Relationship between Rap1 protein phosphorylation and regulation of Ca^{2+} transport in platelets: a new approach

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Although the interrelationship between the two messengers Ca^{2+} and cyclic AMP in platelet function is well documented, its mechanism of action still remains to be established. We investigated here the question of the regulation of platelet Ca^{2+} -ATPases by cyclic AMP through the phosphorylation of the Rap1 protein using a pathological model. We first found experimental conditions where Ca^{2+} transport by platelet membrane vesicles appeared to be dependent on the phosphorylation of the Rap1 protein. Then, we studied platelets of patients with congestive heart failure for their expression of the potential 97 kDa Ca^{2+} -ATPase target of regulation through the Rap1 protein as well as the phosphorylation of the Rap1 protein using the catalytic subunit of the cyclic AMP-dependent protein kinase (C. Sub.). In the first patients studied, we found no significant modification in the expression of the 97 kDa Ca^{2+} -ATPase by Western blotting using the PL/IM 430 monoclonal antibody which specifically recognized this isoform. In contrast, the Rap1

protein was differentially phosphorylated when using 15 $\mu\text{g}/\text{ml}$ of the C. Sub. These results allowed us to use these pathological platelets to study the relationship between the expression of Rap1 protein and the regulation of Ca^{2+} transport by selecting a patient with severe heart failure. We could show a decrease in the expression as well as in the phosphorylation of Rap1 protein and demonstrate a lower effect of C. Sub. on Ca^{2+} transport. Finally, by studying a further series of patients, we could confirm that the decrease in Rap1 protein expression in heart failure, whatever its extent, was variable, and could strictly correlate the expression of Rap1 protein with the stimulatory effect of C. Sub. on Ca^{2+} transport. Besides the evidence for regulation of the expression of the Rap1 protein in platelets from patients with heart failure, these findings constitute a new approach in favour of the regulation of platelet Ca^{2+} transport through the phosphorylation of the Rap1 protein.

INTRODUCTION

The regulation of platelet cytosolic Ca^{2+} concentration involves specific and opposite mechanisms depending upon whether it concerns cell activation or inhibition. Activation of platelets by different physiological stimuli induces transduction signalling processes resulting in an increase in cytosolic Ca^{2+} concentration. This occurs by two phenomena: Ca^{2+} influx from extracellular medium through calcium channels and release of Ca^{2+} ions from intracellular Ca^{2+} storage pools including an inositol 1,4,5-trisphosphate (InsP_3)-sensitive Ca^{2+} pool and/or an InsP_3 -insensitive Ca^{2+} pool [1–4].

Conversely, the restoration of platelet cytosolic Ca^{2+} concentration associated with the inhibition of platelet function, i.e. the return to a basal state, is partly mediated by the activation of a multi-SERCA (SarcoEndoplasmic Reticulum Ca^{2+} -ATPase) system which decreases cytosolic Ca^{2+} concentration, by pumping Ca^{2+} ions into the intracellular Ca^{2+} pools. One of these Ca^{2+} -ATPases was identified as the ubiquitous SERCA 2-b isoform [5], a second one was recently shown to correspond to the SERCA 3 isoform [6,7], whereas a third one was recently detected, specifically recognized by a platelet monoclonal antibody termed PL/IM 430, but which still remains to be identified [8].

In addition to their own activation in the regulation of platelet cytosolic Ca^{2+} concentration, the Ca^{2+} -ATPases were suggested to be up-regulated through cyclic AMP. This hypothesis was initially based on the observation that cyclic AMP inhibits platelet function [1] in association with a decrease in Ca^{2+}

concentration. This might be a consequence of multiple sites [9,10], as well as an action on the phospholipase C and/or the InsP_3 receptor, a stimulation of Ca^{2+} efflux, or an activation of Ca^{2+} -ATPases, the latter being the subject of the present study. Indeed, an interesting early finding was that the addition of cyclic AMP to a cytosolic fraction containing cyclic AMP-dependent protein kinase stimulated Ca^{2+} transport mediated by Ca^{2+} -ATPases and phosphorylated a 22 kDa protein [11]. Various authors subsequently confirmed this finding, using different approaches. First, Fox et al. treated intact platelets with agents enhancing the cyclic AMP level and decreasing the cytosolic Ca^{2+} concentration. They demonstrated that a 22 kDa membrane protein was phosphorylated concomitantly with an increase in Ca^{2+} uptake [12]. Second, this effect of cyclic AMP on both the phosphorylation of a 22 kDa protein and Ca^{2+} uptake was further confirmed by treating isolated membrane vesicles with the purified catalytic subunit (C. Sub.) of the cyclic AMP-dependent protein kinase [13,14]. Many years later, the cyclic AMP-phosphoprotein was identified as a small GTP-binding protein of the Ras family, a Rap1 protein, more precisely the Rap1B [15–17].

