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PLATELET PHOSPHOPROTEIN PHOSPHATASE ACTIVITY

ITS SUBCELLULAR DISTRIBUTION AND REGULATION

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Summary

Phosphoprotein phosphatase (EC 3.1.3.-) activity was found in human platelet homogenates and this activity was stimulated up to 20-fold by pre-incubation with trypsin. Both Mg^{2+} and Mn^{2+} greatly decreased the activity of trypsin-activated phosphatase but the activity of the untreated phosphatase was not affected by increasing the concentration of these divalent cations. It was also shown that the activity of the phosphatase underwent a transient inhibition upon addition of ATP and a permanent one with ATP-γ-S.

Protein phosphorylation reactions catalyzed by protein kinases and regulated by cyclic nucleotides are essential links in the sequence of biochemical events that convert hormone action into specific metabolic responses. Elegant studies on the hormonal control of glycogen metabolism have revealed examples of the physiological importance of protein phosphorylation and dephosphorylation in skeletal muscle [1,2]. The question whether the dephosphorylation of the different phosphoproteins is catalysed by a single phosphatase has not yet been answered because of the existence of multiple molecular forms of the enzyme [3—5]. Ligands and other proteins may control the activity of phosphatase(s) by acting either on the substrate (phosphoprotein) or on the enzyme itself [2, 6—9]. It has also been found that various treatments like ammonium sulphate/ethanol precipitation [4] or trypsin treatment [5] can increase the activity of phosphoprotein phosphatase.

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Glycogen is a major component of blood platelets and is seen as clusters of electron dense particles in electron micrographs. It has been demonstrated that platelet glycogen breakdown occurs in a similar manner to that in skeletal muscle involving the phosphorylation of phosphorylase and activation of phosphorylase kinase by Ca^{2+} [10]. Therefore the investigation of the existence of a phosphatase in platelets would extend our knowledge about glycogen metabolism and focus attention on the importance of dephosphorylation reactions and the manner in which they may be controlled. In this paper we have investigated the subcellular distribution of phosphoprotein phosphatase (EC 3.1.3.-) in platelets and found that the phosphatase activity is predominantly located in the soluble phase and membrane-rich fraction of platelet homogenate. We have also examined the way in which the enzyme may be activated and controlled using procedures developed for the studies of skeletal muscle and liver phosphoprotein phosphatase.

Rabbit skeletal muscle phosphorylase *a* was prepared and assayed in the direction of glycogen synthesis [9]. Protein was determined by the method in [11]. Freshly prepared platelets from human blood [12] were homogenized in a Waring Blender with 2 vols. of 40 mM imidazole/2 mM EDTA/0.5 mM dithiothreitol, pH 7.4. The homogenate was centrifuged at 8000 × *g* for 20 min and passed through glass wool to remove all fatty material, which would otherwise hinder the further fractionation. All operations were carried out at 4° C. The supernatant was layered onto a sucrose gradient to obtain subcellular fractions [13]. After centrifugation (50 000 × g_{av} for 3 h at 4° C in a swing-out rotor) the three major fractions (soluble, membrane-rich and granule-rich) were removed with a pasteur pipette.

Trypsin treatment of the samples was carried out at 30° C in the presence of 0.05 mg/ml bovine pancreas trypsin (trypsin to sample ratio was 1:200). Trypsinization was stopped by the addition of 5-fold excess by weight soybean trypsin inhibitor (the presence of which had no effect on the phosphatase assay). In another set of experiments the different samples were treated with ammonium sulphate and ethanol as described in [4].

The phosphorylation of proteins was studied at 30° C in an incubation medium containing: 8000 × *g* supernatant, 3 mM Mg^{2+}, 0.5 mM ATP or ATP-γ-S (adenosine-5′-*O*-[3-thiotriphosphate], Boehringer), 5 μM dibutyryl cyclic AMP and 5 mM caffeine (final volume 2 ml, protein approx. 10 mg/ml, pH 7.4). Phosphorylase phosphatase activity of the samples was assayed as described earlier [8]. To 0.15-ml aliquots of the different samples was added 0.05 ml phosphorylase *a* (0.1 mg) also containing caffeine (final concentration 5 mM). One unit of phosphatase is that amount of enzyme which converts 1 nmol phosphorylase *a* per min at 30° C.

