effects of NaF and hormones on adenylate cyclase may not be completely independent. Combinations of F⁻ with hormones do not yield additive activities. Harwood and Rodbell (13) have suggested that NaF acts at some point on the pathway by which hormonal interaction with the receptor leads to an increase in the catalytic activity of adenylate cyclase. Recently Najjar and his co-workers (14-16) hypothesized on the mechanism of activation of adenylate cyclase by F⁻ and evidence which supports their hypothesis was seen in the adenylate cyclases from polymorphonuclear granulocytes and blood platelets.

In our previous studies (17, 18), young chicks were used to elucidate the effects of cyclic AMP, biogenic amines and prostaglandins on the central nervous system. We favored young chicks rather than mammals as experimental animals as it was possible to show the remarkable effects of cyclic AMP on locomotor activity quantitatively, and to observe that remarkable effects began to appear in 20 sec to one min after intracerebral administration.

As a continuation of this work, we studied the NaF-stimulated adenylate cyclase of chick brains. Preliminary accounts of this work have already appeared (19, 20).

MATERIALS AND METHODS

Preparation

Two week-old male White Leghorn chicks were decapitated. Telencephalones were removed and homogenized in 5 vol. of the homogenizing medium which contained the following components in the final concentration indicated; 100 mM Na-glutamate, 10 mM MgCl_2 , 20 mM NaH_2PO_4 and 0.1 mM GTP. The pH of the solution was adjusted to 7.0 with NaOH. The homogenate was centrifuged at $2,500 \times g$ for 15 min. The supernatant obtained was centrifuged at $77,500 \times g$ for 40 min. The precipitate was washed once with the same medium by centrifugation. The final pellets were suspended in the same medium and kept at -20°C (designated Mic.). Under this condition, adenylate cyclase activity was stable for more than two months. This fraction was observed to contain mostly microsome-like vesicles with a slight contamination of mitochondria as seen under electron microscopy.

Prior to use, this sample (Mic) was centrifuged at $77,500 \times g$ for 40 min and the precipitate was washed once by resuspension and centrifugation in 20 mM Tris HCl buffer (pH 7.35). The pellets were suspended in 20 mM Tris HCl buffer (pH 7.35) and used for experiments on adenylate cyclase.

For studies on turbidity changes, Mic fraction was dialyzed against 100 vol. of 60% saturated $(\text{NH}_4)_2\text{SO}_4$ (pH 8.8) containing 0.1 mM EDTA at 0°C for 3 hr and then centrifuged at $9,000 \times g$ for 15 min. The pellets were washed 3 times with 20 mM Tris HCl buffer (pH 7.35) by centrifugation. The final pellets were resuspended in 20 mM Tris HCl buffer (pH 7.35) (designated Washed Mic) and used for experiments on turbidity changes. After the treatment with alkaline $(\text{NH}_4)_2\text{SO}_4$, turbidity was always increased to the same plateau by the addition of NaF and MgCl_2 . But before the treatment with alkaline $(\text{NH}_4)_2\text{SO}_4$, turbidity was increased to a maximum and then rapidly decreased by adding NaF and MgCl_2 . The maximum level varied from one experiment to another under the same condition.

Treatment with alkaline $(\text{NH}_4)_2\text{SO}_4$ resulted in approximately a 30% loss in adenylate cyclase activity.

Adenylate cyclase activity

Adenylate cyclase activity was measured by the method of Krishna et al (21) with the modification by Perkins and Moore (11). Unless otherwise noted, the standard assay medium (in a total vol. of 1.0 ml) contained the following components in the final concentration indicated; 40 mM Tris HCl buffer (pH 7.35), 4 mM MgCl_2 , 8 mM theophylline, 5 mM phospho(enol)pyruvate, 125 μg per ml of pyruvate kinase (including 10 μmoles of $(\text{NH}_4)_2$

