# cADP-ribose formation by blood platelets is not responsible for intracellular calcium mobilization

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Human platelet CD38 is a multifunctional ectoenzyme catalysing the synthesis and hydrolysis of cADP-ribose (cADPR), a recently identified calcium-mobilizing agent that acts independently of D-*myo*-inositol 1,4,5-trisphosphate and is known to be expressed by human platelets. The present work shows that ADP-ribosyl cyclase activity is exclusively a membrane activity, of which the major part is located in plasma membranes and a small part in internal membranes. In broken cells, cyclase activity was insensitive to the presence of calcium and was not modulated by agonists such as thrombin or ADP, whereas in intact cells thrombin increased cADPR formation by 30 %, an effect due to fusion of granules with the plasma membrane. In order to assess the role of cADPR as a calcium-mobilizing agent, vesicles were prepared from internal membranes and loaded with $^{45}CaCl_2$. These vesicles were efficiently discharged by $IP_3$ in a dose-dependent manner, but were not responsive to cADPR or ryanodine in the presence or absence of calmodulin. Thus cADPR is unlikely to play a role in intracellular calcium release in human blood platelets.

## INTRODUCTION

Platelet activation leads to a transient rise in free cytoplasmic $Ca^{2+}$, resulting from both $Ca^{2+}$ influx and mobilization of internal stores. The mechanism responsible for the mobilization of intracellular $Ca^{2+}$ stores is well understood for aggregating agents like thrombin, thromboxane $A_2$ and platelet-activating factor on receptors coupled to G-proteins-linked phospholipase C (PLC) [1]. Stimulated PLC induces production of the second messengers diacylglycerol (DAG), an activator of protein kinase C, and D-*myo*-inositol 1,4,5-trisphosphate ($IP_3$), which mediates release of $Ca^{2+}$ from the platelet dense tubular system and thus contributes to the rise in cytosolic free $Ca^{2+}$ concentrations. In the case of ADP, a weak platelet agonist also known to induce the mobilization of intracellular $Ca^{2+}$ stores, it is not clear whether this agent stimulates PLC or not [2,3]. In some studies, ADP has been found to induce increases in $IP_3$ [4], whereas other reports have found ADP to be unable to activate PLC and the subsequent production of $IP_3$ [5,6]. It has been shown recently that platelets from mice deficient in the $\alpha$-subunit of $G_q$ are unresponsive to a variety of aggregating agents, among which is ADP [7]. However, in human platelets, a rapid, transient increase in $IP_3$ and inositol 1,3,4,5-tetrakisphosphate induced by ADP in stopped-flow experiments was not greatly different from that produced by shear forces alone [8]. The compound R59022, which potentiates platelet responses to thrombin by inhibiting DAG kinase and reducing the rate of elimination of DAG, had little effect on responses to ADP, suggesting that DAG is not an important mediator of primary responses to ADP [9]. Thus, in human platelets, PLC activation by ADP is unlikely and the mechanism responsible for the triggering by ADP of $Ca^{2+}$ release from internal stores remains obscure.

It was recently reported that CD38, a 45 kDa single-chain transmembrane glycoprotein involved in relevant cellular events such as activation, differentiation and adhesion, is present in human platelets [10]. The CD38 molecule is a multifunctional ectoenzyme catalysing the formation of cADP-ribose (cADPR) from $NAD^+$ and its hydrolysis to ADPR [11]. cADPR has been found to induce potently the mobilization of $Ca^{2+}$ from intracellular stores in several invertebrate and mammalian cell types and is known to act through an $IP_3$-independent pathway [12,13]. Mounting evidence points to this molecule being an endogenous activator of the $Ca^{2+}$-induced $Ca^{2+}$ release mediated by non-skeletal muscle ryanodine receptors [14,15]. This $Ca^{2+}$-induced $Ca^{2+}$-release process could be further sensitized by an accessory protein such as calmodulin [16,17]. Since several studies have demonstrated the heterogeneity of intracellular $Ca^{2+}$ stores and their sensitivity to cADPR and $IP_3$ [18–21], cADPR may play a role similar to that of $IP_3$ in controlling intracellular calcium signalling [22]. The aim of our study was to determine the location of the ADP-ribosyl cyclase activity of platelets and to assess the role of cADPR as a putative calcium-mobilizing second messenger.

