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STUDIES ON FLUORIDE-PREACTIVATED RABBIT LIVER ADENYLYL CYCLASE

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Summary

Rabbit liver adenylyl (ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1) cyclase was stimulated by preincubation with F^- and Mg^{2+} and the stimulation persisted despite extensive washing and/or detergent solubilization. Optimum preactivation conditions were found to be 4 mM NaF and 2 mM $MgCl_2$; higher or lower concentrations produced submaximum stimulation regardless of preincubation time. In addition to an enhanced catalytic activity, the activated enzyme also exhibited different responses to Ca^{2+} and Cu^{2+} when compared to the basal enzyme. ATP caused a time-dependent inhibition that could be partially prevented or reversed by F^-, but was not completely reversed by washing. This inhibition was not observed when 5′-adenosine(β,γ-imide) triphosphate was the substrate and, furthermore, 5′-adenosine(β,γ-imide) triphosphate blocks inhibition by ATP. The results support, but do not prove, the proposed molecular basis of F^- activation which entails a phosphorylation-dephosphorylation mechanism.

The stimulation of adenylyl cyclase (ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1) by F^- has occupied the attention of numerous investigators since its early recognition by Sutherland and his colleagues [1]. A study of the activation effected by this ion might provide a useful approach to elucidating the mechanism of regulation of adenylyl cyclase.

Hormones require specific receptors presumably on the outer surface of the plasma membrane that have now been clearly separated from the adenylyl cyclase [2,3] and such receptors provide the basis for hormonal specificity. On the other hand, F^- cannot be said to have specific receptor sites and with few exceptions [4] exerts no obvious stimulatory effect on the enzyme when added to intact cells. However, in isolated membrane preparations it usually effects a large stimulation. It is to be assumed, therefore, that F^- activation is exerted

directly on the enzyme molecule or indirectly on a vicinal effector molecule on the internal side of the plasma membrane. In this event, a study of this activation process might provide some insight into the reaction mechanism.

Recently, through a study of the interaction of the phosphorylated form of phosphoglucomutase with nucleophilic reagents, it was observed that phosphate was readily removed from the enzyme through the action of several nuclophiles which nevertheless yielded a fully active dephosphoenzyme [5]. Among these reagents, F^- proved to be highly effective and yielded readily isolable phosphorofluoridate as well as some inorganic phosphate. This novel reaction of F^- with a phosphoenzyme is unlikely to remain an isolated instance. In view of this, the possibility that such a biological effect might hold the clue to the activation of the cyclase was judged reasonable. To this end, a series of studies was performed which indicated that F^- caused permanent activation of this enzyme, which was maintained after thorough removal of the ion by washing. The enzyme did not revert to its original state upon incubation. However, prompt reversal to an F^--activatable state was realized upon incubation in a phosphorylating system containing Mg and ATP [6,7]. It was also shown that during this incubation the cell membranes were phosphorylated by (γ-^{32}P) ATP presumably through membrane-bound protein kinases. Furthermore, some radioactive phosphate was immediately and invariably released upon the addition of F^-. These results were shown in membrane preparations from polymorphonuclear granulocytes and platelets. On this basis it was proposed that adenylyl cyclase exists in two forms: an inhibited phosphorylated form and a stimulated dephosphorylated form [6,7].

This communication reports additional work extending and further conforming these studies. We note that the F^--activated enzyme from rabbit liver is permanently altered in several properties and that the reversal of this activation requires a transferable phosphate. The kinetic parameters typical of adenylyl cyclase can also be explained through possible phosphorylation by ATP which is at once a substrate for the cyclase as well as for the membrane protein kinase.

Materials and Methods

Neutral alumina, creatine phosphate, creatine phosphokinase, theophylline, Lubrol PX, and ATP were purchased from Sigma Chemical Co., St. Louis Mo. 5′-adenosine(β,γ-imido) triphosphate (adenosine imidotriphosphate) was obtained from PL Laboratories, Milwaukee, Wisc. cyclic [^{3}H]AMP, [^{3}H]adenosine imidotriphosphate, and [α-^{32}P]ATP were from New England Nuclear, Boston, Mass. When a particular preparation of [α-^{32}P]ATP gave unacceptably high blanks in the alumina column procedure (see below) it was purified on a column of QAE-Sephadex (Pharmacia Fine Chemicals, Piscataway, N.J.) as described by Short et al. [8].

