Phospholipase C Activity in Platelets From Bernard-Soulier Syndrome Patients

A. McNicol, J. Drouin, K.J. Clemetson, J.M. Gerrard

The levels of glycoprotein (GP) Ib and GPV and phospholipase C activity were measured in platelets from three Bernard-Soulier syndrome patients. The patients' platelets had 46%, 46%, and 24% of control levels of GPIbt and 43%, trace, and 13% of control levels of GPV as determined by immunoblot analysis. Stimulation by thrombin, trypsin, the thromboxane analogue U46619, and the combination of U46619 and trypsin caused the formation of [32P]phosphatidic acid, an index of phospholipase C activity, in [32P]orthophosphate-prelabeled platelets. With all agonists, however, the formation of [32P]phosphatidic acid was markedly reduced in Bernard-Soulier syndrome platelets compared with control platelets. These data indicated a postreceptor defect in phospholipase C activation in Bernard-Soulier syndrome platelets and confirmed earlier observations of potential proteolytic and nonproteolytic mechanisms of platelet activation. (Arterioscler Thromb. 1993;13:1567-1571.)

KEY WORDS • Bernard-Soulier syndrome • platelets • phospholipase C • glycoproteins • protease

Thrombin, a serine protease, is the most potent platelet agonist in vitro, although its mechanism of action remains to be clearly elucidated. Platelets stimulated by thrombin display the kinetic characteristics of both an agonist-receptor interaction and an enzyme-driven process. Recently, two groups have cloned and sequenced a thrombin receptor with a novel mechanism of action. Thrombin binds to a hirudin-like domain on the long extracellular N-terminal of the receptor and proteolytically cleaves a fragment to generate a tethered ligand. The tethered ligand subsequently autostimulates the receptor. Platelets can be stimulated by adding polypeptides corresponding to the terminal region of the tethered ligand.

Intracellularly, thrombin-induced platelet activation is associated with the stimulation of phospholipase C, a resultant increase in cytosolic calcium levels, production of diacylglycerol, and activation of protein kinase C. The effects of thrombin-induced activation of phospholipase C appear to be dual in nature: proteolytic and nonproteolytic. The individual components of this activity can be mimicked by the serine protease trypsin and by a receptor-related agonist, respectively. These effects on phospholipase C activity are additive. Platelet aggregation induced by the tethered ligand polypeptide is accompanied by phospholipase C activity, although whether this mimics both components of activity has not been examined.

There are several other thrombin substrates/binding sites on platelets, although it is unknown whether they contribute to thrombin-induced platelet activation. Of particular interest are glycoprotein (GP) Ib, which contains a binding site for thrombin, and GPV, which is a substrate for the proteolytic action of thrombin on platelet membranes. The precise relevance, if any, of each of these proteins in thrombin-induced platelet activation remains unknown. For example, the presence of GPIb on platelets does not appear to be a prerequisite for thrombin-induced platelet activation. Thrombin-induced platelet activation is reported to occur by both GPIb-dependent and GPIb-independent mechanisms, although GPIb appears to be critical when low concentrations of thrombin are used. Similarly, inhibition of the proteolysis of GPV by anti-GPV antibodies does not have a coincidental inhibitory effect on aggregation. It is possible that GPIb and GPV are either directly or indirectly linked to phospholipase C in platelets.

Bernard-Soulier syndrome (BSs) is an inherited bleeding disorder associated with the absence or dysfunction of GPIb, GPV, and GPIX. BSs platelets are reported to be less sensitive to stimulation by thrombin than controls, providing further indirect evidence for a role for at least one of these proteins in thrombin-induced platelet activation. These platelets, therefore, allow the potential roles of GPIb and GPV in thrombin-induced activation of phospholipase C to be examined.

Methods

Patients

M.R. and M.R.H. are sisters in a previously reported family of French-Canadian origin. Each sister has experienced repeated epistaxes, excessive bleeding after dental extractions, and postpartum hemorrhage. The sisters both have prolonged bleeding times, platelet counts of 30,000 to 60,000, giant platelets, and the absence of ristocetin-induced aggregation.
A young woman with a lifelong bleeding tendency who was raised in foster homes and does not know the identity of her biological parents. She has suffered from repeated epistaxes, gum bleeding, gastrointestinal bleeding requiring multiple units of packed red cells and platelets, and severe menorrhagia. Laboratory investigations showed a platelet count of about 40,000 with a predominance of giant platelets, a prolonged bleeding time, and the absence of platelet aggregation in response to ristocetin.

