Archives of Biochemistry and Biophysics 538 (2013) 130–137

Contents lists available at ScienceDirect

Archives of Biochemistry and Biophysics

journal homepage: www.elsevier.com/locate/yabbi

The membrane potential modulates thrombin-stimulated Ca^{2+} mobilization and platelet aggregation

Letizia Albarrán, Natalia Dionisio, Esther López, Ginés M. Salido, Juan A. Rosado *

Department of Physiology (Cell Physiology Research Group), University of Extremadura, 10003 Cáceres, Spain

ARTICLE INFO

Article history:
Received 13 June 2013
and in revised form 13 August 2013
Available online 27 August 2013

Keywords:
Thrombin
Membrane potential
Ca^{2+} release
Ca^{2+} entry
Aggregation
Human platelets

ABSTRACT

G protein-coupled receptors can be directly modulated by changes in transmembrane voltage in a variety of cell types. Here we show that, while changes in the membrane voltage itself do not induce detectable modifications in the cytosolic Ca^{2+} concentration, platelet stimulation with thrombin or the PAR-1 and PAR-4 agonist peptides SFLLRN and AYPGKF, respectively, results in Ca^{2+} release from intracellular stores that is sensitive to the membrane depolarisation. Direct activation of G proteins or phospholipase C by AlF_4^- and *m*-3M3FBS, respectively, leads to Ca^{2+} release that is insensitive to changes in the membrane potential. Thapsigargin-, as well as OAG-induced Ca^{2+} entry are affected by the membrane voltage, probably as a result of the modification in the driving force for Ca^{2+} influx; however, hyperpolarisation does not enhance thrombin- or OAG-evoked Ca^{2+} entry probably revealing the presence of a voltage-sensitive regulatory mechanism. Transmembrane voltage also modulates the activity of the plasma membrane Ca^{2+}-ATPase (PMCA) most likely due to a decrease in the phosphotyrosine content of the pump. Thrombin-stimulated platelet aggregation is modulated by membrane depolarisation by a mechanism that is, at least partially, independent of Ca^{2+}. These observations indicate that PAR-1 and PAR-4 receptors are modulated by the membrane voltage in human platelets.

Introduction

Platelets, as well as other non-excitable cells, are characterized by the lack of voltage-dependent Na^+ and Ca^{2+} channels. However, human platelets have been reported to express a large number of voltage-dependent K^+ channels that contribute to the maintenance of the membrane potential at between −60 and −70 mV [1,2] and to rectify the depolarizing effect of the cation influx that occurs after platelet stimulation by physiological agonists, such as thrombin [3] or ADP [4].

It is well known that depolarisation can influence Ca^{2+} signals by modification of the driving force for Ca^{2+} entry through the plasma membrane. In addition, depolarisation has been reported to exert other effects in cytosolic Ca^{2+} homeostasis such as the modulation of G-protein-coupled receptors (GPCR).[1] In rat megakaryocytes, depolarisation combined with me tabotropic purinoreceptor ($P2Y_1$) stimulation, resulted in a transient increase in the cytosolic free-Ca^{2+} concentration ($[Ca^{2+}]_i$) due the release of Ca^{2+} from agonist-releasable intracellular compartments through the activation of inositol 1,4,5-trisphosphate (IP_3) receptors [5]. Consistent with this hypothesis, membrane hyperpolarisation has been reported to attenuate IP_3 synthesis in rabbit mesenteric artery [6]. Furthermore, depolarisation has been shown to transiently enhance the amplitude of Ca^{2+} oscillations, while hyperpolarisation either abolished Ca^{2+} oscillations or reduced their amplitude [7]. Modulation of GPCR by membrane voltage has been well characterized in muscarinic receptors, whose affinity for acetylcholine varies in a voltage-dependent fashion [8,9]. The metabotropic glutamate receptors mGluR3 and mGluR1a are also voltage sensitive. The affinity of these receptors toward glutamate upon membrane depolarisation either decreases (mGluR3) or increases (mGluR1a), an effect where the intracellular loops that couple to the G-protein play an important role [10]. Cumulative evidence suggests that the voltage sensitivity of GPCR does not reside downstream to the activation of the receptors but rather in the receptor itself [10,11]. Here we have investigated the effect of membrane potential on Ca^{2+} mobilisation and platelet aggregation induced by stimulation of metabotropic thrombin receptors in human platelets.

