Cytoskeleton-dependent inhibition of the ADP-ribosyl cyclase activity of CD38 in thrombin-stimulated platelets

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Abstract Stimulation of human platelets with thrombin caused a 42% inhibition of the ADP-ribosyl cyclase activity of membrane CD38. This effect was mediated by the activation of the platelet thrombin receptor rather than by proteolysis of CD38, and was not due to a different distribution of the synthesised nucleotide or to a reduced accessibility of CD38 to the substrate. The inhibitory effect of thrombin required actin polymerisation and was not observed when interaction of CD38 with the cytoskeleton was prevented by cytochalasin D. Finally, we analysed whether cADPR could play a role as a Ca^{2+}-mobilising agent in human platelets. Using saponin-permeabilised cells, we found that unlike IP_3, cADPR did not induce any release of Ca^{2+} from intracellular stores. These results indicate that the enzymatic activity of membrane CD38 can be modulated by platelet activation, and that the function of this glycoprotein is probably not related to Ca^{2+} mobilisation.

Key words: ADP-ribosyl cyclase; CD38; Cytoskeleton; Platelet; Ca^{2+} mobilization

1. Introduction

CD38 is a type II transmembrane glycoprotein expressed in a variety of cells and displaying several functional properties [1]. This molecule is an adhesion receptor able to support the interaction of lymphocytes with endothelial cells and to bind to hyaluronate [2,3]. Moreover, CD38 represents a cell-activating receptor that, upon ligation by selected monoclonal antibodies, triggers specific responses including proliferation and cytokine production [4,5]. Finally, the extracellular domain of CD38 is a bifunctional enzyme that catalyses the synthesis of cADPR from NAD^+ and the hydrolysis of cADPR to ADPR [6]. Thus, CD38 is involved in the transient formation of cADPR, which represents a second messenger able to stimulate the release of Ca^{2+} from ryanodine-sensitive, but IP_3-insensitive intracellular stores [7].

During the last years, an increasing amount of information has been produced on the effect of CD38 ligation on cell activation. In contrast, very little is known on the effects of cell activation on the biochemical properties and enzymatic activities of CD38. An increased ADP-ribosyl cyclase activity of CD38 has been reported in HL60 cells treated with retinoic acid, but this effect was due to the induction of CD38 mRNA and expression of new molecules on the cell surface [8]. Evidence for post-translational modifications of soluble and membrane-bound CD38, including ADP-ribosylation and oligomerisation, has also been described and implications for the enzymatic activities have been suggested [9–11]. In sea urchin eggs, the synthesis of cADPR by a soluble ADP-ribosyl cyclase, and the consequent Ca^{2+} mobilisation, was stimulated by nitric oxide, through the activation of a guanylate cyclase and the formation of cGMP [12,13]. However, such a regulation in mammalian cells has not been reported. Recently, a modulation of the ADP-ribosyl cyclase activity of membrane CD38 in response to the activation of muscarinic acetylcholine receptors has been described [14]. In particular, m1 and m3 subtypes of acetylcholine receptors were able to stimulate the ADP-ribosyl cyclase activity of CD38 through the action of a cholera toxin-sensitive G-protein, while m2 and m4 receptor subtypes caused a pertussis toxin-sensitive inhibition of the ADP-ribosyl cyclase activity of CD38 [14].

Human platelets express enzymatically active CD38 on the plasma membrane [15], and we have recently shown that this glycoprotein specifically interacts with the intracellular cytoskeleton upon cell stimulation with thrombin [16]. One of the earliest events in platelet activation is the Ca^{2+} mobilisation from intracellular stores, which is mediated by IP_3 formation, but could also involve other messengers such as cADPR [17]. Therefore, in this work, we investigated the modulation of the ADP-ribosyl cyclase activity of CD38 in intact platelets stimulated with physiological agonists. Surprisingly, we found that thrombin caused a strong inhibition of the ADP-ribosyl cyclase activity in intact cells, which was mediated by the interaction of CD38 with the cytoskeleton. Moreover, we also observed that cADPR did not actually function as a Ca^{2+}-mobilising agent in these cells.

2. Materials and methods

2.1. Materials

Nicotinamide guanine dinucleotide (NGD^+), cyclic ADP-ribose, cytochalasin D, U46619, leupeptin, RGDS peptide, aprotinin, IP_3, and all the platelet agonists used were purchased from Sigma. The thrombin receptor activating peptide (TRAP) and FLUO-3 were from Calbiochem. Microcon 3 filters were from Amicon. Triton X-100 was from ICN. All other reagents were of analytical grade.

