Evidence for a role of *rap1* protein in the regulation of human platelet Ca^{2+} fluxes

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The relationship between the 22–24 kDa cyclic AMP (cAMP)-dependent phosphoprotein previously described as being involved in the regulation of human platelet membrane Ca^{2+} transport and a GTP-binding protein of low molecular mass (*ras*-like protein) was investigated. After isolation of plasma membranes and intracellular membranes, it was found that guanosine 5'-[γ -thio]triphosphate (GTP[S]) bound to plasma membrane proteins ranging in molecular mass from 22 to 29 kDa, but not to intracellular membranes. The major GTP-binding protein appeared as a 24 kDa protein under reduced conditions and a 22 kDa protein under non-reduced conditions. A similar membrane location and electrophoretic mobility were found for both the cAMP phosphoprotein and the protein recognized by a specific anti-*rap1* antibody. The identity between the cAMP phosphoprotein and the *rap1* GTP-binding protein was further examined by studying the functional effect of GTP on plasma membrane Ca^{2+} transport. A maximal GTP[S] concentration of 40 μM was found to: (1) inhibit to the same degree (40%) both Ca^{2+} -ATPase activity and the Ca^{2+} transport function mediated by the Ca^{2+} -ATPase; (2) inhibit the phosphorylation of the 22–24 kDa protein by the catalytic subunit of the cAMP-dependent protein kinase (C.Sub.); and (3) abolish the stimulation of Ca^{2+} uptake induced by C.Sub. It is concluded that the platelet cAMP phosphoprotein is indeed the *rap1* GTP-binding protein, and that it regulates plasma membrane Ca^{2+} transport, thus providing evidence for a new role of a *ras*-related protein.

INTRODUCTION

A large number of cellular processes are triggered by an increase in the intracellular Ca^{2+} concentration in response to a wide variety of stimuli [1]. In human platelets, this elevation in the intracellular Ca^{2+} concentration is due to both Ca^{2+} influx from the extracellular medium and the liberation of intracellular Ca^{2+} from its storage organelle, the dense tubular system, through the action of inositol 1,4,5-trisphosphate [2]. In contrast, the reversibility of platelet activation induced by cyclic AMP (cAMP) is associated with a decrease in the intracellular Ca^{2+} concentration. This could be due, in part, to the activation of Ca^{2+} -ATPases which actively remove intracellular Ca^{2+} , thereby lowering cytosolic Ca^{2+} levels.

A first series of reports agreed with such an effect of cAMP on platelet Ca^{2+} transport. After a prostaglandin E_1 (PGE_1)-induced increase in the cAMP concentration in intact platelets, phosphoproteins of 22 and 24 kDa were detected, and Ca^{2+} uptake into the isolated membrane vesicles was increased [3,4]. The effect of cAMP on Ca^{2+} uptake was also assessed by treating the membrane vesicles with the catalytic subunit of the cAMP-dependent protein kinase (C.Sub.) [5–8]. A phosphoprotein of 22 or 24 kDa (depending upon the authors) was detected, and an increase in Ca^{2+} uptake was observed. These results were comparable with those obtained with the cardiac sarcoplasmic reticulum Ca^{2+} -ATPase, which is modulated by cAMP through the phosphorylation of a 24 kDa protein, phospholamban [9].

However, a second series of reports disagrees with the concept of a regulatory role for cAMP in platelet Ca^{2+} transport. Further studies established that the platelet phosphoprotein differed from

phospholamban [5,10,11]; it was consequently named thrombolamban. Other reports showed that the cAMP-dependent phosphoprotein did not appear to directly affect Ca^{2+} uptake into platelet microsomal fractions [12,13].

We next determined that the cAMP phosphoprotein is in fact mainly associated with plasma membranes, after separation of these membranes from microsomes. Moreover, we found that it appears as a 24 kDa protein under reducing conditions and as a 22 kDa protein under non-reducing conditions [14]. To date, the platelet cAMP phosphoprotein remains unidentified and its regulatory function is not yet established.

The presence of small-molecular-mass GTP-binding proteins (s mg) has been demonstrated in human platelets [15–24]. One of these is phosphorylated *in vivo* in response to a PGE_1 -induced increase in the cAMP concentration in intact platelets, and *in vitro* by the cAMP-dependent protein kinase [24]. Its phosphorylation by cAMP was also reported after purification of the protein, which was identified as a *ras*-like protein [25], but its function remained unknown.