Mature rabbits were killed by exsanguination and the livers quickly excised. They were freed of connective tissue, cut into 5—10-g pieces and frozen at —70°C until used. Alternatively, frozen livers (obtained from Pel Freeze Biologicals, Rogers, Ark.) were used which had no detectable difference in adenylyl cyclase properties.

Preparation of particulate fraction. All steps were carried out at 4°C. After thawing, the liver sample was minced with scissors under 4 vols. (w/v) cold 0.05 M Tris/HCl buffer (pH 7.5). It was then homogenized with 6 up-and-down strokes in a glass/Teflon homogenizer driven at 1000 rev./min, and filtered through 4 layers of cheesecloth. The suspension was centrifuged at 14000 × *g* for 15 min and the supernatant solution removed. The precipitate was resuspended in 8 vols. (w/v) buffer with homogenization and recentrifuged. This washing procedure was repeated twice more. The final precipitate was resuspended in 2 vols. buffer and used on the same day it was prepared, because it was found that frozen/thawed samples gave variable results in many of the experiments. The addition of sucrose, dithiothreitol, or ethylene diamine tetraacetic acid to the homogenization medium produced little or no change in the properties of adenylylate cyclase.

Preparation of detergent-dispersed enzyme. The particulate fraction was washed with 9 vols. cold distilled water. It was then adjusted to contain 0.5% (w/v) Lubrol PX, 5 mM Tris, and approx. 20 mg/ml protein. Use of higher concentrations of Tris or Lubrol resulted in decreased levels of dispersed cyclase. The mixture was homogenized, left on ice for 10 min and homogenized again. It was then centrifuged at 105000 × *g* for 1 h; the supernatant contained virtually all of the cyclase activity.

Preactivation of the enzyme. Unless otherwise noted, preactivation was carried out under the "standard conditions" described below. The particulate suspension obtained above was adjusted to contain 4 mM NaF and 2 mM $MgCl_2$ and incubated at 37°C for 25 min. 20 vols. cold Tris buffer was then added, stirred and centrifuged at 14000 × *g* for 15 min. The supernatant solution was discarded and the precipitate washed 3 more times using 20 vols. cold buffer. The final precipitate was resuspended in 2 vols. buffer. For purposes of comparison with F^--preactivated enzyme, "basal" activity is defined as that obtained following the same preincubation and washing procedure but in the absence of F^-.

Enzyme assay. The activity of adenyl cyclase was assayed at 37°C in a total volume of 110 μl containing 50 mM Tris/HCl (pH 7.5), 4 mM $MgCl_2$, 30 mM theophylline, 0.30 mg particulate or detergent-dispersed protein, either 1.0 mM [α-^{32}P]ATP (20—40 cpm/pmol) or 2.0 mM adenosine imidotriphosphate, and a regenerating system consisting of 20 mM creatine phosphate and 8 units creatine kinase.

Reactions containing [α-^{32}P]ATP were stopped by adding 0.90 ml 95% ethanol/10 mM Tris · HCl (pH 7.5)/0.5 nM cyclic [^{3}H]AMP (40 Ci/mmol). After standing at least 1 h on ice, the assay tubes were centrifuged and the cyclic [^{32}P]AMP content of the supernatant determined by the alumina column method of White and Zinser [9]. Alternatively, cyclic AMP produced from adenosine imidotriphosphate was determined with the protein kinase-binding method of Gilman [10]. In the latter case the reactions were stopped by the addition of 0.90 ml 95% ethanol only.

The efficacy of the ATP regeneration system was checked with thin layer chromatography. In this procedure, assays were terminated with 20 μl 75% (w/v) trichloroacetic acid. The supernatants were recovered by centrifugation and the trichloroacetic acid extracted with diethyl ether. Aliquots were then

chromatographed on PEI-cellulose plates (Baker-Flex, Arthur H. Thomas Co., Philadelphia, Pa.) in 1 M LiCl [11]. It was found that after a 30-min incubation, at least 85% of the [α-^{32}P]ATP was still present. When [^{3}H]adenosine imidotriphosphate was used in the same procedure, results showed that neither particulate nor detergent-dispersed preparations hydrolyzed adenosine imidotriphosphate to any appreciable extent.

