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Differential sensitivity of human platelet $P2X_1$ and $P2Y_1$ receptors to disruption of lipid rafts

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Abstract

ATP-stimulated $P2X_1$ and ADP-stimulated $P2Y_1$ receptors play important roles in platelet activation. An increase in intracellular Ca^{2+} represents a key signalling event coupled to both of these receptors, mediated via direct gating of Ca^{2+}-permeable channels in the case of $P2X_1$ and phospholipase-C-dependent Ca^{2+} mobilisation for $P2Y_1$. We show that disruption of cholesterol-rich membrane lipid rafts reduces $P2X_1$ receptor-mediated calcium increases by ~80%, while $P2Y_1$ receptor-dependent Ca^{2+} release is unaffected. In contrast to artery, vas deferens, bladder smooth muscle, and recombinant expression in cell lines, where $P2X_1$ receptors show almost exclusive association with lipid rafts, only ~20% of platelet $P2X_1$ receptors are co-expressed with the lipid raft marker flotillin-2. We conclude that lipid rafts play a significant role in the regulation of $P2X_1$ but not $P2Y_1$ receptors in human platelets and that a reserve of non-functional $P2X_1$ receptors may exist.

Keywords: $P2X_1$ receptors; $P2Y_1$ receptors; Platelets; Calcium; Lipid rafts; Cholesterol; ADP; ATP

ATP and ADP are released into the circulation from a variety of sources, including the cytoplasm of damaged cells at the site of vascular injury, or following secretion from activated platelets [1] and the endothelium [2,3]. These nucleotides play roles in haemostasis and thrombosis [4,5] via three P2 receptor subtypes expressed on the platelet surface membrane; ATP stimulates $P2X_1$ receptor ion channels, while ADP activates G-protein coupled $P2Y_1$ and $P2Y_{12}$ receptors [5]. $P2X_1$ receptor stimulation causes direct calcium and sodium influx through the receptor channel with an associated membrane depolarisation [4]. $P2Y_1$ receptors couple through Gαq G-proteins to activate phospholipase-Cβ-dependent Ca^{2+} mobilisation and also couple via Rho kinase activation, while $P2Y_{12}$ receptors couple through Gαi with PI3-kinase activation and adenylyl cyclase inhibition as primary signalling events [6]. Transgenic studies have demonstrated a key role of the $P2X_1$ receptor in arterial thrombosis [7,8] and $P2Y_1$ and $P2Y_{12}$ receptor-deficient mice also show modified haemostatic and thrombotic phenotypes [9–12]. In addition, it appears that P2 receptors in platelets show significant cross-talk at the signalling and functional level [13]. It is well established that co-stimulation of $P2Y_1$ and $P2Y_{12}$ receptors is required for full ADP-evoked activation of fibrinogen binding sites on the integrin $\alpha_{IIb}\beta_3$ [5]. We have also shown that $P2X_1$ receptor stimulation speeds subsequent $P2Y_1$ receptor-mediated calcium increases in the platelet [14] which can be explained by both calcium and voltage-dependent mechanisms [15]. However, the mechanisms whereby platelet P2 receptors or their downstream signals interact are poorly understood.

The cell membrane is not homogeneous and differences in lipid composition result in distinct, compartmentalised membrane domains, with different properties and biological

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functions. Lipid rafts are rich in cholesterol and glycosphingolipids, resulting in liquid-ordered microdomains within the liquid-disordered glycerophospholipid membrane bilayer [16,17]. These lipid rafts may facilitate the localised assembly of signalling complexes at the cell membrane and a wide range of proteins, including many signalling molecules and receptors, have been shown to be preferentially associated with lipid rafts [18,19]. We have recently shown that $P2X_1$ receptors in arterial smooth muscle are present in lipid rafts and disruption of the rafts by depletion of membrane cholesterol inhibits $P2X_1$ receptor-mediated responses [20]. Thus, lipid rafts may also play an important role in regulation of $P2X_1$ receptors in platelets. In the present study, we have determined the extent of localisation of $P2X_1$ receptors in human platelet lipid rafts and show that activation of $P2X_1$ and $P2Y_1$ receptor signals displays a marked differential sensitivity to lipid raft disruption.

Methods

Solutions and reagents. Nominally Ca^{2+}-free Hepes-buffered saline (CFHBS) contained (in mM): 145 NaCl, 5 KCl, 1 $MgCl_2$, 10 Hepes, and 10 glucose, titrated to pH 7.35 with NaOH. Acid citrate dextrose contained (in mM): 85 trisodium citrate, 78 citric acid, 111 glucose. ADP, α, β-meATP, and methyl-β-cyclodextrin (MβCD) were from Sigma–Aldrich (Poole, Dorset, UK). ADP was treated with hexokinase as described previously [21] to reduce ATP to negligible levels (<0.001%); assessed by bioluminescent measurements (ATP Assay Kit, Calbiochem–Novabiochem UK Ltd, Beeston, Nottingham) on a Model 400 lumi-aggregometer (Chrono-log Corporation, Havertown, PA, USA).

Cell preparation. Blood was taken from informed, consenting donors into acid citrate dextrose anti-coagulant (1 part ACD: 6 parts blood). The study was approved by the University of Cambridge Human Biology Research Ethics Committee. Platelet-rich plasma (PRP) was prepared by centrifugation for 5 min at 700*g* and treated with aspirin (100 μM), apyrase (0.32 U ml^{-1} type VII), and PGI_2 (0.2–1 μg ml^{-1}). Washed platelet suspensions were prepared by centrifugation for 20 min at 350*g* followed by gentle resuspension in CFHBS with 0.32 U ml^{-1} apyrase.

