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STIMULATION OF THE 23-Kd PROTEIN cAMP DEPENDENT PHOSPHORYLATION BY INOSITOL 1,4,5 TRISPHOSPHATE IN HUMAN PLATELET MEMBRANE VESICLES

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The effect of inositol 1,4,5 trisphosphate (IP_3) has been investigated on the cAMP-induced phosphorylation of the 23-Kd protein involved in platelet calcium fluxes by isolated membrane vesicles. The studies were conducted using the catalytic subunit of the cAMP-dependent protein kinase (C. Sub.). A dose-dependent stimulation of the 23 Kd protein phosphorylation induced by C. Sub. was initiated by IP_3 with a half-maximal effect of 0.5 µM. The maximal effect was observed after 1-2 min. The effect was detected in the absence of Ca^{2+} and in the presence of phosphatase inhibitors.

These results can suggest that the 23 Kd is an associated protein to the IP_3 receptor in human platelets.

The polyphosphoinositide metabolite IP_3 is a novel second messenger in cellular signal transduction (1) acting through a mobilization of intracellular calcium. In recent years, the IP_3 effect has been tested in the platelet system by different ways namely on Ca^{2+}-loaded isolated membrane vesicles with different degrees of purification (2,3,4,5,6) or on permeabilized cells by testing Ca^{2+} dependent functions (7,8). So, the role of IP_3 as a Ca^{2+} mobilizing agent from internal stores can be now considered as well established in the platelet system.

However, the mechanism of action of IP_3-induced calcium release is not yet clearly defined. The presence of a receptor for the molecule has been recently demonstrated in bovine adrenal cortex (9), rat liver microsomes (10), hepatocytes and neutrophils (11). A role for protein phosphorylation has been proposed in regulating the permeability of the sarcoplasmic reticulum to calcium (12,13) and also a role for cAMP-dependent protein phosphorylation in the

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function of calcium channels has been demonstrated using a purified system (14).

In platelets cAMP has been shown to regulate calcium transport by isolated membrane vesicles (15,16,17). The effect is correlated with the phosphorylation of a 23-Kd protein. The phosphorylation state of this protein could play a role in the regulation of IP_3-induced calcium release (6).

The work reported here was designed to investigate whether IP_3 interferes directly with the phosphoprotein. The findings show that IP_3 potentiates the 23-Kd protein phosphorylation due to a subsequent increase in the number of phosphorylable sites. This suggests that the phosphoprotein could be associated to the IP_3 receptor in the human platelet membranes.

MATERIAL AND METHODS

IP_3 was prepared from human erythrocyte [^{32}P] labelled membranes essentially by the method of Downes et al., (18) and modified either as described in (19) or by using an alternative procedure to remove ammonium ions. The column eluate was mixed with AG1-X8 (chloride form) and IP_3 was eluted with 1 M LiCl, and lyophylised LiCl was then removed by anhydrous ethanol (20). A blank consisting of the elution buffer which was used for eluting IP_3 from the AG1-X8 column (formate form) and treated as described above was always prepared in each experiment. IP_3 purity was ascertained by paper chromatography (21) and HPLC. The concentration of the final solution of IP_3 was 150-200 µM as calculated from its radioactivity and the specific radioactivity of Ptd Ins (4,5) P_2. This alternative procedure permitted a reduction in the amount of contaminating Ca^{2+} in the preparation of both IP_3 and the blank to about 100-300 µM instead of 700 µM in the procedure described in (19). This Ca^{2+} concentration was buffered with EGTA ($EGTA/Ca^{2+}$ = 1.24) prior to the assays.

The calcium accumulating membrane vesicles were prepared as previously described (22). Briefly, fresh platelets were isolated and washed in a Tyrode's modified buffer. The cells were then disrupted by controlled ultrasonication and centrifuged at 19,000 g. The supernatant was centrifuged at 100,000 g and the pellet was used as a source of membrane vesicles. The characterization of this fraction has been described (23). The specific activity of the endoplasmic reticulum marker enzyme, the antimycin-insensitive NADH-cytochrome C reductase was enriched by a factor 9, while plasma membranes were enriched by a factor 2.

