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Phosphatidylinositol-3,4,5-trisphosphate stimulates Ca^{2+} elevation and Akt phosphorylation to constitute a major mechanism of thromboxane A_2 formation in human platelets

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ABSTRACT

Phosphatidylinositol trisphosphate (PIP_3) has been implicated in many platelet functions however many of the mechanisms need clarification. We have used cell permeable analogues of PIP_3,1-O-(1,2-di-palmitoyl-sn-glyero-3-O-phosphoryl)-D-myo-inositol-3,4,5-trisphosphate (DiC16-PIP_3) or 1-O-(1,2-di-octanoyl-sn-glyero-3-O-phosphoryl)-D-myo-inositol-3,4,5-trisphosphate (DiC8-PIP_3) to study their effects on activation on washed human platelets. Addition of either DiC8- or DiC16-PIP_3 to human platelets induced aggregation in the presence of extracellular Ca^{2+}. This was reduced by the presence of indomethacin, the phospholipase C inhibitor U73122 and apyrase. DiC8-PIP_3 induced the phosphorylation of Akt-Ser^{473} which was reduced by the Akt inhibitor IV, wortmannin and EGTA (suggesting a dependence on Ca^{2+} entry). In Fura2 loaded platelets DiC8-PIP_3 was effective at increasing intracellular Ca^{2+} in a distinct and transient manner that was reduced in the presence of indomethacin, U73122 and 2-aminoethyl diphenylborinate (2APB). Ca^{2+} elevation was reduced by the non-SOCE inhibitor LOE908 and also by the SOCE inhibitor BTP2. DiC8-PIP_3 induced the release of Ca^{2+} from stores which was not affected by the proton dissipating agent bafilomycin A1 and was more potent than the two-pore channel agonist DiC8-PI[3,5]P_2 suggesting release from an endoplasmic reticulum type store. DiC8-PIP_3 weakly induced the tyrosine phosphorylation of Syk but not of PLCγ2. Finally like thrombin DiC8-PIP_3 induced the formation of thromboxane B_2 that was inhibited by the Akt inhibitor IV. These studies suggest that PIP_3 via Ca^{2+} elevation and Akt phosphorylation forms a central role in thromboxane A_2 formation and the amplification of platelet activation.

1. Introduction

Platelet activation is an essential component of haemostasis and contributes to pathological aspects of many cardiovascular disorders. Elucidation of distinct signalling pathways may help to identify components important in separating haemostasis from thrombotic events and thus provide targets for potential therapy. Ca^{2+} elevation in the cytosol, which is an essential event in platelet activation can occur via a number of different mechanisms. Agonists that act on G protein- coupled receptors result in stimulation of phospholipase C β (PLCβ) or adhesion receptors result in activation of PLCγ2. Both PLCs cleave phosphatidylinositol[4,5]bisphosphate (PIP_2) releasing inositol-1,4,5-trisphosphate (IP_3) and 1,2-diacylglycerol (DAG) [1–3]. IP_3 and DAG serve as important second messengers for elevation of cytosolic Ca^{2+} and activation of protein kinase C (PKC). IP_3 induces Ca^{2+} release from endoplasmic reticulum type stores and this can lead to Ca^{2+} entry utilising the Ca^{2+} sensor protein STIM1 (stromal interaction molecule 1) and the channel Orai1 (Store operated Ca^{2+} entry [SOCE]). Ca^{2+} entry can occur via a non-SOCE route employing the transient receptor potential (TRPC) 6 and 3 channels activated by DAG or by the released agonist ATP via the P2X1 channel [1,4–6]. The SOCE pathway has been suggested as a potential target for anti-thrombotic therapy [7–9].

Plasma membrane PIP_2 can also be modified by phosphoinositide-3 kinase (PI-3K) to form PI[3,4,5]P_3 (PIP_3). This molecule has important second messenger properties. PIP_3 stimulates phosphoinositide dependent protein kinase 1 (PDK1) and Akt (also known as protein kinase B) that contain in their structures PIP_3 binding regions referred to as pleckstrin homology (PH) domains. PIP_3 is known to bind to many PH domain containing proteins (such as PLCγ2) recruiting them to the plasma membrane and aiding their activation. Formation of PIP_3 is

Abbreviations: (DiC16-PIP_3), 1-O-(1,2-di-palmitoyl-sn-glyero-3-O-phosphoryl)-D-myo-inositol-3,4,5-trisphosphate; (DiC8-PIP_3), 1-O-(1,2-di-octanoyl-sn-glyero-3-O-phosphoryl)-D-myo-inositol-3,4,5-trisphosphate; (SOCE), store operated Ca^{2+} entry; (PLC), phospholipase C; (IP_3), inositol-1,4,5-trisphosphate; (DAG), 1,2-diacylglycerol; (PDK1), phosphoinositide dependent protein kinase 1; Akt, (protein kinase B).