Due to the similarities of these observations to those with the cAMP-dependent phosphoprotein, it was tempting to test whether the same protein was involved. Thus the aim of the present work was to establish whether the cAMP phosphoprotein referred to above is identical to a member of the *ras* superfamily of proteins [26,27], and to define its role in the regulation of platelet Ca^{2+} transport. We show that platelet plasma membranes bind the non-hydrolysable GTP analogue guanosine 5'-[γ -thio]triphosphate (GTP[S]) and contain material that reacts with an antibody directed against the *ras*-related *rap1* protein. The relationship between the cAMP phosphoprotein, the GTP-

Abbreviations used: GTP[S], guanosine 5'-[γ -thio]triphosphate; GDP[S], guanosine 5'-[β -thio]diphosphate; p[NH]ppG, guanosine 5'-[β -imido]triphosphate; Ca^{2+} -ATPase, Ca^{2+} -activated Mg^{2+} -dependent ATPase; Temed, *NNN'*-tetramethylethylenediamine; cAMP, cyclic AMP; C.Sub., catalytic subunit of the cAMP-dependent protein kinase; PGE_1 , prostaglandin E_1 ; DTT, dithiothreitol.

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binding protein and the *rap1* protein was then investigated, along with its role in the regulation of Ca^{2+} transport in platelet plasma membranes.

EXPERIMENTAL

Materials

BSA, Hepes, ATP (sodium salt) and C.Sub. from bovine heart were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Products for electrophoresis (glycine, Tris, SDS, acrylamide, bisacrylamide, ammonium persulphate, β -mercaptoethanol and Temed) were from Bio-Rad (Richmond, CA, U.S.A.). $^{45}\text{CaCl}_2$ was purchased from Amersham International (Amersham, Bucks., U.K.). $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and $[\text{S}^{35}]\text{GTP}[\text{S}]$ were from Dupont-NEN Inc. GTP[S], guanosine 5'- $[\beta\text{-thio}]\text{diphosphate}$ (GDP[S]) and guanosine 5'- $[\beta\gamma\text{-imido}]\text{triphosphate}$ (p[NH]ppG), GDP, GTP and ITP were obtained from Boehringer (Mannheim, Germany).

Preparation of platelet membranes

Platelet membrane vesicles were prepared as previously described [28–30]. Briefly, fresh human platelets were isolated and washed in Tyrode's modified buffer. They were then disrupted by controlled ultrasonication in lysis buffer containing 0.8 mg of benzamidine/ml and 0.1 unit of aprotinin/ml, with or without 1 mM-dithiothreitol (DTT), depending upon the experiment. Lysed platelets were centrifuged at 19000 *g* to eliminate unlysed platelets, mitochondria and granules. The supernatant was centrifuged at 100000 *g* and the pellet, containing a mixture of plasma membranes and intracellular membranes, was layered over a 40% (w/v) sucrose cushion and centrifuged again at 100000 *g*. The two membrane fractions obtained at the interface with the sucrose cushion and at the bottom of the tube were characterized. Analysis of the two membrane subfractions by SDS/PAGE demonstrated different profiles, with different proportions of high- and low-molecular-mass proteins. The pellet membrane fraction exhibited weak $[\text{H}^3]\text{concanavalin A}$ labelling, low phosphodiesterase activity and a lower cholesterol/phospholipid ratio. These values were 4–12% of those found in the interface membrane fraction [14,28]. The IgG alloantibody, specific for the platelet surface-oriented glycoproteins IIb/IIIa, gave 90% and 10% cross-reactivity with the interface membrane fraction and the pellet membrane fraction respectively, using a quantitative immunofluorescence technique [28]. Finally, anti-Lek^a antibody, which recognizes glycoprotein IIb, only reacted with the interface membrane fraction. Thus, based on these different characteristics, we concluded that the two subfractions were plasma membranes (interface fraction) and intracellular membranes (pellet fraction).