Rabbit muscle phosphorylase *a* was used as a substrate to test for the possible existence of phosphoprotein phosphatase in platelets. Table I shows the distribution of phosphatase activity in platelet subcellular fractions. The specific activity of phosphatase in the untreated 8000 × *g* supernatant, soluble and membrane-rich fractions was similar. As shown in Table I, preincubation with trypsin resulted in the significant activation of phosphatase. Preincubation with trypsin for 8 min stimulated the specific activity about 20-fold in the membrane-rich fraction, 5-fold in the soluble fraction but

TABLE I

DISTRIBUTION AND ACTIVATION OF PHOSPHORYLASE PHOSPHATASE IN SUBCELLULAR FRACTIONS OF HUMAN PLATELETS

Preparation of 8000 × *g* supernatant and subcellular fractions, trypsin treatment of the samples and ammonium sulphate/ethanol precipitation are described in the text. The results are the average values from three different platelet extracts and are given in specific activities of phosphorylase phosphatase

Time of trypsin treatment	Specific activity of phosphorylase phosphatase (U/mg protein)			
	8000 × *g* supernatant	Soluble fraction	Membrane-rich fraction	Granule-rich fraction
None	0.017	0.021	0.014	0.005
3 min	0.102	0.088	0.236	0.009
8 min	0.123	0.105	0.287	0.011
20 min	0.133	0.081	0.314	0.010
Ammonium sulphate/ ethanol precipitate	0.55	0.78	0.35	—

only 2-fold in the granule-rich one. Further trypsin treatment (20 min) slightly decreased the specific activity of the phosphatase in the soluble fraction. Phosphorylase phosphatase activity was also increased by the combined treatment of ammonium sulphate and ethanol. The average increase was about 30--35 fold, with the exception of the granule-rich fraction where this treatment resulted in the complete loss of the enzyme activity. The soluble phosphatase represented more than 80% of the total activity before the preincubation with trypsin but after prolonged tryptic digestion the amount of phosphatase activity in the membrane-rich fraction almost equaled that of the soluble phase enzyme.

The effect of magnesium and manganese chloride on the phosphatase activity was also investigated (Fig. 1). The 8000 × *g* supernatant was used as a phosphatase preparation before and after preincubation with trypsin. Both Mg^{2+} and Mn^{2+} greatly decreased the activity of trypsin-activated phosphatase approaching the level of the untreated phosphatase. However, the activity of the untreated phosphatase was not affected by increasing the concentration of these divalent cations.

It is known that the phosphatase activity undergoes a reversible inhibition during the protein phosphorylation process (upon addition of Mg^{2+} and ATP) in the protein-glycogen complex isolated from rabbit muscle [14]. Recently, the transient inhibition of phosphatase has also been demonstrated in a muscle extract [8]. To demonstrate this control mechanism for the platelet phosphatase the phosphorylation process was stimulated by Mg^{2+} and ATP in the 8000 × *g* supernatant and the phosphatase activity was tested using exogenous muscle phosphorylase *a* as substrate. As shown in Fig. 2 the phosphatase activity of the undiluted samples rapidly decreased upon addition of Mg^{2+} and ATP causing a total inhibition within 2—3 min.Thereafter the activity increased and reached the original level again after 20 min. However, the activity of phosphatase remained unchanged in the 15-fold dil-