## EXPERIMENTAL

### Chemicals

$^{45}CaCl_2$, [$^{14}$C]$NAD^+$ and cADPR were from Amersham (Little Chalfont, U.K.). Fluorescein isothiocyanate (FITC)-conjugated antibodies, mAbs against CD38 (MHCD3801) and control antibody (mouse IgG1, MG101), were purchased from Tebu (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.). Nicotinamide guanine dinucleotide ($NGD^+$), NADH, ADP, ATP, thapsigargin, calcium ionophore A23187, leupeptin, aprotinin and 4-(amiolinophenyl)-methanesulphonyl fluoride were from Boehringer (Mannheim, Germany); $IP_3$, 3-deoxy-3-fluoro-$IP_3$

Abbreviations used: $IP_3$, D-*myo*-inositol 1,4,5-trisphosphate; PLC, phospholipase C; DAG, diacylglycerol; ADPR, ADP-ribose; FITC, fluorescein isothiocyanate; $NGD^+$, nicotinamide guanine dinucleotide; $NAADP^+$, nicotinic acid adenine dinucleotide phosphate; mAb, monoclonal antibody; t-BuBHQ, 2,5-di-(t-butyl)-1,4-hydroquinone; 3-F-$IP_3$, 3-deoxy-3-fluoro-$IP_3$; cGDPR, cGDP-ribose.

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(3-F-$IP_3$), calmodulin and 2,5-di-(t-butyl)-1,4-hydroquinone (t-BuBHQ) were from Calbiochem (La Jolla, CA, U.S.A.). Bovine $\alpha$-thrombin, ryanodine and *p*-nitrophenol were obtained from Sigma (St. Louis, MO, U.S.A.), while all other reagents were of analytical grade.

### Preparation of washed human platelets

Human platelets were isolated and washed as previously described [23] and resuspended in Tyrode's buffer containing 2 mM $Ca^{2+}$, 1 mM $Mg^{2+}$, 0.35 % (v/v) human serum albumin (Centre de Transfusion Sanguine, Strasbourg, France) and apyrase (2 $\mu$g/ml). In subcellular fractionation experiments, the final resuspending medium was buffer A (25 mM Tris/HCl and 150 mM KCl, pH 7.4) containing 3 mM $MgCl_2$/ATP, 1 mM EGTA and protein inhibitors [10 $\mu$g/ml leupeptin, 10 $\mu$g/ml aprotinin and 10 $\mu$M 4-(amiolinophenyl)-methanesulphonyl fluoride].

### Flow cytometric analysis of platelet CD38

Analyses were performed on washed platelets ($1.5 \times 10^7$ in 100 $\mu$l) treated with 1 unit/ml human $\alpha$-thrombin, 10 $\mu$M ADP or no agonist. Samples containing Triton X-100 permeabilized platelets previously fixed with paraformaldehyde (2 %, v/v) were also examined. The platelet samples were incubated with 2 $\mu$l of anti-CD38 mAb (reference MHCD3801-FITC) or isotype control and analysed on a FACSort fluorescence cytometer (Becton and Dickinson, San Jose, CA, U.S.A.) using Cell Quest software.

### Subcellular fractionation and plasma-membrane isolation

Cell disruption was carried out by nitrogen cavitation [24], unbroken cells were separated from the homogenate by centrifugation, and the supernatant was collected for subcellular fractionation on an alkaline Percoll™ gradient [25]. Alkaline mixtures were centrifuged (79000 ***g***, 15 min, 4 °C), and fractions were harvested from the top of the gradient. After dilution with buffer (20 mM Hepes, 100 mM KCl and 5 mM $MgCl_2$, pH 7.1), fractions were sonicated twice for 15 s on ice, centrifuged (200000 ***g***, 60 min, 4 °C) and resuspended in the same buffer. $Ca^{2+}$-ATPase, NADH dehydrogenase (EC 1.6.99.3) and glucose-6-phosphatase (EC 3.1.3.9) activities were measured to identify the intracellular membranes (endoplasmic reticulum, mitochondria and granules) [25–27], and acid phosphatase (EC 3.1.3.2) activity was measured to localize plasma membranes [28]. The plasma- (fractions 1–3) and intracellular (fraction 11) membrane fractions were centrifuged (200000 ***g***, 60 min, 4 °C) and resuspended in phosphate buffer (20 mM Hepes, 10 mM $KH_2PO_4$, 100 mM KCl and 5 mM $MgCl_2$, pH 7.1). Protein concentrations in the homogenate and in the plasma- and intracellular membrane fractions were determined by bicinchoninic acid assay and adjusted to 2–5 mg/ml.