Protein concentrations were determined by the method of Lowry et al. [12], using crystalline bovine serum albumin (Pentex, Kankakee, Ill.) as standard. Particulate preparations were first dissolved in 1% (w/v) sodium dodecyl sulfate.

Results

The activating effect of F^-

Kinetics of preactivation. In order to study the characteristics of the stable activated state of the F^--activated adenylyl cyclase, it was necessary to determine the F^- concentration required for full activation, in the absence of the substrate ATP, and compare this with activation in the presence of ATP. We shall henceforth refer to the former as preactivation and to the enzyme as preactivated enzyme. This process of F^--preactivation is therefore considered apart from that in which the native unaltered enzyme is exposed to F^- during the catalytic reaction in the presence of ATP.

Samples of liver membranes were preincubated at 37°C for 25 min in different F^- concentrations as indicated, with $MgCl_2$ and Tris/HCl buffer (pH 7.5) (Fig. 1). These were then diluted with cold buffer, washed and the catalytic activity assayed under standard conditions and in the absence of F^-. It is

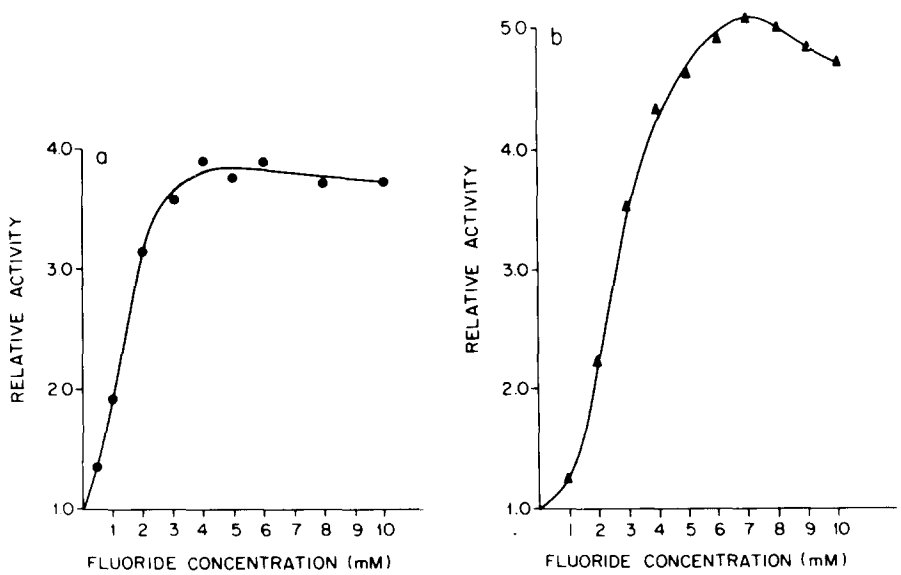

Fig. 1. Effect of various F^- concentrations on the activity of adenyl cyclase. a. F^- was added to the preactivation mixture, then incubated and washed as described in Materials and Methods. b. The enzyme was not preactivated; instead, F^- was added to the assay mixture. Assay time was 10 min in both cases.

apparent that maximal preactivation occurred with 4 mM F^-. Higher concentrations did not increase the level of preactivation.

For the purpose of comparison, Fig. 1b shows data on the requirement of F^- for activation of the native enzyme in the presence of ATP substrate. The presence of F^- in the assay mixture yielded a pronounced sigmoidal curve at increasing F^- concentration. In contrast to that found for preactivation, a higher concentration of F^- (approx. 7 mM compared to 4 mM) was required for maximum stimulation. It is to be stressed that the only difference between the conditions for F^--activation in the two sets of experiments was the presence or absence of ATP.

It was further found, unexpectedly, that despite prolonged preincubation of cyclase preparations with suboptimal concentrations of fluoride, the enzyme never attained maximal stimulation. At 1 mM F^-, activation proceeds slowly and is followed by a decline in activity at a rapid rate (Fig. 2). The nature of this irreversible inactivation of the liver membrane that occurs at low but not at high F^- concentrations is unusual and had not been observed before. This decline in activity seen at 1 mM F^- may explain the sigmoidal nature of the curve shown in Fig. 1b. Thus, low F^- concentrations stimulate the enzyme and inactivate. The latter effect appears to be minimized or overcome at higher F^- concentrations.