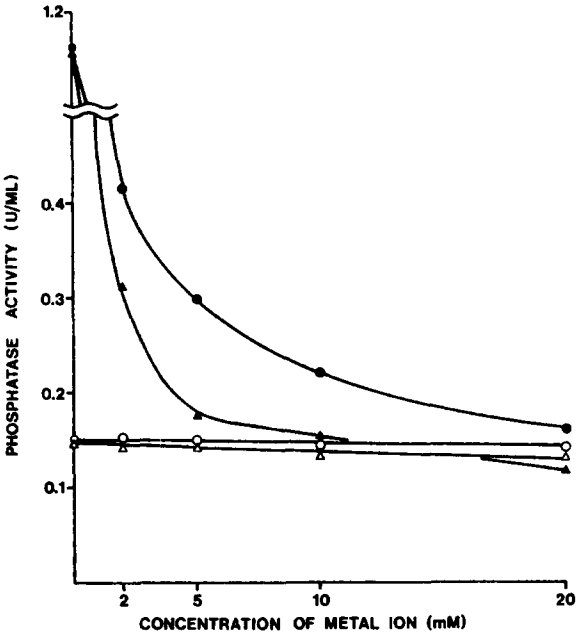

Fig. 1. Effect of magnesium and manganese chloride on phosphorylase phosphatase activity. The phosphatase samples were preincubated with the metal ions for 5 min at 30°C then assayed as described in the text. Open symbols correspond to the 8000 X *g* supernatant and closed symbols indicate a trypsin-activated supernatant (trypsinization for 8 min with 0.05 mg/ml trypsin). Activity of phosphatase in the presence of $MgCl_2$ (○——○, ●——●) and $MnCl_2$ (△——△, ▲——▲).

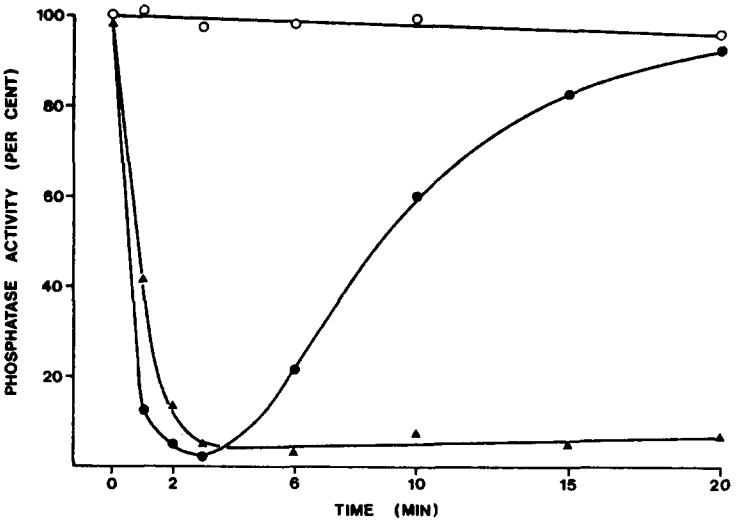

Fig. 2. Reversible inhibition of phosphatase in the 8000 X *g* supernatant. Conditions of the activation process and the phosphatase assay are given in the text. Phosphatase activity is expressed as % initial activity (given as 100%) obtained before addition of Mg^{2+} and ATP. Open symbols represent the enzyme activity of 15-fold diluted samples and closed symbols that of the undiluted samples. Phosphatase activity after addition of ATP (○——○, ●——●), or ATP-γ-S (▲——▲).

uted samples. A permanent inhibition of phosphatase was observed with Mg^{2+} and ATP-γ-S.

The results presented in this paper have shown that the phosphorylase phosphatase activity of human platelets is remarkably high and represents about 10—20% of the value in muscle (data for muscle phosphatase activity taken from Refs. 4, 15). On the other hand, platelet phosphorylase activity is about 1% and phosphorylase kinase less than 0.1% of the value for muscle. These data indicate that the phosphatase in platelets is likely to dephosphorylate phosphoproteins other than those involved in glycogen metabolism. There are reports about several phosphorylation processes in platelets (see Ref. 16 for references) and the relatively high phosphatase activity suggests that the enzyme may also function in the dephosphorylation of these other phosphoproteins. Recently, it has been reported that phosphorylase phosphatase may have other physiological substrates thereby enlarging its regulatory role [17].