2.2. Platelet preparation and analysis of the ADP-ribosyl cyclase activity

Human platelets were isolated by gel filtration on a Sepharose 2B-CL column, in HEPES buffer (10 mM HEPES, 137 mM NaCl, 2.9 mM KCl, 12 mM $NaHCO_3$, pH 7.4) as previously described [15]. Samples of gel-filtered platelets (0.5 ml) were prewarmed at 37°C for 5 min and then treated with buffer or with 10 nM thrombin, 10 μM TRAP, 10 μg/ml collagen or 10 μM U46619. In some experi-

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Abbreviations: NGD^+, nicotinamide guanine dinucleotide; cGDPR, cyclic GDP-ribose; cADPR, cyclic ADP-ribose; ADPR, ADP-ribose; IP_3, inositol 1,4,5-trisphosphate

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ments, platelet samples were incubated with 10 μM cytochalasin D for 2 min before the addition of the agonists. Two minutes after the addition of the agonists, 100 μM NGD^+ was added to the samples to measure the ADP-ribosyl cyclase activity [18]. The concentrations of the nucleotides were evaluated in 100 μl aliquots immediately after the addition of the substrate, and after incubation at 37°C for 60 min. Typically, samples were centrifuged at $13\,000\times g$ for 3 min, and the supernatants were ultrafiltered on Microcon 3 membrane (cut-off 3000 Da). Nucleotides in the supernatants were separated and quantified by reverse phase HPLC using a 25×0.46 cm Supelcosil LC-18T column, as previously described [15]. In some experiments, platelets were lysed with 1/10 volume of 10% Triton X-100 before addition of 100 μM NGD^+. In other experiments, intact platelets were incubated with NGD^+, and, at the end of the incubation time, 1/10 volume of 7 N perchloric acid was added, and samples were vigorously mixed and placed on ice for 5 min. Samples were then centrifuged at $13\,000\times g$ for 3 min and the acid extracts were neutralised and extracted with freon-trioctylamine as described [19]. Nucleotides were then analysed by HPLC.

2.3. Measurement of Ca^{2+} release

Gel-filtered platelets were prepared at a final concentration of 2×10^9 cells/ml in HEPES buffer containing 10 μg/ml leupeptin, 10 μg/ml aprotinin, 2 U/ml creatine phosphokinase, 4 mM phosphocreatine, 0.5 mM ATP, 1 mM $MgCl_2$. Samples (0.4 ml) were prewarmed at 37°C in a Perkin-Elmer spectrofluorimeter, and 6 μM FLUO-3 was added. Platelets were then permeabilised by addition of 50 μg/ml saponin for 1 min. Ca^{2+} release from intracellular stores was stimulated by addition of 3 μM IP_3 or 0.1–10 μM cADPR. FLUO-3 fluorescence was continuously monitored at 490 nm excitation and 535 nm emission.

3. Results and discussion

We investigated the effect of platelet stimulation with the strong agonists thrombin, collagen and the thromboxane A_2 analogue U46619 on the ADP-ribosyl cyclase activity of membrane CD38. All the agonists analysed caused an inhibition of the synthesis of cGDPR from NGD^+ by intact platelets, which was reduced by $42\pm20\%$, $19\pm12\%$, and $25\pm10\%$ in thrombin-, collagen-, and U46619-treated platelets, respectively (Fig. 1). Since the strongest effect was observed in thrombin-stimulated platelets we focused our investigations on this agonist. As thrombin is also a proteolytic enzyme, we considered that the inhibitory effect on the ADP-ribosyl cyclase activity could be due to direct proteolysis of the CD38 molecule, rather than to platelet activation. However, Fig. 1 also shows that TRAP was as effective as thrombin in inhibiting the ADP-ribosyl cyclase activity of CD38 ($44\pm2\%$ inhibition), demonstrating that this effect was mediated by signals generated through the activation of the thrombin receptor. Moreover, we never observed release of ADP-ribosyl cyclase activity in the supernatant of thrombin-stimulated platelets, indicating that this enzyme did not induce membrane CD38 shedding (data not shown).

