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REGULATION OF THE INTRACELLULAR CALCIUM LEVEL IN HUMAN BLOOD PLATELETS: CYCLIC ADENOSINE 3′,5′-MONOPHOSPHATE DEPENDENT PHOSPHORYLATION OF A 22 000 DALTON COMPONENT IN ISOLATED Ca^{2+}-ACCUMULATING VESICLES

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Summary

Two protein kinase activities have been separated from the supernatants of homogenized human blood platelets by DEAE cellulose chromatography. One of them (peak I enzyme) is an efficient stimulator of the uptake of Ca^{2+} into isolated membrane vesicles in the presence of cyclic AMP and ATP. The second (peak II enzyme), although equally active towards histone, exerts only about one third of the activity of the peak I enzyme. The stimulation of Ca^{2+} uptake is accompanied by the phosphorylation of a membrane protein with an apparent molecular weight of 22 000, which appears to play an essential role in the regulation of the intracellular Ca^{2+} level and hence of platelet activity.

Blood platelets are not only of prime importance for spontaneous haemostasis, arterial thrombosis, and perhaps atherogenesis, but they are also an easily accessible model for studying cellular reactions, such as contractility, aggregation, and secretory processes, to external stimuli. It is well established that the mobilization of intracellular Ca^{2+} plays a decisive role in the activation of platelets [1]. On the other hand, it has been shown that activation is prevented or reversed by all measures which result in an increased level of cyclic adenosine 3′,5′-monophosphate (cyclic AMP) within the cell [2]. This obviously is due to the existence in platelets of an ATP-dependent pump, capable of specifically removing Ca^{2+} from the cytoplasm, the activity of which is considerably increased by cyclic AMP in the presence of a protein kinase. In earlier publications [3,4] we have reported in detail the isolation and characterization of Ca^{2+}-accumulating membrane vesicles. In this paper we describe experiments performed on such isolated membrane vesicles which

show that there appears to exist a correlation between the stimulation of Ca^{2+}-uptake and the phosphorylation of a 22 000 dalton protein by means of a cyclic AMP-dependent protein kinase.

Calcium-accumulating membrane vesicles were prepared as described earlier [3], except that they were suspended in a buffer devoid of oxalate, which exerts an inhibitory effect on the protein kinase. Protein kinases were isolated from the supernatant of the homogenate of washed platelets after centrifugation at 100 000 × *g* for 60 min. They were separated on a Biogel A-1.5 m column, which was eluted with a 5 mM phosphate buffer pH 7.0 containing 2 mM EDTA and 1 mM dithiothreitol. Active fractions were further purified by DEAE-cellulose chromatography using for elution a linear KCl ionic strength gradient in the pH 7 buffer mentioned above. All buffers contained 10% glycerol, which stabilizes the enzyme [5]. Enzyme activity was estimated according to Booyse et al. [6] and Huang and Robinson [7]. Two peaks with protein kinase activity were obtained, which in analogy to the isozymes isolated from other tissues [8] are designated as peak I and peak II-enzymes. Peak I elutes at a concentration of about 0.06 M KCl, peak II at 0.15 M KCl. Calcium uptake by the membrane vesicles was measured with a calcium-selective electrode according to Madeira [9] in the buffer described in the legend to Fig. 1.

In order to minimize dephosphorylation by endogenous phosphoprotein phosphatases, phosphorylation experiments on the isolated vesicles were performed in a KH_2PO_4-containing buffer, corresponding to the medium used by Kirchberger and Chu [10] in their work on heart microsomes. Phosphorylated proteins were separated by sodium dodecyl sulphate-polyacrylamide (13%) gel electrophoresis according to a modification by Haslam [11] of the method of Laemmli [12]. Gels were stained with Coomassie-Blue and in parallel experiments, in which [γ-^{32}P]ATP was used as a phosphate donor, were sectioned into 1.5-mm slices, which were counted in a Packard Tri-Carb, Model 2450 liquid scintillation counter. The scintillation solution consisted of 0.3 ml Protosol, 25 mg Permablend III in 10 ml toluene.

