KINETICS OF ACTIVATION OF THYMOCYTE PLASMA MEMBRANE ADENYLATE CYCLASE BY AMP–PPS AND BY OTHER STIMULATORY AGENTS

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1. Introduction

The properties of the enzyme adenylate cyclase are generally studied in membrane fractions, because of the apparent unstability of the enzyme during the purification steps. Despite this limitation, a number of kinetic features of the enzyme activity are known [1,2]. Adenylate cyclase activity is modulated at least in part by the binding of guanine nucleotides to a regulatory site of the enzyme distinct from the catalytic site. We show here that the commercial adenosine triphosphate analogue adenosine-5′-*O*-thiotriphosphate AMP–PPS activates the adenylate cyclase of purified calf thymocyte plasma membranes. The stimulatory effect produced by this compound is additive to that induced by the GTP analogue GMP–P(NH)P.

Activation of adenylate cyclase by GMP–P(NH)P is known to be a slow, time-dependent process [3–6]. We show here that not only GMP–P(NH)P, but also fluoride, as well as AMP–PPS, activate the calf thymocyte plasma membrane enzyme in a time-dependent, slow process, which is unrelated to a progressive permeabilisation of the closed vesicles to the substrate or to the stimulating agents.

2. Materials and methods

2.1. *Preparation of the lymphocyte membrane fractions*

This was performed as described [7]. The plasma membrane pellets were resuspended in TKM buffer containing 10 mM dithiothreitol (DTT) and 2.5 mM theophyllin. They were used immediately, or stored frozen in liquid nitrogen.

2.2. *Adenylate cyclase assay*

The assay was performed according to [8] at 36°C. Membrane samples were thawed just before the assay. Final concentrations in the assay were, unless indicated: 1 mM ATP (containing $1.2–1.8 \times 10^6$ cpm [α-^{32}P]-ATP); 1 mM cAMP; 1.25 mM theophylline; 20 mM creatine phosphate; 70 units/ml creatine kinase; 5 mM DTT; 1 mM EGTA; 5 mM $MgCl_2$; 12.5 mM KCl; 50 mM Tris–HCl, pH 7.3; 0.3–0.7 mg/ml membrane proteins. The assay volume was 100 μl/time point. The reaction was initiated by the addition of labeled ATP, or when preincubation took place, it was measured from the time of addition of labeled ATP, on. The adenylate cyclase activity was strictly proportional to the amount of proteins added. The specific activity was measured over time intervals of 5 min or 10 min, by substracting from each time-point value the value determined in the same sample at the previous time point. Duplicates agreed within less than 5%. All nucleotides and analogues were from Sigma and from Boehringer. [α-^{32}P]ATP (10–20 Ci/mmol) in aqueous solution was from the Radiochemical Centre, Amersham.

2.3. *Radioimmunoassay*

cAMP was extracted from the samples by addition of boiling ethanol. The cAMP supernatant content was determined by radioimmunoassay as in [9], after succinylation of the dessicated supernatant residue. ^{125}I-Labeled succinyl-tyrosyl-methyl ester of cAMP and anti-succinyl cAMP antibodies were prepared by P. Pradelles, Institut Pasteur.

3. Results

3.1. *AMP–PPS-induced activation of adenylate cyclase*

The ability of adenylate cyclase to use AMP–PPS as a substrate was tested by radioimmunoassay measurement of the cAMP produced by membranes incubated with AMP–PPS (Boehringer) as the sole possible substrate. AMP–PPS was found to be a substrate for adenylate cyclase (table 1). To compare AMP–PPS and ATP in this respect, we determined the specific activity of the cAMP produced in systems in which variable proportions of AMP–PPS and ATP were used, by processing the samples in parallel according to [8] and to the radioimmunoassay method [9]. When ATP was the sole substrate of the reaction, the cAMP specific activity measured was close to that of ATP. (Experimental error: ±18%) (table 1). The cAMP specific radioactivity decreased in inverse ratio to that of the AMP–PPS content in the substrate mixture (table 1). AMP–PPS was thus roughly comparable to ATP as a substrate for adenylate cyclase.