The Mg^{2+} concentration most effective in preactivation (with 4 mM F^-) was found to be 2 mM (data not shown). Higher or lower concentrations yielded lower activities. F^- without added $MgCl_2$ did cause definite preactivation but

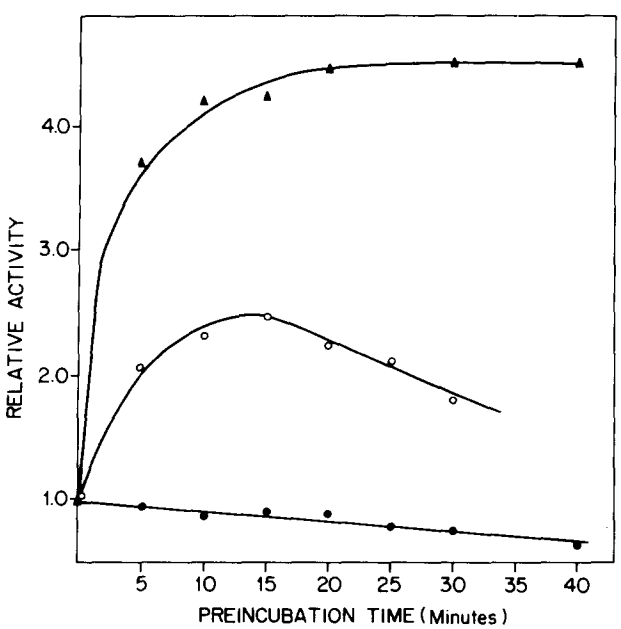

Fig. 2. Effect of preincubation time on the activity of adenylyl cyclase. The mixtures were adjusted to contain either 4 mM NaF (▲———▲) 1 mM NaF (○———○), or no NaF (●———●), and incubated at 37°C for the indicated lengths of time. They were then centrifuged, washed, and assayed as described in Materials and Methods.

at approximately half the maximum level. In view of these results, we elected henceforth to use optimal preactivation concentrations of 2 mM $MgCl_2$ and 4 mM NaF.

We have observed that the stability of both basal and preactivated cyclase is comparable. Thus, at 37°C and in the absence of ATP, both preactivated and native enzymes lose activity at the same rate (approx. 1% per min). This decrease is linear up to 40 min.

Kinetics of catalysis with ATP and adenosine imidotriphoshate. Fig. 3 depicts the kinetics of catalysis of the preactivated cyclase with ATP as substrate as well as with the non-phosphorylating analog adenosine imidotriphosphate. Both reactions were carried out in the presence and absence of F^-. In the absence of F^- in the reaction mixture, there was a progressive decrease of activity with ATP as substrate, but not with adenosine imidotriphosphate. This effect is partially prevented or reversed by including F^- in the assay, an addition that has virtually no effect on the reaction with adenosine imidotriphosphate. It is to be emphasized that fully preactivated cyclase was used for these experiments.

The kinetics depicted in Fig. 3 could be explained by a time-dependent inhibition of cyclase by ATP, a process not apparent when the non-phosphorylating analog, adenosine imidotriphosphate, is used as the substrate. This con-

