Hydrolysis of $NADP^+$ by platelet CD38 in the absence of synthesis and degradation of cyclic ADP-ribose 2′-phosphate

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Abstract CD38 is a multifunctional cell surface ectoenzyme that catalyzes both the synthesis of cyclic ADP-ribose from NAD^+ and its hydrolysis to ADP-ribose. In this work, we investigated the metabolism of $NADP^+$ by CD38 expressed on human platelets. Incubation of either platelet membranes or intact cells with $NADP^+$ resulted in the rapid and time-dependent accumulation of ADP-ribose 2′-phosphate that paralleled the consumption of the substrate. However, under the same conditions, synthesis of cyclic ADP-ribose 2′-phosphate was not observed. By immunoprecipitation experiments, we identified CD38 as the enzyme responsible for the observed $NADP^+$ glycohydrolase activity. The lack of detection of cyclic ADP-ribose 2′-phosphate was not due to its rapid hydrolysis, since direct incubation of platelet membranes with cyclic ADP-ribose 2′-phosphate did not result in the formation of ADP-ribose 2′-phosphate. By contrast, the same membrane samples expressed a significant ability to hydrolyze cyclic ADP-ribose to ADP-ribose. The absence of cyclic ADP-ribose 2′-phosphate hydrolase activity was also confirmed using high concentrations of substrate and by analysing both intact Jurkat T-lymphocytes and immunoprecipitated CD38. These results indicate that CD38, which is a multifunctional enzyme towards NAD^+, displays exclusively a $NADP^+$ glycohydrolase activity and is unable to catalyze both the synthesis and the hydrolysis of cyclic ADP-ribose 2′-phosphate.

Key words: ADP-ribosyl cyclase; CD38; $NADP^+$; Platelet

1. Introduction

Human CD38 is a type II transmembrane glycoprotein involved in several cellular functions, including activation, differentiation and adhesion [1]. Based on the high sequence homology with the ADP-ribosyl cyclase from *Aplysia californica*, an enzyme that converts NAD^+ into cyclic ADP-ribose (cADPR) [2], an enzymatic function for CD38 has been proposed. Indeed, it has been demonstrated that CD38 is actually a multifunctional enzyme [3]. Its extracellular domain possesses both ADP-ribosyl cyclase activity, is able to synthesize cADPR from NAD^+ and cADPR hydrolase activity is able to hydrolyze cADPR to ADP-ribose (ADPR). Thus, CD38 may be responsible for the transient formation of cADPR, which is a potent stimulator of Ca^{2+} release from specific intracellular stores [4]. The sum of these reactions results in a NAD^+ glycohydrolase activity causing the conversion of NAD^+ to ADPR. However, it has recently been demonstrated that formation of cADPR from NAD^+ is an alternative reaction, rather than an obligatory step in the NAD^+ glycohydrolase activity of NAD^+ [5,6].

In addition to NAD^+, human CD38, as well as the ADP-ribosyl cyclase from *A. californica*, can utilize $NADP^+$ as substrate [7]. It has been demonstrated that, at acidic pH and in the presence of nicotinic acid, both enzymes catalyze the exchange of the nicotinamide group of $NADP^+$ with nicotinic acid to produce nicotinic acid adenine dinucleotide phosphate ($NAADP^+$) [7]. This compound acts as an intracellular messenger able to promote Ca^{2+} release from intracellular stores distinct from those sensitive to either inositol 1,4,5-trisphosphate (IP_3) or cADPR [8,9]. Much less is known about the $NADP^+$ metabolism by CD38 at physiological pH. Aarhus and coworkers reported that, in these conditions, the ADP-ribosyl cyclase from *A. californica* converts $NADP^+$ to cyclic ADP-ribose 2′-phosphate (2′-P-cADPR), while CD38 causes hydrolysis of $NADP^+$ to ADP-ribose 2′-phosphate (2′-P-ADPR) [7]. However, the expression of a 2′-P-ADP-ribosyl cyclase and 2′-P-cADPR hydrolase activity by CD38 was not investigated.