### Extraction and estimation of membrane lipid contents

Lipids were extracted from homogenates, plasma membranes (fractions 1–3) and intracellular membranes (fraction 11) according to Folch et al. [29]. The phospholipid mass was obtained by phosphorus determination [30], and the cholesterol content was estimated by an enzymic colorimetric method (reference 310328, Boehringer).

### Assay for $^{45}Ca^{2+}$ release from platelet membrane vesicles

Membrane vesicles (50 $\mu$g) were loaded with calcium by incubation for 30 min at 25 °C in phosphate buffer containing 1 mM ATP and 37 kBq/ml $^{45}CaCl_2$ (free $[Ca^{2+}] = 1$ $\mu$M). $^{45}CaCl_2$ uptake was stopped by addition of 0.4 mM K-EDTA. The induction of $Ca^{2+}$ release by $IP_3$, cADPR and ryanodine was tested essentially as described by O'Rourke et al. [31]. Briefly, 5 $\mu$l of agonist was added, and, after a 5 min incubation at 25 °C, the reaction was stopped by adding 25 $\mu$l of a quench medium (0.6 mM formalin in 50 mM K-EDTA, pH 7.1), preventing $Ca^{2+}$ re-uptake. Assays were terminated by dilution of the reaction mixture in 2.5 ml of phosphate buffer and rapid filtration through nitrocellulose filters (0.45 $\mu$m porosity), after which the tubes and filters were rinsed twice and the radioactivity of the filters was measured by scintillation counting.

### Determination of enzyme activities

In ADP-ribosyl and NADase assays, the platelet homogenate obtained in the initial step of subcellular fractionation was incubated with 50 $\mu$M [$^{14}$C]$NAD^+$ at 20 °C under slight agitation. At varying times, the reaction was terminated by addition of 0.6 mM perchloric acid, and the extract was neutralized with 2 vol. of freon-11/trioctylamine (1:1, v/v) before reverse-phase HPLC analysis. Elution was performed with 25 mM potassium buffer, pH 6.4, containing 2 mM 11-aminoundecanoic acid in water/methanol (94:6, v/v), at an eluent flow rate of 1 ml/min [32]. These conditions allowed simultaneous measurement of the production of cADPR and ADPR and the disappearance of the substrate ($NAD^+$).

GDP-ribosyl cyclase activity was measured by following the production of fluorescent cGDP-ribose (cGDPR) using $NGD^+$ as substrate [33]. Intact platelets, plasma membranes or intracellular membrane fractions were incubated with 400 $\mu$M $NGD^+$ in 20 mM Tris buffer, pH 7.4, at 37 °C or 20 °C under slight agitation. After varying intervals of time, the reaction was stopped by addition of perchloric acid, and cGDPR production was analysed by HPLC as described above. The retention times of standard nucleotides, cGDPR, cADPR, GDPR/ADPR and $NGD^+$/$NAD^+$ were 2.6, 2.85, 5.3 and 7.2 min respectively, under our HPLC conditions.

### Reproducibility

The experiments shown are representative of at least three independent assays using different membrane preparations.

## RESULTS

### Enzyme activities

Using [$^{14}$C]$NAD^+$ as substrate, production of cADPR was detected at very low levels (0.03 nmol of cADPR/min per mg of protein) in platelet homogenates (Figure 1A), whereas levels of cADPR hydrolase or NADase activity were very high (3.8 nmol of ADPR/min per mg of protein) (Figure 1B). In view of the apparent low rate of cADPR production, further studies of ADP-ribosyl cyclase activity were performed using $NGD^+$ instead of $NAD^+$ as substrate. This compound is converted by the same enzyme to cGDPR, which may be detected by fluorimetry. Since cGDPR is not hydrolysed to GDPR and is therefore detectable at higher levels, this provides a convenient and more accurate method of assessing ADP-ribosyl cyclase activity.

