THE ACTIVATION OF ADENYLATE CYCLASE: II. THE POSTULATED PRESENCE OF (A) ADENYLATE CYCLASE IN A PHOSPHO (INHIBITED) FORM (B) A DEPHOSPHO (ACTIVATED) FORM WITH A CYCLIC ADENYLATE STIMULATED MEMBRANE PROTEIN KINASE†

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Summary: Membrane adenylate cyclase (AC) from polymorphonuclear (PMN) leucocytes and platelet membranes are activated several fold by fluoride and prostaglandin E_1 (PGE_1) respectively. Incubation of such activated membranes in a phosphorylating system inhibits cyclase activity. The inhibition can now be relieved by further treatment with fluoride and PGE_1 respectively. These findings suggest that AC exists in an inhibited phospho- and activated dephospho-form. This is supported by the finding that membrane preparations from both sources contain a cyclic adenylate (cAMP) stimulated protein kinase and points to the existence of an adequate membrane phosphorylating system.

INTRODUCTION

It has been shown recently, by Layne and Najjar (1), that the fluoride ion reacts with the phosphorylated form of phosphoglucomutase (phospho-PGM) to result in the liberation primarily of phosphofluoridate and a variable but sizable amount of inorganic phosphate. This is presumed to occur through a nucleophilic attack at the phosphoryl function of the serine phosphate residue of the active site of the phosphoenzyme. The resulting dephospho-PGM was fully active. This indicated that the fluoride ion, as would be expected, did not produce any other covalent or noncovalent bond alteration in the protein. Because of this unique effect of the fluoride ion, it

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was postulated (2) that the fluoride effect in the activation of adenylate cyclase ultimately might well result in the removal of a phosphate group. The implication is that a phosphorylated adenylate cyclase (phospho-AC) is in a less active or inhibited state compared to a dephosphorylated adenylate cyclase (dephospho-AC). Inasmuch as the stimulatory activities of fluoride and hormones on the cyclase are similar and not additive (3, 4), it is postulated that both effects are due to a nucleophilic displacement of the phosphoryl group of the presumed phospho-AC (2). If this is the case, then the resulting dephospho-AC could not be activated by further treatment with either fluoride or hormone. It also follows that the activity of a dephospho-AC upon exposure to a phosphorylating system should be inhibited, presumably because of phosphorylation. This inhibition should then be relieved and the enzyme activity regained following treatment with fluoride or hormone.

This communication reports data that are in agreement with this postulate. We have chosen peritoneal PMN granulocytes and blood platelets which offer the advantage of being discrete cells well suited for radioactive labeling. Furthermore, both have soluble cAMP dependent protein kinases (2, 5). We show further that membrane preparations from both cells possess a cAMP stimulated protein kinase that phosphorylates endogenous membrane substrate as well as exogenous added histone. In both cases we have used ATP as the phosphorylating agent.

MATERIALS AND METHODS

PMN granulocytes were obtained from rabbit peritoneal exudates (6) with no more than 3% contamination with erythrocytes. Pure blood platelets were obtained from dog plasma (7). Other reagents are: ATP γ-^{32}P. 17.4 Ci per mmole, inorganic ^{32}P (^{32}Pi) 0.5 Ci per mmole (New England Nuclear) and cAMP-^{3}H 12.8 Ci per mmole (Schwartz-Mann), PGE_1 was a gift of Dr. John E. Pike (Upjohn Co., Kalamazoo) and histone (Sigma). In the preparation of membranes all manipulations were carried out at 0°-4° C. PMN granulocytes were lysed in 0.34 M sucrose (6, 8),

then homogenized in a teflon glass homogenizer in ice using 2-5 strokes and the membranes collected by centrifugation. The supernatant was saved. The membranes were then washed two times, each with 30 volumes of the sucrose solution by centrifugation at 600 g. This was followed by two washings with tris HCl buffer 40 mM containing 1 mM $MgCl_2$, pH 7.2. The saved supernatant was clarified by centrifugation at 16,000 g for 30 minutes and used as the source of protein kinase.

Platelets were frozen and thawed once in the tris buffer, then homogenized with 20 strokes, washed and the membrane sedimented at 16,000 g for 10 minutes. All further washings follow in all details the stipulations above. cAMP was measured according to Gilman (9) and radioactive counting was done in a scintillation counter (Nuclear of Chicago). Protein was measured by the Lowry procedure (10).

RESULTS AND DISCUSSION

The experimental design with all membrane preparations from both cells were as follows: (a) A portion of each membrane preparation was first tested for adenylate cyclase activity in the presence and absence of fluoride 5 mM or PGE_1 3 μM. Only fluoride was used to activate PMN membranes since the AC enzyme in this preparation does not respond to hormone stimulation (8). PGE_1 as well as fluoride were used as activators of platelet membranes since these have been shown to be potent AC activators of such preparations (11). However, only activation by PGE_1 will be reported here. Preparations that showed 4-7 fold synthesis of cAMP after treatment with the activator were used for the activity modulation experiments. For these experiments, the remaining membrane preparation was incubated for 20 minutes at 30° C with the particular activator and washed. It was then reincubated in a membrane self-phosphorylating system using relatively large quantities of the preparation, 2.1-2.8 mg of protein, containing sufficient activity of cAMP stimulated protein kinase. When smaller quantities of membrane preparation, 0.28-1.0 mg, were used for phosphorylation the reaction mixture was supplemented with

Table 1

THE INHIBITION OF ADENYLATE CYCLASE AFTER INCUBATION IN A PHOSPHORYLATION SYSTEM AND ITS REACTIVATION BY FLUORIDE OR PGE_1