We have previously reported that enzymatically active CD38 is expressed on the plasma membrane of human platelets and translocates to the cytoskeleton upon platelet stimulation with thrombin [10,11]. Mobilization of Ca^{2+} from intracellular stores plays a key role in the biochemical mechanisms of platelet activation induced by a variety of extracellular agonists [12]. This process is known to be promoted by IP_3, produced through the agonist-induced activation of phospholipase C [12]. By contrast, cADPR does not seem to play any role as Ca^{2+}-releasing agent in these cells [13,14]. However, the possibility that some metabolites from $NADP^+$ produced by CD38 may be involved in Ca^{2+} mobilization in platelets cannot be ruled out. $NAADP^+$, which is a potent Ca^{2+}-mobilizing agent, is produced by CD38 through a base exchange reaction at acidic pH and in the presence of high concentrations of nicotinic acid [7–9] and thus, it is unlikely to play a physiological role in platelet activation. By contrast, a role for 2′-P-cADPR, produced by the ADP-ribosyl cyclase from *A. californica* at neutral pH on Ca^{2+} release, has been proposed [15].

The aim of this work was to investigate the metabolism of $NADP^+$ by platelet CD38 at physiological pH, in order to get more insights into the enzymatic activities of this glycoprotein and in search for new messengers regulating Ca^{2+} mobilization in platelets. The results demonstrate that CD38 causes hydrolysis of $NADP^+$ to 2′-P-ADPR, but not synthesis of 2′-P-cADPR. Moreover, we found that 2′-P-cADPR, unlike cADPR, was not a substrate for CD38. These results demonstrate that the expression of the $NADP^+$ glycohydrolase ac-

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Abbreviations: ADPR, ADP-ribose; cADPR, cyclic ADP-ribose; 2′-P-ADPR, ADP-ribose 2′-phosphate; 2′-P-cADPR, cyclic ADP-ribose 2′-phosphate; IP_3, inositol 1,4,5-trisphosphate

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tivity does not require synthesis and degradation of 2′-P-cADPR from $NADP^+$ and argue against a role of 2′-P-cADPR in platelet activation.

2. Materials and methods

2.1. Materials

$NADP^+$, 2′-P-ADPR, 2′-P-cADPR, cADPR were purchased from Sigma. Microcon 3 filters were from Amicon. Sepharose CL-2B and protein A-Sepharose were from Amersham Pharmacia Biotech. Yurkat T-cells and the monoclonal antibody IB4 against CD38 were a gift from Prof. F. Malavasi (Department of Genetics, Biology and Medical Chemistry, University of Turin, Turin, Italy). All other reagents were of analytical grade.

2.2. Platelets and Jurkat T-lymphocytes preparation

Blood was taken from healthy volunteers using citric acid-citrate-dextrose as anti-coagulant. Platelets were prepared by gel-filtration on a Sepharose CL-2B column, equilibrated with HEPES buffer (10 mM HEPES, 137 mM NaCl, 2.9 mM KCl, 12 mM $NaHCO_3$, pH 7.4) as previously described [16]. The platelet count was finally adjusted to 10^9 cells/ml. Jurkat T-lymphocytes were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin and 100 U/ml streptomycin at 37°C in a humidified atmosphere with 5% CO_2. Cells were collected by centrifugation, washed twice with HEPES buffer and finally resuspended at a concentration of 10^7 cells/ml.

2.3. Membrane preparation

Platelets were prepared from concentrates obtained from the local blood bank (Servizio di Immunoematologia e Transfusione, IRCCS Policlinico S. Matteo, Pavia, Italy) as described [10] and finally resuspended at a final count of 2×10^9 cells/ml in hypotonic buffer (10 mM HEPES, pH 7.5, 100 μM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin). Platelets were disrupted by sonication and then centrifuged at $1800\times g$ for 20 min to eliminate intact cells. The supernatant was ultracentrifuged at $100\,000\times g$ for 45 min and the membrane pellet was washed once with hypotonic buffer. The membranes were finally resuspended in HEPES buffer, the protein concentration was determined with the bicinchoninic acid assay and adjusted at 3–7 mg of membrane proteins/ml. Membranes were stored at −70°C until used.

2.4. Immunoprecipitation

Platelet membrane samples (typically 250 μl containing 0.4 mg of membrane proteins) were lysed by addition of an equal volume of ice-cold immunoprecipitation buffer (HEPES buffer containing 2% Nonidet P40, 0.5% sodium deoxycholate, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin). Upon incubation on ice for 15 min, lysates were centrifuged at $13\,000\times g$ for 5 min. The cleared supernatants were incubated with 4 μg of the anti-CD38 monoclonal antibody IB4 or with 4 μg of unrelated mouse immunoglobulins for 2 h at 4°C. Protein A-Sepharose (100 μl of a 50 mg/ml stock solution) was then added and samples were incubated at 4°C for 30 min. The protein A-Sepharose pellets were collected by brief centrifugation, washed twice with ice-cold immunoprecipitation buffer and finally resuspended with 100 μl of HEPES buffer for enzymatic determinations.