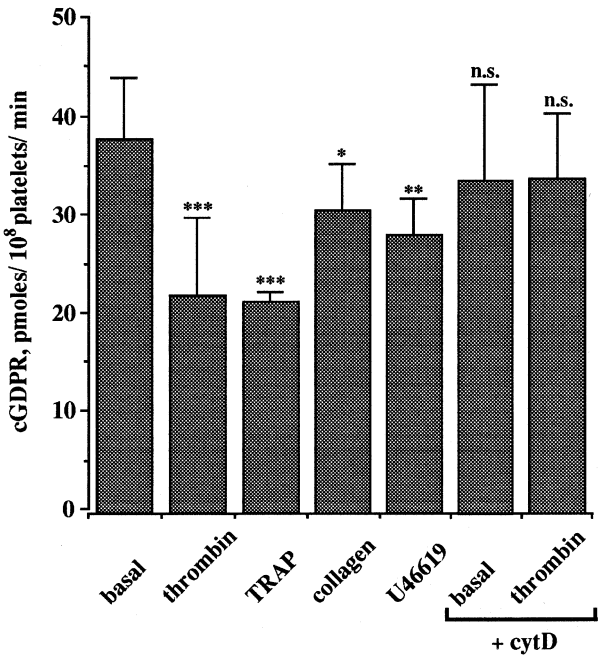

Fig. 1. Effect of platelet stimulation on the ADP-ribosyl cyclase activity of CD38. Gel-filtered platelets were treated with buffer (basal), 10 nM thrombin, 10 μM thrombin receptor activating peptide (TRAP), 10 μg/ml collagen, or 10 μM U46619 for 2 min. In some experiments, gel-filtered platelets were preincubated with 10 μM cytochalsin D (cytD) for 2 min before treatment with buffer (basal) or 10 nM thrombin. The ADP-ribosyl cyclase activity of CD38 was then measured by incubation of the samples with 100 μM NGD^+ for 60 min, and the production of cGDPR was analysed by HPLC, as described in Section 2. Results are the means ± S.D. of three to eight different experiments. $***P<0.001$; $**0.001<P<0.01$; $*0.02<P<0.05$; n.s., not significant.

We have previously shown that thrombin induces the association of about 40% of the platelet ADP-ribosyl cyclase activity with the cytoskeleton [16]. Since this value was very similar to the reduction of the ADP-ribosyl cyclase activity measured in stimulated intact platelets, we considered that the interaction of CD38 with the cytoskeleton could mediate the inhibitory effect of this agonist. Therefore, we analysed the effect of cytochalasin D, which prevents CD38 translocation to the cytoskeleton without affecting platelet aggregation [16,20], on the synthesis of cGDPR by resting and thrombin-activated platelets. We found that in thrombin-stimulated platelets, the inhibition of the ADP-ribosyl cyclase activity of

Table 1
ADP-ribosyl cyclase activity of CD38 in intact and lysed platelets

	Resting platelets	Thrombin-stimulated platelets
Intact platelets	37.8 ± 2.9	28.5 ± 2.3
Perchloric acid-lysed platelets	39.5 ± 1.0	28.6 ± 1.2
Triton X-100-lysed platelets	35.5 ± 2.5	27.8 ± 1.6

Gel-filtered platelets were treated with either buffer (resting platelets) or 10 nM thrombin (thrombin-stimulated platelets) for 2 min at 37°C. Samples of intact platelets were then incubated with 100 μM NGD^+ for 60 min, and then platelets were pelleted by centrifugation and the supernatants processed for HPLC analysis. NGD^+ (100 μM) was also directly added to some other samples, but at the end of the incubation time, platelets were lysed with perchloric acid, and the extracts were processed for HPLC analysis. In contrast, some samples were lysed with Triton X-100 (1% final concentration) before addition of 100 μM NGD^+. Production of cGDPR by each sample was determined by HPLC analysis. Results are expressed as pmol/min of cGDPR produced by 10^8 platelets, and represent the means ± S.D. of three to five experiments.

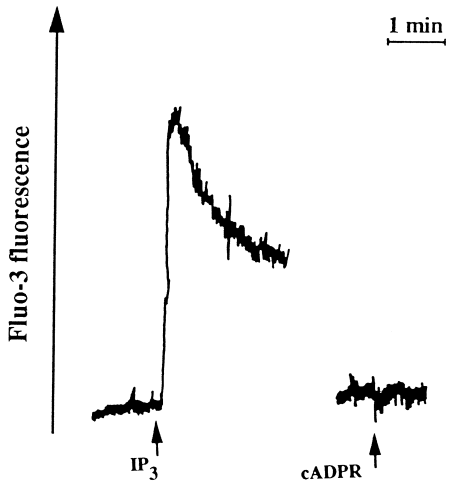

Fig. 2. Ca^{2+} release induced by IP_3 and cADPR. Gel-filtered platelets were permeabilised with 50 μg/ml saponin in the presence of the fluorescent Ca^{2+} probe FLUO-3. Release of Ca^{2+} from internal stores was stimulated by the addition of 3 μM IP_3 or 100 nM cADPR. Typical fluorescent traces, representative of at least three different experiments, are reported.

CD38 was completely prevented by treatment of the cells with cytochalasin D (Fig. 1). Therefore we conclude that the reduced production of cGDPR in thrombin-stimulated platelets is a consequence of the agonist-induced interaction of CD38 with the intracellular cytoskeleton. In this regard, it is interesting to note that collagen and U46619, which were reported to be less potent than thrombin in promoting the interaction of CD38 with the actin filaments [16], also caused a reduced inhibition of the ADP-ribosyl cyclase activity in intact platelets (Fig. 1).