Moreover, we were recently able to suggest that the Ca^{2+} -ATPase target of the regulation through the Rap1B protein would strictly involve the 97 kDa isoform recognized by the PL/IM 430 antibody, among the above-defined platelet multi-SERCA system [18]. However, during these studies, some controversy was raised about this effect of Rap1 protein on platelet Ca^{2+} transport, based on the fact that the C. Sub. effect could be

Abbreviations used: C. Sub., catalytic subunit of the cyclic AMP-dependent protein kinase; Rap, Ras-proximate; SERCA, sarcoendoplasmic reticulum Ca^{2+} -ATPase; InsP_3 , inositol 1,4,5-trisphosphate; PL/IM 430, monoclonal anti-(Ca^{2+} -ATPase) antibody raised using highly purified platelet intracellular membranes as immunogen.

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artefactual and due rather to phosphate contamination of the commercial C. Sub. solution than to the concomitant C. Sub.-induced phosphorylation of a 22 kDa protein by itself [19,20]. Finally, this relationship between intracellular cyclic AMP and Ca^{2+} uptake was investigated by using a kinetic study *in situ*, i.e. again in intact platelets, which shows an increase in the rate and maximal extent of Ca^{2+} uptake [21].

The aim of the present work was to revisit this relationship between the phosphorylation of the Rap1 protein and the regulation of platelet Ca^{2+} transport by using an approach distinct from those previously used and taking advantage of the congestive heart failure model. Indeed, because of the evidence for a down-regulation of myocardial β -adrenoreceptors in this pathology [22], as well as altered platelet α_2 -adrenoreceptors in patients with angina pectoris [23], the regulation of cyclic AMP-phosphoproteins such as the major Rap1B protein in platelets could be modified in heart failure.

For this purpose, we looked for both Rap1 protein expression and phosphorylation in mixed platelet membranes isolated from several patients with heart failure. Because of the detection of variable abnormalities of the Rap1 protein without significant modification of the PL/IM 430-recognizable 97 kDa Ca^{2+} -ATPase expression, we were able to further investigate the relationship between the Rap1 protein and the regulation of platelet Ca^{2+} uptake using this pathological model. By studying patients with variable decreases in Rap1 protein expression, a correlation between its phosphorylation and its effect on Ca^{2+} transport was found, thus again strongly arguing for an effect of Rap1 protein phosphorylation on platelet Ca^{2+} transport.

EXPERIMENTAL

Materials

BSA, Hepes, ATP (sodium salt), the catalytic subunit of the cyclic AMP-dependent protein kinase from bovine heart and the anti-(mouse IgG)-horseradish peroxidase conjugate 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.). $^{45}\text{CaCl}_2$, $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and Enhanced Chemiluminescence (ECL) Western blotting reagents were purchased from the Radiochemical Centre (Amersham, U.K.).

Platelet isolation

Human blood was obtained from normal healthy adult individuals and from patients with severe congestive heart failure due to ischaemic, dilated or hypertrophic cardiomyopathy (class III according to the functional classification of the New York Heart Association). The investigation was performed according to the Declaration of Helsinki, and informed consent was obtained from patients and volunteers. Nine parts of blood were collected with one part of the ACD-C anticoagulant made of 130 mM trisodium citrate, 111 mM glucose and 152 mM citric acid. The blood was centrifuged at 120 g for 15 min at room temperature to obtain platelet-rich plasma. Platelets were then washed using a modified Tyrode's buffer, pH 7.4, containing 130 mM NaCl, 5 mM KCl, 1 mM NaH_2PO_4 , 24 mM NaHCO_3 , 2 mM EDTA- Na_2 , 10 mM glucose, 12.5 mM saccharose and 0.35 % BSA.

Preparation of platelet membranes

Platelet membrane vesicles were prepared as previously described [24]. Briefly, platelets were disrupted by controlled ultra-

sonication in a lysis buffer containing 25 mM Hepes, pH 7, 100 mM KCl, 2 mM MgSO_4 , 25 mM NaCl, 12 mM sodium citrate, 10 mM glucose, 5 mM ATP, 0.35 % (w/v) BSA, 0.8 mg of benzamidine/ml and 0.1 unit of aprotinin/ml. Lysed platelets were centrifuged at 19000 g to eliminate unlysed platelets, mitochondria and granules. The supernatant was centrifuged at 100000 g and the pellet was used as the source of membrane vesicles. This 100000 g mixed membrane fraction has been fully characterized and consists of a mixture of plasma and intracellular membranes [25]. The protein content of the membrane fractions was measured by the method of Bradford using γ -globulins as standard [26].

SDS/PAGE

Aliquots of membrane fractions were solubilized in the following buffer: 50 mM Tris, pH 6.8, containing 1 % SDS, 0.01 % (v/v) Bromophenol Blue and 25 % glycerol. Samples were treated for reduction for 15 min at 60 °C with the addition of 5 % β -mercaptoethanol and applied to SDS/11 or 15 % polyacrylamide slab gels according to the method of Laemmli [27]. Electrophoresis was performed at 40 V overnight.