In order to locate the site of ADP-ribosyl cyclase activity in platelets, the membrane fractions obtained by akaline Percoll™ gradient centrifugation were characterized with specific enzyme markers. As previously reported by Fauvel et al. [25], Percoll™ gradient separation yielded two major membrane fractions. The low-density fractions (1–3) contained plasma membranes in

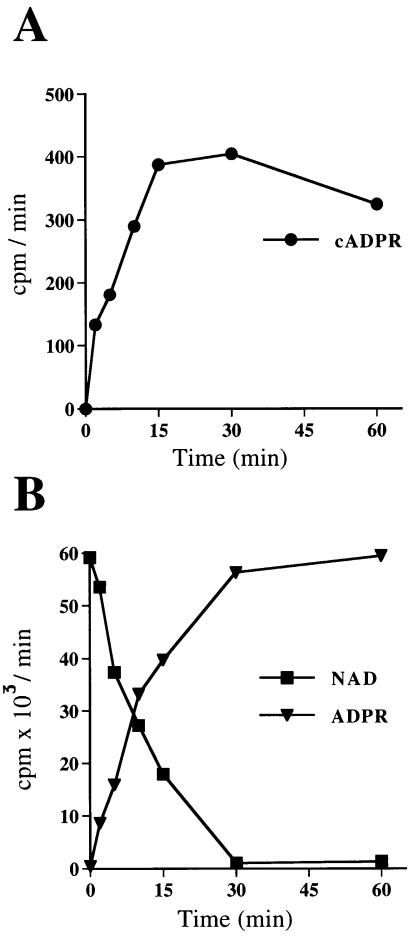


**Figure 1 cADPR synthesis and hydrolysis in platelet homogenates**

Membrane homogenates obtained by nitrogen cavitation of $3.5 \times 10^6$ platelets were incubated in 50 $\mu$M $NAD^+$ containing 18.5 kBq of [$^{14}$C]$NAD^+$/tube at 20 °C under slight agitation. At varying times, the reaction was stopped by addition of $HClO_4$, and the centrifuged extract was neutralized before HPLC analysis. Results are expressed in c.p.m./min for cADPR (**A**) or in c.p.m. $\times 10^3$/min for ADPR and $NAD^+$ (**B**) and correspond to one experiment representative of three identical independent experiments.

**Table 1 Biochemical characterization of platelet subfractions**

Homogenates, plasma membranes (fractions 1–3) and intracellular membranes (fraction 11) were obtained from homogenates by separation on an alkaline Percoll™ gradient. After dilution with buffer, the membrane fractions were centrifuged (200 000 ***g*** for 60 min), and the activities of various enzyme markers and levels of phospholipids and cholesterol were determinated in the homogenates and fractions as described in the Experimental section. Results are representative of three independent experiments, and numbers in parentheses denote the enrichment ratio. Abbreviations: ND, not detected.

| | Homogenate | Plasma membranes | Intracellular membranes |
|---|---|---|---|
| Acid phosphatase ($\mu$mol/min per mg) | 4 | 17 (4.3) | $< 1$ ($< 0.25$) |
| NADH-dehydrogenase (nmol/min per mg) | 8 | 26 (3.3) | 82 (10.3) |
| Glucose-6-phosphatase (nmol/min per mg) | 48 | ND | 110 (2.3) |
| $Ca^{2+}$-ATPase (nmol/min per mg) | 3 | 2 (0.7) | 6 (2) |
| Phospholipids (nmol/mg of protein) | 221 | 1595 (7.2) | 422 (1.9) |
| Cholesterol (nmol/mg of protein) | 74 | 809 (10.9) | 90 (1.2) |
| Cholesterol/phospholipids | 0.33 | 0.51 | 0.21 |