As shown in Fig. 1, Ca^{2+} uptake is stimulated in the presence of cyclic AMP by peak I and not by peak II, if the 2 preparations are applied at the same activity of about 1000 pmol ^{32}P/min, ^{32}P-incorporation determined with histone as a substrate. However, a stimulation is also observed with peak II, provided it is added in a 2—3 times higher concentration. Most likely this is due to contamination of the peak II with the enzyme from peak I; there can be no doubt, though, that peak II contains another protein kinase activity, which is not related to phosphorylations linked to Ca^{2+} transport. To our knowledge there exist no studies on the characteristics of the 2 isoenzymes in relation to Ca^{2+} uptake, except perhaps those of Nilsson et al. [13] who observed an increased phosphorylation of proteins in intestinal microsomes of the rabbit, which was catalyzed by the peak II, but not by the peak I enzyme. These authors speculate that phosphorylation increases binding sites for Ca^{2+}.

Fig. 2 shows that the membrane vesicles incorporate modest amounts of ^{32}P, when incubated with cyclic AMP and [^{32}P]ATP. Within peak I, incorporation is observed mainly into a polypeptide with an apparent molecular weight of 40 000. It remains at present unclear, whether in analogy to the situation in skeletal muscle (cf. Ref. 14), this corresponds to an autophosphorylation of