**Phospholipase C Activity**

All studies were performed on washed platelets isolated from fresh, citrated blood drawn from either BSs patients or normal control subjects. Platelets were pre-labeled at 37°C for 90 minutes in a calcium-free, phosphate-free N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid buffer with [32P]orthophosphate (100 μCi/mL) as previously described. The platelets were then washed and resuspended in the same buffer. Aliquots were incubated (2 minutes) with either the agonist(s) or the vehicle (saline) control; the reactions were then terminated and the phospholipids were extracted, separated, and quantified by radiochromatographic scanning. [32P]Phosphatidic acid, labeled to isotopic equilibrium under the conditions employed, was monitored as an indirect index of phospholipase C activation. The incorporation of label into phospholipids did not differ between the platelets from normal and BSs individuals. In normal individuals, the resting levels of [32P]incorporated in phosphatidic acid were 360 ± 113 cpm (mean ± SEM), which represented 2.6 ± 0.4% of the [32P]incorporated into total phospholipids. In BSs platelets these values were 381 ± 93 cpm and 3.9 ± 0.4%, respectively. All data are expressed as a fold increase of the saline control.

**Immunoblotting**

Platelet samples from BSs patients or from normal control subjects were treated with 2% sodium dodecyl sulfate, 2 mmol/L N-ethylmaleimide, and 50 mmol/L tris(hydroxymethyl)aminomethane-HCl, pH 6.8, and were incubated at 100°C for 5 minutes. Proteins (10 μg) were separated on a 7.5% or 10% polyacrylamide gel with a 3% stacking gel and were transferred to polyvinylidene difluoride membranes as previously described. Membranes were then incubated for 1 hour with rabbit anti-GPV, anti-glycocalicin, or anti-GPIbβ polyclonal antisera. The membranes were incubated (1 in 7500 for 1 hour) with alkaline phosphatase–conjugated goat anti-rabbit antibody (Promega, Madison, Wis) and were visualized by using nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate p-toluidine salt (Bethesda Research Laboratories, Gaithersburg, Md). The color reaction was terminated with water and was quantified by densitometry.

**Results**

Normal platelets stimulated with thrombin (1 U/mL), trypsin (1 μmol/L), and the thromboxane analogue U46619 (1 μmol/L) showed a 24-, 31-, and 7-fold increase in [32P]phosphatidic acid production, respectively (Table). The coaddition of trypsin and U46619 to normal platelets had an approximately additive effect on stimulated [32P]phosphatidic acid levels (Table) as previously observed. Thrombin caused 15.3-, 11.8-, and 7.1-fold increases of [32P]phosphatidic acid levels in platelets from the three BSs patients, M.R., M.R.H., and N.O., respectively. In platelets from M.R., [32P]phosphatidic acid levels were elevated by approximately 15.2-, 2.6-, and 23.6-fold by trypsin, U46619, and the combination of trypsin and U46619, respectively (Table). Trypsin and U46619 stimulated [32P]phosphatidic acid levels by approximately 13.3- and 1.7-fold, respectively, in M.R.H. The coaddition of trypsin and U46619 caused an approximate increase of 14.5-fold (Table). Similarly, trypsin, U46619, and the combination of trypsin and U46619 caused 9.6-, 2.6-, and 11.8-fold increases in the [32P]phosphatidic acid levels in platelets from N.O. (Table).

Immunoblot analysis determined N.O. to have 24% of the normal level of GPIbα (Fig 1A) and trace amounts of GPIbβ (Fig 1B). Previously, M.R. and M.R.H. were each shown to have 46% of the normal level of GPIbα. N.O., M.R.H., and M.R. had 13%, 43%, and trace amounts, respectively, of the normal level of GPV (Fig 2). Neither M.R. nor M.R.H. had detectable levels of GPIX24 and GPIX levels in N.O. were undetermined.