We considered that the reduced accumulation of cGDPR observed in thrombin-stimulated intact platelets could reflect not a real inhibition of the ADP-ribosyl cyclase activity of CD38, but other events, including, for instance, a stimulated influx of the synthesised cGDPR into the platelet, or increased binding to the membrane, as well as the internalisation of CD38 upon binding to the actin filaments. To verify these possibilities, we measured the production of cGDPR in resting and thrombin-stimulated platelets using different approaches. In one set of experiments, intact resting and thrombin-stimulated platelets were incubated with NGD^+, and the total nucleotides, including extracellular, intracellular, and membrane bound pools, were extracted by addition of perchloric acid and analysed by HPLC. Table 1 shows that even when the total cell nucleotides were considered, a reduced amount of cGDPR was present in samples of thrombin-treated platelets compared to resting cells. To verify whether our results could reflect a reduced accessibility of CD38 to the extracellular substrate in thrombin-stimulated platelets, resting and thrombin-aggregated cells were lysed with 1% Triton X-100 before addition of the NGD^+. Treatment with Triton X-100 preserves the interaction of CD38 with the actin filaments, but solubilises the cell membrane and allows NGD^+ to reach potentially internalised active sites of CD38 molecules. Table 1 shows that even when NGD^+ was added after cell membrane disruption, the amount of cGDPR measured in thrombin-stimulated platelets was reduced compared to resting cells. Therefore, we conclude that the reduction of cGDPR observed in thrombin-activated intact platelets was not due to internalisation of CD38 or influx of cGDPR, but to the inhibition of the ADP-ribosyl cyclase activity of the enzyme. Recently, Higashida et al. reported that the inhibition of membrane associated ADP-ribosyl cyclase activity is promoted by activation of m2 and m4 muscarinic acetylcholine receptors by a mechanism involving a pertussis toxin-sensitive G-protein [14]. Our data are in agreement with this report, since the thrombin receptor, which mediates the inhibition of the ADP-ribosyl cyclase activity in intact platelets, is known to be associated to the pertussis toxin-sensitive G-protein G_i [21]. Therefore, our results provide further evidence that the enzymatic activity of CD38 can be modulated by signals generated through G-protein-coupled receptors.

It is known that thrombin stimulation of platelets induces a rapid rise in the intracellular Ca^{2+} concentration [17]. If the function of CD38 in platelets was related to the control of Ca^{2+} movements, one would expect an increase of the ADP-ribosyl cyclase activity upon cell activation rather than an inhibition. However, the presence of cADPR-sensitive Ca^{2+} stores in platelets has not so far been demonstrated. Therefore, we investigated whether cADPR was able to promote the release of Ca^{2+} from internal stores in these cells. Gel-filtered platelets were permeabilised with saponin in the presence of FLUO-3, and then cADPR or IP_3 were added. Fig. 2 shows that while IP_3 caused a significant release of Ca^{2+}, no response to cADPR was observed. This nucleotide displayed no effects even when used at concentrations up to 10 μM (data not shown). These results suggest that, unlike other cells, platelets do not possess cADPR-sensitive Ca^{2+} stores, and, therefore, it is unlikely that this nucleotide plays a role in Ca^{2+} release in platelets.

This evidence raises the question of the physiological function of CD38 in platelets. Recently it has been reported that in addition to NAD^+, CD38 can also use $NADP^+$ as a substrate to produce a different compound through a base exchange reaction involving nicotinic acid, nicotine acid adenine dinucleotide phosphate (NAADP), which displays Ca^{2+}-mobilising activity by acting on intracellular stores distinct from those sensitive to either IP_3 or cADPR [22,23]. The metabolism of $NADP^+$ by platelet CD38 and the possible role of NAADP as a Ca^{2+}-mobilising agent in these cells deserve further investigation. We may, however, hypothesise that the thrombin-induced inhibition of the ADP-ribosyl cyclase activity described in this work may represent a way to switch the enzymatic activity of CD38 to the base exchange reaction to increase the production of NAADP. It is also possible that platelet CD38 may play a role unrelated to Ca^{2+} mobilisation. It is known that the synthesised cADPR is rapidly hydrolysed to ADPR by the same CD38 molecule [6]. Previous studies have demonstrated that ADPR is an inhibitor of platelet activation induced by several agonists [24]. Therefore, it is possible that CD38 contributes to a tonic negative control of platelet function and that the inhibition of the ADP-ribosyl cyclase activity induced by thrombin may represent a way to remove, at least in part, this inhibitory effect.

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