ACCELERATED PUBLICATION

Calmodulin interacts with the platelet ADP receptor $P2Y_1$

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$P2Y_1$ [P2 (purinergic type-2)-receptor 1] is a G-protein-coupled ADP receptor that regulates platelet activation and ADP-induced Ca^{2+} signalling. Studies using $P2Y_1$-knockout mice, G_q-deficient mice or $P2Y_1$-selective inhibitors have previously identified a key role for $P2Y_1$ in pathophysiological thrombus formation at high shear stress. We provide evidence that a positively charged juxtamembrane sequence within the cytoplasmic C-terminal tail of $P2Y_1$ can bind directly to the cytosolic regulatory protein calmodulin. Deletion by mutagenesis of the calmodulin-binding domain of $P2Y_1$ inhibits intracellular Ca^{2+} flux in transfected cells. These results suggest that the interaction of calmodulin with the $P2Y_1$ C-terminal tail may regulate $P2Y_1$-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 ($P2Y_1$).

INTRODUCTION

$P2Y_1$ [P2 (purinergic type-2)-receptor 1] is a G-protein ($G\alpha_q$)-linked seven-transmembrane receptor of the purinergic receptor family that activates platelets in response to ADP and regulates Ca^{2+}-dependent signalling events, initiating shape change and reversible αIIbβ3-dependent aggregation [1,2]. $P2Y_1$-dependent activation is reinforced by a second ADP receptor on platelets, $P2Y_{12}$, which is $G\alpha_i$-linked and promotes irreversible platelet aggregation [1–3]. The major evidence for a key pathophysiological role for $P2Y_1$ in thrombus formation comes from studies in $P2Y_1$-deficient mice, G_q-deficient mice or normal mice treated with $P2Y_1$ antagonists such as MRS-2179 or MRS-2500 [4–12]. Without $P2Y_1$ (and despite normal $P2Y_{12}$ expression), platelets show a decreased propensity to form a stable thrombus. Platelets lacking $P2Y_1$ aggregate only at high ADP concentrations (via $P2Y_{12}$), and do so without shape change or elevation of cytosolic Ca^{2+} levels. $P2Y_1$-null mice show little or no tendency for spontaneous bleeding, but do show markedly increased resistance to thromboembolism *in vivo*, when induced by intravenous injection of ADP or collagen plus adrenaline [6,7]. Most recently, it was shown in mice deficient in $P2Y_1$, or treated with the $P2Y_1$-antagonists MRS-2179 or MRS-2500, that arterial thrombosis was significantly less than controls in $FeCl_3$- 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 $P2Y_1$ and $P2Y_{12}$ 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 GPIb (glycoprotein Ib)–IX–V, which binds von Willebrand factor and initiates thrombus involving other receptors, including the collagen receptor, GPVI, and integrins (chiefly αIIbβ3). Cytoplasmic domains of both GPVI and GPIb–IX–V (subunits GPIbβ and GPV) contain discrete juxtamembrane sequences that directly bind calmodulin, interactions which dissociate upon platelet activation [14–19]. Engagement of GPIbα (the major ligand-binding subunit of GPIb–IX–V) or GPVI leads to secretion of ADP that acts via $P2Y_1$ and $P2Y_{12}$ to increase αIIbβ3-dependent aggregation. It was shown recently [13], that GPIbα-dependent thrombus formation on von Willebrand factor at high shear is specifically impaired by $P2Y_1$ blockade, including elevation of cytosolic Ca^{2+} associated with platelet arrest, whereas $P2Y_{12}$ blockade inhibited formation of larger aggregates. Together, these studies indicate a key role for $P2Y_1$ in thrombus formation under conditions of shear stress in flowing blood, and support the promise of $P2Y_1$ as a future antithrombotic target [1,3,9,11]. The goal of the present study was to determine whether the cytoplasmic domain of $P2Y_1$ binds calmodulin in the same manner as other receptors with analogous sequences (Figure 1), and whether the interaction may regulate $P2Y_1$-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 $P2Y_1$ C-terminal tail sequence Arg^{332}–Arg^{345} (R^{332}RRLSRATRK-ASRR^{345}), 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 $P2Y_{12}$ inhibitor AR-C69931MX

Abbreviations used: AR-C69931MX, N^6-(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; $P2Y_1$, P2 (purinergic type-2)-receptor 1; W-7, *N*-(6-aminohexyl)-5-chloronaphthalene-1-sulfonamide.