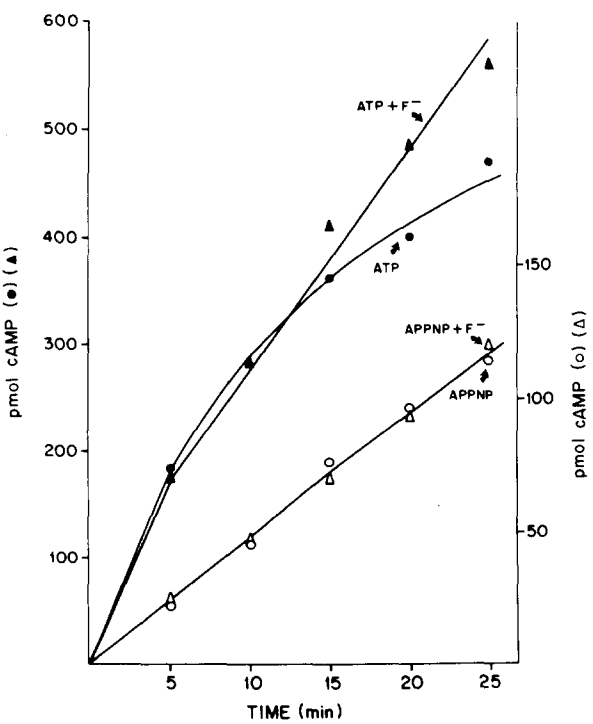

Fig. 3. Time course of the adenylylate cyclase reaction using preactivated enzyme. Assays were conducted with [α-^{32}P]ATP as substrate in the absence, (●———●) or presence (▲———▲) of 7 mM NaF. Alternatively, adenosine imidotriphosphate was the substrate in the absence (○———○) or presence (△———△) of 7 mM NaF. Each point was determined in triplicate. Results obtained in the absence of F^- were corrected for loss of enzymatic activity due to denaturation. cAMP, cyclic adenosine 3′,5′-monophosphate.

tention is based on several observations. First, the decrease in rate with time cannot be due to spontaneous enzyme inactivation since rates have been corrected for the loss of activity with time due to denaturation in the absence of F^-. Despite this correction, the progressive inhibition by ATP and the lack of inhibition by adenosine imidotriphosphate is consistent and highly reproducible. Second, the inhibition is not due to destruction of the substrate, since thin-layer chromatography of the assay mixture indicated that the regenerating system maintained the level of ATP at or near the original concentration for at least 30 min. Third, the addition of cyclic AMP and/or pyrophosphate to the assay mixture, at concentrations up to 10 times that produced by the cyclase reaction, has no effect on the observed catalytic rate. Fourth, there is no indication that a soluble inhibitor is produced, since after 20 min the membranes may be removed from the assay mixture by centrifugation and the supernatant used for a subsequent assay, after the addition of fresh cyclase. The kinetics so obtained are identical to those obtained with a fresh reaction mixture.

The time-dependent inhibition of cyclase by ATP is definitely less in the presence of F^- than in its absence. This suggests that F^- may effect a reversal of this inhibition. This possibility was further investigated by adding F^- to an assay of prestimulated cyclase after the rate had noticeably decreased (data not shown). This results in an immediate increase in the reaction rate. Thus, the decrease in rate is indeed reversed by F^- to a considerable extent. In addition there is, even in the presence of fluoride, another definite and reproducible inhibition when ATP is the substrate. This particular inhibtiion is limited to the initial period of approx. 5 min (Fig. 3). After that, the rate assumes the linear characteristic of a stabilized equilibrium state between that of F^--activation and ATP inhibition. In contrast, the rates with adenosine imidotriphosphate as substrate are linear and virtually identical in the presence or absence of F^-.

The effect of detergents on F^- activation. Detergent-dispersed preparations of cyclase maintain the same characteristics as the native membrane-bound enzyme with respect to their susceptibility to activation by F^-. This applies to preparations solublized in either Triton or Lubrol. Fig. 4 presents data obtained with Lubrol-dispersed liver membranes. One sample contained cyclase that was preactivated with F^- and washed before detergent treatment. The other contained basal cyclase and was not activated before detergent solublization. It is apparent that the basal enzyme which had been dispersed in detergent can still be activated by F^-, albeit to a lesser extent. However, when the enzyme is first preactivated and subsequently treated with detergent, it is not stimulated further by F^-. On the contrary, the addition of F^- served only to inhibit the enzyme to a significant extent. This is possibly the result of the formation of $Mg^{2+} \cdot$ fluorophosphate inhibitory complex [13—15]. Detergent-dispersed cyclase shows a reduction in the catalytic rate with time. This decrease in rate is due entirely to denaturation.

The reversal of the preactivated state by ATP. It was previously reported that the fluoride activation of cyclase in membrane preparations from granulocytes and platelets can be reversed by incubation with ATP. The activity of the enzyme which is suppressed by ATP can again be stimulated by a subsequent incubation with F^- [4,6].

A similar sequence of events can be reproduced with rabbit liver cyclase

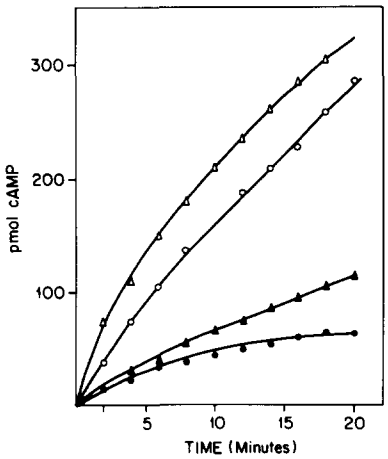

Fig. 4. Activity of detergent-dispersed adenylylate cyclase. Preactivated cyclase was extracted and assayed in the absence (△———△) or presence (○———○) of 7 mM NaF. By comparison, basal cyclase was extracted and assayed in the absence (●———●) or presence (▲———▲) of 7 mM NaF. cAMP, cyclic adenosine 3′,5′-monophosphate.