Cyclic AMP-induced Rap1 protein phosphorylation

Incubations were conducted at 30 °C for 10 min in a reaction mixture containing 10 mM sodium phosphate buffer, pH 7.0, 15 mM MgSO_4 , 100 mM NaF, 6 mM $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (460 MBq/mmol), and 0.1 % Triton X-100 in the presence or absence of the catalytic subunit of the cyclic AMP-dependent protein kinase (enzyme/substrate = 0.02) [28]. Reactions were initiated by the addition of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and stopped by adding an equal volume of solubilization buffer containing 50 mM Tris, pH 6.8, 1 % SDS, 0.01 % (v/v) Bromophenol Blue and 25 % glycerol. Membrane proteins were separated by SDS/PAGE as described above. They were electrotransferred on to nitrocellulose membranes for 2 h at 250 mA and 4 °C and autoradiographed at -80 °C using Kodak X-Omat AR films and intensifying screens.

Western blot procedure

(a) The anti-Rap1 polyclonal rabbit antibody was raised against a peptide encompassing residues 121–137 of the Rap1 proteins. Its preparation and characterization are described in detail elsewhere [29,30]. This antibody recognizes both Rap1A and Rap1B proteins. Nitrocellulose membranes were blocked in TBS (10 mM Tris/HCl, pH 7.5, 150 mM NaCl) containing 5 % (w/v) non-fat dry milk, at 37 °C for 1 h and then incubated with a 1:100 dilution of anti-Rap1 serum in TBS containing 0.5 % non-fat dry milk for 2 h at room temperature. After this step, the nitrocellulose membranes were washed five times with distilled water and then with TBS and 0.1 % Tween 20 for 10 min. Antibody binding was revealed by 1 h of incubation at room temperature of the nitrocellulose membranes with 1850 MBq/ml ^{125}I -labelled Protein A (30000 c.p.m./strip) in TBS and 0.5 % non-fat dry milk. The membranes were then washed twice in TBS and 0.1 % Tween 20. Autoradiography of the dried nitrocellulose membranes was performed at -80 °C using Kodak X-Omat AR films and intensifying screens.

(b) The monoclonal PL/IM 430 antibody was raised using highly purified platelet intracellular membrane vesicles as immunogen, and purified from culture supernatant of the corresponding hybridoma cell line by Protein A affinity chromatography at high ionic strength (3 M NaCl) [8]. Its preparation and characterization are described elsewhere [31]. Briefly, this antibody inhibits Ca^{2+} uptake into platelet membrane vesicles and

Ca^{2+} sequestration into intracellular stores in saponin-permeabilized platelets, and specifically recognizes the 97 kDa Ca^{2+} -ATPase in platelets and different cell lines [32].

Nitrocellulose membranes were treated overnight at room temperature with a quenching solution containing 150 mM NaCl, 10 mM Tris/HCl (pH 7.5), 0.1 % Tween 20 and 5 % non-fat dry milk. The blots were then rinsed with the same solution and incubated with the purified PL/IM 430 antibody at a final concentration of 1 $\mu\text{g}/\text{ml}$ for 2 h at room temperature. Thereafter, the nitrocellulose sheets were extensively washed with the quenching solution and further incubated with the anti-(mouse IgG)-horseradish peroxidase conjugate (1:2000 dilution) for 2 h. After washing steps, antibody binding was detected by using the ECL Western blotting reagents according to the instructions of the manufacturer.

Measurement of Ca^{2+} uptake into platelet membrane vesicles

Calcium uptake by the membrane vesicles was measured 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 or 10 mM MgCl_2 , 0.5 mM $^{45}\text{CaCl}_2$ and 0.5 mM EGTA. The final concentration of free Ca^{2+} was 1.6 μM as calculated according to Fabiato and Fabiato [33]. The reaction was started by adding 5 mM ATP to the incubation medium and stirring at 37 °C. At various intervals, 0.1 ml aliquots were withdrawn and filtered through Millipore filters (HAWP 0.45- μm pore size) previously soaked in a 2 mg/ml BSA solution. The filters were washed three times with 5 ml of 0.1 M CaCl_2 and counted for radioactivity in 10 ml of scintillation liquid.

RESULTS

Relationship between platelet Ca^{2+} transport and Rap1 protein phosphorylation

Effect of C. Sub. dialysis

We first carried out experiments to re-explore the relationship between Rap1 protein phosphorylation and the regulation of Ca^{2+} transport, by testing different experimental conditions. Because one argument for an effect of phosphate ions present in the catalytic subunit of the cyclic AMP-dependent protein kinase (C. Sub.) over that of concomitant C. Sub.-induced protein phosphorylation on Ca^{2+} uptake was based on the fact that dialysed C. Sub. was inactive (whereas it fully phosphorylated a 22 kDa protein [20]), we proceeded with dialysis of the C. Sub. preparation. Then, we tested its effect on isolated platelet membranes for specific Rap1 protein phosphorylation as previously characterized [16,18] (Figure 1a) and Ca^{2+} uptake under conditions again previously used and characterized as allowing total Ca^{2+} uptake mediated by the multi-SERCA system [24,25,34,35] (Figure 1b). Figure 1(a) shows that we did not succeed in maintaining a full C. Sub. activity to phosphorylate the Rap1 protein. Indeed, the comparison between lanes 2 and 3 obviously shows significant reduction in phosphorylation of the Rap1 protein after dialysis. In agreement with this finding was the result of Figure 1(b) showing the absence of any significant effect of dialysed C. Sub. on total Ca^{2+} uptake, whereas non-dialysed C. Sub. induced the twofold stimulation previously observed.