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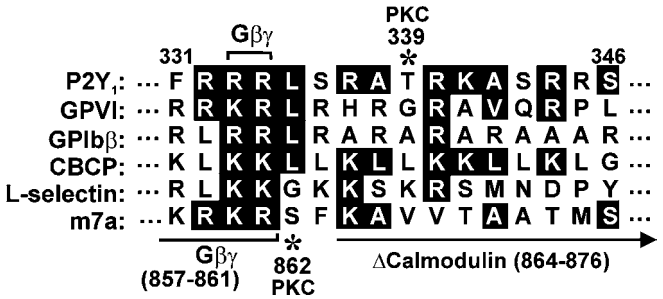

Figure 1 Comparison of calmodulin-binding sequences

Juxtamembrane calmodulin-binding sequence within the $P2Y_1$ C-terminal cytoplasmic tail, compared with known calmodulin-binding sequences in platelet GPVI [15–17], GPIbβ [14], a non-physiological calmodulin-binding control peptide that forms amphipathic α-helix [20], leucocyte adhesion receptor, L-selectin [21] and the G-protein-coupled glutamate receptor m7a [22]. Identical residues or conservative substitutions are highlighted. The G$\beta\gamma$-binding region (residues 857–861) and the site of a deletion (residues 864–876) that blocks calmodulin binding are shown on the m7a sequence [22] compared with the G-protein-binding site at Arg^{333}/Arg^{334} of $P2Y_1$ [23]. Asterisks show known PKC-dependent phosphorylation sites in m7a (Ser^{862}) [24] and $P2Y_1$ (Thr^{339}) [25,26].

[N^6-(2-methylthioethyl)-2-(3,3,3-trifluoropropylthio)-β,γ-dichloromethylene-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 $P2Y_1$ C-terminal tail sequence (R^{332}RRLSRATRKASR^{345}) 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 0–3.0 nmol of $P2Y_1$ peptide in 0.1 M Tris/HCl and 4 M urea, pH 7.5, in the presence of either 1 mM Ca^{2+} 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)–$P2Y_1$ fusion proteins

cDNA corresponding to the $P2Y_1$ C-terminal tail sequence (Phe^{325}–Leu^{373}) was amplified by PCR from full-length cDNA encoding wild-type human $P2Y_1$ in a pCDNA3 vector (Invitrogen, Carlsbad, CA, U.S.A.) [6]. Forward and reverse primers included unique restriction sites (EcoR1 and BamH1 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 EcoR1 and BamH1 and inserted into the pMAL-C2X vector (New England Biolabs) encoding MBP N-terminal to the $P2Y_1$ tail insert. A calmodulin-deletion mutant of MBP–$P2Y_1$ tail lacking residues Arg^{337}–Asn^{349} was prepared using a blunt-end ligation method as previously described [27]. The correct sequence of the vectors was confirmed by sequencing. MBP alone, or wild-type or mutant MBP–$P2Y_1$ C-terminal tail fusion proteins expressed in *Escherichia coli* strain BL21 were purified on amylose–agarose and dialysed into TS buffer (0.01 M Tris/HCl and 0.15 M NaCl, pH 7.4) using standard methods [15]. Subcloning of wild-type or calmodulin-deleted $P2Y_1$ tail sequences in GST (glutathione S-transferase) vector (pGEX-4T-1; Amersham Pharmacia), expression and purification of fusion proteins on glutathione–Sepharose was performed using similar methods.

Pull-down assays with $P2Y_1$ fusion proteins

The ability of the full-length $P2Y_1$ C-terminal tail expressed as a MBP-fusion protein to bind calmodulin in human platelet cytosol was assessed using a pull-down assay, as previously described [15]. Briefly, MBP alone, MBP–$P2Y_1$ wild-type C-terminal tail, or MBP–$P2Y_1$ calmodulin-deletion mutant was incubated with cytosol and rocked for 16 h at 4 °C. An equivalent volume of amylose beads (1:1 suspension in TS buffer) was added, and the mixture incubated for a further 4 h. Beads were washed with TS buffer and immunoblotted with anti-calmodulin antibody (Upstate Biotechnology, Lake Placid, NY, U.S.A.) or anti-MBP antibody (New England Biolabs) as previously described [15]. Pull-downs using GST alone, GST–$P2Y_1$ wild-type C-terminal tail or GST–$P2Y_1$ calmodulin-deletion mutant from human platelet lysates [28] with glutathione–Sepharose beads were performed using essentially the same methods, and immunoblotted with anti-Gβ_2 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.). Blots were visualized using ECL® (enhanced chemiluminescence; Amersham, Bucks., U.K.).

