THE OCCURRENCE OF FLUORIDE STIMULATED MEMBRANE PHOSPHOPROTEIN PHOSPHATASE†

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Received June 5,1973

Summary: Membrane preparations from rabbit peritoneal granulocytes and dog blood platelets possess an active phosphoprotein phosphatase. The enzyme is stimulated by fluoride and to a lesser extent by prostaglandin E_1 (PGE_1). It dephosphorylates ^{32}P labeled, catalytically active phosphoglucomutase (PGM) and labeled endogenous membranes to yield, in both cases, inorganic phosphate. It is inactive towards denatured PGM, denatured endogenous membranes and thymus histone labeled with ^{32}P.

INTRODUCTION

In a preceding article (1) it was shown that membrane adenylate cyclase (AC), preactivated by sodium fluoride or prostaglandin E_1 (PGE_1), was strongly inhibited following incubation with preparations containing protein kinase, cyclic adenylate (cAMP) and ATP. It was assumed that the inhibited enzyme exists as a phosphorylated adenylate cyclase (phospho-AC). The inhibited AC enzyme could again be activated by retreatment with the activator to produce what was assumed to be dephosphorylated enzyme (dephospho-AC). This assumption was prompted by the observation that fluoride is a highly effective dephosphorylating agent for phosphorylated phosphoglucomutase (2). It was further shown that fluoride is also capable of dephosphorylating membrane preparations that were phosphorylated by ATP γ-^{32}P through a protein kinase (1,3).

The question arose as to whether fluoride and presumably hormones also act directly on the cyclase or perchance on a protein phosphatase which in turn can activate the cyclase by dephosphorylation. This would fit into the postulated multienzyme system in which the cyclase and phosphatase would be working

†Supported by Grant AI-09116, National Institutes of Health, U.S. Public Health Service and Grant GB-31535 X, National Science Foundation.

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in concert with the cAMP stimulated membrane protein kinase (3). In this event, regulation of the adenylate cyclase system might be more responsive to physiological modulation. Such a possibility was deemed worthy of investigation. Consequently, a study was initiated to explore the possible existence of a membrane phosphoprotein phosphatase in polymorphonuclear (PMN) granulocytes and platelets. The AC of the former is stimulated only by fluoride and the AC of platelets is stimulated best by PGE_1 (4). These two individual cell systems can be prepared in pure form and labeled with ^{32}P by safe and simple methodology. Their preparation requires no damaging agents that might alter the relationship of membrane components (5).

MATERIALS AND METHODS

Rabbit peritoneal PMN granulocyte and blood platelet preparations, and the assay for adenylate cyclase, activation by fluoride as well as relevant methods and materials, are described in the preceding article (1).

Phosphorylation of PGM with glucose-6-^{32}P (G6-^{32}P) was performed through the exchange reaction (6)

$$\text{phospho-PGM+G6-}^{32}\text{P} \longrightarrow \text{dephospho-PGM+G1-P,6-}^{32}\text{P}$$

$$\text{dephospho-PGM+G1-P,6-}^{32}\text{P} \longrightarrow \text{phospho (}^{32}\text{P)-PGM+G1-P}$$

Inorganic phosphate was identified as the molybdate by partition in organic solvents (7) and by chromatography (8).

RESULTS AND DISCUSSION

Fluoride Activated Membrane Phosphoprotein Phosphatase

(a) Exogenous protein substrate:

Membrane preparations from PMN granulocytes, showing fluoride activatable adenylate cyclase, were preincubated with and without 5mM sodium fluoride. These were thoroughly washed in tris buffer 50mM containing 1mM $MgCl_2$ (1) and each incubated with exogenous ^{32}P labeled phospho-PGM as substrate. The liberation of inorganic phosphate (^{32}Pi) was followed with time. Two controls were run concurrently. In one control, native ^{32}P-PGM was incubated with boiled membranes. In the other, boiled ^{32}P-PGM was incubated with native mem-

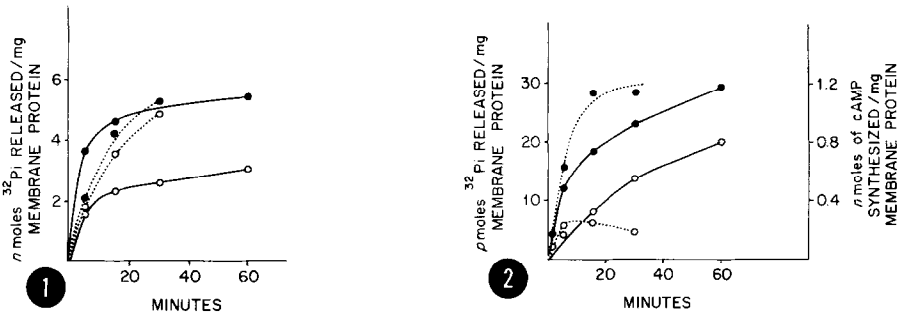

Fig. 1. The dephosphorylation of PGM by PMN and platelet membranes.

Membranes from each cell type were prepared as described in the preceding article (1). A portion of PMN membranes, 10 mg/ml, was preactivated (●—●) in a mixture containing 5mM NaF in tris-HCl buffer 40mM, PH 7.2, and 10mM $MgCl_2$ at 30°C for 30 minutes. The control (O—O) had an equivalent amount of chloride added. The final volume was 1.0 ml. These were then washed off 3X each with 40 volumes of 40mM tris-HCl PH 7.2 containing 1mM $MgCl_2$. Samples of each, 0.85 mg, were incubated at 30°C with 0.45 µmole of ^{32}P-PGM as substrate in the same reaction mixture as above but lacking fluoride. Platelet membranes, 3 mg of protein, were similarly incubated in the same buffer also lacking fluoride but in the presence (●--●) or absence (O--O) of 3µM PGE_1. At the indicated time period 0.1ml of each reaction mixture was removed, mixed with 0.1 ml of 5% bovine serum albumin, precipitated with 10 µl of 70% perchloric acid and centrifuged. 0.1ml aliquot of the supernatant was counted.

