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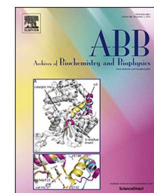

Relationship between calcium mobilization and platelet α- and δ-granule secretion. A role for TRPC6 in thrombin-evoked δ-granule exocytosis

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ABSTRACT

Changes in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) regulate granule secretion in different cell types. Thrombin activates PAR1 and PAR4 receptors and promotes release of Ca^{2+} from distinct intracellular stores, which, in turn, activates store-operated Ca^{2+} entry (SOCE). A crucial step during platelet function is the release of physiological agonists stored in secretory granules to the extracellular compartment during activation. We aim to study the role of Ca^{2+} mobilization from the extracellular compartment or from different intracellular stores in platelet granule secretion. By using flow cytometry, we have found that α- and δ-granules are secreted in thrombin-stimulated platelets in the absence of extracellular Ca^{2+}, and in a concentration-dependent manner. Our findings show that thrombin-stimulated granule secretion depends on Ca^{2+} mobilization from intracellular stores. Analysis of the kinetics of granule secretion reveals that platelet stimulation with thrombin results in rapid release of α-granules which precedes the secretion of δ-granules. Incubation of platelets with a specific antibody, which recognizes the extracellular amino acid sequence 573–586 of TRPC6, inhibited thrombin-evoked δ-granule exocytosis. Our results indicate that the mechanisms underlying thrombin-induced α- and δ-granule secretion show differences in dependency on Ca^{2+} mobilization.

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1. Introduction

Platelet activation, shape change and secretion are dependent on changes in cytosolic calcium concentration ($[Ca^{2+}]_c$) [1,2]. Several mechanisms have been proposed to describe how Ca^{2+} may regulate platelet granule secretion: 1) activation of Ca^{2+}-dependent PKC isoforms [3], and subsequent phosphorylation of actin depolymerizing proteins; 2) activation of Ca^{2+}-dependent actin filament-severing proteins, such us scinderin [4,5]; 3) induction of dense core granule secretion by Rab27 through the activation of the Ca^{2+}-binding protein Munc13-4 [6,7]; 4) modulation of platelet dense granule secretion by Rap1GAP2/synaptotagmin-like protein 1 [8], as well as Rab8/synaptotagmin-like protein 4 [9]. Different autocrine and paracrine molecules have been found stored in both platelet α- and dense (δ)-granules, hence discharge of these granules is required for full platelet activation. Platelet α-granules contain both membrane bound proteins that reach the plasma membrane upon exocytosis and soluble proteins that are released into the extracellular medium. Membrane bound proteins include integrins (such as α_{IIb}, α_6, β_3), immunoglobulin family receptors, leucine-rich repeat family receptors (such as the GPIb-IX-V complex) and tetraspanins. Releasable factors include paracrine substances, such as von Willebrand factor (vWF), PAF4 or vitronectin [10]. On the other hand, δ−granules contain mostly autocrine platelet agonists such as Ca^{2+}, ADP, ATP and serotonin (5-HT). ADP secreted evokes a transient Ca^{2+} release from the dense tubular system (DTS) that induces short-term platelet activation and aggregation that may be rapidly reversed [11,12], while stronger platelet agonists, like thrombin, evoke Ca^{2+} release either from acidic granules or from the DTS depending on the concentration of the agonist [11,13]. The differential regulation of Ca^{2+} release from internal stores by distinct agonists suggests that certain processes

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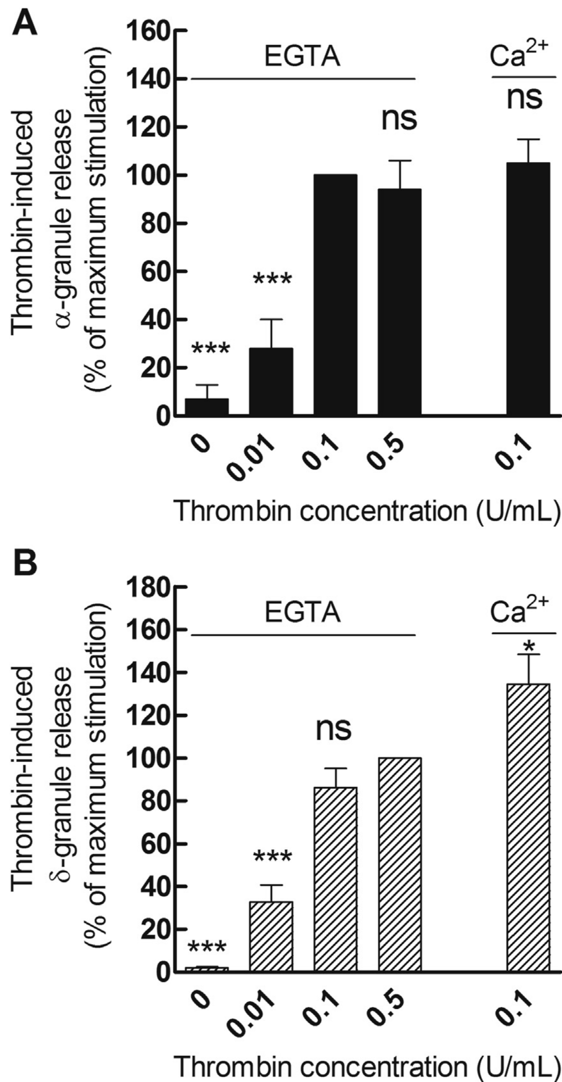