(Table I). Samples of membrane-bound, preactivated cyclase were incubated with or without ATP or other additions as shown, then washed and assayed. It is seen that ATP effects a considerable though incomplete inhibition of cyclase activity. Under these conditions, maximal inhibition of the preactivated enzyme was produced with 2 mM ATP. Lower concentrations produced less inhibition, while higher concentrations yielded no further increase in inhibition. The enzyme so inhibited can be stimulated with F^-.

The extent of inhibition afforded by preincubation with ATP was somewhat variable and never exceeded 40—50%. This incomplete inhibition is possibly due to disturbance of the structural relationships in the membrane preparation resulting in limited interaction of the ATP with the enzyme(s). It might also be due to a partial reversal of the inhibition by membrane-bound phosphoprotein phosphatases, if indeed phosphorylation is involved. In constrast to the inhibition caused by ATP, preincubation of the membranes with adenosine imidotriphosphate has no effect on the cyclase activity. Presumably, adenosine imidotriphosphate cannot participate in a phosphorylation reaction. Furthermore, as

TABLE I

Representative data on the effect of preincubation with ATP and/or adenosine imidotriphosphate on cyclase activity. Samples contained 10 mg/ml particulate protein and were preincubated at 37°C for 25 min in the presence of the ATP-regenerating system. They were then washed, resuspended, and assayed for 10 min as described in Materials and Methods. Results are the average of at least three separate experiments.

Additions to preincubation mixture	Relative specific activity
—	1.00
1 mM ATP	0.59
2 mM adenosine imidotriphosphate	0.97
1 mM ATP + 2 mM adenosine imidotriphosphate	0.93

the table shows the addition of adenosine imidotriphosphate along with ATP prevents the inhibition caused by the latter.

When membrane preparations containing native instead of prestimulated cyclase were subjected to similar treatment with ATP or adenosine imidotriphosphate, ATP inhibited the basal activity of the native cyclase to about the same extent as the preactivated enzyme. Similarly, adenosine imidotriphosphate did not inhibit basal activity.

In addition to an inhibition of cyclase activity, it was also found that ATP diminishes the extent to which F^- stimulates the basal activity of the native enzyme (Table II). The presence of ATP in an incubation mixture containing F^- results in the diminution of F^- stimulation. By contrast, adenosine imidotriphosphate exerts no such inhibitory effect. However, the analog cannot block ATP inhibition of F^--stimulation.

Comparative effects of basal and F^--stimulated activity. Preincubation with ATP, as mentioned above, inhibits both the basal cyclase and preactivated cyclase to the same extent. By contrast, Ca^{2+} and Cu^{2+} exert different effects on the catalytic activity of both types of cyclase, native and preactivated. Fig. 5 shows the different response of the two enzyme preparations to increasing concentrations of Ca^{2+}. The preparation that had been preactivated with fluoride shows increased inhibition with increasing concentrations Ca^{2+} in the reaction mixture. However, the basal activity under the same conditions show first a stimulation followed by inhibition as the Ca^{2+} concentration is increased.

Differences in the activity of cyclase occasioned by the addition of calcium to the reaction mixture with and without fluoride have been previously reported [16,17]. However in the present study, it was found that calcium had no effect on the extent of F^--activation when it was included in the preactivation medium in the absence of substrate. Thus, preincubation of cyclase with F^-, Mg^{2+}. and various concentrations of Ca^{2+} (up to 0.26 mM) produced the same degree of stimulation as did preincubation with F^- and Mg^{2+} alone. This was true even when suboptimal concentrations of F^- and Mg^{2+} were used. These data indicate that calcium inhibits the F^--activated form of cyclase but not the mechanism of F^--activation.