SO₄) 0.8 mM cyclic AMP, 0.4 mM [³H]AMP (5.2 μ Ci per mole) and the enzyme sample (usually 0.4–0.9 mg protein). The assay medium was incubated at 30°C for 20 min and then placed in a boiling water bath for 5 min. In a control reaction [³H]ATP was added, after boiling for 5 min, to the tube containing all of the components except [³H]ATP. The tube was then re-placed in the boiling bath for 5 min. After cooling in ice, the samples were centrifuged to remove heat-denatured protein. The supernatant fluid was transferred to Dowex 50 W-X8, mesh 100–200, column (1.0 ml of column vol.). Elution of cyclic AMP from the column was as described by Krishna et al (21). Carrier ATP (2 μ moles) was added to the 3.5 ml of cyclic AMP fraction. After Zn(OH)₂-BaSO₄ precipitation, a portion of the supernatant fluid was analyzed for radioactivity with Bray's solution (22) and another used to determine the absorbance at 260 m μ , which provided a measure for the recovery of cyclic AMP throughout the entire procedure. The enzyme activity was linear with time for 25–30 min. In tables and figures, the values of adenylate cyclase activity are corrected for recovery and expressed as nanomoles of cyclic AMP formed in 20 min per mg of protein at 30°C. All assays were performed in duplicate.

Turbidity changes

Washed Mic sample was incubated with NaF (test ions) and MgCl₂ in 20 mM Tris HCl buffer (pH 7.35) at 30°C. Turbidity changes were estimated by recording O.D. at 660 m μ continuously. After preincubation at 30°C, the reaction was started by adding NaF and MgCl₂ simultaneously or by adding Washed Mic samples. A decrease in O.D. at 660 m μ due to dilution was corrected.

Protein was determined by the method of Lowry et al. (23) with bovine serum albumin as a standard.

Chemicals

Cyclic AMP was a gift from Takeda Pharmaceutical Co., Ltd. [³H]ATP was obtained from New England Nuclear Corp. ATP, GTP (Type II-S), ITP, UTP, CTP, ADP, IDP, pyruvate kinase (type I) and phospho(enol)pyruvate were purchased from Sigma Chemical Co. Other reagents used were all commercial products of analytical grade.

RESULTS

The activation of adenylate cyclase and the turbidity changes of microsome suspension induced by F⁻ and Mg²⁺

The enzyme was previously incubated with F⁻ and Mg²⁺ in 20 mM Tris HCl buffer (pH 7.35) at 30°C and the enzyme activity was measured with 10 times dilution in the absence of F⁻ (Fig. 1). The activity increased with the time of the previous incubation and was higher than that of untreated enzyme measured with F⁻ in the assay medium. When the enzyme was previously incubated with F⁻ and Mg²⁺ at 30°C for 30 min and thoroughly washed with Tris HCl buffer (pH 7.35) by repeated centrifugation, it showed a high activity without a addition of F⁻. The activity was not further enhanced by adding F⁻ to the assay medium (Fig. 1, inserted graph).

FIG. 1. Activation of adenylate cyclase by the previous incubation with NaF and MgCl_2 . The enzyme preparation (6.25 mg of protein per ml) was incubated at 30°C in the presence of 4.0 mM NaF plus 4.0 mM MgCl_2 . The incubation was started by adding NaF and MgCl_2 and at the time indicated 0, 1-ml aliquots were withdrawn and added to the assay medium (a final vol. of 1.0 ml) for measurement of adenylate cyclase activity in the absence of NaF. The activity at the zero time was measured in the presence (—) and absence (●) of 4.0 mM NaF. In the inserted graph, the enzyme preparations (0.32 mg of protein per ml) were incubated in the presence of either 4.0 mM NaF plus 4.0 mM MgCl_2 (F, Mg^{2+}) or 4.0 mM MgCl_2 only (Mg^{2+}), or in the absence of NaF and MgCl_2 (None). After incubation at 30°C for 30 min the enzyme preparations were washed twice with 20 mM Tris HCl buffer (pH 7.35) by centrifugation. The final pellets were assayed for adenylate cyclase activity in the presence and absence of 4.0 mM NaF. Values represent the average from duplicate incubations.

FIG. 2. Effect of NaF and MgCl_2 on the turbidity of microsome suspensions. Turbidity changes were examined by measurement of O.D. at 660 mμ. Washed Mic samples (75 μg of protein per ml) were incubated at 30°C in 20 mM Tris HCl buffer (pH 7.35). At the time indicated by the arrow a or b, NaF and MgCl_2 were added at 3.5 mM each (A) or 9.3 mM each (B). At the time indicated by the arrow c, the incubation mixtures were stirred. In the absence of washed Mic samples the addition of NaF and MgCl_2 had no effect on O.D. at 660 mμ (C).