It is known that phosphatase exists in a large molecular weight form in various tissues. It has been demonstrated that ammonium sulphate/ethanol precipitation or tryptic digestion considerably increases its activity converting it into lower molecular weight form(s) [4, 5, 15]. The mechanism of the activation involves the removal or disruption of inhibitory proteins associated with phosphatase. This activation of phosphatase was observed in the different subcellular fractions and the original 8000 × *g* supernatant of the platelet homogenate. However, the extent of the activation was not the same in every fraction indicating a variation in inhibitor content. Divalent cations (Mg^{2+} and Mn^{2+}) did not influence the activity of phosphatase in the 8000 × *g* supernatant but did inhibit the trypsin-activated samples. It is interesting that they decreased the phosphatase activity to the level of the untreated samples. The mechanism of Mg^{2+} inhibition has been investigated using purified muscle phosphatase and it appears to be due to its binding to specific sites on phosphatase [6]. The lack of inhibition in the original 8000 × *g* supernatant could be explained by the masking of the metal ion binding sites of phosphatase with the associated proteins.

Platelet phosphorylase phosphatase is also under a phosphorylation regulatory mechanism. It was found that the activity of phosphatase underwent a transient inhibition upon addition of ATP, and a permanent one with ATP-γ-S. Phosphorylated proteins were transiently formed in the presence of ATP causing a reversible inhibition. On the addition of ATP-γ-S thiophosphorylated proteins were formed and remained in this form. Since they are resistant to the action of phosphatase [18, 19], the result is a permanent inhibition. No inhibition was found in the 15-fold diluted samples suggesting that the inhibition involves the interaction of phosphatase with some of the phosphorylated protein components in the incubation medium and not in modification of the phosphatase itself. This control mechanism of phosphatase has been reported for muscle gel-filtrated extracts [8] and the authors suggest that phosphorylase kinase [19] and heat-stable inhibitor protein [7] are the most likely candidates for this inhibitory action. The actual inhibitory protein(s) which control the activity of phosphatase in the platelet are at present unknown.

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References

1 Krebs, E.G. (1972) Curr. Top. Cell Reg. 5, 99—133
2 Nimmo, H.G. and Cohen, P. (1977) Adv. Cycl. Nucl. Res. 8, 145—266
3 Killilea, S.D., Brandt, H., Lee, E.Y.C. and Whelan, W.J. (1976) J. Biol. Chem. 251, 2363—2368
4 Brandt, H., Capulong, Z.L. and Lee, E.Y.C. (1975) J. Biol. Chem. 250, 8038—8044
5 Kato, K., Kobayashi, M. and Sato, S. (1974) Biochim. Biophys. Acta 371, 89—101
6 Martensen, T.M., Brotherton, J.E. and Graves, D.J. (1975) J. Biol. Chem. 248, 8323—8328
7 Nimmo, G.A. and Cohen, P. (1978) Eur. J. Biochem. 87, 353—365
8 Gergely, P., Dombradi, V. and Bot, Gy. (1978) FEBS Lett. 93, 239—241
9 Bot, Gy., Kovacs, E. and Gergely, P. (1977) Acta Biochim. Biophys. Acad. Sci. Hung. 12, 355—341
10 Akkerman, J.W.N. (1978) Thromb. Haemost. 39, 712—724
11 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
12 Baenziger, N.L. and Majerus, P.W. (1974) Methods Enzymol. 31, 149—155
13 Taylor, D.G. and Crawford, N. (1974) FEBS Lett. 41, 317—320
14 Detwiler, T.C., Gratecos, D. and Fischer, E.H. (1977) Biochemistry 16, 4818—4823
15 Antoniw, J.F., Nimmo, H.G., Yeaman, S.J. and Cohen, P. (1977) Biochem. J. 162, 423—433
16 Haslam, R.L., Davidson, M.M.L., Davies, T., Lynham, J.A. and McClenaghan, M.D. (1978) Adv. Cycl. Nucl. Res. 9, 533—552
17 Burchell, A., Foulkes, J.G., Cohen, P.T.W., Condon, G.D. and Cohen, P. (1978) FEBS Lett. 92, 68—72
18 Gratecos, D. and Fischer, E.H. (1974) Biochem. Biophys. Res. Commun. 58, 960—967
19 Gergely, P., Vereb, Gy. and Bot, Gy. (1974) Biochim. Biophys. Acta, 429, 809—816