2.5. Enzymatic analysis

Measurements of enzymatic activities of CD38 were performed on 100 μl samples containing 50 μg membrane proteins, 10^8 intact platelets, 10^7 Jurkat T-cells or immunoprecipitates. Samples were prewarmed at 37°C and reactions were started by addition of the nucleotide substrate (typically, 100 μM $NADP^+$, 100 μM 2′-P-cADPR or 100 μM cADPR). Immediately after addition of the substrates or after incubation at 37°C for the time indicated in the figure legends, samples were centrifuged at $13\,000\times g$ for 5 min. The supernatants were ultrafiltered on Microcon 3 membranes (cut-off 3000 D) and then analyzed by reverse-phase HPLC using a 25×0.46 cm Supelcosil LC-18T column as described [10]. The amount of nucleotides was determined using a calibration curve obtained by analyzing a known amount of standard nucleotides.

3. Results and discussion

Analysis by reverse-phase HPLC of the nucleotides produced upon incubation of platelet membranes with 100 μM $NADP^+$ revealed a progressive decrease of the substrate concentration, which was paralleled by a time-dependent increase of a new nucleotide that co-migrated with authentic 2′-P-ADPR (Fig. 1A). In these experiments, we also investigated the possible production of 2′-P-cADPR upon incubation of platelet membranes with $NADP^+$. Under the chromatographic conditions used, 2′-P-cADPR eluted at about 1.9 min, well separated from 2′-P-ADPR which was eluted at 2.9 min (data not shown). However, as shown in Fig. 1A, production of 2′-P-cADPR was never observed, even after 2 h incubation of platelet membranes with $NADP^+$. Therefore,

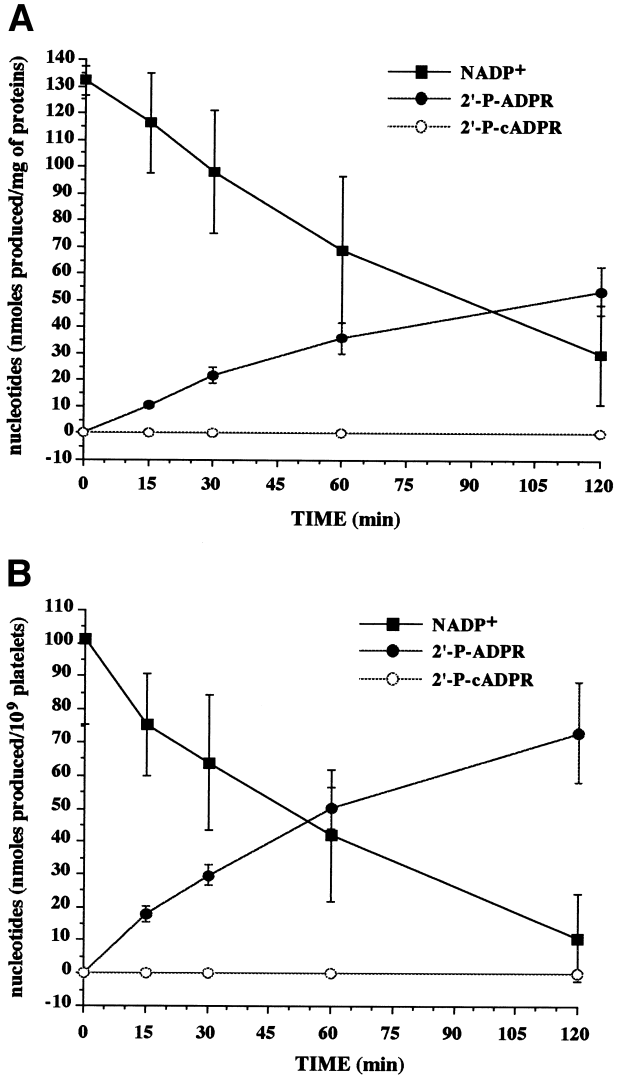

Fig. 1. Metabolism of $NADP^+$ by platelet membranes and intact cells. (A) Platelet membrane preparations. (B) Intact gel-filtered platelets were incubated with 100 μM $NADP^+$ at 37°C. At increasing time points, samples were centrifuged and ultrafiltered on Microcon 3 membranes. 20 μl aliquots were then subjected to reverse-phase HPLC analysis. $NADP^+$, 2′-P-ADPR and 2′-P-cADPR were identified by co-elution with standard nucleotides and quantitated using a calibration curve obtained with known amounts of each nucleotide analyzed. Results are the mean ± S.D. of four different experiments.