The phosphorylation assays were conducted as described in (17) : the 100,000 g membrane vesicle fraction was incubated at 37°C for 10 minutes in a reaction mixture containing 10 mM $MgSO_4$, 100 mM NaF, 6 mM [$\gamma^{32}P$] ATP (7.4 nCi/nmole), 25 mM sodium phosphate buffer pH 7.0, in the presence or the absence of the C. Sub. (enzyme/substrate ratio : 0.01). The phosphorylation reactions were initiated by the addition of ATP and stopped by the addition of the sample buffer (v/v) at a final concentration of 0.0025 M Tris pH 6.8, 2.5% SDS, 0.001% v/v bromophenol blue, 5% v/v sucrose, 5% v/v β -mercaptoethanol. After dissociation for 10 minutes in boiling water, samples were applied to SDS/polyacrylamide gradient gels 5-20% (24). Electrophoresis were performed at 30 volts overnight. The gels were stained with Coomassie brilliant blue and dried. They were then autoradiographed using Kodak X OMAT AR films at -80°C with intensifying screens. Autoradiographs were densitometrically scanned on an LKB Ultroscan laser densitometer to determine the relative area of each peak.

RESULTS AND DISCUSSION

The characterization of the cAMP platelet phosphoprotein has been previously described (17,23,25). The incubation of the platelet membrane vesicles with the C. Sub. resulted in a phosphate incorporation into a 23-Kd protein (Fig. 1 lane 2). It occurs to a smaller extent when using cAMP and the addition of the protein kinase inhibitor totally suppressed the phosphorylation reaction (23). The intensity of the response increased up to 20 µg/ml of C. Sub (17). The time course of the phosphorylation reached a plateau value within 10 min (25) and the phosphorylation remained maximal for 60 min suggesting the absence of a dephosphorylation process under our experimental conditions. These results were obtained without addition of EGTA in the incubation medium i.e. with contaminating Ca^{2+} ions. In order to determine whether the 23-Kd protein was also a target for a Ca^{2+}-dependent protein kinase, we proceed to cAMP-protein phosphorylation in the absence or the presence of Ca^{2+}. Table 1 shows that the phosphorylation intensities were not modified by the addition of either EGTA, or of Ca^{2+}.

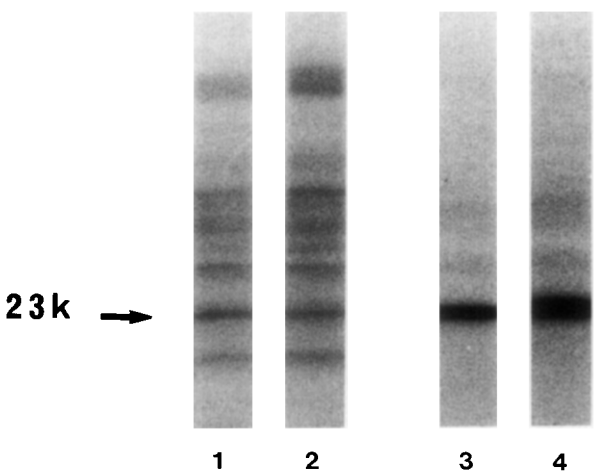

Fig. 1 . Effect of IP_3 on the cAMP dependent 23-Kd protein phosphorylation. The membrane vesicles were phosphorylated in the absence of C. Sub. (lane 1) or in the presence of C. Sub. (15 µg/ml) (lane 3) or of 1 µM IP_3 (lane 2) or both (lane 4). Shown are the autoradiographs of phosphoproteins separated by 5-20% acrylamide gel electrophoresis.
A greater phosphorylation intensity is observed in lanes 1 and 2 by comparison with lanes 3 and 4 : this is due to a longer exposure time of the autoradiograms to detect the autophosphorylation and the IP_3 effect.