	Phosphorylation Reaction			cAMP pmoles/mg/15 min.	
Experiment	Kinase added*	ATP added	Membrane source (mg protein)	Control	Activator
			(PMN)	(-Fluoride)	(+Fluoride)
1	Membrane	-	0.28	280	250
	Cytoplasm	+	0.28	130	245
2	Membrane	-	2.10	100	128
	Cytoplasm	+	2.10	27	115
3	Fractionated cytoplasm	-	2.30	126	142
	Fractionated cytoplasm	+	2.30	57	103
	Cytoplasm	+	2.30	57	131
			(Platelets)	(-PGE_1)	(+PGE_1)
4	Membrane	-	2.80	68	79
	Membrane	+	2.80	42	78
	Fractionated cytoplasm	+	2.80	31	75
5	Membrane	-	1.00	102	112
	Cytoplasm	+	1.00	39	108
6	Membrane	-	2.40	68	75
	Membrane	+	2.40	62	160
	Cytoplasm	+	2.40	40	120

*Where "membrane" is shown, no additions were made. The membranes served as the source of kinase. Fractionated cytoplasm refers to precipitated protein at 0.5 ammonium sulfate saturation.

Washed PMN or platelet membranes, 6-8 mg/ml, were preactivated with 5 mM fluoride or 3 μM PGE_1 respectively for 20 minutes at 30° C in tris buffer 40 mM containing 10 mM $MgCl_2$ pH 7.2. These were washed 3 x each with about 40 volumes of the buffer. The phosphorylation reaction was carried out at 30° C for 30 minutes and consisted of: ATP 1mM, $MgCl_2$ 10 mM, theophylline 4 mM, cAMP 1 μM and membrane 0.28-2.8 mg protein. In addition, cytoplasm, fractionated cytoplasm or bovine serum albumin (BSA) for the controls were added in 3 mg amounts where indicated. Final volume 0.5 ml. The presumably phosphorylated membranes were washed 3 x as before and cyclase activity assayed for 15 minutes at 30° C with and without the fluoride or PGE_1 for PMN and platelet membranes respectively as previously described (2, 9).

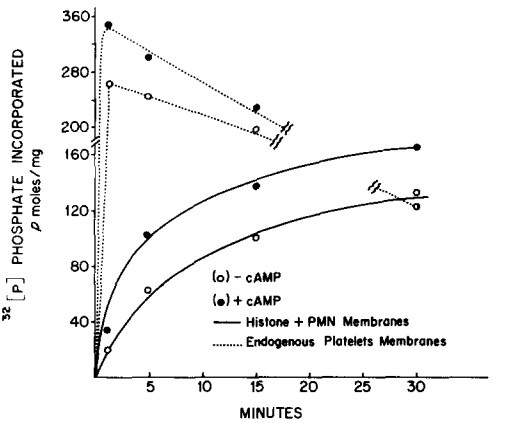

Fig. 1: The phosphorylation of exogenous (histone) and endogenous substrates with membrane protein kinase with and without cAMP. The reaction mixture at pH 6.75, 30° C, 1.0 ml, consisted of ATP γ-^{32}P 1 mM (0.1 mCi) $MgCl_2$ 10 mM and phosphate buffer 10 mM. PMN membranes 3.2 mg or platelet membranes 10 mg were added as the source of protein kinase. Where indicated, cAMP 1 μM and histone 3 mg were added. At various times, 0.2 ml was centrifuged for ^{32}P incorporation. To 0.1 ml of the supernatant, 2 mg BSA were added, followed by 2 ml 6% TCA. The precipitate, dissolved in 0.2 ml of 1 N NaOH, was reprecipitated with TCA. This washing procedure was further repeated 2 × and the precipitate dissolved in alkali for radioactive histone assay. Membranes as endogenous substrates were sedimented, dissolved in alkali, washed 3 × by TCA as above and counted.

cytoplasmic kinase. In each case, ATP was the phosphate donor. In such a reaction system it has been shown that the use of ATP γ-^{32}P, as the phosphoryl donor, resulted in substantial incorporation of ^{32}P in these membrane preparations (2). The phosphorylated membrane preparation was then washed and again assayed for cAMP synthesis with and without activator, fluoride or PGE_1 as the case may be. Table I shows results representative of several experiments. Two results that have direct bearing on the conclusions are revealed in each experiment. Since the starting material in each case is a membrane AC preparation mostly activated by pretreatment with fluoride or PGE_1 the activity of the enzyme, as expressed in cAMP synthesis, is near maximal. This relatively high activity is not significantly altered by further treatment with activator unless the first activation is incomplete. Following incubation with a phosphorylating system, the activity shows definite inhibition and less cAMP is formed. However, upon subsequent treatment with

the activator fluoride or PGE_1 the activity is regained and approaches the original level of cAMP synthesis.

We have assumed thus far that exposure of the enzyme to a phosphorylating system results in the phosphorylation of adenylate cyclase. This assumption is based on the fact that treatment of both membrane preparation, with ATP γ-^{32}P, with or without added exogenous substrate protein, results in the phosphorylation of either endogenous or exogenous substrate. This is shown in Fig. 1.

The figure shows that membrane preparations from PMN granulocytes (and platelets not shown) contain an active protein kinase that phosphorylates histone. Both preparations can phosphorylate endogenous acceptor protein (PMN not shown). The membrane bound kinase from both sources is stimulated by added cAMP in the low μM concentrations ordinarily used for soluble protein kinases. Both membrane preparations (PMN not shown) cause a decline in endogenous phosphate incorporation with time. This is suggestive of the presence of a membrane bound phosphatase (12).

While the final proof of the occurrence of a phospho-AC rests on the isolation of a pure and soluble enzyme, the data presented here strongly support this postulate. Furthermore, the finding of phosphoprotein phosphatase that is stimulated by fluoride and prostaglandin E_1 and presented in the accompanying article (12) adds further support for this hypothesis.

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