On the previous incubation with F^- and Mg^{2+} , turbidity of the enzyme suspension was greatly increased and small fragment-like precipitates were observed. Fig. 2 shows the effect of F^- and Mg^{2+} on turbidity of the microsome suspension (Washed Mic sample). Turbidity was rapidly increased by adding F^- and Mg^{2+} and reached the plateau in about 6 min. When the microsome preparation was denatured by heating at 100°C for 10 min, the increase in turbidity induced by F^- and Mg^{2+} was not observed. The percent increase in the turbidity was dependent on the concentration of protein which had a negative correlation (Fig. 3).

Table I shows a specificity for F^- in both the activation of the enzyme and the increase

FIG. 3. Effect of the concentration of microsomes on the turbidity change induced by NaF and MgCl_2 . Washed Mic samples were incubated at varying concentrations at 30 C in the presence of 9.3 mM NaF plus 9.3 mM MgCl_2 (A), 3.5 mM NaF plus 3.5 mM MgCl_2 (B) or 1.0 mM NaF plus 1.0 mM MgCl_2 (C). The turbidity change was expressed as percent increase in O.D. at 660 μm . Values represent the average from duplicate incubations.

FIG. 4. Activation of adenylate cyclase and the turbidity change of microsomes induced by MgCl_2 plus varying concentrations of NaF. The enzyme preparations (8.0 mg of protein per ml) were incubated in the presence of 3.5 mM MgCl_2 plus varying concentrations of NaF. After incubation at 30 C for 30 min, 0.1-ml aliquots were removed and added to the assay medium for measurement of adenylate cyclase activity (●—●). For measurement of the turbidity change (○—○), washed Mic samples (75 μg of protein per ml) were incubated at 30 C in the presence of 3.5 mM MgCl_2 plus varying concentrations of NaF. Each point indicates the difference in the values obtained with NaF plus MgCl_2 (3.5 mM) and with 3.5 mM MgCl_2 only. Values represent the average from duplicate incubations.

in the turbidity. Only fluoride ions produced a strong activation of the enzyme and a great increase in the turbidity. Among various cations, Li^+ had inhibitory effects both on the turbidity change and on activation of adenylate cyclase caused by F^- .

Both the activation of the enzyme and the increase in the turbidity were examined at varying concentrations of F^- (Fig. 4). The dose-response curves were quite similar and half the maximal effects were obtained at 1.1–1.2 mM F^- on both the activation and the turbidity change.

Figs. 5 and 6 show the effects of Mg^{2+} concentration. To examine the effect of Mg^{2+} concentration on the activation of the enzyme, the enzyme preparation was previously

TABLE 1. Effects of various ions on adenylate cyclase and turbidity of microsome suspensions

	Relative potency	
	Adenylate cyclase	Optical density At 660 m μ
NaF	100%	100%
KF	96	100
NH ₄ F	105	94
CsF	96	95
LiF	64	32
NaBr	2	9
NaI	3	0
NaSCN	10	0
NaCN	16	0
NaNO ₃	2	0
NaCl	(0)	(0)

Enzyme preparations (8.6 mg of protein per ml) were incubated in the presence of various ions (3.5 mM) indicated plus 3.5 mM MgCl₂. After incubation at 30°C for 30 min, 0.1-ml aliquots were removed and added to the assay medium for measurement of adenylate cyclase activity. In experiments on turbidity changes, washed Mic samples (75 μ g of protein per ml) were incubated in the presence of various ions (3.5 mM) indicated plus 3.5 mM MgCl₂. Both to the activation of the enzyme and to the turbidity changes, the potency of NaF was expressed as 100% and the potency of NaCl was expressed as 0%, as Tris HCl buffer (pH 7.35) was used in incubation. Values represent the average from duplicate incubations.

incubated with F⁻ plus varying concentrations of Mg²⁺, and washed with Tris HCl buffer by repeated centrifugation. The activity was then measured in the presence of 4.0 mM Mg²⁺. The enzyme was partially activated by the previous incubation with F⁻ only, as shown in Fig. 6. However, Mg²⁺ was essential for the turbidity change induced by F⁻. Half the maximal effects were obtained at 1.6 mM Mg²⁺ on the activation of the enzyme and at 1.0 mM Mg²⁺ on the increase in the turbidity.