Platelet aggregation

Platelet aggregation was performed in a whole-blood Lumi-Aggregometer (Chrono-Log, Havertown, PA, U.SA) using human citrated platelet-rich plasma as previously described [28]. Aggregation was induced by addition of ADP (5 μM). Samples were preincubated with TS buffer or the calmodulin inhibitor W-7 (50–150 μM) for 3 min at 37 °C, prior to adding ADP. Assay mixtures included the $P2Y_{12}$ inhibitor ARC369931MX (50 nM).

Intracellular Ca^{2+} measurements

As a functional test of the effect of calmodulin site deletion on ADP responses, intracellular Ca^{2+} measurements in 1321N1 cells transfected with either $P2Y_1$ wild-type or the calmodulin-deleted mutant lacking residues Arg^{337}–Asn^{349} [recombinant human $P2Y_1$ in pEGFP-N3 vector (Clontech)] were carried out as described previously [29,30].

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 $P2Y_1$. We recently reported that two platelet-adhesion receptors, GPIb–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 $P2Y_1$, which also initiates platelet aggregation [1–11,13], contains an analogous sequence in the juxtamembrane region of its cytoplasmic C-terminal tail (Figure 1). This sequence of $P2Y_1$ 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, $P2Y_{12}$ (F^{304}RNLSLISMLKCPNSAT^{320}) [32], does not contain a consensus calmodulin-binding sequence. Other G-protein-coupled receptors contain a calmodulin-binding site in the same region of

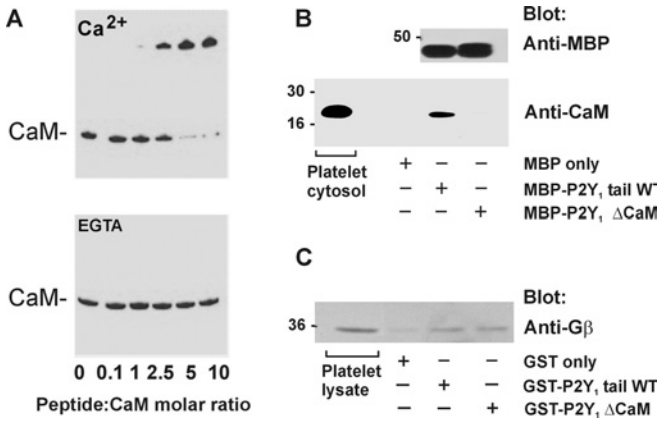

Figure 2 Gel-shift assay (A) and pull-down experiments (B and C)

(**A**) Non-denaturing gel-shift assay of purified calmodulin (CaM) and increasing concentrations of the $P2Y_1$-related peptide in the presence of Ca^{2+} (upper panel) or EGTA (lower panel). Proteins were stained with Coomassie Blue. Data are representative of four separate experiments. (**B**) Pull-down of calmodulin from platelet cytosol by amylose beads and MBP alone, MBP–$P2Y_1$ wild-type C-terminal tail or MBP–$P2Y_1$ ΔCaM 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 Gβ subunits from platelet lysate by glutathione–Sepharose beads and GST alone, GST–$P2Y_1$ wild-type C-terminal tail or GST–$P2Y_1$ ΔCaM mutant. Samples were analysed by Western blotting with anti-Gβ antibody and visualized using ECL®.

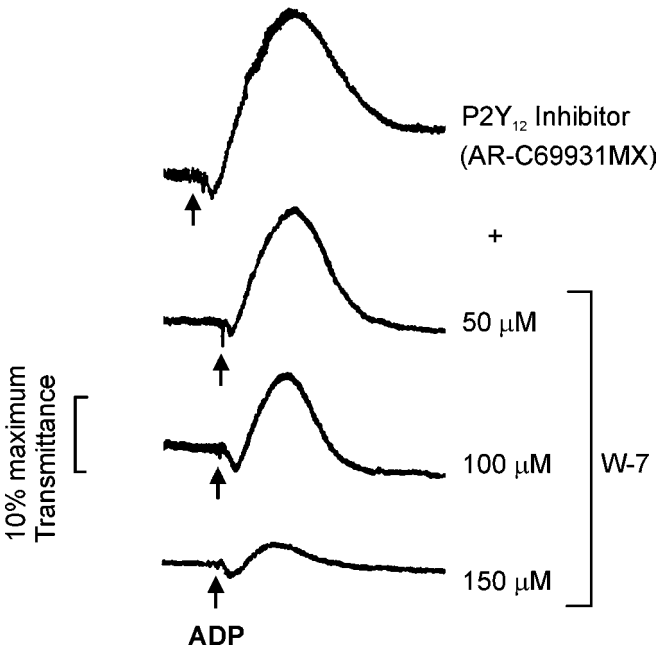

Figure 3 Inhibition of ADP-induced platelet aggregation by the calmodulin inhibitor W-7

W-7 (50–150 μM) was preincubated with citrated platelet-rich plasma for 3 min at 37 °C in the presence of the $P2Y_{12}$ receptor antagonist AR-C69931MX (50 nM) before addition of ADP (5 μM).

the cytoplasmic C-terminal tail as $P2Y_1$, including the glutamate receptor m7a [22,24] (Figure 1).

