ACCELERATED PUBLICATION

Calmodulin interacts with the platelet ADP receptor P2Y₁

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P2Y₁ [P2 (purinergic type-2)-receptor 1] is a G-protein-coupled ADP receptor that regulates platelet activation and ADP-induced Ca²⁺ signalling. Studies using P2Y₁-knockout mice, G₁₁-deficient mice or P2Y₁-selective inhibitors have previously identified a key role for P2Y₁ in pathophysiological thrombus formation at high shear stress. We provide evidence that a positively charged juxtamembrane sequence within the cytoplasmic C-terminal tail of P2Y₁ can bind directly to the cytosolic regulatory protein calmodulin. Deletion by mutagenesis of the calmodulin-binding domain of P2Y₁ inhibits intracellular Ca²⁺ flux in transfected cells. These results suggest that the interaction of calmodulin with the P2Y₁ C-terminal tail may regulate P2Y₁-dependent platelet aggregation.

Key words: calmodulin, G-protein-coupled platelet ADP receptor, maltose-binding protein (MBP), pathophysiological thrombus formation, platelet aggregation, P2 (purinergic type-2)-receptor 1 (P2Y₁).

INTRODUCTION

P2Y₁ [P2 (purinergic type-2)-receptor 1] is a G-protein (G₁₁)-linked seven-transmembrane receptor of the purinergic receptor family that activates platelets in response to ADP and regulates Ca²⁺-dependent signalling events, initiating shape change and reversible αIβ(3)-dependent aggregation [1,2]. P2Y₁-dependent activation is reinforced by a second ADP receptor on platelets, P2Y₁₂, which is G₁₂-linked and promotes irreversible platelet aggregation [1–3]. The major evidence for a key pathophysiological role for P2Y₁ in thrombus formation has been reviewed and a recent study shows that P2Y₁-deficient mice, G₁₂-deficient mice or normal mice treated with P2Y₁ antagonists such as MRS-2179 or MRS-2500 [4–12]. Without P2Y₁ (and despite normal P2Y₁2 expression), platelets show a decreased propensity to form a stable thrombus. Platelets lacking P2Y₁ aggregate only at high ADP concentrations (via P2Y₁₂), and do so without shape change or elevation of cytosolic Ca²⁺ levels. P2Y₁-null mice show little or no tendency for spontaneous bleeding, but do show markedly increased resistance to thrombosis in vivo, when induced by intravenous injection of ADP or collagen plus adrenaline [6,7]. Most recently, it was shown in mice deficient in P2Y₁, or treated with the P2Y₁-antagonists MRS-2179 or MRS-2500, that arterial thrombosis was significantly less than controls in FeCl₃- or laser-induced arterial wall injury models (high-shear conditions); however, venous thrombosis (lower shear) was only slightly inhibited in these models [10,12]. Combined blockade of P2Y₁ and P2Y₁₂ is essentially additive when inhibiting thrombus formation under shear stress [8,10,12,13].

Recent studies support the notion that calmodulin plays a central role in the initiation of platelet thrombus formation. In human platelets, aggregation at high shear stress is triggered by the adhesion receptor GPⅡb–Ⅲa–Ⅴ (subunits GPIbβ and GPV) and integrins αIβ(3). Calmodulin interacts with the platelet ADP receptor P2Y₁ (chiefly αIβ(3). Cytoplasmic domains of both GPⅡb–Ⅲa–Ⅴ (subunits GPIbβ and GPV) contain discrete juxtamembrane sequences that directly bind calmodulin, interactions which dissociate upon platelet activation [14–19]. Engagement of GPⅡb (the major ligand-binding subunit of GPⅡb–Ⅲa–Ⅴ) or GPⅡb leads to secretion of ADP that acts via P2Y₁ and P2Y₁₂ to increase αIβ(3)-dependent aggregation. It was shown recently [13], that P2Y₁-dependent thrombus formation on von Willebrand factor at high shear is specifically impaired by P2Y₁ blockade, including elevation of cytosolic Ca²⁺ associated with platelet arrest, whereas P2Y₁₂ blockade inhibited formation of larger aggregates. Together, these studies indicate a key role for P2Y₁ in thrombus formation under conditions of shear stress in flowing blood, and support the promise of P2Y₁ as a future antithrombotic agent [1,2,10,12]. The goal of the present study was to determine whether the cytoplasmic domain of P2Y₁ binds calmodulin in the same manner as other receptors with analogous sequences (Figure 1), and whether the interaction may regulate P2Y₁-dependent platelet activation.