Used at 0.5–2 mM, AMP–PPS enhanced the adenylate cyclase specific activity, as compared to that measured in the presence of equal concentrations of ATP, by a factor of 3–4 (table 2).

Association of AMP–PPS and GMP–P(NH)P in the assay induced an increase in the plateau value of the enzyme specific activity which was about the sum of the increase produced by each component (table 2).

3.2. *Kinetics of activation of adenylate cyclase by the stimulatory agents AMP–PPS, GMP–P(NH)P and NaF*

In basal conditions and except for the first 2–10 min, the production of cAMP was linear for 1–2 h (fig.1). (If, however, the ATP regenerating system was omitted from the incubation medium, the adenylate cyclase specific activity decreased linearly with time, reaching 50% of the control value after 10 min incubation.)

When AMP–PPS was added to the assay mixture, a 20–30 min accelerating phase in enzyme specific activity was observed (fig.1).

A similar phenomenon of slow activation of the

Table 1
Assessment of AMP–PPS as a substrate for adenylate cyclase

	Substrate	cAMP (pm.mg^{-1} min^{-1}) RIA	cAMP (pm.mg^{-1} min^{-1}) Salomon	ATP (cpm.pm^{-1})	cAMP (cpm.pm^{-1})
1	ATP 1.1 mM	9.2	14	13	17
	AMP–PPS 1 mM	57.7	–	–	–
	AMP–PPS 1 mM + ATP 0.1 mM	46	31.3	130	5.5
2	ATP 1 mM	17.6	17.4	13	13.9
	AMP–PPS 1 mM + ATP 2 μM	62.5	62.7	6500	14.1[a]
	AMP–PPS 0.1 mM + ATP 2 μM	8.3	6.5	6500	134

[a] The very low cAMP specific radioactivity measured in this case may be due to uncertainty in ATP concentration if, indeed, AMP–PPS (1 mM) is contaminated to some extent by ATP, the ATP concentration becomes higher than 2 μM, and its specific radioactivity is consequently lower

Membranes were incubated for 20–40 min at 36°C in the assay mixture described in section 2, with the following modifications: 12.5 nM cAMP; 0.2 mM isobutylmethylxanthine (Aldrich), (expt. 1); or 5 mM theophyllin (expt. 2); and the substrates indicated in the first column. Production of cAMP, in pmol mg^{-1}. min^{-1}, was determined by radioimunnoassay (second column in this case, no labeled ATP in the assay) and by the method [8] (third column; in this case, radioactive ATP, of various specific radioactivities, indicated in the fourth column, was used). Aliquots, 100 μl, were withdrawn in each case at 10 min intervals, and the specific radioactivity of the cAMP produced was determined, and indicated in the fifth column

Table 2
Effects of GMP–P(NH)P and/or AMP–PPS on adenylate cyclase

ATP	AMP–PPS	GMP–P(NH)P	Adenylate cyclase spec. act. (pm.mg^{-1}. min^{-1})
0.6 mM	–	–	26
1.1 mM	–	–	36
2.1 mM	–	–	34.5
0.1 mM	0.5 mM	–	85
0.1 mM	1.0 mM	–	139
0.1 mM	2.0 mM	–	200
1.0 mM	–	0.001 mM	271
1.0 mM	–	0.010 mM	290
1.0 mM	–	0.1 mM	264
0.1 mM	0.5 mM	0.1 mM	316
0.1 mM	1.0 mM	0.1 mM	406
0.1 mM	2.0 mM	0.1 mM	454

Membranes were incubated for 20–50 min at 36°C in the assay mixture containing the nucleotides ATP, AMP–PPS and/or GMP–P(NH)P at the concentrations indicated in the first three columns. The adenylate cyclase specific activity values, (pmol cAMP. mg^{-1}. min^{-1}) were the plateau values (determined after 40–50 min incubation). The concentration of AMP–PPS was taken into account for the calculation of the enzyme specific activity