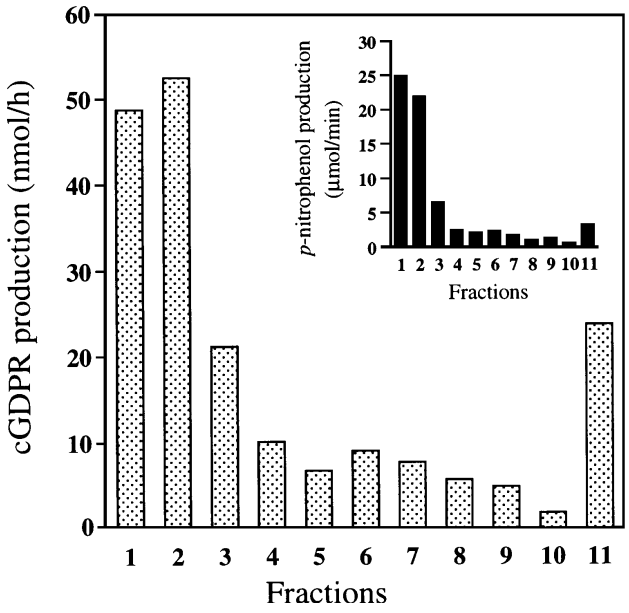


**Figure 2 GDP-ribosyl cyclase activity in platelet membrane fractions**

Platelet homogenates ($4 \times 10^{10}$ platelets) were fractionated on an alkaline Percoll gradient, and the 11 fractions recovered were centrifuged (200 000 ***g***, 60 min) and resuspended in 4 ml of phosphate buffer. Each fraction (100 $\mu$l each) was incubated with 200 $\mu$M $NGD^+$ for 60 min at 20 °C under slight agitation, after which fluorescent GDPR production was analysed by HPLC and expressed in nmol/h. Inset, acid phosphatase activity was measured to localize the plasma membrane and expressed in mmol/min.

which the acid phosphatase activity was 17 $\mu$mol/min per mg of protein (Table 1) and the enrichment level relative to the homogenate was 4.3 fold. The high-density fraction (11) corresponding to the intracellular membranes, comprising elements of the dense tubular system and mitochondrial and granule membranes, was characterized by measuring the activities of the three specific enzymes. NADH-dehydrogenase, glucose-6-phosphatase and $Ca^{2+}$-ATPase were found to be enriched in this fraction 10.3, 2.3 and 2 times, respectively, with respect to the homogenate, whereas these activities were very low in plasma membranes (Table 1). Another important difference between plasma and intracellular membranes is the relative content of phospholipids and cholesterol and in particular the molar cholesterol-to-phospholipid ratio. Table 1 lists the total phospholipids and cholesterol contents of the homogenates and the plasma and intracellular membranes. Phospholipids were enriched 7.2- and 1.9-fold in plasma and intracellular membranes respectively. The molar cholesterol-to-phospholipid ratio increased from 0.33 in homogenates to 0.51 in plasma membranes and diminished to 0.19 in intracellular membranes.

In all subsequent membrane preparations, the acid phosphatase activity as a plasma-membrane control (Figure 2, inset) and the NADH-dehydrogenase activity as an intracellular membrane control (results not shown) were measured along the Percoll™ gradient. GDP-ribosyl cyclase activity (70 %) was found in the plasma membrane (fractions 1–3), and significant activity (13 %) was found in the intracellular membranes (fraction 11) (Figure 2). In addition, GDP-ribosyl cyclase activity was measured in the cytosol obtained after platelet disruption by nitrogen cavitation and high-speed centrifugation (200 000 ***g***, 60 min). The cytosol (supernatant) showed, however, very low

