European Journal of Cell Biology 93 (2014) 127–136

Contents lists available at ScienceDirect

European Journal of Cell Biology

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

Epithelial sodium channel modulates platelet collagen activation

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ARTICLE INFO

Article history:
Received 23 August 2013
Received in revised form 30 January 2014
Accepted 24 February 2014

Keywords:
Epithelial sodium channel (ENaC)
Amiloride (AMR)
Adhered platelets
Intermediate filaments (IF)
Dystrophin-associated proteins (DAP)
Platelet migration

ABSTRACT

Activated platelets adhere to the exposed subendothelial extracellular matrix and undergo a rapid cytoskeletal rearrangement resulting in shape change and release of their intracellular dense and alpha granule contents to avoid hemorrhage. A central step in this process is the elevation of the intracellular Ca^{2+} concentration through its release from intracellular stores and on throughout its influx from the extracellular space. The Epithelial sodium channel (ENaC) is a highly selective Na^+ channel involved in mechanosensation, nociception, fluid volume homeostasis, and control of arterial blood pressure. The present study describes the expression, distribution, and participation of ENaC in platelet migration and granule secretion using pharmacological inhibition with amiloride. Our biochemical and confocal analysis in suspended and adhered platelets suggests that ENaC is associated with Intermediate filaments (IF) and with Dystrophin-associated proteins (DAP) via α-syntrophin and β-dystroglycan. Migration assays, quantification of soluble P-selectin, and serotonin release suggest that ENaC is dispensable for migration and alpha and dense granule secretion, whereas Na^+ influx through this channel is fundamental for platelet collagen activation.

Introduction

Blood platelets are small anucleated cell fragments derived from megakaryocytes that prevent blood loss after vessel injury by means of adhering to the exposed subendothelial Extracellular matrix (ECM), collagen being the most thrombogenic component (Baumgartner and Haudenschild, 1972). In response to ECM components, platelets undergo fast cytoskeletal reorganization that results in a change of shape, the release of intracellular dense and alpha granule contents, and adhesion to the damaged vessel, thus stopping hemorrhage. Secondary mediators released from the dense and α-granules strengthen the activated state, recruiting more platelets to the site of injury. Adhesion of platelets to the damaged endothelium promotes the formation of actin-based structures such as filopodia, lamellipodia, stress fibers, and a contractile ring, which that centralize the granules, forming a structure denominated granulomere. Independent of the signaling pathway triggered by any of the physiological platelet agonists, a sustained and significant increase in intracellular calcium concentration $[Ca^{2+}]_i$ occurs. This increase consists of the release of compartmentalized calcium through the store-operated Ca^{2+} entry (SOCE) (Alonso et al., 1991) and the entry of extracellular Ca^{2+} at the plasma membrane through the stromal interaction molecule (STIM1), a sensor in the dense tubular system, and Orai1, the major store-operated Ca^{2+} (SOC) channel in the plasma membrane or the Na^+/Ca^{2+} exchanger (Braun et al., 2009), contributing to hemostatic platelet responses.

The Epithelial sodium channel/Degenerin (ENaC/Deg) family comprises cation-selective ion channels found widely expressed in animals from hydra and nematodes to vertebrates. ENaC are highly selective Na^+ channels assembled from three homologous subunits termed α, β, and γ. These channels are expressed at the apical membrane of Na^+ transporting epithelia, where they facilitate Na^+ reabsorption from the luminal space. ENaC-mediated Na^+ reabsorption in the distal nephron is important for fluid volume homeostasis and control of the arterial blood pressure (Kellenberger and Schild, 2002). In the airways, ENaC contribute to the maintenance of the airway surface-liquid and mucociliary clearance (Mall et al., 2004). ENaC were initially characterized in the apical membrane of native epithelia as highly selective for

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http://dx.doi.org/10.1016/j.ejcb.2014.02.003
0171-9335/

sodium over potassium and sensitive to amiloride (Palmer and Frindt, 1986).

ENaC subunits also play a role in the myogenic response, where vascular smooth muscle cells contract in response to stretching (Drummond et al., 2008), suggesting that ENaC is a mechanosensitive channel. Little is known regarding how extracellular signals are transmitted to the pore of the channel and subsequently alter channel gating. It has been proposed that these channels are tethered through their intra- and extracellular regions to the cytoskeleton and the Extracellular matrix (ECM), respectively (Chalfie, 2009).

ENaC subunits possess two membrane-spanning helices (TM1 and TM2), resulting in cytoplasmic amino and carboxyl termini. The cytoplasmic domains have sites that are phosphorylated by specific kinases, bear specific motifs that direct protein–protein and protein–lipid interactions that affect channel gating and/or trafficking, and have sites that may directly influence channel gating or trafficking. In addition, carboxyl termini serve to link the channel to the cytoskeleton by binding α-spectrin and possibly actin (Copeland et al., 2001). The Dystrophin glycoprotein complex (DGC) is an oligomeric complex involved in both mechanical stabilizing and signaling roles in mediating interactions among the cytoskeleton, membrane, and ECM. It is thought that the DGC provides proper anchoring and/or clustering to ion channels by its direct or indirect binding to ion transport systems at the plasma membrane (Dalloz et al., 2003). In addition to dystrophin, dystroglycan and syntrophins are also components of the DPC core. The syntrophin family of adaptor proteins is composed of the following five members: syntrophins α-; β1-; β2-; γ1-, and γ2- (Adams et al., 1993). Each syntrophin contains a **P**ost-synaptic density protein-95, a **D**rosophila disc large tumor suppressor, and a **Z**onula occludens-1 protein (zo-1) PDZ domain and two Pleckstrin homology (PH) domains. PDZ domains bind to the C-terminal tails of many different classes of transmembrane protein, but can also participate in homotypic interactions with other PDZ domains containing proteins, including neuronal Nitric oxide synthase (nNOS), as well as in the regulation and localization of various ionic channels and membrane-binding proteins (Brenman et al., 1996).