Membrane fractionation. Human platelets and cells were fractionated using a detergent-free method adapted from [22]. After addition of PGI_2 (0.2–1 μg ml^{-1}), washed platelet suspensions were centrifuged at 350*g* for 20 min. The platelet pellet was then lysed (on ice, 20 min) in 2 ml of 500 mM sodium carbonate (pH 11). Lysed platelets were then subjected to three 20 s bursts of sonication. Homogenates were brought to 45% sucrose by addition of an equal volume of 90% sucrose in MBS (25 mM MES and 150 mM NaCl, pH 6.5) and loaded in an ultracentrifuge tube. A discontinuous sucrose gradient was layered on top of the sample by placing 4 ml of 35% sucrose prepared in MBS with 250 mM Na_2CO_3 and then 4 ml of 5% sucrose (also in MBS/Na_2CO_3). The gradient was centrifuged at 39,000 rpm on a TH-641 rotor in a Sorvall OTD65B ultracentrifuge (Kendro Laboratory Products Plc, Bishop's Stortford, UK) for 16 h at 4 °C. After centrifugation, eleven fractions of 1 ml were collected from the top to the bottom of each tube run on a gel and blotted with anti-$P2X_1$ antibody (Alamone, Jerusalem, Israel). Cholesterol measurements were assessed with the Amplex Red cholesterol assay kit (Molecular Probes Europe BV, Leiden, The Netherlands). Each experimental condition was repeated three times.

$[Ca^{2+}]_i$ measurements. Platelets were loaded with fura-2 by incubation with 2μM fura-2AM for 45 min at 37 °C. Ratiometric fluorescence measurements from washed platelet suspensions were conducted at 37 °C in a Cairn spectrofluorimeter system (Cairn Research Limited, Faversham, Kent, UK) and converted to $[Ca^{2+}]_i$ as described previously using a dissociation constant for Ca^{2+} of 224 nM [23]. For studies of $P2X_1$-evoked Ca^{2+} influx, 2 mM $CaCl_2$ was added to the cuvette 30 s prior to 10 μM α,β-meATP [24]. Ca^{2+} mobilisation evoked by $P2Y_1$ receptors was monitored using a submaximal concentration of ADP (1 μM) in the absence of external Ca^{2+} following addition of 1 mM EGTA and 2 mM $MgCl_2$. ADP-evoked Ca^{2+} responses were studied in the absence of external Ca^{2+} to avoid secondary activation of $P2X_1$ receptor-evoked Ca^{2+} influx due to ATP release (C.Y.Fung and M.P.Mahaut-Smith, unpublished observations). Under the conditions of our experiments without added prostaglandins the contribution of $P2Y_{12}$ receptors to the ADP-evoked Ca^{2+} response is not significant [25,26].

Results

Association of platelet $P2X_1$ receptors with both lipid raft and non-raft domains

Many proteins are not randomly inserted into the cell surface membrane but show association with other molecules in the formation of signalling domains. Lipid rafts form one such type of microdomain and they can be isolated following solubilisation and separation by sucrose density ultracentrifugation [16]. Platelet cell lysates were prepared using detergent-free conditions, ultracentrifuged on a discontinuous sucrose gradient, and fractions were collected and analysed by Western blot. Analysis of platelet fractions for the lipid raft marker flotillin-2 [27] showed that it was localised in fraction 4. Cholesterol is known to be enriched in lipid rafts and fraction 4 was found to contain ~40% of the total cellular cholesterol, yet less than 10% of the total cellular protein. These observations are consistent with fraction 4 corresponding to the lipid raft microdomain. In platelets, ~20% of the $P2X_1$ receptor was detected in lipid raft fraction 4, with the remainder in the non-raft fractions 8–11 (Fig. 1). This is in contrast

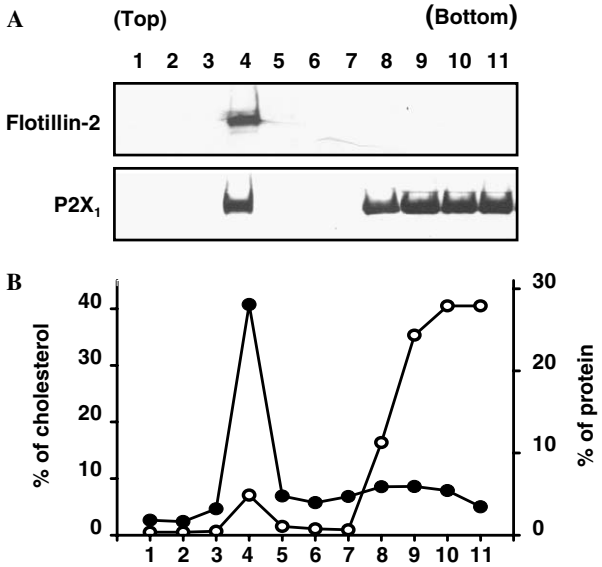

Fig. 1. $P2X_1$ receptors are localised in lipid raft and non-raft fractions in human platelets. (A) Platelets were lysed and separated on a sucrose density gradient; the 11 fractions (1 top of the gradient–11 bottom of the gradient) were immunoblotted for the lipid raft marker flotillin-2 and the $P2X_1$ receptor. (B) Fractions were also assayed to determine the cholesterol (closed circles) and protein (open circles) content.