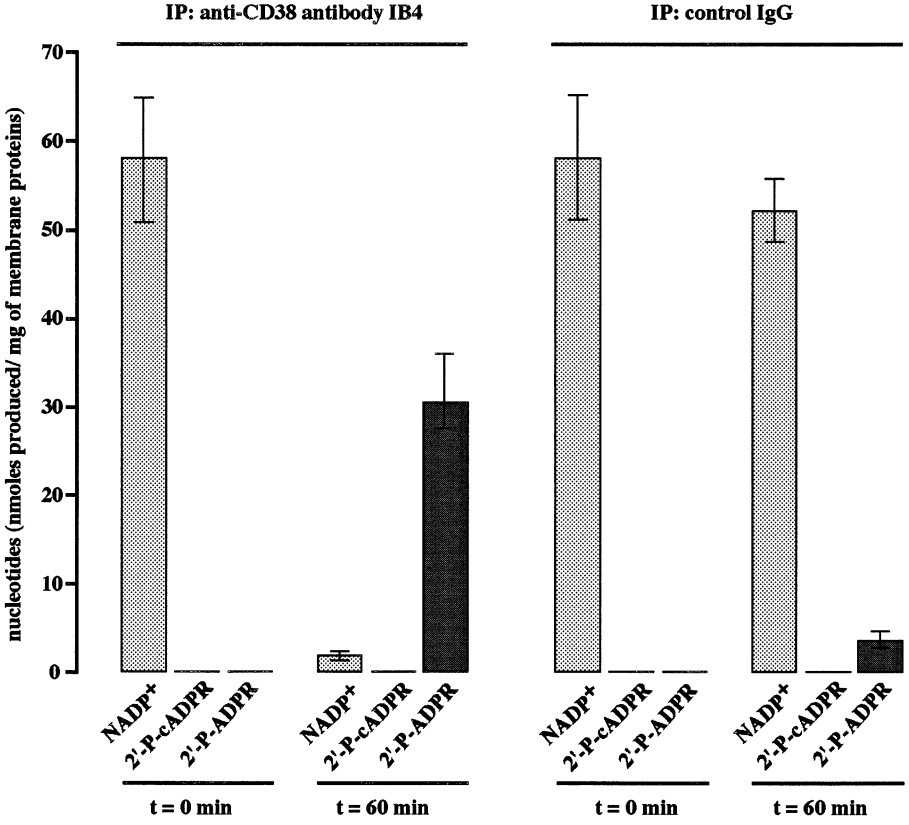

Fig. 2. Hydrolysis of $NADP^+$ by immunoprecipitated CD38. Platelet membranes were solubilized with immunoprecipitation buffer and incubated with the anti-CD38 monoclonal antibody IB4 or with unrelated mouse immunoglobulins (IgG) as control. Immunoprecipitates were incubated at 37°C with 100 μM $NADP^+$. The amounts of $NADP^+$, 2′-P-cADPR and 2′-P-ADPR were determined immediately after the addition of the substrate ($t = 0$) and after incubation for 60 min. Results are expressed as nmol of nucleotides produced by immunoprecipitated materials obtained from 1 mg of solubilized membrane proteins and are the mean ± S.D. of three separated experiments.

platelet membranes are able to hydrolyze $NADP^+$ to 2′-P-ADPR without a detectable production of 2′-P-cADPR. We next analyzed whether the $NADP^+$ glycohydrolase activity detected in platelet membranes was due to an ectoenzyme. Upon incubation of intact human platelets with $NADP^+$, we observed a time-dependent accumulation of 2′-P-ADPR similar to that produced by membrane preparations (Fig. 1B). Moreover, also in this case, synthesis of 2′-P-cADPR was not observed (Fig. 1B). To demonstrate that the ectoenzyme responsible for the hydrolysis of $NADP^+$ to 2′-P-ADPR was the CD38 molecule, platelet membranes were solubilized and immunoprecipitated with the anti-CD38 monoclonal antibody IB4 or with unrelated immunoglobulins as control. The immunoprecipitates were mixed with 100 μM $NADP^+$ and the concentrations of $NADP^+$, 2′-P-cADPR and 2′-P-ADPR were measured by HPLC analysis immediately upon addition of the substrate and after incubation at 37°C for 60 min. Fig. 2 shows that CD38 immunoprecipitated with the IB4 monoclonal antibody was able to convert $NADP^+$ to 2′-P-ADPR without accumulation of 2′-P-cADPR. Very little enzymatic activity was detected when immunoprecipitates obtained with unrelated immunoglobulins were tested and could be due to the aspecific precipitation of a small amount of CD38 molecules (Fig. 2).