To examine the effect of a low concentration of Ca²⁺ on the activity, the enzyme activity was measured in the presence of EGTA or EGTA plus Ca²⁺ (Fig. 7). In these experiments two preparations of the enzyme were used; one was the enzyme previously activated by the incubation with

FIG. 5. Turbidity change induced by NaF plus varying concentrations of MgCl₂. Washed Mic samples (75 μ g of protein per ml) were incubated at 30°C in the presence of 3.5 mM NaF plus varying concentrations of MgCl₂ (●---●) or MgCl₂ only (○). ---, the difference between both curves. Values represent the average from duplicate incubations.

FIG. 6. Activation of adenylyl cyclase by NaF plus varying concentrations of MgCl_2 . The enzyme preparations (0.37 mg of protein per ml) were incubated in the presence of 4.0 mM NaF plus varying concentrations of MgCl_2 . After incubation at 30°C for 30 min the enzyme preparations were washed twice with 20 mM Tris HCl buffer (pH 7.35) by centrifugation. Adenylyl cyclase activity of the final pellets was measured in the standard manner in the presence ($\circ$ — $\circ$) and absence ($\cdots$) of 4.0 mM NaF. $\bullet$ — $\bullet$, the activity of the enzyme incubated without NaF.

F^- , followed by washing, another was the enzyme untreated with F^- . On the activity of the latter, the effect of Ca^{2+} was examined in the presence and absence of F^- in the assay medium. As shown in Fig. 7, the presence of EGTA in the assay medium resulted in a marked reduction in the activities of these preparations. Reduction in the activities due to EGTA was completely restored by the simultaneous addition of Ca^{2+} . The maximal activities of these preparations were obtained at a final concentration of $1\text{--}3 \times 10^{-6}$ M free Ca^{2+} and were slightly higher than those obtained when EGTA was omitted from the assay medium (open circles in Fig. 7).

Studies on the reversibility of the activation and the turbidity change

The increase in turbidity induced by F^- and Mg^{2+} was rapidly reversed by adding ATP

FIG. 7. Effect of Ca^{2+} on the activity of adenylyl cyclase. The activity of adenylyl cyclase was measured in the presence of varying concentrations of free Ca^{2+} . Low concentrations of free Ca^{2+} (10^{-8} M) were obtained by adding Ca-EGTA complex (1:1 mole ratio) and EGTA to the assay medium. The concentration of Ca-EGTA plus EGTA was fixed at 0.5 mM. Final concentrations of free Ca^{2+} in the assay medium containing 4.0 mM MgCl_2 at pH 7.35 were calculated by the method of Portzehl et al (24). The points on each curve from left to right ($<10^{-8}$ M Ca^{2+}) in figure show the activities measured with (total Ca^{2+})/(total EGTA) ratio of 0, 0.5, 0.9, 0.99, 0.999, 0.9999 and 1.0. In the experiments with higher concentrations of Ca^{2+} (10^{-5} – 10^{-3} M), CaCl_2 was added to the assay medium at the concentrations indicated without EGTA. Curve A indicates the activity of adenylyl cyclase which was previously incubated at 30°C for 30 min in the presence of 4.0 mM NaF plus 4.0 mM MgCl_2 , and then washed twice with 20 mM Tris HCl buffer (pH 7.35) by centrifugation. The activity was measured in the absence of NaF in the assay medium. Curves B and C indicate the activity of untreated adenylyl cyclase in the presence (B) and absence (C) of 4.0 mM NaF in the assay medium. $\cdots$ $\circ$ $\cdots$, the activity measured in the absence of EGTA.

FIG. 8. Effect of ATP on the turbidity change induced by NaF plus MgCl_2 . Washed Mic samples ($75 \mu\text{g}$ of protein per ml) were incubated at 30°C in 20 mM Tris HCl buffer (pH 7.35) (total vol. 2.95 ml.) At the time indicated by the arrow a or d, NaF and MgCl_2 (arrow a) or MgCl_2 only (arrow d) were added at 3.5 mM each. At the time indicated by the arrow b, ATP (0.2 ml, pH 7.35) was added at 4.3 mM. At the time indicated by the arrow c, the incubation mixture was stirred.

FIG. 9. Effects of varying concentrations of ATP on the turbidity change induced by NaF plus MgCl_2 . Washed Mic samples ($75 \mu\text{g}$ of protein per ml) were incubated at 30°C in the presence of 3.5 mM NaF plus 3.5 mM MgCl_2 . After O.D. at 660 m μ increased to the plateau, ATP were added to the incubation mixtures at varying concentrations. Percent reversals were calculated from the decreased values in O.D. at 660 m μ observed for 4 min immediately after the addition of ATP. Values represent the average from duplicate incubations.