SDS/polyacrylamide-gel electrophoresis

The protein content of the membrane fractions was measured by the method of Bradford [31]. Aliquots of membrane fractions were solubilized in 50 mM-Tris, pH 6.8, containing 1% SDS, 0.01% (v/v) Bromophenol Blue and 25% glycerol for 15 min at 60 °C. Reduced samples were produced by the addition of 5% β -mercaptoethanol. Samples were analysed by SDS/PAGE in either 11% or 15% gels [32].

Membrane protein phosphorylation

Phosphorylations were carried out on membranes isolated from platelets lysed in the absence of DTT in order to eliminate the appearance of the cAMP-phosphorylatable 24 kDa β -subunit of glycoprotein 1b [14]. Incubations were conducted at 30 °C for 10 min in a reaction mixture containing 10 mM-sodium phosphate

buffer, pH 7.0, 25 mM-MgSO₄, 100 mM-NaF, 6 mM- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (460 MBq/mmol) and 0.1% Triton, in the presence or the absence of C.Sub. (enzyme/substrate ratio of 0.02) [6]. Reactions were initiated by the addition of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and stopped by the addition of an equal volume of solubilization buffer. Membrane proteins were separated by SDS/PAGE as described above, electrotransferred on to nitrocellulose membranes for 2 h at 60 V and 4 °C [33] and autoradiographed using intensifying screens for 24–48 h with Kodak X-Omat AR films at –80 °C.

GTP binding to membrane proteins

The nitrocellulose membrane was incubated for 30 min at 25 °C in a buffer containing 50 mM-Tris/HCl, pH 7.5, 0.3% Tween 20, 0.012 mM-MgCl₂, 1 mM-DTT and 5% (w/v) non-fat dried milk, and then for 45 min at 25 °C with $[\text{S}^{35}]\text{GTP}[\text{S}]$ (1 $\mu\text{Ci/ml}$; 48.5 TBq/mmol) or $[\alpha\text{-}^{32}\text{P}]\text{GTP}$ (1 $\mu\text{Ci/ml}$; 111 TBq/mmol) in the same buffer. The membrane was then washed four times for 15 min with 10 mM-Tris/HCl buffer, pH 7.5, containing 0.2% Tween 20 and 150 mM-NaCl. It was finally rinsed with distilled water and air-dried. GTP binding was revealed by autoradiography for 24–72 h using Kodak X-Omat AR films at –80 °C. The specificity of GTP binding was assessed by preincubating the nitrocellulose membrane with 20 μM -GTP, -GDP[S], -p[NH]ppG or -ITP in binding buffer for 5 min at 25 °C prior to the addition of the labelled GTP.

Western blot procedure

Polyclonal rabbit antibodies were raised against a peptide encompassing residues 121–137 of the *rap1* protein. Its preparation and characterization are described in detail elsewhere [34]. Nitrocellulose membranes were blocked in TBS (150 mM-NaCl, 10 mM-Tris/HCl, pH 7.5) containing 5% (w/v) non-fat dried milk at 37 °C for 1 h, and then incubated with a 1:100 dilution of anti-*rap1* antiserum in TBS containing 0.5% non-fat dried milk for 2 h at room temperature. After this step, the nitrocellulose membranes were washed five times with distilled water and then with TBS/0.1% Tween 20 for 10 min. Antibody binding was revealed by 1 h of incubation of the nitrocellulose membranes at room temperature with ^{125}I -labelled Protein A (1850 MBq/mg; 30000 c.p.m./strip) in TBS/0.5% non-fat dried milk. The nitrocellulose membranes were then washed twice in the same buffer, and autoradiography of the dried membranes was performed with Kodak X-Omat AR films, using intensifying screens, for 12–72 h at –80 °C.

Measurement of Ca^{2+} -ATPase activity

These experiments, as well as those on Ca^{2+} uptake (see below), were conducted on isolated membranes using lysis buffer containing DTT, which was required to preserve enzyme activity. Ca^{2+} -ATPase activity was measured as previously described [35] by incubating plasma membrane vesicles at 37 °C and pH 8 in 0.2 ml of reaction mixture containing 20 mM-Hepes, 100 mM-KCl and 0.5 mM-EGTA, with or without 0.5 mM- CaCl_2 and 2 mM-MgCl₂. The final concentration of free Ca^{2+} was 1.6 μM , as calculated using a computer program kindly provided by Dr. L. Brass (University of Pennsylvania, Philadelphia, PA, U.S.A.). The reaction was initiated by the addition of 0.5 mM-ATP (final concentration) and stopped with 2 ml of Malachite Green reagent, used for the colorimetric determination of P_i [36].