Table I

Characterization of the IP_3 effect on the cAMP-induced 23-Kd protein phosphorylation

Preincubation 10 min	Addition	Relative phosphorylation
-	-	19
C. Sub.	-	100
C. Sub. + EGTA (124 µM)	-	101
C. Sub. + Ca^{2+} (100 µM)	-	105
C. Sub.	IP_3 (1 µM)	147
C. Sub.	Blank	105
C. Sub.	IP_3 (20 µM)	156
C. Sub. + IP_3 (1 µM)	-	156
C. Sub.	IP_2 (5 µM)	74
C. Sub. + EGTA (124 µM)	IP_3 (20 µM)	140
C. Sub. + Ca^{2+} (100 µM)	IP_3 (20 µM)	138
* Na_2VO_3 (5 µM)	IP_3 (20 µM)	134
* -NaF	IP_3 (20 µM)	120

Platelet membranes were phosphorylated with $\gamma^{32}P$ ATP and proteins were separated by polyacrylamide gel electrophoresis as in Fig. 1. The kinetics of the IP_3 effect were tested under the different experimental conditions and the maximal effect is expressed. The relative phosphorylation was determined by densitometry as described under experimental and the values correspond to percent of stimulation by 15 µg/ml C. Sub.

* The IP_3 effect was tested after one hour preincubation time.

The effect of IP_3 on the 23-Kd protein phosphorylation is shown on Fig. 1. IP_3 alone did not induce any stimulation of the phosphorylation (lane 2). In contrast, after 10 min phosphorylation of the 23-Kd protein by a submaximal concentration of the enzyme (15 µg/ml), the addition of 1 µM IP_3 (our preparation) resulted in an increase of this phosphorylation (lane 4). Fig. 2A shows the time course of the increase in the ^{32}P incorporation induced by IP_3. The stimulation effect appeared within 30 sec and reached its maximum at about 1.5 min. The maximal phosphorylation corresponded to about 150% of the value obtained with bidistilled water as a control or with the blank (desalted elution buffer) (Table I). The dose-response curve for the stimulation of the cAMP-mediated phosphorylation of the 23-Kd protein is shown on fig. 2B. Maximal stimulation was observed at 1 µM IP_3. Similar results were obtained using commercially available IP_3 preparations. However, higher IP_3 concentrations were needed to obtain the maximal effect (20 µM IP_3) (Table I). So, both simultaneous addition of IP_3 (fig. 2B) and the catalytic subunit of the cAMP-dependent protein kinase or successive additions (first the enzyme and the IP_3) (fig. 2A) led to a stimulation of the 23-Kd protein phosphorylation.

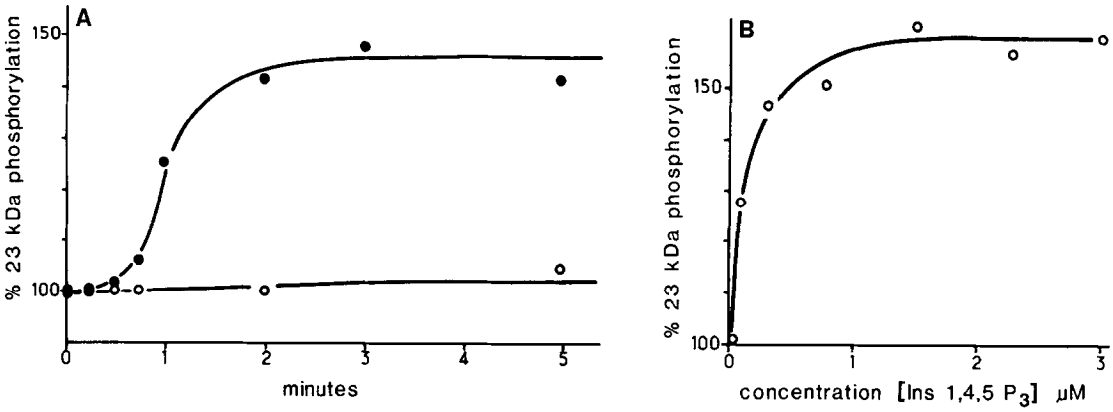

Fig. 2A . Kinetics of the effect of IP_3 on the cAMP-dependent 23-Kd protein phosphorylation.
Proteins were phosphorylated for 10 minutes in the presence of the C. Sub. (15 µg/ml) and then IP_3 was added.