(Fig. 8) and the fragment-like precipitates disappeared. The reversal rates were calculated from the decreased value in O.D. at 660 m μ obtained for 4 min immediately after the addition of ATP and expressed as percent of the value in O.D. at 660 m μ increased by 3.5 mM F^- plus 3.5 mM Mg^{2+} . The reversal percent plotted as a function of ATP concentration was sigmoid (Fig. 9). Two apparent K_{es} for ATP appeared on the double reciprocal plots. Fifty percent reversal was obtained at 3.1 mM ATP. ADP and adenosine were less effective than ATP. AMP and Pi were ineffective (Table 2). GTP, ITP, UTP and CTP were as effective as ATP. A high concentration of EDTA had a slight effect. But it showed a different time course and O.D. at 660 m μ decreased slowly after addition of EDTA. These results suggested that the triphosphonucleotide itself has a reversing effect on the turbidity change induced by F^- and Mg^{2+} .

The effect of ATP on the enzyme activated by F^- was then examined (Table 3). The enzyme was activated by the incubation with 4 mM F^- and 4 mM Mg^{2+} in 20 mM Tris HCl buffer (pH 7.35), followed with washing by repeated centrifugation. The activated enzyme was reincubated with 10 mM ATP at 30°C for 15 min. After dialyzing against Tris HCl buffer (pH 7.35) (10,000 volumes), the activity was measured both in the presence and in the absence of F^- . The reincubation with ATP did not result in a significant change in the activity. However, the activity of the enzyme reincubated with ATP was slightly enhanced

TABLE 2. Effects of various nucleotides on the turbidity change induced by NaF plus $MgCl_2$

	Concentration	Per Cent Reversal
ATP	4.3 mM	68.2%
ADP	4.3	47.5
AMP	4.3	7.1
cyclic AMP	1.2	-6.7
Adenosine	4.2	33.8
Pi	2.0	1.3
GTP	4.3	54.8
UTP	4.3	66.8
ITP	4.3	62.5
CTP	4.3	66.2
EDTA	3.2	-0.9
EDTA	4.7	21.1

Washed Mic samples (75 μ g of protein per ml) were incubated at 30° C in the presence of 3.5 mM NaF plus 3.5 mM $MgCl_2$. After O.D. at 660 m μ increased to the plateau, compounds indicated were added to the incubation mixtures. Percent reversals were calculated as for Fig. 9. Values represent the average from duplicate incubations.

TABLE 3. Effect of ATP on adenylate cyclase previously activated by NaF plus $MgCl_2$

Treatment	Adenylate cyclase activity (N-moles per mg protein, 20 min)	
	-F ⁻	+F ⁻
Without treatment	3.75	7.50
Incubation with NaF + $MgCl_2$ (F ⁻ -Enz)	13.21	13.1
Reincubation with ATP (ATP-Enz)	14.73	16.55
Reincubation with ATP ; Mg^{2+} (ATP, Mg^{2+} -Enz)	13.00	15.00

The enzyme preparations (0.4 mg of protein per ml) were incubated in the presence of 4.0 mM NaF plus 4.0 mM $MgCl_2$ in 20 mM Tris HCl buffer (pH 7.35). After incubation at 30° C for 30 min, the enzyme preparations were washed twice with 20 mM Tris HCl buffer (pH 7.35) by centrifugation. The pellets were suspended in 20 mM Tris HCl buffer (pH 7.35) (2.4 mg of protein per ml) (F⁻-Enz) and further incubated at 30° C for 15 min in the presence of 10 mM ATP plus 4.0 mM $MgCl_2$ (ATP, Mg^{2+} -Enz) or 10 mM ATP only (ATP-Enz). After incubation, these three samples were dialyzed against 20 mM Tris HCl buffer (pH 7.35) at 4° C for 7 hr. Adenylate cyclase activities were measured in the presence and absence of 4.0 mM NaF. Values represent the average from duplicate incubations.