So, these findings mean that, in our hands, the dissociation between the effect of C. Sub. and that of phosphate on Ca^{2+} uptake cannot be obtained using dialysed C. Sub. Consequently, we were led to develop the following approaches.

Figure 1 Relationship between platelet Rap1 protein phosphorylation (a) and Ca^{2+} transport (b)

(a) Effect of dialysis on C. Sub.-induced Rap1 protein phosphorylation. Mixed platelet membranes were isolated from fresh platelets after lysis and differential centrifugation at 19000 g and 100000 g as described in the Experimental section. Membrane proteins (50 μg) were phosphorylated in the absence or presence of 15 $\mu\text{g}/\text{ml}$ of catalytic subunit of the cyclic AMP-dependent protein kinase (C. Sub.), either directly or after dialysis. Dialysis was performed at 4 °C using Millipore filter VSWP (0.025 μm pore size) for 2 h in 25 mM Hepes, pH 8, 100 mM KCl. Reactions were stopped, and samples were reduced using 5 % β -mercaptoethanol. Proteins were separated on SDS/11 % PAGE, electrotransferred and submitted to autoradiography. Lane 1, phosphorylation in the absence of C. Sub.; lane 2, dialysed C. Sub.-induced Rap1 protein phosphorylation; lane 3, control C. Sub.-induced Rap1 protein phosphorylation. The Figure is typical of two experiments. (b) Effect of dialysed C. Sub. on Ca^{2+} uptake into membrane vesicles. Mixed platelet membranes (100000 g pellet) were isolated from normal individuals. They were incubated in the reaction mixture containing 20 mM Hepes buffer, pH 7.4, 100 mM KCl, 5 mM MgCl_2 , 0.5 mM $^{45}\text{CaCl}_2$ and 0.5 mM EGTA in the absence or presence of 15 $\mu\text{g}/\text{ml}$ of C. Sub. used either before or after dialysis. Ca^{2+} uptake was started by adding 5 mM ATP to the incubation medium and stirring at 37 °C. Aliquots were withdrawn at the times indicated and membrane vesicles were treated for Ca^{2+} uptake measurements as described in the Experimental section. Results are expressed as percentages of maximal Ca^{2+} uptake in membrane vesicles measured at 15 min and in the presence of non-dialysed C. Sub., arbitrarily taken as 100%. ■, Ca^{2+} uptake into membrane vesicles in the absence of C. Sub.; ▲, Ca^{2+} uptake into membrane vesicles in the presence of non-dialysed C. Sub.; □, Ca^{2+} uptake into membrane vesicles in the presence of dialysed C. Sub. The Figure is typical of two experiments.

The priming effect of C. Sub.

To begin with, we tried to dissociate the effect of C. Sub. from that of phosphate ions and focused on the Ca^{2+} uptake mediated by the PL/IM 430-recognizable SERCA target of the Rap1 protein. For this purpose, we took advantage of the following previous observations about the platelet Ca^{2+} -ATPase system: (i) the PL/IM 430-recognizable 97 kDa Ca^{2+} -ATPase accounts for a large part of Ca^{2+} uptake among the multi-SERCA [36] and

Figure 2 Relationship between platelet Rap1 protein phosphorylation and Ca^{2+} uptake: evidence for Ca^{2+} uptake as a function of Rap1 protein phosphorylation

Mixed platelet membrane vesicles (100 000 *g* pellet) were isolated from four normal individuals. They were incubated in the reaction mixture containing 20 mM Hepes buffer, pH 7.4, 100 mM KCl, 10 mM MgCl_2 , 0.5 mM $^{45}\text{CaCl}_2$ and 0.5 mM EGTA in the absence or presence of 15 $\mu\text{g}/\text{ml}$ of the C. Sub. The final concentration of free Ca^{2+} was 1.6 μM . The reaction was started by adding 5 mM ATP to the incubation medium and stirring at 37 °C. Aliquots were withdrawn at the times indicated and membrane vesicles were treated for Ca^{2+} uptake measurements as described in the Experimental section. Results are expressed in nmol/mg of protein and are the means $\pm$ S.D. of four experiments. ■, Ca^{2+} uptake into membrane vesicles in the absence of C. Sub.; □, Ca^{2+} uptake into membrane vesicles in the presence of C. Sub.

requires higher ATP concentrations than the SERCA 2-b and SERCA 3 isoforms [8]; (ii) Mg^{2+} -ATPase activities are present in mixed membrane fractions which can mask the SERCA activities [34]. So, we postulated that this masking of Ca^{2+} uptake by increasing MgCl_2 should be particularly effective as regards the PL/IM 430-recognizable 97 kDa Ca^{2+} -ATPase. In such a case, it should be possible to look for some priming role of the Rap1 protein phosphorylation to induce Ca^{2+} uptake, thus allowing the subsequent effect of phosphate ions on the Ca^{2+} transport, i.e. dissociating the two effects in a sequential manner. Thus, we isolated mixed platelet membrane fractions and tested the effect of C. Sub. on Ca^{2+} uptake under the experimental conditions of Figure 1(b), except for MgCl_2 concentration, which was increased by a factor of two.