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enzyme was observed in the presence of NaF. Adenylate cyclase was stimulated up to 10-fold by the addition of 10 mM NaF, higher concentrations of fluoride progressively leading to a decrease in specific activity. Omission of the ATP regenerating system did not affect the fluoride-stimulated, adenylate cyclase apparent specific activity, for at least 1 h. The specific activity of adenylate cyclase sharply increased upon addition of NaF to the assay mixture until a plateau value was reached within 30–40 min (fig.1). As a way to increase the permeability of the membrane vesicles to the components of the assay mixture, membranes were submitted to the action of sodium deoxycholate [10]. To membranes submitted to 0.03% DOC for 20 min in a preincubation medium containing ATP, fluoride and tracer labeled ATP were added together at the onset of incubation. A same 30 min accelerating phase of the enzyme specific activity was still observed, the plateau value being 70% that reached in membranes preincubated without DOC, and then treated with fluoride (table 3). Exactly the same accelerating phase and plateau value of the enzyme specific activity (70% control) were observed when preincubation was omitted, fluoride, DOC and ATP being added together. By analogy with DOC, we studied the effect of alamethicin, a channel forming ionophore (Upjohn Co.) [11], on the kinetics of adenylate cyclase activation by fluoride. Alamethicin, 0.1 μg/μg membrane protein, was added to membranes, either in preincubation for 20 min with ATP, fluoride being added at the onset of incubation, or to non-preincubated membranes, together with ATP and fluoride. In both cases, the alamethicin and fluoride-treated membranes displayed a much higher adenylate cyclase activity than

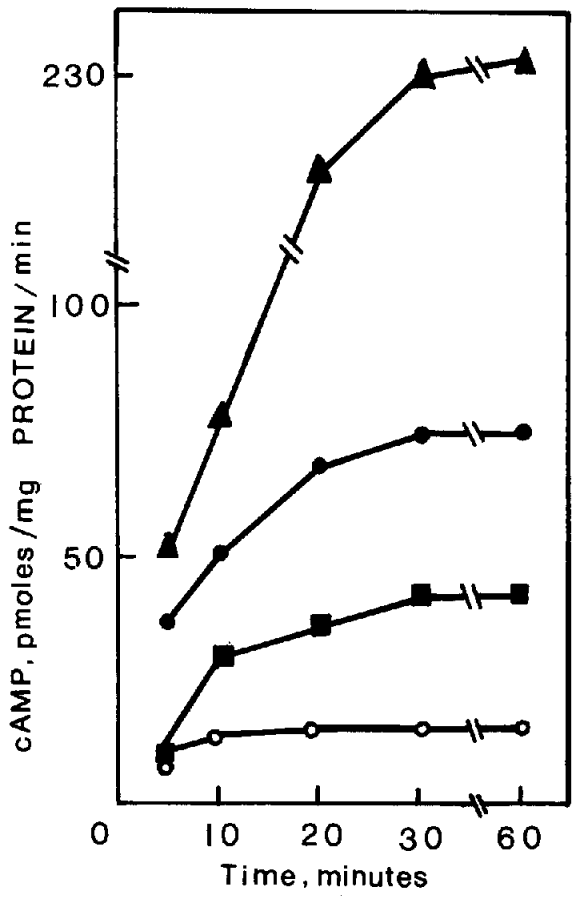

Fig.1. Membranes were incubated at 36°C in an assay medium containing 1 mM ATP and the other components listed in Section 2, without any other addition (○——○), in the presence of 1 mM AMP–PPS (■——■), 10 mM NaF (●——●) or 10^{-5}M GMP–P(NH)P (▲——▲).

Table 3
Effect of DOC or of alamethicin on fluoride stimulated adenylate cyclase specific activity

	Spec. act. (pm. mg^{-1}. min^{-1})
Membranes not treated with fluoride	28
Membranes treated with 10 mM NaF	180
Membranes treated with NaF 10 mM and 0.03% DOC	126
Membranes treated with NaF 10 mM and alamethicin	486

Membranes were incubated for 20–50 min at 37°C in the assay mixture described in section 2, without addition, or with 10 mM NaF, 0.03% DOC, or alamethicin (0.1 μg/μg membrane protein). The adenylate cyclase specific activities refer to the plateau values (determined after 40–50 min incubation)

control membranes (alcohol and fluoride treated) (table 3), but exactly the same 30 min accelerating phase in enzyme specific activity was observed in alamethicin, and control, fluoride treated membranes.