Fig. 2. The rate of dephosphorylation of ^{32}P labeled PMN membranes, partially preactivated with fluoride, as compared to the nonactivated control.

Radioactive PMN membranes were prepared by incubation of PMN cells with ^{32}Pi as described elsewhere (3). The labeled membranes, 5 mg per ml, were preactivated (●—●) by incubation at 37°C for 15 minutes with 5mM sodium fluoride, 10mM $MgCl_2$, in tris-HCl buffer 40 mM PH 7.2 . An amount of NaCl equivalent to the fluoride was added to the control (O—O). During this incubation no more than 10% of the label was released. The membranes were sedimented at 2000 g in the cold and washed 2X each with 100 volumes of the tris buffer. The washed membranes, 3 mg per ml, were again incubated at 37°C in tris buffer lacking fluoride. At the indicated time, 0.2 ml of each reaction mixture was centrifuged and 0.1 ml of the supernatant counted. cAMP was assayed (3,8) separately with (●--●) and without (O--O) fluoride using an unlabeled aliquot as described in the text.

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branes. The latter control showed little or no liberation of ^{32}Pi. The former containing native PGM showed some liberation of ^{32}Pi due to the possible presence of reactive groups in the boiled membrane preparation (2). These values were deducted from those obtained in the fluoride activated and nonactivated experimental samples of native membranes. The phosphatase activity of platelet membranes were studied with and without the presence of PGE_1. Fig. 1 is illustrative of this type of experiment.

It is clear that a phosphoprotein phosphatase exists in both membrane preparations and that it is stimulated by fluoride and PGE_1. It was completely inactive towards denatured PGM. The extent of fluoride stimulation varied with the preparation, 30-250%. PGE_1 stimulation of the platelet membrane phosphatase was definite but much less in magnitude yielding 12-15% above the control (3). Repeated attempts to elicit activity towards ^{32}P labeled histone were not successful. Membrane preparations showed slow phosphatase activity towards *p*-nitrophenyl phosphate, but this was not stimulated by fluoride. However, there was definite and rapid phosphatase activity toward ^{32}P labeled phosphofluoridate yielding ^{32}Pi. This explains our repeated failure to isolate the anticipated phosphofluoridate as a product of fluoride activation of membrane preparations. In all instances, only inorganic phosphate was produced.

(b) Endogenous membrane substrate:

Viable and motile PMN granulocytes were labeled by incubation at 37° with $^{32}P_i$ in tris buffer PH 7.2 for 30 minutes(3).These labeled membrane preparations served as endogenous substrates for the autogenous membrane phosphatase. After thorough washing in isotonic tris NaCl buffer, they were lysed and homogenized as described in the preceding article (1). An aliquot of the labeled and thoroughly washed membranes was preincubated with fluoride. The control received an equivalent addition of chloride during the preincubation period. Both samples were again washed and incubated in tris buffer PH 7.2 under identical conditions. Samples were assayed for the liberated ^{32}Pi at the times indicated. cAMP synthesis was assayed in PMN samples of membranes prepared from the same cell stock preparation before labeling with ^{32}P.

Fig. 2 shows that the phosphatase is active against its endogenous substrate and that again it is stimulated by fluoride. As with exogenous PGM substrate, the continuous presence of fluoride is not required. It can be washed away with full retention of the stimulatory effect. Not shown in the figure is that the addition of an aliquot of heat inactivated membranes does not result in any more liberation of ^{32}Pi than is released in its absence.

All ^{32}P released into the medium was identified as ^{32}Pi (7,8). The figure also shows that the synthesis of cAMP increased simultaneously with the release of ^{32}Pi. This occurred in the early phase of the reaction, both in the fluoride activated and the non-activated control samples. This is consonant with the notion that a membrane phosphoprotein phosphatase might function as the possible immediate activator of the adenylate cyclase enzyme. The cessation and subsequent decline of cAMP accumulation, often observed, may be due to cAMP diesterase.

We have consistently observed that as the fluoride stimulated phosphatase activity increases with time, the fluoride effect becomes progressively less evident (figures 1 and 2). A possible interpretation is that once a portion of the enzyme is activated, the remaining inactive and presumably phosphorylated enzyme may act as its own substrate and in time become fully activated.

Of potential significance is the finding that the enzyme is inactive towards denatured protein, whether PGM or endogenous membrane, as substrate. The inactivity of the enzyme towards denatured endogenous membrane may, however, result from lack of membrane-membrane interaction. Its lack of activity toward histone may be due to the fact that this substrate is in the denatured state.

It should be stressed that the demonstration of a phosphoprotein phosphatase rests primarily on the dephosphorylation of native PGM to yield inorganic phosphate. The dephosphorylation of endogenous membrane, which also yields inorganic phosphate, is assumed to result from the action of the same enzyme on a phosphoprotein substrate, since both reactions are stimulated by fluoride. The possibility that the enzyme exists in an inhibited phospho- and stimulated dephospho-form is only supported by the data presented in this communication but not proven. Final proof must await the isolation of the pure enzyme.

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