Figure 2 shows the results of these experiments conducted on platelet membrane vesicles isolated from normal individuals. Whereas the Ca^{2+} uptake was very low under these conditions, as expected, the time course of the effect of C. Sub. was strikingly different. (i) It appeared to be delayed in comparison with our previous findings (Figure 1b), i.e. not observed in the first minutes. In contrast, and very interestingly, the stimulation of Ca^{2+} uptake began to be visualizable clearly after 10 min incubation, the corresponding time for a maximal effect of C. Sub. on the phosphorylation of the Rap1 protein [35–37]. (ii) It was much more pronounced, as it increased by a factor of about 8, reaching a Ca^{2+} uptake of 80 nmol/mg of protein at 30 min, in agreement with the specific unmasking of the effect of C. Sub. on its target Ca^{2+} -ATPase. So, the most likely interpretation for these results is that the phosphorylation of the Rap1 protein induced by C. Sub. appears as a condition for platelet Ca^{2+} transport and occurs prior to the effect of phosphate ions, thus constituting new experimental conditions under which to study the relationship between Rap1 protein phosphorylation and the regulation of Ca^{2+} transport.

Figure 3 Expression of PL/IM 430-recognizable 97 kDa Ca^{2+} -ATPase and Rap1 protein phosphorylation in platelet membranes isolated from a normal individual or from patients with heart failure

Mixed platelet membrane proteins were isolated from either a normal individual or from three patients with congestive heart failure. They were then either treated for detection of the 97 kDa platelet Ca^{2+} -ATPase or for Rap1 protein phosphorylation as described in the Experimental section. (a) Immunodetection of the 97 kDa platelet Ca^{2+} -ATPase; 50 μg of platelet membrane proteins were submitted to SDS/15%-PAGE. Proteins were electrotransferred for 2 h at 250 mA on to nitrocellulose membranes and the blots were treated for immunodetection of the 97 kDa platelet Ca^{2+} -ATPase as follows: the nitrocellulose membranes were blocked by incubation with a quenching solution containing 150 mM NaCl, 10 mM Tris/HCl, pH 7.4, 0.1 % Tween 20 and 5 % non-fat dry milk. They were then incubated with 1 $\mu\text{g}/\text{ml}$ purified PL/IM 430 monoclonal antibody for 2 h and revelation of antibody binding was performed using the ECL kit. Lane C, normal individual platelet membrane proteins; lane 1, patient 1 platelet membrane proteins; lane 2, patient 2 platelet membrane proteins; lane 3, patient 3 platelet membrane proteins. This Figure is typical of two experiments. (b) Cyclic AMP-induced Rap1 protein phosphorylation. Platelet membrane proteins (50 μg) were phosphorylated under the conditions described in the Experimental section in the presence (lanes +) or absence (lanes –) of 15 $\mu\text{g}/\text{ml}$ of the catalytic subunit of the cyclic AMP-dependent protein kinase. The reactions were stopped and membrane proteins were separated on SDS/PAGE (15 % gel). They were electrotransferred on to nitrocellulose membranes as described above and autoradiographed for 2 days at –80 °C. Lane C, normal individual platelet membrane proteins; lane 1, patient 1 platelet membrane proteins; lane 2, patient 2 platelet membrane proteins; lane 3, patient 3 platelet membrane proteins. This Figure is typical of two experiments.

Comparative expression of the platelet 97 kDa Ca^{2+} -ATPase and Rap1 protein phosphorylation in heart failure

In parallel, we looked for a pathological model in which to investigate the role of the phosphorylation of the Rap1 protein on the PL/IM 430-recognizable 97 kDa Ca^{2+} -ATPase, and tested the usefulness of the congestive heart failure model. Thus, we screened a series of three patients with severe heart failure (patients 1, 2 and 3) for their expression of the 97 kDa Ca^{2+} -ATPase and their exogenous phosphorylation of the Rap1 protein. For this purpose, we isolated mixed platelet membranes (100 000 *g* fraction) [8,16] and performed, in parallel, Western blotting experiments using the PL/IM 430 purified monoclonal antibody and Rap1 protein phosphorylation using C. Sub., both technical approaches being well documented [8,16,18,28,38]. Figure 3 shows the comparative results of such experiments performed on three patients (lanes 1 to 3) and one normal individual (lane C). Figure 3(a) shows the results of the Western blottings of the 97 kDa Ca^{2+} -ATPase, whereas Figure 3(b) shows the Rap1 protein phosphorylation obtained in the presence (lanes +) or in the absence (lanes –) of 15 $\mu\text{g}/\text{ml}$ C. Sub. We can see that the expression of the 97 kDa Ca^{2+} -ATPase protein (Figure 3a) was present in the platelet membranes isolated from a normal individual and the patients, in spite of some possible variations between individuals. Figure 3(b) shows that the specific Rap1 protein phosphorylation in the platelet membranes isolated from the same patients differed because, when compared with the normal individual, it appeared similar in patient 3, but higher in patient 2 and lower in patient 1. Moreover, while the Rap1 protein phosphorylation in patients 2 and 3 could also refer to

some variations between individuals, the striking decrease in Rap1 phosphorylation found in patient 1 clearly by-passed this variation.