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accepted as an important component in the ADP-P2Y12 receptor mediated amplification of platelet activation [10–12] though details of how amplification occurs are not fully established. PIP_3 has been implicated as having a role for Ca^{2+} entry in T cells, mast cells and rabbit platelets via a store independent mechanism [13–15]. In these studies cell permeable analogues of PIP_3 such as 1-O-(1,2-di-palmitoyl-sn-glyero-3-O-phosphoryl)-D-myo-inositol-3,4,5-trisphosphate (DiC16-PIP_3) or 1-O-(1,2-di-octanoyl-sn-glyero-3-O-phosphoryl)-D-myo-inositol-3,4,5-trisphosphate (DiC8-PIP_3), have been reported to cause entry of Ca^{2+} in rabbit platelets, T-cells and mast cells. Further it has been reported that PIP_3 analogues stimulate Ca^{2+} entry in HEK-293 cells transfected with multiple TRPC family members [15] and that they may bind to these channels [16]. Thus generation of PIP_3 may provide an additional mechanism of Ca^{2+} elevation where the details require further evaluation.

We therefore decided to explore the effects of cell permeable analogues of PIP_3 in platelets and in particular its activity in Ca^{2+} elevation, Akt phosphorylation, aggregation and thromboxane formation. Our results indicate that PIP_3 analogues added to washed human platelets induce aggregation, Ca^{2+} elevation, phosphorylation of Akt and thromboxane generation. The Ca^{2+} mobilisation has distinct characteristics and together with the Akt pathway represents a major mechanism for thromboxane generation.

2. Materials and methods

2.1. Reagents

DiC8-PIP_3, DiC16-PIP_3, DiC8-[3,4]PIP_2, DiC8-[4,5]-PIP_2 and DiC8-[3,5]-PIP_2 were obtained from CellSignals Inc. Columbus, Ohio USA. Akt inhibitor IV (Calbiochem) and U73122 were obtained from Merck Biosciences (Nottingham, UK). Fura2-AM was from Molecular Probes (Oregon, USA). BTP-2 (N-(4-[3,5-bis(trifluoromethyl)-1H-1yl]phenyl)-4-methyl-1,2,3-thiodiazole-5-carboxamide) was from Calbiochem (Nottingham, UK). LOE-908 (3,4-dihydro-6,7-dimethoxy-a-phenyl-N,N-bis[2-(2,3,4-trimethoxyphenyl)ethyl]-1-isoquinolineacetamide hydrochloride) was from Tocris (Bristol, UK). 2 APB (2-Aminoethyl diphenylborinate) was from Sigma-Aldrich UK. Phospho-Akt antibody recognising phospho-Ser^{473} (clone 11E61) was obtained from Upstate (Lake Placid, USA). Pan-Akt antibody (C67E7) recognising Akt1, Akt2 and Akt3 proteins was obtained from Cell Signalling Technology (Danvers, MA, USA). Goat anti-mouse antibody linked to HRP was purchased from GE Healthcare (UK). Antibodies to PLCγ2 and Syk were from Santa Cruz Biotechnology Inc., Dallas, USA. Antiphosphotyrosine antibody 4G10 was obtained from TCS Biologicals, Bucks, United Kingdom. Collagen related peptide (CRP) was obtained from Professor Richard Farndale (University of Cambridge, UK). All other chemicals including bovine serum albumin (BSA) were obtained from Sigma-Aldrich (Dorset, UK).