The $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase activity was calculated by subtracting the values obtained in the presence of both Mg^{2+} and Ca^{2+} from those obtained in the presence of Mg^{2+} but without Ca^{2+} . The basal Mg^{2+} -ATPase activity was estimated by subtracting the values obtained with ATP from those obtained without ATP.

Measurement of Ca²⁺ uptake into membrane vesicles

Ca²⁺ uptake by membrane vesicles was measured using freshly isolated plasma membrane vesicles by incubating 100 µg of platelet membranes in 1 ml of reaction mixture containing 20 mM-Hepes buffer, pH 7.4, 100 mM-KCl, 5 mM-MgATP, 0.5 mM-⁴⁵CaCl₂ and 0.62 mM-EGTA. The final concentration of free Ca²⁺ was 1 µM, as estimated by Fabiato & Fabiato [37]. The reaction was started by adding ATP to the incubation medium and stirring at 37 °C. At various times, 0.1 ml aliquots were withdrawn and filtered through Millipore filters (HAWP 0.45 µm pore size) previously soaked in a BSA solution (2 mg/ml). The filters were washed with 3 × 5 ml of 0.1 M-CaCl₂ and counted for radioactivity in 10 ml of scintillation liquid.

RESULTS

Detection and membrane localization of GTP-binding proteins in platelets

Previous studies conducted on isolated platelet plasma membranes and intracellular membranes showed that the platelet cAMP phosphoprotein was located in plasma membranes, and migrated on SDS/PAGE as a 24 kDa protein under reducing conditions and as a 22 kDa protein under non-reducing conditions [14]. We therefore investigated the GTP-binding ability of plasma membrane proteins using a nitrocellulose filter overlay technique after separation of membrane proteins by SDS/PAGE, under both reducing and non-reducing conditions (Fig. 1). These studies were carried out using either [α -³²P]GTP (Fig. 1a) or the non-hydrolysable analogue [³⁵S]GTP[S] (Fig. 1b), both of which resulted in the detection of GTP-binding proteins. However, a greater number of proteins was detected with the non-hydrolysable GTP analogue. Moreover, other nucleotides such as ITP or non-hydrolysable GTP analogues such as p[NH]ppG or GDP[S] totally abolished GTP binding (results not shown). The [³⁵S]GTP[S] binding study allowed the detection of five different polypeptides under both reducing and non-reducing

Fig. 1. GTP binding to platelet plasma membranes

Platelet plasma membranes were prepared in the absence of DTT as described in the Experimental section. Membrane proteins (50 µg) were electrophoresed under reducing (R) or non-reducing (NR) conditions on SDS/PAGE (11% gels) and electrotransferred on to nitrocellulose membranes. The membranes were then incubated with either [α -³²P]GTP or [³⁵S]GTP[S] as described in the Experimental section. [α -³²P]GTP binding was revealed by autoradiography for 24 h (a), and [³⁵S]GTP[S] binding was revealed by autoradiography for 48 h (b). The identification of the molecular masses was by means of standard labelled markers. Numbers on the left sides of the figure represent the molecular masses of the observed GTP-binding proteins. The autoradiogram is typical of five different experiments.

Fig. 2. Immunodetection of rap1 protein in platelet membranes

Platelet plasma membranes were prepared as described in Fig. 1. They were electrophoresed on SDS/PAGE (15% gels) under reducing or non-reducing conditions, electrotransferred on to a nitrocellulose membrane and incubated with the anti-rap1 serum as described in the Experimental section. Antibody binding was revealed by incubation with ¹²⁵I-Protein A. (a) Immunoblot of the plasma membrane proteins: dose-response analysis. Membrane proteins (25 and 50 µg) were electrophoresed under reducing (R) or non-reducing (NR) conditions. Recombinant bacteria were used as a control (Bi). Antibody binding was revealed after 24 h of autoradiography. (b) Immunoblot of mixed, plasma membrane and intracellular membrane proteins. The different membrane fractions (50 µg) were electrophoresed under reducing conditions, and the proteins were then electrotransferred and treated as described in the Experimental section. Antibody binding was revealed after 72 h of autoradiography. Lane A, 100 000 g mixed membranes; lane B, plasma membranes; lane C, intracellular membranes. This Figure is typical of two different experiments.

conditions, ranging in molecular mass from 22 to 30 kDa. The most heavily labelled bands corresponded to a protein of molecular mass 22 kDa and 24 kDa under non-reducing and reducing conditions respectively. This suggests a shift in the migration of this protein on reduction, as previously observed for the cAMP phosphoprotein.