by F⁻ in the assay medium (Table 3). Further, the presence of ATP in the previous incubation with F⁻ and Mg^{2+} had no significant effect on the activation by F⁻. When the enzyme previously activated by F⁻ plus Mg^{2+} was incubated with ATP, the specific activity of the enzyme sometimes increased due to loss of the protein during the washing. Table 4 shows

the effect of incubation with various triphospho-nucleotides on the activities of the enzyme previously activated by F^- . The enzyme was incubated with 4 mM F^- and 4 mM Mg^{2+} in 20 mM Tris HCl buffer (pH 7.35) at 30°C for 30 min and then triphospho-nucleotides were added to the incubation mixture. After a further 20 min incubation, the enzyme was washed with Tris HCl buffer (pH 7.35) by repeated centrifugation (nucleotides diluted about 50,000-fold). The activity was then measured both in the presence and in the absence of F^- . Among these triphospho-nucleotides, ITP was quite effective for reversing the activity as shown in Table 4. UTP was less effective. Further the effects of IDP, IMP and inosine were examined (Table 4, Expt. 2) and it was found that only ITP was effective. IDP and IMP had a slight effect. The activity of the enzyme incubated without F^- was also reduced by incubation with ITP (Table 4, Expt. 2). As shown in Table 4, the activation of adenylate cyclase by NaF seemed to be completely reversed by ITP, but the enzyme activity reversed by ITP was not enhanced again by adding NaF to the assay medium. Fig. 10 shows the reversing effect of ITP on the activity at varying concentrations. The activity enhanced by F^- was reduced

TABLE 4. Effects of various nucleotides on adenylate cyclase previously activated by NaF plus $MgCl_2$

1st. Addition	2nd. Addition (10 mM)	Adenylate cyclase activity (N-moles per mg protein, 20 min)	
		F ⁻	+F ⁻
Expt. 1			
Control (unincubated)		3.11	5.37
4.0 mM NaF + 4.0 mM MgCl ₂	None	11.06	10.80
	ATP	9.72	10.98
	GTP	10.88	11.80
	ITP	4.05	4.40
	UTP	7.57	9.21
	CTP	10.81	11.75
Expt. 2			
Control (unincubated)	—	3.31	5.36
4.0 mM NaF + 4.0 mM MgCl ₂	No-incubation	9.34	8.37
	ITP	2.01	1.93
	IDP	5.86	5.82
	IMP	7.15	6.73
	Inosine	8.90	7.98
4.0 mM MgCl ₂ only	ITP	1.82	2.05

Enzyme preparations (0.52 mg of protein per ml in Expt 1, 0.35 mg of protein per ml in Expt 2) were incubated in the presence of 4.0 mM NaF plus 4.0 mM $MgCl_2$. After incubation at 30°C for 30 min, nucleotides indicated were added at 10 mM to the incubation mixtures. After further incubation at 30°C for 20 min, the enzyme preparations were washed twice with 20 mM Tris HCl buffer (pH 7.35) by centrifugation. The final pellets suspended in 20 mM Tris HCl buffer (pH 7.35), were assayed for adenylate cyclase activity in the presence and absence of 4.0 mM NaF. A different preparation was used for each experiment. Tris HCl buffer (pH 7.35) was used in incubation. Values represent the average from duplicate incubations.

FIG. 10. Effects of varying concentrations of ITP on adenylate cyclase previously activated by NaF plus $MgCl_2$. The experiments on adenylate cyclase were carried out under conditions similar to those described in the legend to Table 4. The solid line indicates the activity of adenylate cyclase which was kept at $0^\circ C$ for 48 hr after the final pellets were re-suspended. The effect of ITP on the turbidity change induced by NaF plus $MgCl_2$ (---○---) was examined under conditions similar to those described in the legend to Fig. 9. Cont.=Activity of untreated enzyme.

with increase in the concentration of ITP. Half the maximal effect was obtained at about 3.5 mM ITP. This value was very similar to that obtained for the reversing effect of ITP on the turbidity enhanced by F^- (Fig. 10). The effect of ITP was also observed on the enzyme sample which was previously incubated with F^- and Mg^{2+} and then washed by centrifugation. The enzyme previously activated by F^- was very stable. However, the enzyme which was previously activated by F^- and reversed by ITP, was unstable and its activity decreased gradually on standing at $0^\circ C$ (Fig. 10, solid line).

Some incubation systems were tested to observe conditions in which a lower concentration of ITP may reverse the activity previously enhanced by F^- . On the enzyme sample which had been treated with F^- plus Mg^{2+} and then with ATP under the same conditions as for Table 4, a low concentration of ITP (1 mM) was rather effective. Above 50% of the activity enhanced by F^- was reversed by further incubation with 1 mM ITP at $30^\circ C$ for 20 min. After treatment with 1 mM ITP, the activity of adenylate cyclase was slightly enhanced (15–20%) by 4.0 mM NaF in the assay medium.