Calmodulin binds to a $P2Y_1$-based peptide

Using a gel-shift assay, a synthetic peptide based on the juxtamembrane sequence of the $P2Y_1$ C-terminal tail sequence, Arg^{332}–Arg^{345} (R^{332}RRLSRATRKASRR^{345}), 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 Ca^{2+}, 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-$P2Y_1$ peptide interaction under these conditions is Ca^{2+}-dependent.

Calmodulin binds to MBP–$P2Y_1$ C-terminal tail fusion proteins

We expressed two MBP fusion proteins containing the C-terminal region of $P2Y_1$ to see whether they could bind calmodulin, as did the $P2Y_1$-based synthetic peptide. One of the fusion proteins consisted of MBP–$P2Y_1$, which contained the full-length wild-type cytoplasmic tail of $P2Y_1$ (Phe^{325}–Leu^{373}). A second MBP–$P2Y_1$ fusion protein was a calmodulin-deletion mutant lacking residues Arg^{337}–Asn^{349} within the calmodulin-binding sequence (MBP–$P2Y_1$ ΔCaM). 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–$P2Y_1$ wild-type tail, but not the MBP–$P2Y_1$ ΔCaM 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–$P2Y_1$ wild-type tail and MBP–$P2Y_1$ ΔCaM 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 $P2Y_1$ C-terminal tail involving residues Arg^{337}–Asn^{349}. The cytoplasmic tail of seven-transmembrane receptors is involved in G-protein binding, and the calmodulin-binding site of $P2Y_1$ is proximal to the residues (Arg^{333} and Arg^{334}) 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–$P2Y_1$ wild-type tail and GST–$P2Y_1$ ΔCaM pull-down samples with an antibody raised against amino acids 1–300 of the N-terminus of human $G\beta_2$ (Santa Cruz Biotechnology), which recognizes $G\beta_1$–$G\beta_4$ and, to a lesser extent, $G\beta_5$, confirmed that the $P2Y_1$ calmodulin-deleted mutant was still able to bind $G\beta\gamma$ subunits (Figure 2C).

Calmodulin inhibition by W-7 inhibits $P2Y_1$-dependent platelet aggregation

To determine whether there is a functional role for calmodulin in regulating $P2Y_1$ receptor function, we first tested the effect of the calmodulin inhibitor W-7 on $P2Y_1$-dependent platelet aggregation. Assays were carried out under conditions where the other ADP receptor on platelets, $P2Y_{12}$, was blocked using the $P2Y_{12}$-selective inhibitor AR-C69931MX. This inhibitor is an ATP analogue which selectively blocks the human ADP-receptor $P2Y_{12}$, but has no effect on $P2Y_1$ [33]. In AR-C69931MX-treated platelets, ADP acts on $P2Y_1$ and induces reversible aggregation, without $P2Y_{12}$-dependent reinforcement leading to stable aggregates [33]. Aggregation occurs up to ~1 min, but is then reversed. $P2Y_1$-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 Ca^{2+}-dependent platelet shape change and aggregation in response