These types of experiments were also conducted with intracellular membranes, which exhibited negligible levels of GTP-binding proteins (results not shown), as previously observed for the cAMP phosphoprotein [14].

Thus a GTP-binding protein seemed to exhibit similar electrophoretic migration characteristics and was located in the same membrane fraction as the cAMP phosphoprotein identified in our previous report.

Identification and localization of ras family GTP-binding proteins in platelet membranes

We then examined whether GTP-binding proteins of the ras family were expressed in the same membrane fraction. Membrane proteins were submitted to immunoblotting using antibodies raised against different members of the ras family, such as the *rab1*, *rab2*, *rap1* and *rap2* proteins. Of the different antibodies used, only polyclonal anti-rap1 antibodies reacted, in a dose-

Fig. 3. Identity between the cAMP phosphoprotein, the major GTP-binding protein and *rap1*

Plasma membranes (50 μ g) were first phosphorylated with C.Sub. (20 μ g/ml) as described in the Experimental section. Reduced or non-reduced phosphorylated proteins were then submitted to SDS/PAGE, electrotransferred on to a nitrocellulose membrane and submitted to autoradiography. After 3 weeks (to allow 32 P radioactivity to decay), the nitrocellulose membrane was incubated with [35 S]GTP[S] for measurement of GTP binding as in Fig. 1. The same nitrocellulose membrane was used, again after waiting (3 months) to allow the decay of 35 S radioactivity, for immunoblotting with the antibody directed against the *rap1* protein, as in Fig. 2. (a) C.Sub.-induced protein phosphorylation, (b) [35 S]GTP[S] binding, (c) immunoblotting. R, reducing conditions; NR, non-reducing conditions.

Fig. 4. Effect of GTP[S] on C.Sub.-induced phosphorylation

Plasma membrane proteins (50 μ g) were preincubated or not with 40 μ M-GTP[S] for 5 min, and then incubated in the presence of C.Sub. (20 μ g/ml) as described in the Experimental section. The phosphoproteins were reduced, electrophoresed on SDS/PAGE and autoradiographed for 48 h. (a) Membrane proteins preincubated with GTP[S]; (b) untreated membrane proteins. Lanes 1, C.Sub.-induced protein phosphorylation; lanes 2, controls. This Figure is typical of three different experiments.

dependent manner, with isolated platelet plasma membrane proteins (Fig. 2a). We could not detect *rap2* protein, recently identified in a crude membrane fraction [38]. In addition, it can

be seen that the electrophoretic migration of the protein recognized by the antibody again depends upon the conditions (reducing or non-reducing). Fig. 2(b) demonstrates that *rap1* protein was associated with the mixed membrane fraction (lane A), but this appeared to be due to the plasma membranes (lane B), as the intracellular membranes hardly react with the antibody (lane C). Thus the *rap1* protein from platelets appeared as a 24 kDa species under reducing conditions and as a 22 kDa protein under non-reducing conditions, and exhibited the same membrane location as both the cAMP phosphoprotein and the major GTP-binding protein.

Relationship between the cAMP phosphoprotein, a low-molecular-mass GTP-binding protein and the *rap1* protein in platelets

The above results led us to study the potential identity between the cAMP phosphoprotein, the GTP-binding protein and *rap1* protein, using the following approach. On the same membrane preparation we successively performed cAMP-induced phosphorylation, comparative electrophoresis under reducing and non-reducing conditions, transfer of the phosphorylated proteins on to a nitrocellulose membrane, binding of [35 S]GTP[S] and immunoblotting with anti-*rap1* antibody. This was based on: (1) similar GTP binding whatever the phosphorylation state of the phosphoprotein (results not shown); and (2) the fact that the anti-*rap1* antibody used recognized neither the GTP-binding site nor the phosphorylation site [34].