MATERIALS AND METHODS

General reagents

Amylose–agarose was purchased from New England Biolabs (Beverly, MA, U.S.A.). A synthetic peptide based on the human P2Y₁ C-terminal tail sequence Arg₁₃₂–Arg₁₄₅ (R₁₃₂RRLSRATRK ASRR₁₄₅), purified by reverse-phase HPLC and characterized by MS, was from Mimotopes (Clayton, VIC, Australia). The calmodulin inhibitor W-7 [N-(6-aminohexyl)-5-chloronaphthalene-1-sulfonamide] was obtained from Calbiochem (La Jolla, CA, U.S.A.). The P2Y₁₂ inhibitor AR-C679931MX

Abbreviations used: AR-C699931MX, N²-(2-methylthioethyl)-2-(3,3,3-trifluoropropylthio)-β,γ-dichloromethylene-ATP, ECL (Amersham), enhanced chemiluminescence; GP, glycoprotein; GST, glutathione S-transferase; MBP, maltose-binding protein; 2-MeSADP, 2-methylthio-ADP; PKC, protein kinase C; P2Y₁, P2 (purinergic type-2)-receptor 1; W-7, N-(6-aminohexyl)-5-chloronaphthalene-1-sulfonamide.

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and P2Y1 (Thr339) [25,26]. Purified on amylose–agarose and dialysed into TS buffer (0.01 M β-mercaptoethanol), 10% glycerol, 1.0 M KCl, 1.0 mM EDTA, 1.0 mM dithiothreitol, pH 7.5, [Nα–(2-methylthioethyl)-2-(3,3,3-trifluoropropylthio)-β,γ-di-chloromethylene-ATP], was the gift of Dr Shaun Jackson (Australian Centre for Blood Diseases, Clayton, Monash University, VIC, Australia).

Gel-shift assay

The capacity of a synthetic peptide based on a P2Y1 C-terminal tail sequence (R352RLRSATRKRASRR365) to form a complex with purified calmodulin was examined using a gel-shift assay previously used to identify calmodulin-binding peptides [13,14,19,21,22]. Briefly, 0.3 nmol of bovine calmodulin (Sigma, St Louis, MO, U.S.A.) was mixed with 0.3–3.0 nmol of P2Y1 peptide in 0.1 M Tris/HCl and 4 M urea, pH 7.5, in the presence of either 1 mM Ca2+ or 10 mM EGTA. After 30 min at 22°C, 0.5 vol of 50% (v/v) glycerol containing two drops of 0.1% Bromophenol Blue was added to each sample prior to resolution on 12.5%–polyacrylamide gels containing 4 M urea, and stained with Coomassie Blue.

Preparation of MBP (maltose-binding protein)–P2Y1 fusion proteins

cDNA corresponding to the P2Y1 C-terminal tail sequence (Phe325–Leu373) was amplified by PCR from full-length cDNA encoding wild-type human P2Y1 in a pCDNA3 vector (Invitrogen, Carlsbad, CA, U.S.A.) [6]. Forward and reverse primers included unique restriction sites (EcoRI and BamHI respectively) in non-complementary 5′ ends for subcloning of the amplified fragment into an MBP-fusion vector. The PCR fragment was purified using a QIAquick PCR purification kit (Qiagen, Doncaster, VIC, Australia), digested with EcoRI and BamHI and inserted into the pMAL-C2X vector (New England Biolabs) encoding MBP N-terminal to the P2Y1 tail insert. A calmodulin-deletion mutant of MBP–P2Y1 tail lacking residues Arg337–Asn349 [recombinant human P2Y1 (Thr339)] was prepared in pEGFP-N3 vector (Clontech) [24] and P2Y1 (Thr339) [25,26].

RESULTS AND DISCUSSION

In the present study we show that the cytosolic regulatory protein calmodulin directly interacts with a juxtamembrane positively charged sequence within the C-terminal cytoplasmic tail of P2Y1. We recently reported that two platelet-adhesion receptors, GP Ib–IX–V and GPVI, which bind von Willebrand factor or collagen respectively to initiate platelet aggregation, bound directly to calmodulin via positively charged juxtamembrane sequences within their cytoplasmic domains [14,15]. In view of the functional role of these receptors, it was noteworthy that the G-protein-coupled ADP receptor P2Y1, which also initiates platelet aggregation [1–11,13], contains an analogous sequence in the juxtmembrane region of its cytoplasmic C-terminal tail (Figure 1). This sequence of P2Y1 contains positively charged and hydrophobic residues spaced in a manner similar to calmodulin-binding sequences in other proteins that form amphipathic helices (reviewed in [31]). The corresponding juxtamembrane region of the other platelet ADP receptor, P2Y12 (FPRNLRLSISLMLKPNSAT129) [32], does not contain a consensus calmodulin-binding sequence. Other G-protein-coupled receptors contain a calmodulin-binding site in the same region of...
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The MBP–P2Y1 wild-type tail, but not the MBP–P2Y1 to abolish calmodulin binding of the m7a receptor (Figure 1) corresponding calmodulin-deletion mutation previously reported.