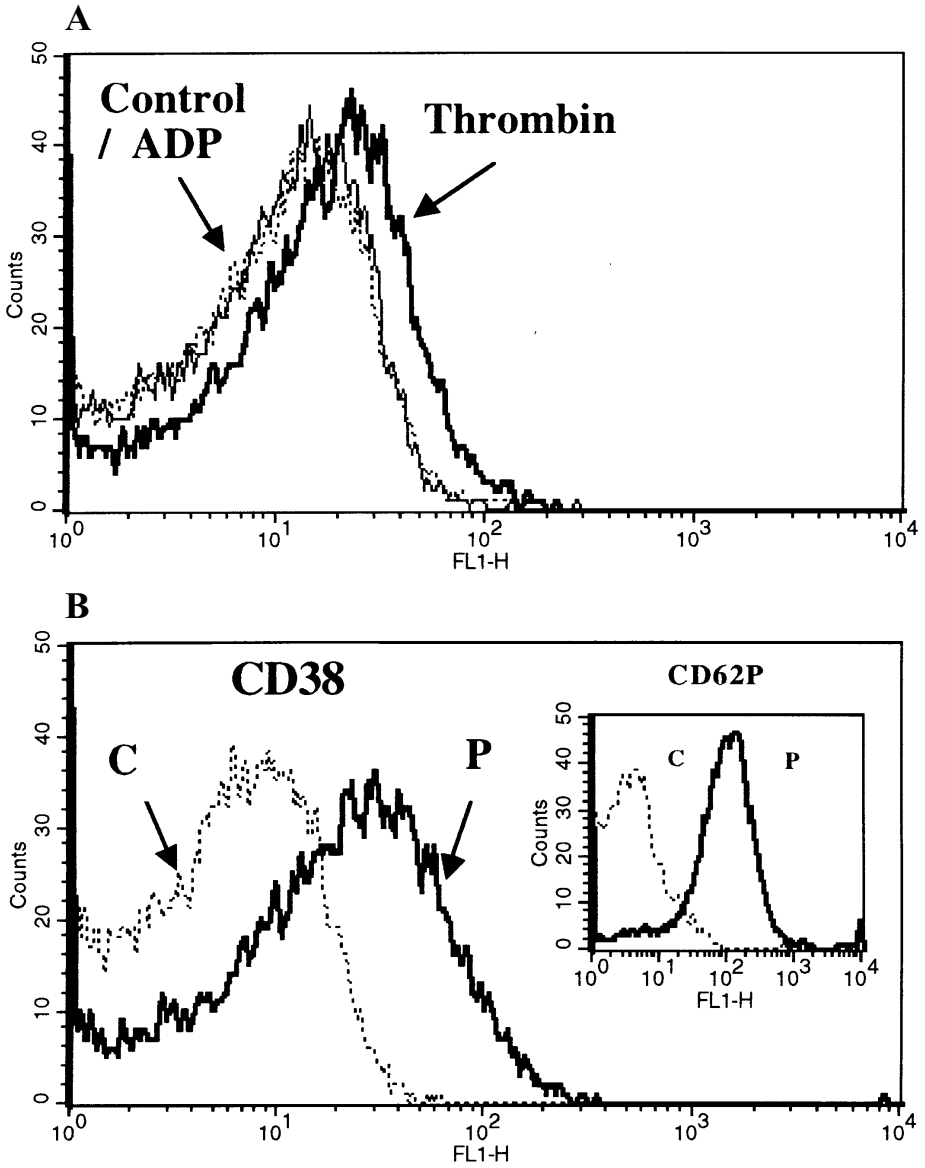


**Figure 3 Flow cytometric analysis of platelet CD38**

Washed platelets ($1.5 \times 10^7$) were incubated with an FITC-coupled anti-CD38 mAb (FL1-H), after treatment with 1 unit/ml thrombin (thick solid line), 10 $\mu$M ADP or no agonist (dashed lines) (**A**). In order to observe intracellular labelling, washed platelets were fixed with 2% (v/v) paraformaldehyde before Triton X-100 permeabilization. Permeabilized (P) (solid line) and non-permeabilized (C) (dashed line) platelets were incubated with the anti-CD38 mAb (**B**). Inset, permeabilization was controlled using an mAb specific for the granular protein CD62P (solid line). FL1-H, fluorescence in channel 1.

GDP-ribosyl cyclase activity (12 pmol/min per $10^9$ platelets), representing less than 1% of the total membrane activity (results not shown). These findings indicate that cyclase activity is almost exclusively a membrane activity of which the major part is located in plasma membranes. This enzymic activity was insensitive to the presence of calcium and was not modulated by agonists such as thrombin or ADP (results not shown). As shown before, GDP-ribosyl cyclase activity could also be detected on intact washed platelets, suggesting this activity to be extracellular. The basal activity (1172 pmol/min per $10^9$ platelets) rose 34% after platelet stimulation with 1 unit/ml thrombin (1551 pmol/min per $10^9$ platelets), whereas 5 $\mu$M ADP had no effect (results not shown).

### Flow cytometric analysis of platelet CD38

The CD38 signal obtained with an anti-CD38 mAb directly coupled to FITC (MHCD3801-FITC) increased in thrombin- but not in ADP-stimulated platelets (Figure 3A), whereas intracellular labelling of CD38 was detected in permeabilized fixed platelets (Figure 3B). As a control of permeabilization, we used an mAb specific for the granular protein CD62P (Figure 3B, inset). These results suggest that the thrombin-induced increase in GDP-ribosyl cyclase activity was due to the appearance of additional CD38 molecules at the cell surface rather than to modulation of the specific activity of the enzyme.

To confirm that CD38 catalysed the synthesis of cGDPR, we

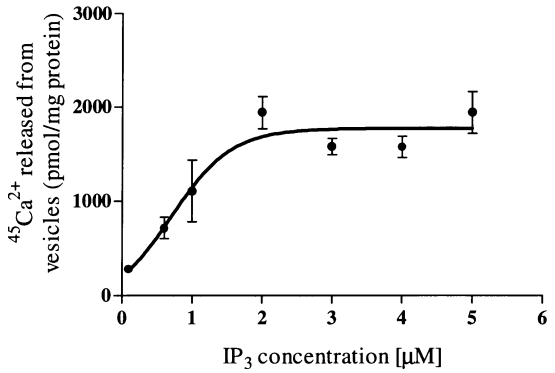


**Figure 4 Dose-dependent release of $^{45}Ca^{2+}$ induced by $IP_3$**

Membrane vesicles (50 $\mu$g) were loaded for 30 min at 25 °C with 18.5 kBq of $^{45}CaCl_2$ (free $[Ca^{2+}] = 1$ $\mu$M). Calcium-loaded vesicles were discharged by addition of agonist, the reaction was stopped by rapid filtration, and residual $^{45}Ca^{2+}$ remaining in the vesicles was measured by scintillation counting and expressed in pmol/mg of vesicle protein. Points are the mean of three determinations, and bars show the S.E.M.