Fig. 3 shows the results of such an experiment, which clearly demonstrates identity in the migration profiles of the cAMP phosphoprotein (Fig. 3a), the predominant GTP-binding protein (Fig. 3b) and the *rap1* protein (Fig. 3c) under both reducing (lanes R) and non-reducing (lanes NR) experimental conditions. However, to further establish that the three functions examined reside on the same protein, we undertook experiments in order to test whether (1) cAMP-dependent phosphorylation influences recognition by anti-*rap1* antibodies and (2) GTP binding affects cAMP-dependent phosphorylation. No modification in the recognition signal of the anti-*rap1* antibody was observed as a function of the phosphorylation state of the protein (results not shown). In contrast, Fig. 4 shows that incubation of the membrane proteins with GTP[S] for 5 min (conditions which allow the GTP binding [39]) prior to phosphorylation resulted in marked inhibition of phosphorylation intensity. This is in contrast with the results obtained by Hoshijima *et al.* [24], who found that the GTP[S]-bound form of smg p21 was also phosphorylated by the cAMP-dependent protein kinase. However, their experiments were conducted on a purified sample of smg p21 rather than on platelet membranes; GTP can induce a conformational change in the protein in the latter, or steric hindrance can prevent the accessibility of the phosphorylation site. In any case, our results suggest that the three functions examined reside on the same protein.

Effect of GTP[S] on plasma membrane Ca^{2+} transport

Since the cAMP phosphoprotein was identified as the *ras*-related *rap1* GTP-binding protein, it became possible to investigate its role in the regulation of Ca^{2+} transport, using the analogue GTP[S].

Effect on Ca^{2+} -ATPase activity. Ca^{2+} -ATPase activity is responsible for Ca^{2+} transport across membrane vesicles in which the enzyme is inserted. Its characterization was previously described [14,40]. Fig. 5(a) shows the effect of GTP[S] on the time course of this Ca^{2+} -ATPase activity. The pretreatment of platelet plasma membranes with 40 μ M-GTP[S] for 5 min before the addition of ATP caused a decrease in Ca^{2+} -ATPase activity within a few minutes. This decrease was dependent upon the

Fig. 5. Effect of GTP[S] on plasma membrane Ca^{2+} -ATPase activity

(a) Effect of GTP[S] on the time course of plasma membrane $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase activity. The membranes were preincubated for 5 min at room temperature in the presence or in the absence of GTP[S] in order to allow nucleotide binding [39]. The time courses of $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase activity measured in the absence (●) or the presence (△) of $40 \mu\text{M}$ -GTP[S] are shown. (b) Dose-dependent effect of GTP[S] on $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase activity and on Mg^{2+} -ATPase activity. Membranes were treated with GTP[S] as above. The $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase activity (●) and the Mg^{2+} -ATPase activity (▲) were measured as described in the Experimental section. Results are expressed as percentages of the enzyme activities measured in the absence of GTP[S] and are means $\pm$ S.E.M. of four different experiments.

Fig. 6. Effect of GTP[S] on Ca^{2+} uptake into plasma membrane vesicles

Ca^{2+} uptake was measured as described in Experimental section in membrane vesicles pretreated or not with GTP[S] as in Fig. 5. (a) Effect of GTP[S] on the time course of Ca^{2+} uptake into plasma membrane vesicles. ●, Ca^{2+} uptake measured in the absence of GTP pretreatment; △, Ca^{2+} uptake measured in the presence of $40 \mu\text{M}$ -GTP[S]. (b) Dose-dependent effects of GTP[S] on Ca^{2+} uptake by membrane vesicles. The membranes were pretreated with GTP[S] and Ca^{2+} uptake was measured after a 45 min incubation. Results are expressed as a percentage of Ca^{2+} uptake obtained in the absence of GTP[S], and are means $\pm$ S.E.M. of four different experiments.

concentration of GTP[S] (from 0 to $40 \mu\text{M}$), and reached a maximum level of 40 % (Fig. 5b). The inhibition observed was specific for GTP[S], as no modification of the enzyme activity could be observed with GDP (results not shown). It was also specific for Ca^{2+} -ATPase activity, as the Mg^{2+} -ATPase activity measured in parallel was not affected (Fig. 5b).