In a time-dependent manner very similar to the fluoride-induced activation, GMP–P(NH)P stimulated adenylate cyclase. The specific activity of the enzyme sharply increased for 30–60 min or even longer (fig.1). The plateau value reached was studied as a function of GMP–P(NH)P concentration (table 2). The accelerating phase was unchanged over the whole range of concentrations studied.

4. Discussion

To our knowledge, the enhancement of adenylate cyclase specific activity by AMP–PPS has not yet been reported. We have shown that this thiotriphosphate analogue of ATP is indeed a substrate for adenylate cyclase. It also works as a stimulating agent. The question may be raised of a possible contamination of the compound by GMP–PPS, which has been shown to activate turkey erythrocyte adenylate cyclase, in the presence of hormone [12]. Indeed, adenosine triphosphate analogues are produced by modification of yeast AMP (Boehringer), and unless this compound is submitted to several chromatographies, it is usually contaminated by traces of GMP. Further work is in progress to clarify this point. By analogy, we have found that some batches of AMP–P(NH)P induced a marked increase in adenylate cyclase specific activity (unpublished results). In such cases, a contamination of the AMP–P(NH)P batch by less than 0.1% of GMP–P(NH)P would be sufficient to explain the stimulatory effect. Such an effect was not additive with that obtained with GMP–P(NH)P. On the contrary, in the case of AMP–PPS adenylate cyclase stimulation, we have found that the AMP–PPS and the GMP–P(NH)P-induced effects were additive. If the active compound in the AMP–PPS batch was to be GMP–PPS, this observation would suggest that GMP–PPS and GMP–P(NH)P, although being probably bound by the same subunit, have different and cooperative effects on the enzyme – if, indeed, their target is the enzyme. However, the active compound may actually be AMP–PPS, binding to the catalytic site or any adenosine nucleotide binding site. In this case, the additivity of the effects of AMP–PPS and GMP–P(NH)P would not be a problem 'per se'. Although a dephosphorylation of the enzyme has been proposed as a mechanism of activation [13], it is tempting to speculate, in the case of AMP–PPS, on a thiophosphorylation of the enzyme, which would in some way mimic a very stable phosphorylation. The conditions chosen for the adenylate cyclase assay were indeed conditions for which some membrane proteins of our thymocyte plasma membranes were phosphorylated (unpublished results). Phosphorylation, or thiophosphorylation, of adenylate cyclase is thus not excluded.

As long as the mechanism by which AMP–PPS activates adenylate cyclase remains unknown, it is difficult to speculate on the 'lag' phase observed upon establishment of the activated state. However, very similar kinetics of activation were observed when

GMP–P(NH)P or fluoride were used as stimulating agents. A progressive permeabilisation of the closed vesicles to the substrate and stimulatory compounds was first considered. In the case studied here, the membrane fragments were completely closed vesicles [7], and their polarity was mostly right-side out, as plasma membranes ectoenzymes were detected without any latency [7]. The catalytic site of the adenylate cyclase thus faced the inside of the vesicles. If a progressive permeabilisation of the vesicles accounted for the 'lag' phase observed, then an alteration of the vesicles making them freely permeable to the compounds present in the assay should abolish this 'lag' phase. On the contrary, we have shown that this lag phase was not shortened by treatment of membranes with a detergent, or with the channel-forming ionophore, alemethicin. The stimulating agents thus did not provoke an instant increase in enzyme specific activity, even when vesicles were freely permeable to molecules of small size. Such a fact was known for GMP–P(NH)P adenylate cyclase stimulation [3–6] but had not yet, to our knowledge, been reported in the case of fluoride or AMP–PPS activation of the enzyme, or not yet been recognized as such, most probably because the linearity of the reaction with time had not been assessed on a sufficient time range [14,15]. The 'lag' phase observed upon activation of adenylate cyclase by AMP–PPS, GMP–P(NH)P or NaF thus seems to be explained by progressive, physical or chemical, modification(s) of the enzyme at the level of the catalytic site. However, it must be stressed that the enzyme is studied here not as an isolated, purified molecule, but as a component of intact membrane vesicles. This membranous environment may well play a major role on the adenylate cyclase state of activity.

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