So, this first examination seemed encouraging to us, as it provided an opportunity of testing a cell system showing a modulation of the phosphorylation of the Rap1 protein.

Correlation between Rap1 protein expression, phosphorylation and stimulation of Ca^{2+} transport in platelets from a patient with severe heart failure

These data prompted us to immediately test the effect of C. Sub. on the Ca^{2+} transport system described in Figure 2 in platelet membrane vesicles isolated from one patient (patient 4) with severe dilated cardiomyopathy, and we studied in parallel these platelets for Rap1 protein phosphorylation and the effect of C. Sub. on Ca^{2+} uptake.

In addition, because the apparent decrease in exogenous phosphorylation of the Rap1 platelet protein could be due either to a variation in the endogenous phosphorylation or to a decrease in the Rap1 protein expression, we also used platelets from this patient to study comparatively Rap1 protein expression and phosphorylation in order to discriminate between the two alternatives.

(a) Comparative Rap1 protein phosphorylation and expression

Mixed 100000 *g* platelet membranes were isolated and treated either for Rap1 protein phosphorylation as in Figure 3(b) or for Rap1 protein expression by Western blotting using a previously well-characterized anti-Rap1 antibody, clearly shown to recognize specifically platelet Rap1 protein by both Western blotting and immunoprecipitation [16,38].

The results of these experiments are presented in Figures 4(a) and 4(b) respectively. Clearly, a strong decrease in Rap1 protein phosphorylation (which appears here as a doublet band due to incomplete reduction) was observed in the platelet membranes isolated from this patient (lane 4 +) as compared with that of the platelet membranes of the normal individual (lane C +). However, the Rap1 protein phosphorylation was not null, because some residual Rap1 protein phosphorylation was visible on this Figure (for which we deliberately used longer autoradiographic exposure times than those used in Figure 3).

The further examination of the Rap1 protein expression in the platelets from the same patient (Figure 4b) established that this decrease appeared well correlated with its phosphorylation, as it drastically decreased (compare lane 4 to lane C). So, the most plausible explanation for the decrease in Rap1 protein phosphorylation is that it referred to a lower Rap1 protein expression. Finally, the analysis of the 97 kDa Ca^{2+} -ATPase expression in the same patient again showed no marked difference compared with the platelets from the normal individual (results not shown).

(b) Comparative effect of C. Sub. on Ca^{2+} uptake into membrane vesicles

The results, presented in Figure 4(c), show the comparative time courses of Ca^{2+} uptake into the membrane vesicles isolated from platelets of the same normal individual and patient 4, in the presence or absence of 15 $\mu\text{g}/\text{ml}$ C. Sub. It is clear that whereas similar low Ca^{2+} uptake was visualizable in the absence of C. Sub., the effect of C. Sub. was greatly decreased in membrane vesicles isolated from platelets of this patient. Indeed, in contrast with platelet membrane vesicles isolated from a normal individual, no significant effect of C. Sub. was detectable at 10 min. Also, the very low expression of Rap1 protein in platelet

Figure 4 Comparative Rap1 protein phosphorylation, expression and effect of C. Sub. on Ca^{2+} uptake in platelet membranes isolated from a normal individual and a patient with severe heart failure