Effect of GTP[S] on Ca^{2+} transport. We next examined the effect of GTP[S] on the Ca^{2+} transport function. Fig. 6(a) shows that Ca^{2+} uptake was inhibited in the presence of $40 \mu\text{M}$ -GTP[S]. Inhibition was dependent on the GTP[S] concentration, in the same range as for the Ca^{2+} -ATPase assay (Fig. 6b), and the same 40 % maximum inhibition level was also observed. GDP had no effect on Ca^{2+} transport, emphasizing the specificity of the inhibitory effect of GTP[S].

In a previous work [35] we showed that GTP can serve as a substrate for the Ca^{2+} -ATPase, although it was much less potent than ATP; the non-hydrolysable substrate GTP[S] thus may

inhibit Ca^{2+} -ATPase activity by this mechanism. In order to exclude this possibility, we carried out experiments on the effect of GTP[S] on the formation of the Ca^{2+} pump aspartylphosphate intermediate ($\text{E} \sim \text{P}$), and found that a 10-fold higher concentration of GTP[S] ($500 \mu\text{M}$) only induced a maximal 20 % inhibition of the formation of this complex (results not shown).

Effect of both C.Sub. and GTP[S] on Ca^{2+} transport. As GTP[S] binds to the cAMP phosphoprotein and modulates its phosphorylation, we finally tested the effects of both GTP[S] and C.Sub. on Ca^{2+} uptake. Fig. 7 shows that incubation with C.Sub. ($15 \mu\text{g}/\text{ml}$) induced an increase in Ca^{2+} uptake by membrane vesicles, as previously observed [14]. GTP[S] alone, when added 5 min before the onset of the reaction, elicited inhibition of Ca^{2+} uptake as shown in Fig. 6. The addition of both effectors (GTP[S] added 5 min before C.Sub.) resulted in the abolition of the C.Sub.-induced stimulation, which agrees with the inhibition by GTP[S] of the cAMP-induced phosphorylation of the

Fig. 7. Comparative effects of GTP[S] and C.Sub. on Ca^{2+} uptake by plasma membrane vesicles

Membrane vesicles were pretreated for 5 min in the presence or the absence of $40 \mu\text{M}$ -GTP[S] and tested for Ca^{2+} uptake as described in Fig. 6. ●, Control; △, Ca^{2+} uptake in the presence of $40 \mu\text{M}$ -GTP[S]; ○, Ca^{2+} uptake in the presence of $15 \mu\text{g}$ of C.Sub./ml; □, Ca^{2+} uptake in the presence of $40 \mu\text{M}$ -GTP[S] plus $15 \mu\text{g}$ of C.Sub./ml. Results are means $\pm$ S.E.M. of three different experiments.

22–24 kDa protein (Fig. 4). Again, this can be explained by a GTP-induced conformational change in the protein, which would prevent the further stimulation of the Ca^{2+} pump. It is concluded that the binding of GTP to the *rap1* protein, as well as its phosphorylation by cAMP-dependent protein kinase, regulates platelet Ca^{2+} transport.

DISCUSSION

This paper demonstrates that the major cAMP-dependent phosphoprotein present in isolated plasma membranes from human platelets is the *ras*-related *rap1* GTP-binding protein. This identification is based on the electrophoretic mobility of the cAMP phosphoprotein (i.e. the shift in its migration on SDS/PAGE depending on the use of reducing or non-reducing conditions), the use of *rap1*-specific anti-peptide antibodies, and studies of the interactions between cAMP-dependent phosphorylation and GTP binding on the *rap1* protein.

The possible relationship between the cAMP phosphoprotein and a *ras* family GTP-binding protein constitutes a new field of investigation. First, among the different 'small molecular mass' (smg) GTP-binding proteins, smg p21 was shown to be phosphorylated in response to a PGE_1 -induced increase in the cAMP level [23,24], and it was recently demonstrated that the purified protein was identical with the *rap1* B protein [41]. Secondly, a study published while the present work was in progress suggested that the main platelet cAMP phosphoprotein present in isolated membranes has sequence similarity with the *ras* family of GTP-binding proteins [42].