Fig. 1. Thrombin induces α- and δ-granule secretion in the absence and presence of extracellular Ca^{2+}. Human platelets were stimulated with various concentrations of thrombin (0.01–0.5 U/mL) in a Ca^{2+}-free medium or with 0.1 U/mL thrombin in the presence of 1.2 mM extracellular Ca^{2+}, and P-selectine exposure (A) and mepacrine release (B) were estimated as described in methods. Data are presented as mean ± S.E.M. and expressed as percentage of the maximum stimulation. ns, non significant; ***, $P < 0.001$ as compared to the maximal response (one-way analysis of variance combined with the Dunnett test).

involved in platelet function require depletion of both stores, while others may require smaller or more restricted increases in $[Ca^{2+}]_c$. Exocytosis of platelet granules require the reorganization of the cortical actin cytoskeleton located beneath the plasma membrane. Such reorganization allows the molecular interaction between proteins of the exocytotic machinery, including SNAP23, SNAP25, syntaxin-11, VAMP3, VAMP7 or VAMP8, and accessory proteins required for membrane coupling such as munc13-1, munc13-4 and GRP27. Their interaction would facilitate membrane fusion and the subsequent release of the granule content to the extracellular medium [6,14–16]. We have reported a similar mechanism during the activation of SOCE in platelets, where the actin cortical cytoskeleton located nearby the plasma membrane must be reorganized in order to allow molecular contact between proteins located within the DTS and the plasma membrane [17–20]. With the experiments reported here we have explored the role of Ca^{2+} release from the different intracellular stores, as well as Ca^{2+} entry, in the exocytosis of α- and δ-granules, in an attempt to discriminate between the mechanisms underlying exocytosis of both secretory granules.

2. Materials and methods

2.1. Materials

Apyrase (grade VII), aspirin, thrombin, thapsigargin (TG), dimethyl sulfoxide (DMSO), ethylene glycol tetraacetic acid (EGTA), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), sodium citrate, mepacrine®, D-glucose, adenosine diphosphate (ADP), bafilomycin, 2,5-di-[tert-butyl]-1,4-hydroquinone (TBHQ), 1,2-Bis [2-aminophenoxy] ethane-N,N,N′,N'-tetraacetic acid tetrakis [acetoxymethyl ester] (BAPTA-AM) and bovine serum albumin (BSA) were from Sigma (Poole, Dorset, U.K.). Anti-TRPC6 antibody and control antigen peptide (CAP) were from Alomone Laboratories (Jerusalem, Israel). Anti-CD62P-PE antibody, anti-CD41-a PerCP (clone HIP8) were from Becton Dickinson Biosciences, CA. All other reagents were of analytical grade.

2.2. Selection of patients and platelet sample preparation

Washed platelets were prepared as described elsewhere [21,22]. Briefly, blood was obtained from healthy drug-free volunteers and mixed with acid/citrate dextrose anticoagulant containing (in mM): 85 sodium citrate, 78 citric acid and 111 D-glucose. Platelet-rich plasma (PRP) was then prepared by centrifugation for 5 min at 700 × *g* and aspirin (100 μM) and apyrase (40 μg/mL) were added. Platelets were collected by centrifugation at 350 × *g* for 20 min, and resuspended in HEPES-buffered saline (HBS) containing, (in mM): 145 NaCl, 10 HEPES, 10 D-glucose, 5 KCl, 1 $MgSO_4$, pH 7.45 supplemented with 0.1% w/v bovine serum albumin and 40 μg/mL apyrase. Dimethyl BAPTA loaded was achieved by incubation of platelets with 10 μM dimethyl BAPTA-AM for 30 min. Cells were then resuspended in fresh HBS.

2.3. Cytometry analysis of platelet granule content and secretion

Platelet secretion was detected using a flow cytometer (FASCcan cytometer, Becton–Dickinson, CA). Briefly, approximately 1×10^6 washed platelets were suspended in 50 μl HBS and stained with anti-CD41 (1:60) and anti P-selectin (CD62P) (1:60) antibodies and 10 μM of mepacrine® at 37 °C for 30 min. Dimethyl BAPTA-loaded and control platelets were stimulated with different agonists (0.01, 0.1 and 0.5 U/mL thrombin, 10 nM and 1 μM TG, 10 μM ADP, 1 μM bafilomycin A1, 20 μM TBHQ) in the absence (100 μM EGTA added) or the presence of extracellular Ca^{2+} (final concentration 1.2 mM). The reaction was stopped 10 min after platelet stimulation and the samples were analysed by FACS. Platelets were selected first by their size (FSC) and complexity (SSC), and later by the presence of CD41 on their cell surface, which is widely used as an specific marker of the megakaryocytic lineage [23]. δ-granule secretion was estimated as the reduction of mepacrine fluorescence since it is specifically stored in these granules [24–26]. Mepacrine fluorescence was calculated as mean fluorescence intensity (M.F.I. = mepacrine fluorescence − endogenous fluorescence) and expressed as percentage of maximum stimulation. α-granule secretion was assessed by detection of P-selectin exposed on the cell surface, which is specifically stored in these granules [27], and expressed as percentage of maximum stimulation.

For the analysis of the time-course of platelet granule secretion samples were taken 5 s before and 1, 5, 10, 30 and 60 s after the addition of thrombin, fixed by incubation with ice-cold 3% (w/v) formaldehyde in PBS for 10 min and P-selectin exposure, as well as

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