FEBS Letters 568 (2004) 70–78 FEBS 28471

Proteolytic cleavage of platelet endothelial cell adhesion molecule-1 (PECAM-1/CD31) is regulated by a calmodulin-binding motif ☆

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Received 24 February 2004; revised 25 April 2004; accepted 30 April 2004

Available online 19 May 2004

Edited by Beat Imhof

Abstract Homophilic engagement of platelet endothelial cell adhesion molecule-1 (PECAM-1/CD31) induces 'outside-in' signal transduction that results in phosphorylation events and recruitment and activation of signalling molecules. The formation of signalling scaffolds with PECAM-1 are important signalling events that modulate platelet secretion, aggregation and platelet thrombus formation. In this study, we describe a novel interaction between PECAM-1 and cytosolic calmodulin (CaM) in platelets. Reciprocal co-immunoprecipitation studies revealed that cytosolic CaM is constitutively associated with PECAM-1 in resting, thrombin activated and aggregated human platelets. Our studies demonstrate that CaM directly interacts with a PECAM-1 peptide (594–604) C595A containing the sequences ^{594}KAFYLRKAKAK^{604}. This CaM:PECAM-1 interaction has a threefold higher affinity than CaM:GPVI interaction. It is potentiated by the addition of calcium ions, and dissociated by the CaM inhibitor, trifluoperazine. Treatment of platelets with CaM inhibitors triggers cleavage of PECAM-1 in a time- and dose-dependent manner. Furthermore, this membrane proximal portion of PECAM-1 is conserved across mammalian species and the helical representation of basic/hydrophobic residues reveals a charge distribution analogous to other CaM-binding motifs in other proteins. Taken together, these results suggest that this highly charged cluster of amino acids in the PECAM-1 cytoplasmic domain directly interacts with CaM and this novel interaction appears to regulate cleavage of PECAM-1.

Keywords: Calmodulin; Platelet endothelial cell adhesion molecule-1; Platelet

1. Introduction

Platelet endothelial cell adhesion molecule-1 (PECAM-1/CD31) belongs to the immunoglobulin (Ig)-immunoreceptor tyrosine-based inhibitory motif (ITIM) supergene family and is known to mediate calcium-independent homophilic cell–cell binding [1–5]. Other specific heterophilic ligands have been proposed including integrin $\alpha_v\beta_3$ and ectoenzyme CD38 [6,7]. PECAM-1 contains six extracellular Ig-like domains, a transmembrane domain and a cytoplasmic domain. PECAM-1 is expressed on the surface of myeloid cells, naïve B cells, naïve T cells, natural killer cells, mast cells, platelets and endothelial cells. Given its abundant expression, PECAM-1 has been demonstrated to be involved in the initial formation and stabilisation of cell–cell contacts at the lateral junctions of endothelial cells, in the maintenance of vascular permeability, modulation of transendothelial migration of monocytes and neutrophils and formation of new blood vessels in angiogenesis [8–14]. In the vasculature, it also serves as a virulence-associated endothelial receptor for binding *Plasmodium falciparum*-infected erythrocytes [15].

Engagement of PECAM-1 induces 'outside-in' signalling events that induce tyrosine phosphorylation of the PECAM-1 cytoplasmic domain with protein-tyrosine phosphatase (PTP) recruitment and activation that results in selective dephosphorylation and 'inside-out' signalling that serves to disrupt cell adhesion. The mechanisms of initiating and terminating 'outside-in' signalling through PECAM-1 are important new avenues of investigation [16]. Examination of PECAM-1-deficient mice has revealed that PECAM-1 serves as a major negative regulator of immunoreceptor tyrosine-based activatory motif-associated signalling pathways, involving collagen GPVI/FcR γ-chain in platelets, B-cell receptor antigen complex on B cells, and FcRI-mediated signalling on mast cells [17–20]. PECAM-1 is active in transmembrane signalling mediated by the direct recruitment and activation of specific PTPs to phosphorylated ITIMs [1,4,21–24]. Apart from PTPs, PECAM-1 associates with other cytoplasmic signalling and cytoskeletal molecules including β-catenin, γ-catenin, phospholipase C (PLC)-γ1, and phosphatidylinositol 3-kinase (PI 3-kinase) [25–28]. However, it remains unclear whether these molecules directly interact with the PECAM-1 cytoplasmic domain and how these interactions are regulated.

☆ This work was supported by grants from the National Heart Foundation and National Health and Medical Research Council of Australia.

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[1] Recipient of an NHMRC Dora Lush Ph.D. scholarship.
[2] Recipient of an NHMRC Senior Research Fellowship.

0014-5793/$22.00 © 2004 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/j.febslet.2004.04.094

Recent studies have demonstrated that a combination of the extracellular domain and a cluster of highly conserved charged amino acid residues in the juxtamembrane region of PECAM-1 was sufficient to confer efficient localisation of the molecule to cell–cell borders of PECAM-1-expressing cells [29]. We hypothesized that these highly charged residues may be important in cytoskeletal reorganisation via the direct recruitment of signalling molecules and/or cytoskeletal components. One of the candidate molecules that was investigated was calmodulin (CaM).