Materials and methods

Materials

Fura-2 AM and calcein-AM were from molecular probes (Leiden, The Netherlands). Apyrase (grade VII), acetylsalicylic acid,

* Corresponding author. Address: Department of Physiology, University of Extremadura, Av. Universidad s/n, Cáceres 10003, Spain. Fax: +34 927 257110.
E-mail address: jarosado@unex.es (J.A. Rosado).

[1] *Abbreviations used:* GPCR, G-protein-coupled receptors; HBS, HEPES-buffered saline; TBST, tween 20; $P2Y_1$, purinoreceptor.

0003-9861/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.abb.2013.08.007

dimethyl-BAPTA/AM, thrombin, thapsigargin (TG), 2,4,6-trimethyl-N-(meta-3-trifluoromethyl-phenyl)-benzenesulfonamide (m-3M3FBS) and BSA were from Sigma (Madrid, Spain). Anti-PMCA monoclonal antibody (5F10) was from affinity bioreagents (Neshanic Station, NJ, USA). Horseradish peroxidase-conjugated anti-mouse IgG antibody was from Amersham, (Buckinghamshire, UK). Protein A-agarose was from Upstate Biotechnology (Lake Placid, NY, USA). Anti-phosphotyrosine monoclonal antibody (4G10) was from upstate biotechnology (Lake Placid, NY, USA). Inositol triphosphate (IP3) ELISA Kit was from Biocompare (GA, USA). Enhanced chemiluminescence detection reagents were from Pierce (Cheshire, UK). All other reagents were of analytical grade.

Platelet preparation

Blood was collected from healthy volunteers in accordance to the declaration of Helsinki and mixed with one-sixth volume of acid/citrate dextrose anti-coagulant containing (in mmol/l): 85 sodium citrate, 78 citric acid and 111 D-glucose. Platelet-rich plasma was prepared by centrifugation for 5 min at 700*g* and acetylsalicylic acid (100 μmol/l) and apyrase (40 μg/mL) were added [12]. Platelets were collected by centrifugation at 350*g* for 20 min and resuspended in HEPES-buffered saline (HBS) containing (in mmol/l): 145 NaCl, 10 HEPES, 10 D-glucose, 1 $MgSO_4$, pH 7.45 and supplemented with 0.1% w/v BSA and 40 μg/mL apyrase. For dimethyl-BAPTA loading, platelets were incubated for 30 min with 10 μmol/l dimethyl-BAPTA/AM. Platelets were then collected by centrifugation at 350*g* for 20 min and resuspended in HBS.

Platelet viability

Platelet viability was assessed by calcein-loading of platelets and the trypan blue exclusion technique. For calcein-loading, platelets, treated with the agents used, were incubated for 30 min with 5 μmol/l calcein-AM at 37 °C. Two milliliter aliquots were used for fluorescence recording (spectrophotometer Varian Ltd., Madrid, Spain) at λ ex: 494 and λ em: 535 nm. ~95% of platelets were viable in our platelet suspensions, a result further confirmed by the trypan blue exclusion technique.