DISCUSSION

Adenylate cyclase of chick brain microsomes was found to be activated by NaF, but was not activated by biogenic amines. The rate of activation by NaF was dependent on a concentration of F^- and Mg^{2+} . Although the enzyme was slightly activated by only NaF without addition of Mg^{2+} , Mg^{2+} was essential for the maximal activation by F^- . The activation by F^- and Mg^{2+} was irreversible and was not reversed by dilution, washing or dialysis (Fig. 1, Table 3) of the activated enzyme. Similar observations were originally reported by Perkins and Moore on adenylate cyclase of rat brains (11), and by Schramm and Naim on

adenylate cyclase of rat parotid glands (8).

Bradham and his co-workers (25, 26) reported the implication of a low concentration of Ca^{2+} in the activity of adenylate cyclase from bovine brains. We obtained similar results on adenylate cyclase of chick brains, and calculated the free Ca^{2+} concentration required for the maximal activity of adenylate cyclase by using EGTA- Ca^{2+} buffer (24) (Fig. 7). The maximal activities of untreated enzyme measured in the presence and absence of NaF were obtained at a final concentration of 1.3×10^{-6} M free Ca^{2+} . Even on the enzyme previously activated by F^- and Mg^{2+} , a low concentration of free Ca^{2+} ($1-3 \times 10^{-6}$ M) was required for its maximal activity (Fig. 7). At the optimal concentration of free Ca^{2+} , the activities were slightly greater than those measured in the absence of EGTA.

The turbidity change induced by F^- and Mg^{2+} was dependent on the concentrations of F^- and Mg^{2+} and was specific for F^- (Figs. 4 and 5, Table 1). Both the turbidity change of microsomes and the activation of the enzyme induced by NaF plus MgCl_2 were similar with respect to the requirement of F^- , and had similar dose-response curves to F^- (Fig. 4). However, for the requirement of Mg^{2+} , dose-response curves for both processes were not coincident. (Figs. 5 and 6).

The turbidity change induced by F^- and Mg^{2+} was reversed by triphospho-nucleotides (Table 2). The addition of EGTA decreased the turbidity due to F^- plus Mg^{2+} , but the time course was somewhat slower than that by triphospho-nucleotides. Therefore, the effect of triphospho-nucleotides on turbidity does not appear to be caused by removal of metal ions due to a chelating action of nucleotides on Mg^{2+} or other divalent cations. The preparation in which turbidity was increased by F^- plus Mg^{2+} or reversed by further incubation with ATP was gathered by centrifugation and was examined under electron microscopy with OsO_4 stain or PTA (phospho-tungstic acid)-negative stain. However, no significant changes were observed on the membranes. We previously reported (19) that the interaction of fluorochrome, 2-p-toluidinylnaphthalene-6-sulfonate (TNS) with microsomes was changed by F^- plus Mg^{2+} or ATP. The fluorescence of TNS was rapidly increased by NaF and MgCl_2 and was seen to reverse to the original level with addition of ATP. The changes in fluorescence by NaF plus MgCl_2 or ATP may be attributed to reversible changes in the membrane itself from a hydrophilic to a hydrophobic state.

The mechanism of the activation of adenylate cyclase by F^- is unknown. The stimulatory effect of NaF on adenylate cyclase is nonspecific and the enzyme from all mammalian tissues can be stimulated. These observations suggest that NaF acts at a site common to all adenylate cyclases. Johnson and Sutherland (27) reported the following observations. Adenylate cyclase from the rat brain dispersed by detergents exhibited a relatively high specific activity and was inhibited by NaF. When the dispersed enzyme was re-aggregated by removing detergents, the stimulation of the activity by NaF was restored. Harwood and Rodbell (13) recently reported that NaF inhibited the hormonal activation of adenylate cyclase from rat adipocyte at temperatures below 25°C . They suggested that NaF acts at some point on the pathway by which hormonal interaction with the receptor leads to an increase in the catalytic activity of the enzyme. NaF, as reported by Yoshida et al (28), has an inhibitory