In this study, we demonstrate that cytosolic CaM, a calcium sensory protein, is specifically co-precipitated with PECAM-1 from lysates of resting platelets, activated platelets and/or aggregated platelets. We further show with synthetic peptides based on the cytoplasmic domain of PECAM-1 that positionally located positively charged residues, ^{599}RKAKAK^{604}, in the juxtamembrane region mediate the direct interaction of PECAM-1 with cytosolic CaM derived from platelets or with purified CaM. This CaM:PECAM-1 peptide interaction is potentiated by the addition of calcium ions. This interaction of PECAM-1 with cytosolic CaM appears to regulate intracellular receptor cleavage, as CaM inhibitors induce cleavage of PECAM-1 in platelets.

2. Materials and methods

2.1. General reagents

Bovine serum albumin, prostaglandin E_1, bovine CaM, phenylmethylsulfonyl fluoride (PMSF), sodium orthovanadate, leupeptin, streptavidin–agarose beads and human thrombin were purchased from Sigma Chemical Company (St. Louis, MO). Prestained markers were obtained from Invitrogen (Carlsbad, CA). Protein G–Sepharose beads and cyanogen bromide (CNBr)-activated Sepharose beads were purchased from Amersham Pharmacia Biotech AB (Uppsala, Sweden).

2.2. Antibodies

A rabbit polyclonal antibody against human PECAM-1 (SEW16) and a mouse monoclonal antibody against domain 1 of human PECAM-1 (PECAM-1.3) were a kind gift of Dr. Peter Newman (Blood Research Institute, Milwaukee, WI). The polyclonal SEW41 anti-PECAM-1 cytoplasmic domain antibody was generated by immunising rabbits with purified recombinant PECAM-1 cytoplasmic domain protein. The polyclonal SEW41 IgG antibody specifically recognised the cytoplasmic domain of PECAM-1 when tested with transfected cell lines expressing PECAM-1:ICAM-1 chimeras with only positive reactivity in immunoprecipitates in the presence of the PECAM-1 cytoplasmic domain. This polyclonal anti-PECAM-1 antibody recognises PECAM-1 in either a native or denatured form. A mouse monoclonal antibody directed against human CD151 (11B1) was a kind gift from Professor Leonie Ashman (University of Newcastle, Newcastle, NSW). These antibodies have been previously characterised [1]. Mouse monoclonal anti-CaM IgG_1 antibody was obtained from Upstate Biotechnology (Lake Placid, NY). Normal mouse IgG_1 antibody was obtained from Sigma.

2.3. Preparation of washed platelets

Human platelets were obtained from healthy volunteers that were free of any anti-platelet drug medication for the preceding seven days. Platelets were washed according to a previously described method [1].

2.4. Platelet activation/aggregometry studies

Washed platelets (1×10^8/ml) were either left unstimulated, or stimulated with 1 U/ml thrombin (Sigma Chemical Company, St. Louis, MO) for 5 min at 37 °C without stirring. Platelet aggregation was induced by 1 U/ml thrombin with stirring (1000 rpm) in the presence of 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 100 μg/ml fibrinogen for 1 min at 37 °C in a Payton dual-channel aggregation module (Paton Scientific, Adelaide, Australia). Platelets were also pre-incubated with 20 g/ml c7E3 Fab (Reopro) (Centocor B.V., Leiden, Netherlands) for 10 min at 37 °C with stirring (1000 rpm) prior to activation with 1 U/ml thrombin in the presence of 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 100 μg/ml fibrinogen for 1 min at 37 °C in the dual-channel aggregometer.

2.5. Preparation of platelet extracts

Platelets subjected to activation or aggregation were lysed by the addition of 0.5 ml of Triton lysis buffer [15 mM HEPES, pH 7.4, containing 1% (v/v) Triton X-100, 10 mM EGTA, 145 mM NaCl, 0.1 mM $MgCl_2$, 1 mM PMSF, 20 μg/ml leupeptin, and 2 mM sodium orthovanadate], and the lysate constantly mixed for 1 h at 4 °C. Triton-soluble platelet fractions were isolated by centrifugation at $15\,000 \times g$, 4 °C for 15 min.