In this connection, detergent-dispersed preparations behaved differently. It was found that Ca^{2+} inhibited the activities of both preactivated and native enzyme to the same extent. In fact, in terms of percent activity vs. Ca^{2+} concentration, superimposable curves were obtained for both forms of enzyme,

TABLE II

Representative data on the effect of ATP and adenosine imidotriphosphate on preactivation by F^-. Preincubation and assay conditions were the same as in the legend for Table I. Results are the average of at least three separate experiments.

Additions to preincubation mixture	Relative specific activity
—	1.00
2 mM NaF	3.31
2 mM NaF + 1 mM ATP	2.23
2 mM NaF + 2 mM adenosine imidotriphosphate	3.22
2 mM NaF + 1 mM ATP + 2 mM adenosine imidotriphosphate	2.16

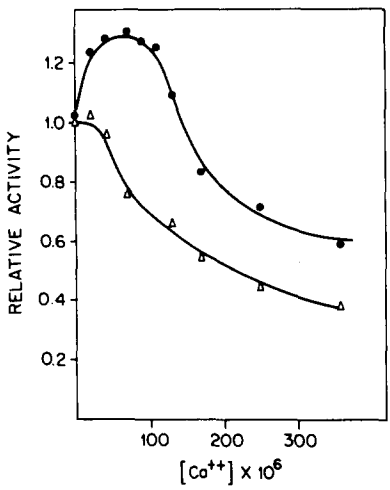

Fig. 5. Relative activities of basal (●———●) and preactivated cyclases (△———△) with increasing concentrations of calcium. Samples were assayed for 10 min.

identical to that depicted in Fig. 5 for preactivated membrane-bound enzyme.

The effects of adding Cu^{2+} to the assay mixture are also different with respect to the two states of the membrane-bound enzyme (Fig. 6). Both the activated and non-activated preparations were increasingly inhibited as the

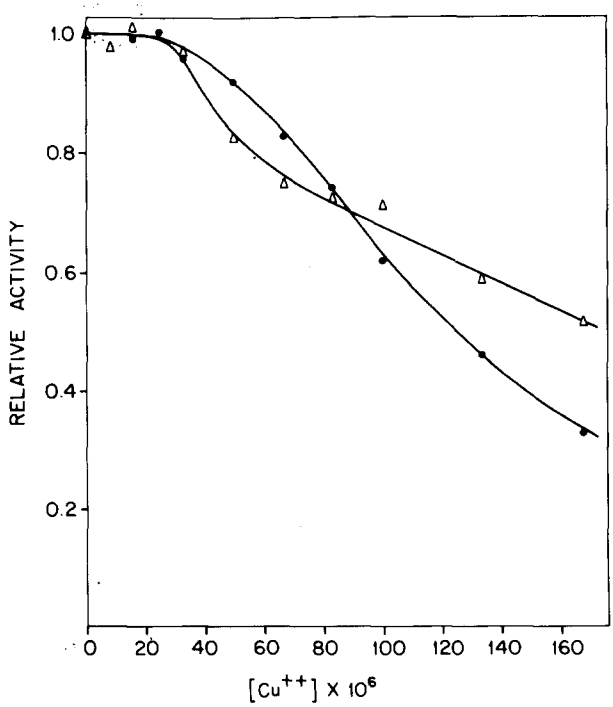

Fig. 6. Relative activities of basal (●———●) and preactivated cyclase (△———△) with increasing concentrations of copper. Samples were assayed for 10 min.

concentration of Cu^{2+} in the assay was raised. However, the pattern of inhibition was reproducibly different in the two cases.

Discussion

The F^--activated adenylate cyclase is an altered enzyme with newly acquired properties and an activity that is considerably higher than that of the basal enzyme. This activated state is stable and is not readily reversed by dialysis or extensive washing of the membrane [6,18—20]. It may be argued that simple ionic binding may effect a change in conformation of the enzyme to produce an activated state. However, such non-covalent binding of the halide should be subject to equilibrium effects. Considerable reduction in the F^- concentration by washing of the membrane preparations should result in a parallel reduction in the amount of F^- bound to the enzyme. Consequently, a high degree of reversion to the native conformation of the enzyme would occur resulting in a lower level of catalysis approaching the basal activity. At the same time, other newly acquired properties (see below) would also revert. However, this situation does not occur. On this basis, we consider the activating effect of F^- to be the result of a dephosphorylation of the cyclase or an associated regulatory molecule. This is modeled after the dephosphorylation of phosphoglucomutase by F^- [5].