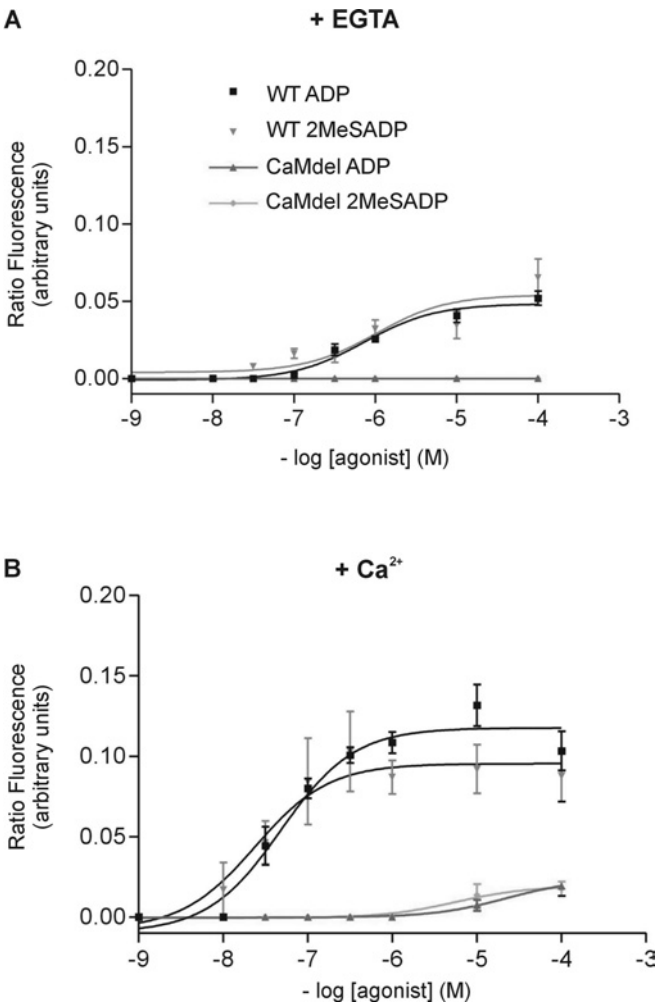

Figure 4 Concentration-dependent increases in intracellular Ca^{2+} concentration in cells transfected with wild-type (WT) or calmodulin-deleted (CAMdel) $P2Y_1$

$[Ca^{2+}]_i$ was induced by ADP or 2MeSADP in either the absence (**A**) or presence (**B**) of 2 mM Ca^{2+}. Each curve represents the mean for three independent experiments and the error bars represent the S.E.M.

to agonists such as collagen via a myosin light-chain kinase-dependent mechanism [18,34,35]. Accordingly, our results do not mean that W-7 only inhibits $P2Y_1$-associated calmodulin, but are consistent with calmodulin playing a critical role in $P2Y_1$-dependent aggregation.

Calmodulin-binding site deletion inhibits ADP-induced increases in intracellular Ca^{2+} in $P2Y_1$-transfected cells

A transient increase in intracellular Ca^{2+} occurs downstream of $P2Y_1$ ligation following treatment of platelets or $P2Y_1$-transfected cells with ADP [29]. To determine more specifically whether calmodulin binding has a role in $P2Y_1$ function, cells were transfected with either wild-type $P2Y_1$ or calmodulin-disrupted $P2Y_1$. Flow cytometry confirmed that similar levels of either wild-type ($9.68 \pm 0.51\,\%$) or mutant receptor ($8.54 \pm 0.38\,\%$) were expressed relative to untransfected cells ($0.10 \pm 0.21\,\%$). Cells expressing wild-type $P2Y_1$ or calmodulin-deleted $P2Y_1$ were then stimulated with ADP or the highly selective $P2Y_1$ agonist 2MeSADP (2-methylthio-ADP; 10^{-9}–10^{-4} μM) in the presence or absence of 2 mM Ca^{2+}. In the absence of Ca^{2+}, wild-type $P2Y_1$-transfected cells demonstrated a small increase in intracellular Ca^{2+} in response to both ADP and 2MeSADP, whereas cells transfected with calmodulin-deleted $P2Y_1$ failed to respond to either agonist (Figure 4A). In the presence of 2 mM extracellular Ca^{2+}, ADP and 2MeSADP elicited a concentration-dependent increase in intracellular Ca^{2+} in wild-type cells, which was strongly inhibited in the $P2Y_1$ calmodulin-deleted mutants (Figure 4B). These data demonstrate that deletion of the calmodulin-binding region from the cytoplasmic tail of $P2Y_1$ significantly inhibits the increase in intracellular Ca^{2+} induced by ADP or 2MeSADP, indicating a requirement for calmodulin in $P2Y_1$ 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 $P2Y_1$. Overexpression of $P2Y_1$ 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 $P2Y_1$ receptors are internalized through a pathway distinct from that of $P2Y_{12}$ receptors [30]. Calmodulin dissociation from activated $P2Y_1$ 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 $P2Y_1$ C-terminal tail may regulate aspects of $P2Y_1$-dependent platelet aggregation. The calmodulin-binding sequence is proximal to functional sites for G-protein association (Arg^{333}–Arg^{334}) [23] and PKC (protein kinase C)-dependent phosphorylation (Ser^{339}) [25] in the C-terminal tail of $P2Y_1$ (Figure 1). Further studies are warranted in order to unravel the functional role of calmodulin-mediated events at the cytoplasmic face of $P2Y_1$ and their relationship to calmodulin-dependent regulation of other receptors that initiate thrombosis.

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