Dystroglycan is a matrix receptor that spans the plasma membrane linking the cytoplasmic components of the DGC to the ECM. Dystroglycan proteolysis produce two subunits, denominated α-dystroglycan and β-dystroglycan; α-dystroglycan binds to ECM proteins, including some laminins, perlecan, agrin, and the neurexins (Michele and Campbell, 2003). β-Dystroglycan associates with α-dystroglycan at the plasma membrane, whereas the β-Dg transmembrane anchors the α-dg to the cell membrane and is linked to the actin cytoskeleton via dystrophin, or its isoforms, or its paralogue, utrophin, providing structural integrity. In addition, Dg is ideally located for transducing signals from the ECM to the inside of cells, in a similar manner to integrins (Ilsley et al., 2001).

In platelets, several classes of ion channels have been identified, including Adenosine triphosphate (ATP)-gated P2X1 channels, Orai1 Store-operated Ca^{2+} (SOC) channels (Schmidt et al., 2011), voltage-gated $K_v1.3$ channels (McCloskey et al., 2010), ionotropic glutamate receptors of the 2-**A**mino-3-(3-hydroxy-5-**M**ethyl-isoxazol-4-yl) **P**ropanoic **A**cid (AMPA) (Morrell et al., 2008), and kainate subtypes (Sun et al., 2009), and connexin-specific Gap junction (GJ) channels (Vaiyapuri et al., 2012). However, the expression of ENaC in platelets has not been fully characterized, and it is within this context that, in the present communication, we sought to establish its presence, distribution, and association. Confocal analysis showed the redistribution and co-localization of ENaC from resting to adhered platelets from the cytoplasm to the plasma membrane and the granulomere zone. Immunoprecipitation assays indicated that ENaC is associated with desmin and vimentin, as well as with α-syntrophin. Platelet migration assays, as well as alpha and dense granule secretion, were not importantly disturbed with ENaC inhibition. In contrast, pharmacological treatment with amiloride severely affected Na influx in collagen-activated platelets. These results demonstrate ENaC expression in platelets and suggest their involvement in platelet activation.

Materials and methods

Platelet preparation

Platelets were obtained by venopuncture from healthy donors who had not received any drug during the 10 days prior to sampling and who gave consent for the procedure to be carried out. Blood was immediately mixed with citrate anticoagulant including dextrose at pH 6.5 (93 mM sodium citrate, 70 mM citric acid, and 140 mM dextrose) at a blood:anticoagulant ratio of 9:1. Platelet-rich plasma was obtained from total blood by centrifugation at $100 \times g$ for 20 min at room temperature, and was subsequently mixed with an equal volume of citrate anticoagulant and centrifuged at $400 \times g$ for 10 min (White, 1983). The platelet pack was suspended and washed twice with Hank's balanced saline solution (HBSS) without calcium (137 mM NaCl, 5.3 mM KCl, 1 mM $MgCl_2$, 0.28 mM $Na_2HPO_4{\cdot}12\ H_2O$, 0.87 mM NaH_2PO_4, 0.44 mM KH_2PO_4, 4.1 mM $NaHCO_3$, and 5.5 mM glucose) and counted in a hematocytometer.

Antibodies

Monoclonal antibodies are referred to as mAb, while polyclonal antibodies are denominated pAb. α-ENaC pAb Catalogue (Cat.) no. sc-21012, actin mAb no. sc-8432, α-tubulin mAb Cat. no. sc-5286, NOS3 pAb Cat. no. sc-654, α-syntrophin pAb Cat. no. sc-13757, β-dystroglycan pAb Cat. no. sc-30405, desmin pAb Cat. no. sc-7559, vimentin pAb Cat. no. sc-7557, PYK2 pAb Cat. no. sc-9019, FAK pAb Cat. no. sc-557, and p-Tyrosine (p-Tyr) agarose beads Cat. no. sc-24957 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA), while α-syntrophin pAb (P6) was the kind gift of D. Mornet (Rivier et al., 1997).

Preparation of inhibitors

A 2-μM Amiloride (AMR) solution (2×) (Sigma Chemical Co., St. Louis, MO, USA) was directly dissolved in HBSS, a 5-μM/mL KB-R7943 solution (2×), a 10-μM BAPTA solution (2×), a 10-μM PP2 solution (2×), a 10-μM W7 solution (2×), and a 10-μM Genistein (2×) (Tocris-Cookson, Ellisville, MO, USA) was prepared in HBSS from concentrated solutions diluted in Dimethyl sulfoxide (DMSO).

Treatment of platelets with inhibitors

Resting platelets in suspension were incubated with the same volume of the drugs to obtain the previously mentioned final concentrations of AMR 2 μM, KB-R7943 5 μM, BAPTA (10 μM), PP2 (10 μM), W-7 (N-[6-aminohexyl]-5-chloro-1-naphthalenesulfonamide) (10 μM), Genistein (10 μM), and Na_3VO_4 (1 M) for 60 min at room temperature.

RNA isolation

Resting platelets were suspended in TRIzol® Reagent, and total RNA was isolated according to the product insert.

Reverse transcriptase-PCR

cDNA was synthesized from total RNA (3 mg) by Oligo (dT) 20 (5 mM) primed reverse transcriptase according to the product insert using ThermoScript™ Reverse Transcriptase. Primers for