2.2. Preparation of Fura-2 labelled human platelets

Blood was taken from donors (using local Research Ethics Guidelines) who denied taking any medication for the last 9 days, into 0.1 vol. 3.2% trisodium citrate and platelet rich plasma (PRP) was isolated after centrifugation of the blood at 200 × *g* for 20 min [4,17]. Fura-2 labelling was carried out at 37 °C with 3 μM Fura-2AM for 1 h in the dark. The PRP was then cooled to room temperature, acidified to pH 6.5 with 0.3 M citric acid and centrifuged at 1200 × *g* for 20 min. The platelets were washed in 36 mM citric acid, 1 mM EDTA, 5 mM glucose, 5 mM KCl, 103 mM NaCl pH 6.5 and 100 nM PGE_1. After centrifugation the platelet pellet was resuspended at 1×10^8 cells/ml in a Hepes medium consisting of 10 mM Hepes, 140 mM NaCl, 5 mM KCl, 1 mM MgCl, 5 mM glucose, 0.42 mM NaH_2PO_4, 12 mM $NaHCO_3$, pH 7.35. In some experiments (e.g. involving DiC8-(3,5)-PIP_2) Fura2 labelling was carried out using the above Hepes buffer plus 0.35% BSA, 1.5 mM EDTA, 1 μM PGE_1 (at 5×10^8/ml) and incubated at 37 °C for 45 min in the dark. At the end the suspension was allowed to cool to room temperature and a further 0.1 μM PGE_1 (final) plus 1/5 volume acid citrate dextrose (ACD) was added, the cells centrifuged, re-suspended in the Hepes buffer without EDTA or BSA and allowed to equilibrate for 45 min at room temperature at 1×10^8 cells/ml. Both methods gave comparable results. Ca^{2+} studies were carried out using 2 ml suspensions at 37 °C with continuous stirring using a purpose built spectrofluorimeter (Cairns Research Ltd., Faversham, Kent, UK). Fluorescence measurements were made with excitation at 340 and 380 nm and emission at 510 nm. $[Ca^{2+}]_i$ is reported as the 340 nm/380 nm ratio ($R_{340/380}$). Where indicated ratio values were converted to nM Ca^{2+} using the equation of Grynkiewicz et al. [18] as described by Sage in [19].

2.3. Preparation of platelets for aggregation and Akt-Ser^{473} phosphorylation studies

Freshly prepared platelets were isolated as described above (without Fura2 loading) and were re-suspended in the Hepes tyrode medium at 2 (or for Akt experiments at 5) × 10^8 cells/ml. Incubations were carried out in aggregation cuvettes using a Peyton dual channel aggregometer using 500 μl suspensions at 37 °C with mixing. Reagents were added for the times stated in the Results section and aggregations measured for 5 min. In experiments involving Akt-phosphorylation the reactions were stopped by addition of equal volume of 2× Laemmli sample buffer and equal aliquots analysed by SDS-PAGE and Western blotting.

Western blotting was carried out using 7% SDS-PAGE gels, followed by transfer to PVDF membranes and blocked overnight with 5% BSA in TBS-Tween (20 mM Tris pH 7.4, 500 mM NaCl, 0.2% Tween). The PVDF membranes were washed 4 times in TBS-Tween followed by incubation with primary antibody (e.g. phospho-Akt-Ser^{473} and the (pan) Akt antibody at a dilution of 1/1000 or as indicated) in TBS-Tween for 2 h at room temperature or overnight at 4 °C. After 4 washes, the membranes were incubated with an appropriate second antibody (e.g. goat anti-mouse usually 1/10,000) conjugated to horse-radish peroxidase in TBS-Tween for 1 h followed by four washes and detection using ECL reagents (GE Healthcare, Little Chalfont, UK).

2.4. Measurement of PLCγ2 and Syk tyrosine phosphorylation

Platelets were obtained as described in [20] and finally resuspended in modified-Tyrode buffer (comprising 134 mM NaCl, 0.34 mM Na_2HPO_4, 2.9 mM KCl, 12 mM $NaHCO_3$, 20 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid, 5 mM glucose, 1 mM $MgCl_2$; pH 7.3) at 5×10^8 cells/ml. For estimation of PLCγ2 and Syk tyrosine phosphorylation (used as a measure of protein activation) platelets were first pretreated with 9 μM Integrilin to inhibit aggregation through integrin αIIbβ$_3$. Platelets were then stimulated with DiC8-PIP_3 with 1 mM Ca^{2+} in the presence or absence of 10 μM dasatinib (Src family kinase inhibitor; LC Labs, Woburn MA, USA) at 37 °C with stirring at 1200 rpm in a Born lumi-aggregometer. Reactions were terminated by addition of 2× ice-cold NP-40 lysis buffer. Platelet lysates were precleared with protein A–Sepharose [21]. An aliquot was dissolved with SDS sample buffer for detection of total tyrosine phosphorylation. Lysates were incubated with the indicated antibodies and protein A–Sepharose. Precipitated proteins or whole-cell lysates were separated by SDS-PAGE, electrotransferred, and Western blotted as described above to detect PLCγ2 and Syk proteins and their tyrosine phosphorylation using the antiphosphotyrosine antibody 4G10 .

2.5. Measurements of thromboxane B_2

Washed platelets were prepared at 4×10^8 cells /ml. Following preincubation with 1 mM $CaCl_2$, washed platelets (4×10^8/ml) were