The platelet cytosol was still able to bind Gβγ subunits (Figure 2C).

Figure 2 Gel-shift assay (A) and pull-down experiments (B and C)

(A) Non-denaturing gel-shift assay of purified calmodulin (CaM) and increasing concentrations of the P2Y1-related peptide in the presence of Ca2+ (upper panel) or EGTA (lower panel). Proteins were stained with Coomasie Blue. Data are representative of four separate experiments. (B) Pull-down of calmodulin from platelet cytosol by amylose beads and MBP alone, MBP–P2Y1 wild-type C-terminal tail or MBP–P2Y1ΔCaM mutant. Samples of platelet cytosol or precipitates were analysed by Western blotting with anti-calmodulin antibody (lower panel) or fusion proteins were probed with anti-MBP antibody (upper panel) and visualized using ECL®. (C) Pull-down of Gβγ subunits from platelet lysate by glutathione–Sepharose beads and GST alone, GST–P2Y1 wild-type C-terminal tail or GST–P2Y1ΔCaM mutant. Samples were analysed by Western blotting with anti-Gβγ antibody and visualized using ECL®.

Calmodulin binds to a P2Y1-based peptide

Using a gel-shift assay, a synthetic peptide based on the juxtamembrane sequence of the P2Y1 C-terminal tail sequence, Arg332–Arg345 (R332RRLSRatkASRR345), was shown to bind calmodulin. The peptide induced a dose-dependent shift in migration of purified bovine calmodulin on polyacrylamide gels in the presence of Ca2+ generating a single band representing a calmodulin–peptide complex (Figure 2A). In contrast, in the presence of EGTA, there was significantly less calmodulin–peptide complex (Figure 2A), suggesting the calmodulin–P2Y1 peptide interaction under these conditions is Ca2+-dependent.

Calmodulin binds to MBP–P2Y1 C-terminal tail fusion proteins

We expressed two MBP fusion proteins containing the C-terminal region of P2Y1 to see whether they could bind calmodulin, as did the P2Y1-based synthetic peptide. One of the fusion proteins consisted of MBP–P2Y1, which contained the full-length wild-type cytoplasmic tail of P2Y1 (Phe325–Leu373). A second MBP–P2Y1 fusion protein was a calmodulin-deletion mutant lacking residues Arg337–Asn349 within the calmodulin-binding sequence (MBP–P2Y1ΔCaM). This deletion was based on a corresponding calmodulin-deletion mutation previously reported to abolish calmodulin binding of the m7a receptor (Figure 1) [24]. The MBP–P2Y1 wild-type tail, but not the MBP–P2Y1ΔCaM mutant, could specifically pull down calmodulin from platelet cytosol as shown by Western blotting precipitates with an anti-calmodulin antibody (Figure 2B). MBP alone was used as a specificity control. Blotting the MBP–P2Y1 wild-type tail and MBP–P2Y1ΔCaM pull-down samples with anti-MBP antibody [15] confirmed there was equivalent bait in each lane (Figure 2B).

These results suggest there is a single calmodulin-binding site in the P2Y1 C-terminal tail involving residues Arg337–Asn349. The cytoplasmic tail of seven-transmembrane receptors is involved in G-protein binding, and the calmodulin-binding site of P2Y1 is proximal to the residues (Arg333 and Arg334) responsible for G-protein association [23]. We therefore investigated whether deletion of the calmodulin-binding site interferes with binding of G-protein subunits. Probing GST–P2Y1 wild-type tail and GST–P2Y1ΔCaM pull-down samples with an antibody raised against amino acids 1–300 of the N-terminus of human Gβ2 (Santa Cruz Biotechnology), which recognizes Gβ2, Gβ4, and, to a lesser extent, Gβ1, confirmed that the P2Y1 calmodulin-deleted mutant was still able to bind Gβγ subunits (Figure 2C).