**Discussion**

The biochemical consequences of thrombin-induced platelet activation have been the subject of numerous studies and, in common with other agonists, involve stimulation of phospholipase C.22,23 However, in the case of thrombin there appears to be a dual mechanism of action, one proteolytic and one nonproteolytic. Several isoforms of phospholipase C have been identified, and it is possible that these individual phospholipase Cs are activated by different mechanisms.
The cloning and detection of a functional thrombin receptor in platelets has led to a novel model of thrombin-induced platelet activation involving the autostimulation of the proteolytically truncated thrombin receptor. The stimulatory effects of thrombin can be mimicked by the tethered peptide generated by the proteolytic action of thrombin on the receptor.

Platelets stimulated by this peptide stimulate phospholipase C activity. Indeed, Huang and colleagues suggest that this accounts for all phospholipase C activity in thrombin-stimulated platelets and that saturable thrombin binding is of no consequence. In contrast, however, the high-affinity thrombin binding site GPIb is reported to be critical when low doses of thrombin are used, and indeed a GPIb-dependent mechanism of thrombin-induced platelet activation is proposed.

To examine whether GPIb or the known thrombin substrate GPV has any function in thrombin-induced phospholipase C activity, [32P]phosphatidic acid was measured in platelets from the three BSs patients. Compared with platelets from normal control subjects, platelets from BSs patients had levels of approximately trace to 46% GPIb and trace to 30% GPV. These levels are potentially indicative of the close association of these two glycoproteins on the platelet membrane. Such an intimate relationship among GPIb and GPV and GPIX has recently been reported. Many of the initial studies examining the glycoprotein profile of BSs platelets indicate either much lower levels or a total absence of these glycoproteins, particularly GPIb. The presence of a significant amount of GPIb on platelets from BSs patients may not be uncommon.
Thrombin-stimulated BSs platelets demonstrated phospholipase C activity as monitored by the formation of [32P]phosphatidic acid. The activity was less than that in normal control platelets, although this observation may reflect a delayed response. The differing kinetics are attributed to the reduced levels of GPIb, which may play a regulatory role to accelerate thrombin-induced platelet activation. However, the addition of the serine protease trypsin or the thromboxane mimetic U46619 also caused diminished phospholipase C activity in the BSs platelets. This may indicate a defect at a common event at or before the level of phospholipase C activation. Platelets from BSs patients are reported to have an altered membrane phospholipid profile, and their membranes are easily deformable. Either of these abnormalities may prevent association of phospholipase C with its phosphoinositide substrate, thereby reducing its effectiveness in signal transduction.

The coaddition of trypsin and the thromboxane analogue U46619 caused an additive formation of [32P]phosphatidic acid in BSs platelets, similar to that reported in normal platelets. This is consistent with the concepts that platelets can be stimulated in both a proteolytic and nonproteolytic manner and that BSs platelets share this capability. Furthermore, it is consistent with a defect in BSs platelets distal to surface receptors.

The present study did not directly examine or quantify the levels of the novel autoproteolytic receptor on BSs platelets, although Gralnick and colleagues report normal numbers of these receptors on the platelets of two BSs patients. Although this receptor appears to serve as the proteolytic substrate on platelets on which thrombin acts, with the resultant activation of phospholipase C, it should be noted that the receptor was less sensitive to proteolysis by trypsin than by thrombin, whereas trypsin is as potent as thrombin at stimulating platelet aggregation, secretion, and phospholipase C activity. This may indicate that the novel receptor is not the sole substrate required for phospholipase C activation and subsequent platelet activation. The novel receptor has the classic seven transmembrane domains consistent with the observed activation of GTP-binding proteins in thrombin-stimulated platelets. However, Torti and Lapeña offer evidence for the activation of a phospholipase C subtype in thrombin-stimulated platelets believed to occur independently of GTP-binding proteins, which is again indicative of another thrombin receptor. Furthermore, Seller and colleagues suggest that the cloned thrombin receptor activates a phospholipase A2-mediated pathway rather than a phospholipase C-mediated pathway of platelet activation.

In conclusion, the present study demonstrated that thrombin stimulates phospholipase C in BSs platelets, which have decreased levels of GPIb and GPV, although the level of stimulation appears to be substantially less than in control platelets. However, phospholipase C activity in response to trypsin and the thromboxane analogue U46619 were likewise decreased in BSs platelets. It is therefore probable that there is a common defect in BSs platelets distal to all surface receptors rather than a specific abnormality due to the diminished levels of GPIb and GPV.

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References


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