These studies do not prove that a phosphorylation-dephosphorylation mechanism is operative in regulating the activity of adenylyl cyclase. However, our results are in accord with this notion for several reasons:

(a) Liver adenylyl cyclase is stimulated by preincubation with F^- and the stimulation persists despite extensive washing and/or detergent solublization. This activated state differs in several properties from the non-activated state. For example, increasing concentrations of Ca^{2+} first stimulate, then inhibit the basal cyclase. However, in the same range of concentrations the preactivated enzyme is only inhibited. Also, copper ions inhibit both basal and activated enzyme preparations. However, the kinetics of inhibition are reproducibly different.

(b) There is a time-dependent inhibition of adenylyl cyclase by ATP (Fig. 3). This inhibition is only partially reversed by washing. F^- prevents or reverses this inhibition, though not completely (Table II).

(c) If there is a time-dependent inhibition of cyclase by ATP that is the result of a phosphorylation event, then this inhibition would not be observed if adenosine imidotriphosphate were used as substrate instead of ATP. Furthermore if, as noted in Fig. 3, the lower rate in the absence of F^- is due to inhibition by the ATP substrate, then assays of preactivated cyclase with adenosine imidotriphosphate substrate should show identical rates in the presence and absence of F^-. In Fig. 3, the rate of cyclic AMP production from adenosine imidotriphosphate after correction for denaturation, is indeed linear and is virtually unaffected by the presence of F^-.

(d) Unlike ATP, preincubation with adenosine imidotriphosphate followed by washing produces no significant inhibition of cyclase. In fact, adenosine imidotriphosphate can block ATP inhibition of cyclase in this type of experiment (Table I). The imido analog does not contain a transferable phosphate

and could not inhibit the enzyme if the inhibited state entails phosphorylation.

(e) On the premise that the effect of ATP can be translated into time-dependent phosphorylation (inhibition) and that of F^- as time-dependent dephosphorylation (activation), then the presence of both agents in a reaction mixture containing preactivated cyclase would be reflected in altered kinetics of the reaction. Since the enzyme is fully preactivated, F^- could not activate it further and thereby affect the intial rate. However, ATP would produce a time-dependent increase in phospho-enzyme formation. Initially, therefore, the inhibition is minimal but progressive. This is also clearly evident in Fig. 3. In the presence of F^-, minimal inhibition occurs during the first 10 min or so, to be followed by straight line kinetics. This attained steady state represents an equilibrium concentration of presumably active dephosphorylated enzymes and inactive phosphorylated enzyme.

(f) In a similar vein, it was found that the F^- concentration required for maximum stimulation of cyclase is greater in the presence of ATP than in its absence (Fig. 1). This can be readily rationalized in the context of the phosphorylation-dephosphorylation concept. Such a situation would be expected in that a higher F^- concentration would be required to overcome the additional competing phosphorylation (inhibition) reaction occurring in the presence of ATP.

In general, the results are consistent with the proposal that the regulation of adenylate cyclase involves phosphorylation (inhibition) and dephosphorylation (activation) events. Additional support for such a scheme has been found in other laboratories. Ho and Sutherland [21,22] reported that hormonal stimulation of adipocytes generated a substance called "feedback regulator". This substance stimulated the phosphorylation of membrane protein as well as added histone. In addition, it strongly inhibited the activity of adenylyl cyclase. This inhibition was observed with ATP or the phosphorylating analog adenosine-5′-(α,β-methylene)triphosphate. It was less pronounced when both ATP and adenosine imidotriphosphate were added together to the reaction mixture. Adenosine imidotriphosphate alone inhibited only at a very high concentration of the "feedback regulator".

The proposed mechanism would involve either the enzyme itself or an associated regulatory molecule. The enzymes required for such a scheme, the cyclase, protein kinase, and phosphoprotein phosphatase are present in plasma membranes. This scheme represents only a working hypothesis that is consonant with known facts and adequately explains the kinetic behavior of the cyclase reaction. It can be formulated in a highly simplified manner:

$$\text{Phosphorylation: (inhibition)} \quad \underset{\text{ATP, protein kinase(s)}}{\overset{F^-,\ \text{hormones, phosphoprotein, phosphatase(s)}}{\rightleftharpoons}} \quad \text{Dephosporylation: (activation)}$$

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