measured GDP-ribosyl cyclase activity in the presence of anti-CD38 mAb. GDP-ribosyl cyclase activity was completely inhibited when intact platelets were incubated with 5 $\mu$g/ml mAb MHCD3801-FITC (results not shown), indicating that platelet surface GDP-ribosyl cyclase activity is essentially associated with CD38 molecules.

### Release of $^{45}Ca^{2+}$ from platelet membrane vesicles

In order to assess the putative role of cADPR as a calcium-mobilizing agent, we prepared internal platelet membrane vesicles that we then loaded with $^{45}CaCl_2$. A medium containing 10 mM phosphate and 1 mM ATP gave optimal $Ca^{2+}$ accumulation in vesicles, the maximal $Ca^{2+}$ uptake of 5–6 nmol/mg of protein reaching a plateau after 20 min at 25 °C. This $Ca^{2+}$ accumulation was dose-dependently inhibited by thapsigargin or t-BuBHQ (results not shown). Calcium-loaded vesicles were efficiently discharged by $IP_3$, in a dose-dependent manner with $EC_{50} = 0.8$ $\mu$M (Figure 4). In response to 5 $\mu$M $IP_3$ or the same dose of its non-hydrolysable analogue 3-F-$IP_3$, calcium-loaded vesicles discharged about 30% of their ionophore A23187-releasable $Ca^{2+}$, whereas cADPR and ryanodine in a range of 0.1 to 100 $\mu$M had no effect (Table 2), alone or in the presence of calmodulin, over a concentration range of 1 to 100 $\mu$g/ml. These results were not influenced by changes in extracellular calcium concentration. $MgCl_2$, a known inhibitor of the ryanodine receptor, has been shown to block cADPR-induced $Ca^{2+}$ release in sea-urchin homogenates [34]. In our assay, an absence of $MgCl_2$ did not, however, improve $Ca^{2+}$ release in response to cADPR or ryanodine stimulation (results not shown).

**Table 2 Release of calcium from intracellular membrane vesicles**

$^{45}Ca^{2+}$ release was determined as described in the legend to Figure 4, and results are expressed as mean values ± S.E.M. from three separate experiments. The percentages are relative to the release induced by A23187.

| Pharmacologic agent | Concentration ($\mu$M) | $^{45}Ca^{2+}$ release (pmol/mg) | Percentage |
|---|---|---|---|
| $IP_3$ | 5 | 2154 ± 226 | 38.2 |
| 3-F-$IP_3$ | 5 | 1943 ± 129 | 34.5 |
| cADPR | 0.1–100 | 200 ± 101 | 3.6 |
| cADPR + calmodulin | 0.5–5 | 52 ± 18 | 0.9 |
| Ryanodine | 0.1–100 | 177 ± 33 | 3.1 |
| A23187 | 5 | 5633 ± 85 | 100 |
| Buffer | – | 244 ± 105 | 4.3 |

## DISCUSSION

The results presented here confirm the presence of the CD38 molecule on human platelets [10]. CD38 was expressed mainly on plasma membranes, although an additional non-negligible pool was found on intracellular membranes. Membrane fractions prepared in this study displayed characteristics similar to those reported by Fauvel et al. [25], plasma membranes originating from the entire surface membrane and intracellular membranes corresponding to the dense tubular system. However, it was impossible to assess the level of contamination of the dense-tubular-system fraction by granule or mitochondrial membranes. ADP-ribosyl cyclase activity, essentially localized in the membrane fractions (i.e. plasma and intracellular membranes), was insensitive to the presence of $Ca^{2+}$ and was not modulated by agonists such as ADP or thrombin. In contrast, the ADP-ribosyl cyclase activity of intact platelets, which was primarily extracellular, could be slightly increased by thrombin but not by ADP stimulation. Using flow cytometry, we found the increase following thrombin stimulation to result from platelet secretion and fusion of granule membranes with the plasma membrane. Thus the rise in ADP-ribosyl cyclase activity induced by thrombin was due to the appearance of additional CD38 molecules on the cell surface rather than to modulation of the specific activity of the enzyme.