Measurement of intracellular free-calcium concentration ($[Ca^{2+}]_i$)

Platelets were incubated at 37 °C with 2 μmol/l fura-2 AM for 45 min and resuspended in HBS. Fluorescence was recorded from 1 ml aliquots of magnetically stirred platelet suspensions (10^8 - platelets/ml) at 37 °C using the fluorescence Spectrophotometer (Varian Ltd., Madrid, Spain) with λ ex: 340 and 380 and λ em: 505 nm. Changes in $[Ca^{2+}]_i$ were monitored using the fura-2 340/380 fluorescence ratio and calibrated according to the method of Grynkiewicz et al. [13,14]. Ca^{2+} release and influx was estimated using the integral of the rise in $[Ca^{2+}]_i$ for 2.5 min after addition of $CaCl_2$[15]. To compare the rate of decay of $[Ca^{2+}]_i$ to basal values after platelet stimulation between different treatments we used the constant of the exponential decay. Traces were fitted to the equation $y = A(1-e^{-K_1T})e^{-K_2T}$, where K_2 is the constant of the exponential decay.

Inmunoprecipitation

Inmunoprecitation was performed as described previously [16]. Briefly, 250 μl aliquots of platelet suspension were lysed and immunoprecipitated by incubation with 2 μg of anti-PMCA antibody and 25 μl of protein A-agarose overnight at 4 °C on a rocking platform.

Western blotting

Proteins were separated by 10% SDS–PAGE and electrophoretically transferred, for 2 h at 0.8 mA cm^2, in a semi-dry blotter (Hoefer Scientific, Newcastle, UK) onto nitrocellulose for subsequent probing. Blots were incubated overnight with 10% (w/v) BSA in tris-buffered saline with 0.1% Tween 20 (TBST) to block residual protein binding sites. Membranes were incubated with anti-phosphotyrosine antibody diluted 1:1000 in TBST for 2 h. To detect the primary antibody, blots were incubated with horseradish peroxidase-conjugated anti-mouse IgG antibody diluted 1:10,000 in TBST and exposed to enhanced chemiluminescence reagents for 5 min. Blots were exposed to photographic films and the optical density was estimated using scanning densitometry.

Platelet aggregation

The percentage, rate and lag-time of aggregation in washed platelets were monitored using a Chronolog (Havertown, PA, USA) aggregometer at 37 °C under stirring at 1200 rpm [17].

IP_3 ELISA kit

Platelets were treated with *m*-3M3FBS at different conditions and then homogenized in 20 mmol/l Tris–HCl pH 7.5; 2 mmol/l EDTA; 150 mmol/l NaCl; 0.5% Triton X-100. IP_3 production determinations were performed with an IP_3 ELISA Kit following manufacturer's instructions. The data were presented as fold-increase.

Determination of phosphatidylserine externalization

The PS exposure of resting and stimulated platelets was determined according to a previously published procedure [18]. Briefly, platelets were stimulated in HBS and samples of platelet suspensions (500 μl) were transferred to 500 μl ice-cold 1% (w/v) glutaraldehyde in phosphate-buffered saline (PBS) for 10 min. Platelets were then incubated for 10 min with annexin V-FITC (0.6 μg/ml) in PBS supplemented with 0.5% (w/v) BSA. After incubation, platelets were collected by centrifugation for 60 s at 3000*g* and resuspended in PBS. Platelet staining was measured using a fluorescence spectrophotometer. Samples were excited at 496 nm and emission was recorded at 516 nm.

Statistical analysis

Data are shown as mean ± SEM. Analysis of statistical significance was performed using one-way analysis of variance (ANOVA) combined with the Dunnet test. $P < 0.05$ was considered to be significant for a difference.

Results

The membrane potential of resting human platelets has been estimated at between −60 and −70 mV [1]. In order to test the effect of membrane potential on thrombin-evoked Ca^{2+} mobilisation we performed a series of experiments in the presence of the K^+ ionophore, valinomycin (3 μmol/l) and increasing extracellular K^+ concentrations ($[K^+]_o$; 1, 10, 45 and 135 mmol/l). Assuming that, in the presence of valinomycin, the membrane is selectively permeable to K^+ and that the intracellular K^+ concentration has been estimated in human platelets as 135 mmol/l [2], we used the Nernst equation to estimate the membrane potential at the $[K^+]_o$ indicated above as −130, −69 (in the physiological range), −29 and 0 mV. In human platelets we found that treatment with 3 μmol/l valinomycin did not significantly alter the ability of