Mixed platelet membrane fractions (100000 *g* pellet) were isolated from a normal individual and from a patient with severe congestive heart failure. They were then treated in parallel either for Rap1 protein phosphorylation, Rap1 protein expression or for Ca^{2+} uptake measurements. (a) Cyclic AMP-induced Rap1 protein phosphorylation. Platelet membrane proteins (50 μg) were phosphorylated in the presence (lanes +) or absence (lanes -) of 15 $\mu\text{g}/\text{ml}$ of the catalytic subunit of cyclic AMP-dependent protein kinase under the conditions described in the Experimental section and previously used in Figure 3. Reactions were stopped, phosphorylated proteins were separated by SDS/15%-PAGE, electrotransferred on to nitrocellulose membranes and autoradiographed for 8 days at -80°C . Lane C, normal individual platelet membrane proteins; lane 4, patient 4 platelet membrane proteins. The Figure is typical of two experiments. (b) Immunodetection of the platelet Rap1 protein. Platelet membrane proteins (50 μg) were isolated, electrophoresed on SDS/15%-PAGE gels and electroblotted as in Figure 3. Nitrocellulose membranes were blocked in TBS (10 mM Tris/HCl, pH 7.5, 0.15 M NaCl) containing 5% (w/v) non-fat dry milk, at 37°C for 1 h and then incubated with a 1:100 dilution of anti-Rap1 serum in TBS containing 0.5% non-fat milk for 2 h at room temperature. After this step, the nitrocellulose membranes were washed five times with distilled water and then with TBS and 0.1% Tween for 10 min. Antibody binding was revealed by 1 h incubation at room temperature of the nitrocellulose membranes with ^{125}I -labelled Protein A as described in the Experimental section. Autoradiography of the dried nitrocellulose membrane was performed for 1 day at -80°C . Lane C, normal individual platelet membrane proteins; lane 4, patient 4 platelet membrane proteins. This Figure is typical of two experiments. (c) Effect of C. Sub. on Ca^{2+} uptake into membrane vesicles. Mixed membrane vesicles were incubated and treated for Ca^{2+} uptake measurements under the conditions of Figure 2. ●, Ca^{2+} uptake by control platelet membrane vesicles in the absence of C. Sub.; ■, Ca^{2+} uptake by control platelet membrane vesicles in the presence of 15 $\mu\text{g}/\text{ml}$ C. Sub.; ○, Ca^{2+} uptake by patient 4 platelet membrane vesicles in the absence of C. Sub.; □, Ca^{2+} uptake by patient 4 platelet membrane vesicles in the presence of 15 $\mu\text{g}/\text{ml}$ C. Sub. This Figure is typical of two experiments.

membranes of this patient appeared to correlate with a further delay in the response towards C. Sub., which only became detectable at 20 min and remained considerably decreased. So again, phosphate ions, present in similar amounts in the different samples, obviously are not the sole factor responsible for stimulation of Ca^{2+} uptake.

Figure 5 Comparative Rap1 protein expression and effect of C. Sub. on Ca^{2+} uptake in platelet membranes isolated from a further series of patients with heart failure

Rap 1 protein expression: platelet membranes were isolated from a normal individual and from different patients with heart failure. Membrane proteins (50 μg) were separated on SDS/15%-PAGE, electroblotted, and treated for Western blotting and immunodetection as in Figure 4(b). The intensities of immunoreactive bands were estimated by densitometric measurements using a laser densitometer. For each experiment, the control values obtained for Rap1 protein expression in normal platelets were arbitrarily taken as 100%. The results (bars 5 to 7) are expressed as percentages of Rap1 protein expression versus the control (bar C). Bars 5 to 7 correspond to platelet membranes isolated from patients 5, 6 and 7 respectively. Effect of C. Sub. on Ca^{2+} uptake into membrane vesicles: mixed platelet membrane vesicles were incubated and treated for Ca^{2+} uptake measurements up to 30 min in the absence or presence of 15 $\mu\text{g}/\text{ml}$ C. Sub. under the conditions of Figure 4(c). For each experiment, the control values obtained for C. Sub.-induced stimulation of Ca^{2+} uptake into normal individual platelet membrane vesicles were arbitrarily taken as 100%. The results (bars 5 to 7) are expressed as percentages of C. Sub.-induced stimulation of Ca^{2+} uptake versus the control (bar C). Bars 5 to 7 correspond to platelet membranes isolated from patients 5, 6 and 7 respectively.

Correlation between the Rap1 protein levels and the stimulation of Ca^{2+} uptake in a further series of patients with heart failure

Finally, we studied another series of three different patients with characterized heart failure, and tested in parallel their platelet membranes for Rap1 protein expression and the effect of C. Sub. on Ca^{2+} uptake, for which we used the experimental conditions of Figure 4(b) and Figure 2 respectively. Moreover, the expressions of Rap1 proteins were estimated by densitometric measurements of the radioactive bands from the same immunoblots and were expressed as percentages of Rap1 protein expression compared with that of the normal individual. The results of the effect of 15 $\mu\text{g}/\text{ml}$ of C. Sub. on the Ca^{2+} uptake by platelet membrane vesicles from the same patients were expressed as percentages of the stimulation observed at 30 min. Figure 5 shows the results of these studies conducted on the same day. Clearly, the analysis of these data allows the conclusion that this last study: (i) confirmed the results presented in Figure 3 obtained using a first series of patients, because the platelet Rap1 protein expressions can be decreased in patients with heart failure, and that this decrease varied greatly, in that it did not exceed 20% in patient 5 (lane 5) but reached about 60% in patient 7 (lane 7); (ii) demonstrated the direct relationship between these variable expressions of Rap1 protein and the stimulatory effect of C. Sub. on Ca^{2+} uptake.

Thus, this so far undescribed opportunity of testing pathological platelets which express abnormal and variable Rap1 protein levels constitutes a further argument for the close

relationship between the phosphorylation of this protein and the regulatory effect on platelet Ca^{2+} transport.

DISCUSSION

This paper first describes an abnormality in the Rap1 protein expression and phosphorylation in platelets from patients with heart failure, and secondly establishes a correlation between this abnormality and the stimulatory effect of C. Sub. on Ca^{2+} uptake by membrane vesicles.