These results, that indicate that platelet CD38 catalyzes the hydrolysis of $NADP^+$ to 2′-P-ADPR without apparent generation of 2′-P-cADPR, are consistent with previously reported data, showing that the only nucleotide detectable upon incubation of purified CD38 with $NADP^+$ at physiological pH is 2′-P-ADPR [7]. On the other hand, we have previously shown that, when tested with NAD^+ as substrate, platelet CD38 produces a small amount of cADPR in addition to ADPR [10]. Therefore, our data suggest that NAD^+

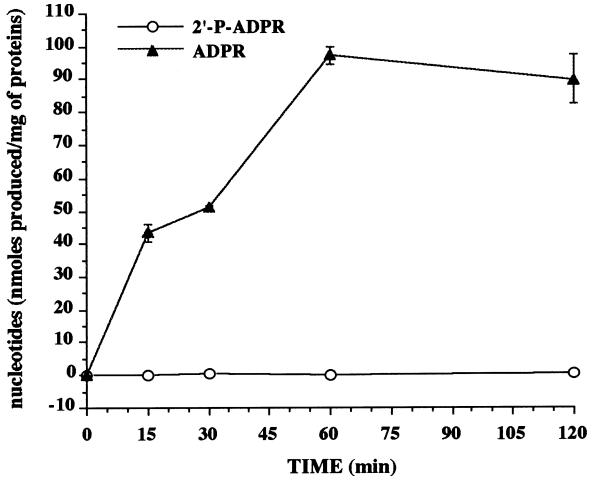

Fig. 3. 2′-P-cADPR and cAPRP hydrolase activity in platelet membranes. Platelet membranes (100 μl, 50 μg of proteins) were incubated at 37°C with 100 μM cADPR or 100 μM 2′-P-cADPR for increasing times. The production of ADPR and 2′-P-ADPR was evaluated by HPLC analysis. Results are the mean ± S.D. of three separated experiments.

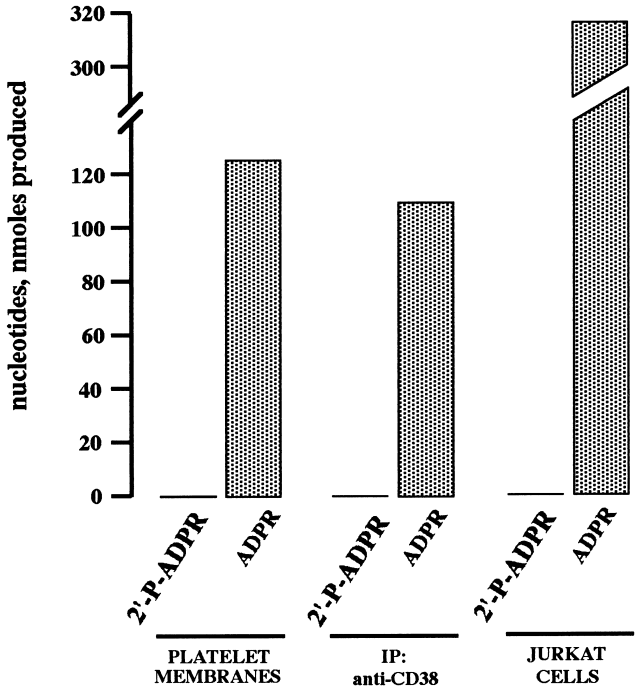

Fig. 4. CD38 does not possess 2′-P-cADPR hydrolase activity. Platelet membranes, immunoprecipitates obtained with the anti-CD38 monoclonal antibody IB4 and intact Jurkat T-cells were incubated at 37°C in the presence of 1 mM 2′-P-cADPR or 1 mM cADPR. After 60 min incubation, the concentration of 2′-P-ADPR and ADPR was determined by HPLC analysis. Results are expressed as nmol of nucleotides produced by 1 mg of membrane proteins, by CD38 immunoprecipitated from 1 mg of solubilized membrane proteins or by 10^8 intact Jurkat T-lymphocytes, respectively, and are the mean of two separated determinations.