Fig. 2B . Concentration dependence of IP_3-induced stimulation of the cAMP-dependent phosphorylation of the 23-Kd protein.
In these experiments both IP_3 and C. Sub. were added simultaneously just before the addition of (γ^{32}P-ATP). Reactions were stopped after 10 min. incubation. The quantitation of the phosphorylation of the 23-Kd protein was made by densitometry.

Because IP_3 was labelled with trace[^{32}P] it was verified that increased phosphorylation came from [^{32}P] ATP rather than [^{32}P] IP_3. The amount of [^{32}P] present in added IP_3 was found to be less than 0.1% of the amount of [^{32}P] present in the incubation medium. It was also found that IP_2 (our preparation) did not increase the C. Sub.-induced phosphorylation of the 23-Kd protein (Table I).

IP_3-induced stimulation of the 23-Kd protein phosphorylation was also tested using different concentrations of the C. Sub. (from 2 to 20 µg/ml) but the maximal effect was obtained with concentrations starting from 5 µg/ml (data not shown).

The stimulation of the 23-Kd protein phosphorylation could be due to a stimulation of the protein kinase(s) activities, an inhibition of phosphatase activity, or an increase in the phosphorylation sites.

The 23-Kd protein could be the target for a cAMP dependent protein kinase, a Ca^{2+}-dependent protein kinase, or a protein kinase C. An activation of the cAMP dependent protein kinase can be ruled out for two reasons : 1) In the absence of added catalytic subunit, the phosphorylation of the 23-Kd protein was

not stimulated by IP_3 as shown on the fig. 1 ; 2) the kinetic of the 23-Kd protein phosphorylation induced by C. Sub. (maximal at 10 min) and that of the IP_3-induced phosphorylation (maximal at 1.5 min) were different.

The 23-Kd protein is not a target for a Ca^{2+}-dependent protein kinase. This was verified again by testing the effect of IP_3 in the presence or the absence of EGTA. An increase in protein phosphorylation was still observed (Table I). The possibility of a stimulation by a protein kinase C can also be eliminated because the 23-Kd protein does not appear phosphorylated by the enzyme (23).

So the hypothesis of an IP_3-stimulation of protein kinase activities can be eliminated.

Furthermore the increase in the phosphorylated state was still observed when the IP_3 stimulation of the 23-Kd protein was tested in the absence of NaF or in the presence of sodium vanadate (5 μM) thus eliminating also the possibility of an effect on a phosphatase activity (Table I).

Thus we were left with the suggestion that IP_3 stimulated the cAMP dependent 23-Kd protein phosphorylation by another mechanism. It is now known that IP_3 binds with high affinity to isolated microsomes (9,10). Comparison of the dose-responses curves of the IP_3 effect on the Ca^{2+} release (6) and on the stimulation of the protein phosphorylation reveals that the latter occurred at lower concentrations. Moreover a small delay was observed between the IP_3-induced Ca^{2+} release and the IP_3-induced protein phosphorylation. Thus, the stimulation of the phosphorylation of the 23-Kd protein by IP_3,would occur subsequently to the effect on Ca^{2+} release. This could be due to the binding of IP_3 to the membrane vesicles causing a conformational change of the protein, increasing its ability to be phosphorylated.

Thus, there is no evidence to implicate directly the 23-Kd protein phosphorylation induced by IP_3 in the Ca^{2+} release mechanism. Indeed, recent reports show that IP_3-induced Ca^{2+} release is not under metabolic control : it is insensitive to change in temperature (7), it does not require ATP in hepatocytes (26) and platelets (27) and, in muscle cells, non hydrolysable analogues were as effective as ATP itself in releasing Ca^{2+} (28).

In conclusion, the present results suggest that the 23-Kd protein could be part of the IP_3 receptor in platelet internal membranes. This new hypothesis is now under investigation.

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