Calmodulin inhibition by W-7 inhibits P2Y1-dependent platelet aggregation

To determine whether there is a functional role for calmodulin in regulating P2Y1 receptor function, we first tested the effect of the calmodulin inhibitor W-7 on P2Y1-dependent platelet aggregation. Assays were carried out under conditions where the other ADP receptor on platelets, P2Y12, was blocked using the P2Y12-selective inhibitor AR-C69931MX. This inhibitor is an ATP analogue which selectively blocks the human ADP-receptor P2Y12, but has no effect on P2Y1 [33]. In AR-C69931MX-treated platelets, ADP acts on P2Y1 and induces reversible aggregation, without P2Y12-dependent reinforcement leading to stable aggregates [33]. Aggregation occurs up to ~1 min, but is then reversed. P2Y1-dependent platelet aggregation induced by ADP in the presence of AR-C69931MX was inhibited by the calmodulin inhibitor W-7 (50–150 µM), with maximal inhibition at ~150 µM (Figure 3), concentrations of W-7 previously shown to inhibit other calmodulin-mediated events in platelets or other cells [17,20,34,35]. W-7 has been reported to inhibit Ca2+-dependent platelet shape change and aggregation in response...
Calmodulin binding has a role in P2Y1 function, cells were trans-fected with ADP [29]. To determine more specifically whether calmodulin-binding site deletion inhibits ADP-induced increases in intracellular Ca²⁺, a transient increase in intracellular Ca²⁺ was induced by ADP or 2MeSADP in either the absence (A) or presence (B) of 2 mM Ca²⁺. Each curve represents the mean for three independent experiments and the error bars represent the S.E.M.

**Calmodulin-binding site deletion inhibits ADP-induced increases in intracellular Ca²⁺ in P2Y1-transfected cells**

A transient increase in intracellular Ca²⁺ occurs downstream of P2Y₁ ligation following treatment of platelets or P2Y₁-transfected cells with ADP [29]. To determine more specifically whether calmodulin binding has a role in P2Y₁ function, cells were transfected with either wild-type P2Y₁ or calmodulin-disrupted P2Y₁. Flow cytometry confirmed that similar levels of either wild-type (9.68 ± 0.51 %) or mutant receptor (8.54 ± 0.38 %) were expressed relative to untransfected cells (0.10 ± 0.21 %). Cells expressing wild-type P2Y₁ or calmodulin-disrupted P2Y₁ were then stimulated with ADP or the highly selective P2Y₁ agonist 2MeSADP (2-methylthio-ADP; 10⁻⁹–10⁻⁴ μM) in the presence or absence of 2 mM Ca²⁺. In the absence of Ca²⁺, wild-type P2Y₁-transfected cells demonstrated a small increase in intracellular Ca²⁺ in response to both ADP and 2MeSADP, whereas cells transfected with calmodulin-deleted P2Y₁ failed to respond to either agonist (Figure 4A). In the presence of 2 mM extra-cellular Ca²⁺, ADP and 2MeSADP elicited a concentration-dependent increase in intracellular Ca²⁺ in wild-type cells, which was strongly inhibited in the P2Y₁ calmodulin-deleted mutants (Figure 4B). These data demonstrate that deletion of the calmodulin-binding region from the cytoplasmic tail of P2Y₁ significantly inhibits the increase in intracellular Ca²⁺ induced by ADP or 2MeSADP, indicating a requirement for calmodulin in P2Y₁ function.

Calmodulin regulates surface expression of GPVI [17] and GPV of the GPIb–IX–V complex [19], which initiate thrombus formation at high shear [18]. Calmodulin binding to the C-terminal cytoplasmic domain could also regulate surface expression or internalization of P2Y₁. Overexpression of P2Y₁ on mouse platelets results in reduced bleeding time and increased reactivity to ADP; these results emphasize the potential importance of receptor expression levels in relation to thrombotic states in humans [36]. Activated P2Y₁ receptors are internalized through a pathway distinct from that of P2Y₁ receptors [30]. Calmodulin dissociation from activated P2Y₁ would provide a mechanism for regulating surface expression, as for G-protein-coupled receptors on other cells [22].

Together, these results suggest that the interaction of calmodulin with the P2Y₁ C-terminal tail may regulate aspects of P2Y₁-dependent platelet aggregation. The calmodulin-binding sequence is proximal to functional sites for G-protein association (Arg²¹³–Arg²³⁴) [23] and PKC (protein kinase C)-dependent phosphorylation (Ser³³⁹) [25] in the C-terminal tail of P2Y₁ (Figure 1). Further studies are warranted in order to unravel the functional role of calmodulin-mediated events at the cytoplasmic face of P2Y₁ and their relationship to calmodulin-independent regulation of other receptors that initiate thrombosis.

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