effect on Na^+ , K^+ -ATPase which exists in many species of microsomes, and the inhibitory effect of NaF on Na^+ , K^+ -ATPase is irreversible and the activity of Na^+ , K^+ -ATPase is not restored by washing with the buffer medium. These observations as well as the present results on turbidity change support the possibility that NaF may act on some component of membranes common to all species and therefore induce a change in a membranous environment. Adenylate cyclase which exists in a membrane as an integral component may conform to an active form by changes in a membranous environment surrounding the enzyme. The effect of ITP was remarkable on reversing the activity of adenylate cyclase enhanced by NaF and MgCl_2 (Table 4). Recently, many workers have shown that GTP and its analog [Gpp(NH)p] enhance the response of adenylate cyclase to peptide hormones (e.g. glucagon and TSH), catecholamines and prostaglandins in different tissues (29–36). In some of these tissues, nucleotides other than GTP were also shown to have a similar, but less potent effect on adenylate cyclase activity. There are however, considerable differences in the relative potency of various nucleotides depending on tissues and hormones for stimulation. In the case of TSH-stimulated adenylate cyclase of thyroid membrane, ITP was demonstrated to be much more effective than GTP and other nucleotides (33). In contrast, a high concentration (10^{-3} M) of ITP had a potent inhibitory effect on F^- -stimulated adenylate cyclase in this tissue, but GTP had little effect on the enzyme activity with F^- .

There are a few reports (33, 35, 36) concerning the effect of nucleotides on F^- -stimulated adenylate cyclase. The present results on F^- -stimulated adenylate cyclase of chick brain appear to be similar to results found with the thyroid. In studies on adenylate cyclase of the thyroid, however, the effects of nucleotides on the enzyme activity were examined by adding these nucleotides directly to the incubation mixtures for adenylate cyclase assay. In the present studies, the enzyme sample was preincubated with various nucleotides and then thoroughly washed with a large volume of buffer solution to remove the direct effect of these nucleotides on adenylate cyclase assay. Therefore, the effect of ITP in the present studies may be due to a different mechanism from that involved in the case of the thyroid.

The effect of ITP on F^- -stimulated adenylate cyclase was observed only at high concentrations (Fig. 10). This effect of ITP however, was not caused by removal of metal ions from the sample due to chelating action of ITP on Mg^{2+} , Mn^{2+} or Ca^{2+} which did have some effect on the enzyme activity, because the stability constants of complexes of ITP with these metal ions are almost the same as those of ATP and AMP with these ions.

The reversal of turbidity by ITP was a rapid process, preceded to the decline in the enzyme activity elevated by F^- . With respect to the reversing effect of ITP on both adenylate cyclase activity and membrane turbidity, both dose response curves were quite similar (Fig. 10). These observations support the possibility that ITP induces a reversal of structure change of the membrane due to F^- and consequently may produce the activity change of adenylate cyclase. The reversal of the enzyme activity was observed, however, only with ITP among the various nucleotides which had the same potency on reversal of membrane turbidity (Table 4). This discrepancy remains to be elucidated. One possible explanation is as follows. The effects of nucleotides on membrane turbidity were determined only in

the presence of nucleotides while adenylate cyclase was assayed after extensive washing of the samples which had been incubated with nucleotides. Nucleotides other than ITP may be washed away and only ITP may remain bound to the membrane preserving the membrane in a certain state. Other components in membranes may also be lost by extensive washing. This would result in an instability and unresponsiveness to re-exposure to F^- of the enzyme, of which activity was reversed by incubation with ITP.

We have not yet determined whether the effect of ITP on the enzyme activity and membrane turbidity may be accompanied by phosphorylation of some components in the membrane. However, IDP and ADP were also partially effective for the enzyme activity and membrane turbidity, respectively. Thus interaction of the nucleotide with the membrane appears to be an important process to induce its effects. Recently Najjar and his co-workers (14–16) reported that adenylate cyclase existed in an inhibited phospho- and activated dephospho-form, and a phospho-form was dephosphorylated by a membrane phospho-protein phosphatase which was stimulated by F^- , and that the dephospho-form was phosphorylated by a protein kinase. If we consider the present results in light of this hypothesis, there is the possibility that F^- or ITP might induce a change in a membrane structure so that a membrane-phosphoprotein phosphatase or -protein kinase might act on phospho- or dephospho-residues of adenylate cyclase macromolecules.

To elucidate the mechanism of activity change of adenylate cyclase induced by F^- and ITP and its relation to the change in turbidity, further studies are underway.

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