Our present findings thus agree with and complete these studies. First, the characteristics of smg p21 are also found for the cAMP phosphoprotein studied here (main GTP-binding protein and *rap1* protein identification), and it appears now to be located in plasma membranes. Secondly, the cAMP phosphoprotein appears as a *rap1* protein amongst the *ras*-related proteins. Thirdly, as the *rap* proteins are cysteine-rich compared with *ras* proteins, the characteristic shift in the migration of the cAMP phosphoprotein, initially supposed to be due to an intrachain disulphide bond [14] is now explained, and consequently constitutes a previously undescribed new characteristic of the *rap1* protein.

The second feature of this paper is the evidence for a new role for a low-molecular-mass platelet GTP-binding protein, which follows from the identity between the *rap1* GTP-binding protein and the cAMP phosphoprotein. As the latter was initially described as regulating Ca^{2+} transport, we consequently investigated a possible role for GTP in plasma membrane Ca^{2+} transport. GTP[S] induced inhibition of the plasma membrane Ca^{2+} pump, as well as inhibiting Ca^{2+} -ATPase activity, which mediates Ca^{2+} uptake. Therefore it can be suggested that the *rap1* protein exerts a more complex role than the positive regulation of Ca^{2+} -ATPase activity through the action of cAMP-dependent phosphorylation. Indeed, *rap1* protein would also be responsible for the negative regulation of the Ca^{2+} -ATPase through the binding of GTP. As both effectors (cAMP and GTP) can result in some conformational change in the *rap1* protein, a possible alternative is that one of them results in a positive interaction between the Ca^{2+} -ATPase and *rap1* protein, and the other one results in a negative interaction between the two proteins.

Consequently, this work provides evidence that implicates the cAMP phosphoprotein *rap1* in the regulation of Ca^{2+} transport, thus confirming previous work (see [3,4,5,8] and the Introduction section). Furthermore, preliminary results obtained with platelets from patients suffering from cardiac insufficiency showed a direct relationship between the amount of *rap1* protein and the cAMP-induced stimulation of Ca^{2+} uptake by the isolated membrane fraction. An alternative hypothesis of an indirect effect of cAMP was based on the similar phosphorylation activity of dialysed and non-dialysed C.Sub. [13], which we have never been able to reproduce (results not shown). In addition, these authors used experimental conditions (low C.Sub. concentrations, short phosphorylation time and high Ca^{2+} concentrations) which did not allow the detection of an effect of cAMP. Moreover, this hypothesis could not explain either the regulation of the effect of C.Sub. upon the Ca^{2+} concentration [8,14] or, most importantly, the fact that both intact platelets treated with PGE_1 [3] and isolated membrane vesicles treated with a cytosolic extract containing the cAMP protein kinase took up more Ca^{2+} than in the absence of the kinase [43].

This role of GTP as a regulator of intracellular Ca^{2+} in human platelets confirms recent evidence of GTP-induced regulation of Ca^{2+} -ATPase activities in different cell types [44,45]. Similarly, it agrees with another recent study conducted in yeast which also suggests that the *ras*-related *YPT1* gene product plays a role in the regulation of Ca^{2+} concentration [46,47].

Finally, analysis of the present results emphasizes the importance of the role of GTP-binding proteins in human platelet activation. Indeed, initial studies showed a G-protein regulation of phospholipase C, leading to signal transduction [48]. This results in the production of $\text{Ins}(1,4,5)\text{P}_3$ which is responsible for the liberation of Ca^{2+} from its storage organelle. Here we provide evidence that the *rap1* GTP-binding protein inhibits plasma membrane Ca^{2+} -ATPase activity, the function of which is to deplete the cell of Ca^{2+} . Thus the increase in Ca^{2+} concentration observed during platelet activation would be the result of at least two different pathways.

Furthermore, besides its role in platelet activation by binding GTP, *rap1* protein, when phosphorylated by cAMP, would contribute to the decrease in the Ca^{2+} concentration associated with platelet inhibition. Thus the *rap1* protein appears to play a complex role in the regulation of platelet Ca^{2+} fluxes and, by binding GTP or being phosphorylated, it will either stimulate or inhibit platelet activation.

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