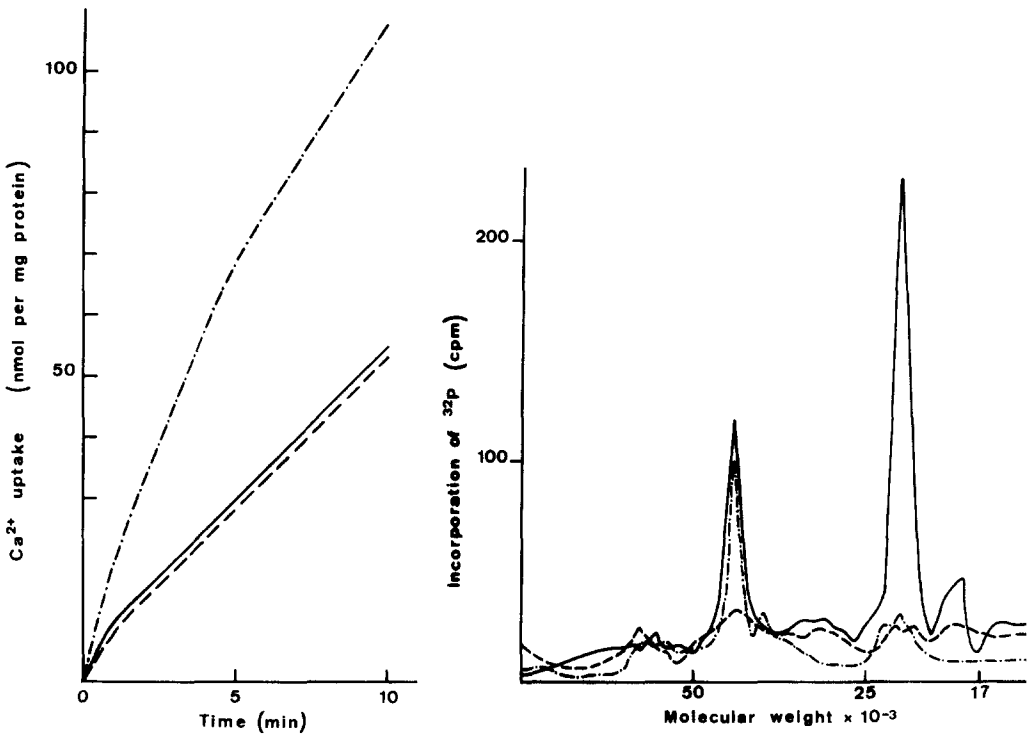

Fig. 1. Activation of calcium-uptake by isolated membrane vesicles in the presence of cyclic AMP and 2 protein kinase containing fractions isolated from platelets. Membrane vesicles (1 mg protein) were incubated for 5 min at 30° C in 0.6 ml of the following medium: 30 mM KCl, 5 mM $MgCl_2$, 5 mM ATP, 5 μM cyclic AMP, 50 mM KH_2PO_4, 40 mM histidine-HCl pH 6.8, in the presence or absence of the protein kinase preparations peak I or peak II, both with an activity of 1150 pmol ^{32}P/min, measured with histone as a substrate. Calcium uptake was measured with a Ca^{2+}-selective electrode, by adding 0.4 ml of the above mentioned incubation mixture to 3.6 ml of a reaction mixture consisting of 120 mM KCl, 5 mM $MgCl_2$, 5 mM ATP, 50 mM KH_2PO_4, 40 mM histidine-HCl pH 6.8 and a Ca-EGTA buffer to give a final Ca^{2+} concentration of 1 μM (cf. Ref. 19), with which the electrode was equilibrated before the experiment. Uptake proceeded under constant stirring at room temperature and was automatically recorded. —, membrane vesicles alone; – · – · –, membrane vesicles + peak I; – – – membrane vesicles + peak II.

Fig. 2. Phosphorylation of calcium-accumulating membrane vesicles analyzed by sodium dodecyl sulphate-polyacrylamide (13%) gel electrophoresis. Membrane vesicles (1.2 mg protein) and peak I protein kinase (240 μg protein, activity 2128 pmol ^{32}P/min) either alone or in combination were incubated in 1 ml of a solution containing 30 mM KCl, 5 mM $MgCl_2$, 50 mM KH_2PO_4, 5 μM cyclic AMP, 0.5 mM ATP with $6 \cdot 10^6$ c.p.m. [γ-^{32}P]ATP, 40 mM histidine-HCl, pH 6.8 for 10 min at 30° C. Reaction was terminated by adding 20% trichloroacetic acid [15]. The precipitates were dissolved in 2% sodium dodecyl sulphate in the presence of 2% dithiothreitol, heated to 100° C for 3 min and separated by polyacrylamide gel electrophoresis (13% gel). The gels were cut into 1.5-mm thick sections and the radioactivity determined by liquid scintillation. Shown is the distribution of ^{32}P radioactivity in membrane vesicles (– – – –), in peak I enzyme (– · – · –) incubated alone or after incubation of both together (—).

the catalytic subunit of the peak I enzyme, or whether this material represents an impurity, e.g. actin.

In the presence of both vesicles and protein kinase in the incubation mixture, a strong and specific incorporation of ^{32}P into a polypeptide band with an apparent molecular weight of 22 000 is observed. This band is found exclusively in the membrane vesicles. Thus, in the presence of cyclic AMP the peak I enzyme essentially phosphorylates one protein. It is tempting to relate this

protein to the Ca^{2+}-transporting activity of the membrane preparation. It should be noted that peak II enzyme preparations of comparable activity towards histone, are significantly less active towards this protein.

These results are in good agreement with those of Tada et al. [15], who described a protein of a molecular weight of 22 000 in the sarcoplasmic reticulum of heart muscle, which is phosphorylated by a cyclic AMP-dependent protein kinase isolated from heart tissue. They named this phosphoprotein "phospholamban" and assigned it a regulatory function for the Ca^{2+} pump mechanism, an assumption, which received further support from the work of Kirchberger and Chu [10] and Tada et al. [16]. Thus, we have shown for the first time that a "phospholamban"-like protein is also present in blood platelets, i.e. a non-muscle cell. Our results, which are obtained on isolated, Ca^{2+}-accumulating membrane vesicles also extend the observations made by Haslam et al. [11,17], who found an increased phosphorylation of polypeptides with apparent molecular weights of 24 000 and/or 22 000, if the adenylate cyclase of intact platelets prelabeled with ^{32}P was stimulated by prostaglandin E_1. These phosphopeptides appear to be membrane-bound [18].

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