Thus, the first essential feature of this work is that it suggested a new way to investigate the relationship between the phosphorylation of the platelet Rap1 protein and stimulation of Ca^{2+} uptake by intracellular Ca^{2+} -ATPases. Indeed, as explained in the Introduction, a controversy was raised, based on an artefactual effect of C. Sub., although these findings did not take into account similar results of the effect of cyclic AMP on stimulation of the Ca^{2+} transport process, examined by approaches on intact platelets which did not involve the C. Sub. [11,12,21]. Also, it did not consider the fact that there is increasing evidence that all the different mechanisms involved in the regulation of cytosolic Ca^{2+} concentration, such as Ca^{2+} channels [39], plasma membrane-type Ca^{2+} -ATPase [40], the intracellular SERCA 1 isoform of Ca^{2+} -ATPase [41], and the InsP_3 -induced Ca^{2+} release [42], are regulated by cyclic AMP in different cell types.

Here, we studied a cell system with variable expression of the Rap1 protein and phosphorylation and showed a correlation between a down-regulation of Rap1 protein and a decrease in the effect of cyclic AMP to regulate Ca^{2+} uptake. Moreover, and interestingly, these findings are just complementary to our recent study of another platelet pathology, where we found an increase in Ca^{2+} uptake associated with an increase in endogenous Rap1 protein phosphorylation, in studies conducted without using C. Sub., i.e. where contaminating phosphate was absent [38]. So, our present interpretation of the controversy is as follows: the question of whether studies using C. Sub. in phosphate buffer measured phosphate- and/or C. Sub.-induced phosphorylation effectively could raise the problem of the difficulty of dissociating the two effects. However, first, one approach used to solve this question was to proceed with the dialysis of C. Sub., but we failed to maintain full phosphorylation after this step (Figure 1a). Secondly, a consensus does exist between authors to show that platelet Rap1 protein is phosphorylated by the C. Sub. Thirdly, the unmasking of the effect of C. Sub. depends upon the experimental conditions (not detectable or transient in Ca^{2+} uptake studies without Ca^{2+} -EGTA buffers) and it appears here as a condition for Ca^{2+} uptake, such as the effect of the phosphorylation of the phospholamban in the cardiac SERCA 2 isoform, because in its dephosphorylated form phospholamban inhibits Ca^{2+} uptake, but it allows Ca^{2+} uptake in its phosphorylated form [43]. As a consequence, we conclude that the present study largely confirms the relationship between the phosphorylation of Rap1 platelet protein and the stimulation of Ca^{2+} transport.

However, this new evidence for a relationship between the phosphorylation of the platelet Rap1 protein and the stimulation of Ca^{2+} transport could raise the question of the location of the proteins involved. Indeed, as regards the platelet Rap1 protein, literature data, including our investigation after cell fractionation, establish its association with plasma membranes [15–17,44,45]. In apparent contrast, the 97 kDa platelet Ca^{2+} -ATPase, which is recognized by PL/IM 430, reacts with a monoclonal antibody raised against intracellular platelet membranes; so, the question of a plasma membrane association is raised. Nevertheless, the technique used to obtain these membranes involved

neuraminidase treatment which can result in some platelet activation, i.e. a redistribution of organelles, such as the migration of Ca²⁺ pools located next to the plasma membrane facing the intracellular space. In agreement with this hypothesis, the results of preliminary experiments suggest a translocation of the PL/IM 430-recognizable 97 kDa Ca²⁺-ATPase from plasma membranes towards intracellular membranes upon platelet activation (G. Berger, T. Kovacs, E. Corvazier, J. Enouf and E. Martin-Cramer, unpublished work). As a consequence, even if a colocalization still remains to be established, the available data cannot eliminate the relationship between the platelet Rap1 protein and PL/IM 430-recognizable Ca²⁺-ATPase, based on different locations of the proteins.

Finally, the other essential feature of this work concerns the evidence for abnormal platelet Rap1 protein expression in congestive heart failure. Rap1 proteins were recently described in different cell types [46], and the study of their roles is still under investigation. In particular, we know very little about the regulation of their expression in cell functions, especially in haematopoietic cells such as platelets which, intriguingly, overexpress this type of protein. The present finding of the down-regulation of Rap1 expression, well established here by both Western blotting using a specific anti-peptide antibody and its phosphorylation, is particularly interesting because it constitutes, as far as we know, the first example of down-regulation of the expression of the platelet Rap1 protein. Indeed, our previous investigations in this context led us to observe up-regulation of Rap1 protein in the two different pathological models studied, associated with an increase in cytosolic Ca²⁺ concentration, namely in platelets from hypertensive rats [18] and on proliferation of smooth-muscle cells induced by a growth factor, the platelet-derived growth factor [30]. So, interestingly, Rap1 proteins appear to be up-regulated in pathological situations associated with an increase in cytosolic Ca²⁺, but in heart failure this type of protein appears to be down-regulated, as are cardiac β -adrenergic receptors which lead to a decrease in cyclic AMP concentration. Thus the present study still emphasizes the interrelation between the two messengers constituted by Ca²⁺ ions and cyclic AMP.

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