The role of cADPR as a calcium-mobilizing agent acting through the $Ca^{2+}$-induced $Ca^{2+}$-release pathway and as a physiological regulator of the ryanodine receptor has been established in various vertebrate and invertebrate tissues [12–15]. Other studies [18–22] have demonstrated the heterogeneity of intracellular $Ca^{2+}$ stores and their variable sensitivity to ryanodine, cADPR or $IP_3$. In the case of platelets, our results showed $IP_3$ to induce the dose-dependent discharge of internal membrane vesicles loaded with $Ca^{2+}$. In the present study, $IP_3$ was able to mobilize only 40% of the total $Ca^{2+}$ content of the vesicles, whereas A23187 induced 100% $Ca^{2+}$ release. Among several hypotheses that could be advanced to explain this low $Ca^{2+}$ release, vesicles fractions derived from the dense tubular system are frequently contaminated with plasma membranes capable of accumulating non-mobilizable calcium [25]. This may occur in particular during sonication, when the membrane vesicles formed can be either right-side-out or inside-out [35].

cADP-ribose and ryanodine had no effect in the presence or absence of calmodulin or $MgCl_2$. Moreover, in Western-blotting experiments, mAbs directed against the ryanodine receptor failed to reveal any labelling, whereas an mAb anti-$IP_3$ receptor gave a well defined band of 260 kDa (results not shown). Hence platelets do not seem to express the ryanodine receptor, and it may be for this reason that cADPR is unable to trigger intracellular $Ca^{2+}$ release in these cells.

How then could ADP induce $Ca^{2+}$ release through a mechanism independent of both $IP_3$ and $Ca^{2+}$-induced $Ca^{2+}$ release? Over the past few years, a number of groups have proposed new second messengers thought to be involved in $Ca^{2+}$ release from internal stores. One of these is nicotinic acid adenine dinucleotide phosphate ($NAADP^+$), a metabolite of $NADP^+$ in reactions catalysed by CD38, which induces $Ca^{2+}$ release from stores

different from those triggered by $IP_3$ and cADPR in sea-urchin eggs [36,37]. Another putative second messenger is sphingosine 1-phosphate, a metabolite of sphingolipids present in platelets [38], which has been shown to mobilize $Ca^{2+}$ from internal stores via an $IP_3$-independent pathway [39,40]. O'Rourke et al. [35] have demonstrated that other inositol phosphates, such as inositol 1,3,4,5-tetrakisphosphate and inositol 1,2,3,4,5,6-hexakisphosphate, are likewise involved in intracellular calcium fluxes originating from pools distinct from those sensitive to $IP_3$ in platelets. These findings could provide an interesting alternative to explain the occurrence of $Ca^{2+}$ release independent of $IP_3$. Futhermore, recent investigations have shown that cytochalasin, an inhibitor of actin polymerization, mediates the discharge of $Ca^{2+}$ from peripheral storage sites in neutrophils [41]. This $Ca^{2+}$ release from peripheral stores in neutrophils was observed in response to stimuli that act through cross-linking of surface molecules such as integrins and Fc receptors, thus causing cell shape change and actin re-arrangement. Such cytoskeletal modifications are well documented in platelets [42] and deserve attention with a view to understanding how $Ca^{2+}$ could be released independently of $IP_3$. Nevertheless, the recent report from Offermans et al. [7] describing platelet activation in $G\alpha_q$ knock-out mice indicates a role of low amounts of $IP_3$ produced during ADP-induced platelet aggregation in mice. Possible species differences have to be assessed, but undetectable $IP_3$ levels in human platelets could still account for the observed ADP-induced calcium mobilization.

The CD38 molecule is known to be involved in many blood cells in different processes, including cellular differentiation, inflammation, cell growth and apoptosis [11]. However, its exact role in platelets is as yet unknown. Recent studies [43] indicate that T-cells adhere to endothelial cells and that this adhesion is mediated by CD38 under dynamic conditions, where integrin functions are minimized, which would suggest that CD38 ligands behave like selectins. In the light of these findings, it is not unreasonable to suppose that platelet CD38 could be involved in cell-cell interactions, although the physiological role of such a function will require further study.

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