and $NADP^+$ are both substrates for CD38, but are differently metabolized. Moreover, when NAD^+ is the substrate, CD38 behaves like a multifunctional enzyme and the synthesized cADPR is rapidly hydrolyzed to ADPR [3]. Therefore, it is possible that the lack of detection of 2′-P-cADPR upon incubation of platelet CD38 with $NADP^+$ reflects its rapid hydrolysis to 2′-P-ADPR through a 2′-P-cADPR hydrolase activity. A similar enzymatic activity was reported to be expressed by a $NAD(P)^+$ glycohydrolase from canine spleen [15]. Therefore, we incubated platelet membranes with 2′-P-cADPR as substrate and the production of 2′-P-ADPR was evaluated by HPLC analysis. As shown in Fig. 3, formation of 2′-P-ADPR was not observed after up to 2 h incubation. By contrast, incubation of the same platelet membrane preparations with cADPR resulted in a rapid and time-dependent synthesis of ADPR (Fig. 3). Therefore, platelet CD38, which displays cADPR hydrolase activity, does not hydrolyze 2′-P-cADPR to 2′-P-ADPR.

The only enzyme described to possess 2′-P-cADPR hydrolase activity is a canine spleen $NAD(P)^+$ glycohydrolase [15]. In this case, however, the K_m for 2′-P-cADPR was calculated to be about 140 μM. Since our enzymatic determinations were performed using 100 μM of substrate, we could not rule out the possibility that these conditions were not appropriate to detect 2′-P-cADPR hydrolase activity of CD38. Therefore, we performed new experiments in which platelet membranes, as well as CD38 immunoprecipitated with the IB4 monoclonal antibody were incubated with 1 mM 2′-P-cADPR or 1 mM cADPR. Fig. 4 shows that even when high concentrations of substrate were used, production of 2′-P-ADPR was not observed. Under the same conditions, production of ADPR from cADPR was evident. Finally, the 2′-P-cADPR hydrolase activity of CD38 was also tested using Jurkat T-cells instead of platelets, since these cells express a higher number of CD38 molecules than platelets. As shown in Fig. 4, Jurkat T-cells, that significantly hydrolyzed cADPR to ADPR, failed to synthesize 2′-P-ADPR when incubated with 1 mM 2′-P-cADPR (Fig. 4). These results clearly indicate that CD38 does not express 2′-P-cADPR hydrolase activity.

In conclusion, our results demonstrate that CD38, which is a multifunctional enzyme towards NAD^+, expressing ADP-ribosyl cyclase, cADPR hydrolase and NAD^+ glycohydrolase activities, is able to metabolize $NADP^+$ exclusively through a $NADP^+$ glycohydrolase activity. Based on the multifunctionality of CD38, the NAD^+ glycohydrolase activity has been considered to represent the sum of the ADP-ribosyl cyclase and cADPR hydrolase activities [3,17,18]. However, this model has recently been hampered by accurate kinetic measurements of the enzymatic reaction mechanism, leading to the conclusion that the synthesis of cADPR from NAD^+ is alternative to the production of ADPR and that cADPR is not an obligatory intermediate during the NAD^+ glycohydrolase activity [5,6]. The results presented in this work, demonstrating the production of 2′-P-ADPR from $NADP^+$ by CD38 in the absence of synthesis and hydrolysis of 2′-P-cADPR, provide further evidence supporting this model.

cADPR and 2′-P-cADPR differ exclusively for the phosphate group in the 2′-position of the ribose moiety. It is thus surprising that while cADPR is a substrate for CD38, 2′-P-cADPR is not. This may reflect the inability of 2′-P-cADPR to interact with the active site of CD38 due to the phosphate group. It is therefore possible that upon binding of $NADP^+$ and cleavage of the nicotinamide-ribose bond, a conformational change of the CD38 molecule occurs to produce an enzyme 2′-P-ADPR intermediate that necessarily proceeds toward the release of 2′-P-ADPR. However, in the absence of structural data on the three dimensional conformation of CD38 in association with the different nucleotides, this possibility remains exclusively speculative.

The ability of 2′-P-cADPR to act directly as a Ca^{2+}-mobilizing agent is still controversial [7,15]. In any case, our results showing that 2′-P-cADPR is not produced in intact platelets by CD38 (or by other still unidentified enzymes) indicate that it is unlikely for this molecule to play a role in the regulation of Ca^{2+} mobilization in platelets. Considering that these cells are not sensitive to cADPR either [13,14], the physiological role of CD38 in platelets does not seem to be related to the control of Ca^{2+} mobilization.

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