2.6. Immunoprecipitation and Western blot studies of platelet lysates

Following fractionation of Triton-soluble platelet supernatant, lysates were precleared with 50 μl of a 50% slurry of CNBr-activated Sepharose beads (Amersham Pharmacia Biotech AB, Uppsala, Sweden) for 15 min at 4 °C with constant mixing and centrifuged at 4000 rpm for 5 min. Immunoprecipitation (IP) studies from Triton-soluble precleared platelet fractions were performed with 10 μg of normal mouse IgG_1, PECAM-1.3 IgG or anti-CaM IgG_1 for 2 h at 4 °C with constant mixing. A 50% slurry (50 μl) of Protein G–Sepharose beads (Amersham Pharmacia Biotech AB, Uppsala, Sweden) was added and incubated for a further hour at 4 °C with constant mixing. The beads were then washed five times with IP buffer [50 mM Tris, pH 7.4, containing 150 mM NaCl and 1% (v/v) Triton X-100] and centrifuged at 4000 rpm for 5 min. Bound proteins were eluted from the beads by addition of 30 μl SDS reducing buffer and samples were boiled for 10 min. Eluted proteins were resolved on either a 10% or 12.5% SDS–PAGE. For Western blot studies, proteins were transferred to polyvinylidene difluoride (PVDF) membrane by semidry Western blotting. Membranes were blocked by a 1 h incubation at room temperature with blocking buffer [20 mM Tris, pH 7.4, containing 3% (wt/v) bovine serum albumin and 0.05% (v/v) Tween 20] and immunoblotted with anti-human PECAM-1 (SEW16) (10 μg/ml) and anti-CaM (1:2000). Blots were visualised using the appropriate horseradish peroxidase-coupled anti-rabbit or anti-mouse secondary antibody (1:10 000) (Silenus, Hawthorn, Australia). Following washing with Tris-buffered saline [TBS; 20 mM Tris, pH 7.4, containing 150 mM NaCl and 0.05% (v/v) Tween 20], blots were developed with enhanced chemiluminescence detection system according to the manufacturer's instructions (Amersham, Buckinghamshire, UK).

2.7. Surface plasmon resonance studies

Streptavidin-coated BIAcore sensor chips SA (BIAcore AB, Uppsala, Sweden) were conditioned with 1 M NaCl in 50 mM NaOH according to the manufacturer's instructions. All experiments were carried out in HBS buffer (0.01 M HEPES, pH 7.4, containing 0.15 M NaCl, 3 mM EDTA, and 0.005% (v/v) superfactant) with a flow rate of 5 μl/min and a constant temperature of 25 °C. For each series of experiments, 30 μl of biotinylated PECAM-1-(594–604) peptide or biotinylated GPVI (294–309) peptide (100 μg/ml) was injected into the respective chip. The sensor surface was regenerated with 0.1 M HCl following each protein run. For determination of affinity constants for the interaction of bovine CaM with PECAM-1 or GPVI peptides, 30 μl of different concentrations of bovine CaM (131–2630 nM) was injected into the sensor chip. Equilibrium-binding isotherms were evaluated in the BIAcore evaluation program. Data were analysed by linear regression analysis using Graph Pad Prism Version 2.0.

2.8. Calmodulin ELISA

Microtiter plates (Nunc Maxisorb, MEDOS) were coated overnight at 4 °C with 50 μl of bovine CaM diluted in 0.05 M carbonate buffer, pH 9.6, at 10 μg/ml and blocked with 2% (wt/v) bovine serum albumin diluted in PBS for 1 h at 37 °C. Plates were washed with PBS containing 0.05% (v/v) Tween 20 (PBS/Tween), and a dose–response (0–20 μg/ml) of respective biotinylated PECAM-1 peptides (wild-type PECAM-1 594–604, RK → AA and KAK → AAA mutated or scrambled forms of PECAM-1 594–604 peptide) diluted with PBS/Tween and incubated for 2 h at room temperature. The plates were then washed and incubated for 1 h with streptavidin–alkaline phosphatase diluted 1:1000 in PBS/Tween. After additional washings, 50 μl of *p*-nitrophenyl phosphate substrate (Sigma) diluted in diethanolamine buffer at 1 mg/ml was applied. Absorbances were read after 20 min at 405 nm. To

test the effect of CaM inhibitors on the CaM–peptide interaction, parallel assays included a final concentration of 5–1200 μM trifluoperazine (Calbiochem, Richmond, CA).

2.9. Peptide synthesis

Biotinylated synthetic peptides ± Tyr(PO_4) based on cytoplasmic domain sequences of human PECAM-1 were synthesised and prior to cleavage the resin was coupled with 1.5-fold molar excess of NHS-long chain (LC) biotin (Pierce, Rockford, IL) at the N-terminus and purified by reverse-phase high-pressure liquid chromatography (HPLC) to >90% purity. All peptides were characterised by ion spray mass spectroscopy to confirm the expected molecular-mass for both the non-phosphorylated and phosphorylated PECAM-1 cytoplasmic domain peptides. These biotinylated 25-mer and 11-mer peptides were generated by Chiron Mimotopes (Clayton, Vic., Australia). The scrambled PECAM-1 594–604 peptide sequence was KYKARFKLAKA, while RK → AA PECAM-1 594–604 peptide sequence was KAYFLAAAKAK and KAK → AAA PECAM-1 594–604 peptide sequence was KAYFLRKAAAA. All lyophilised biotinylated peptides were prepared as 1 mg/ml solutions and dissolved in 10 mM sodium phosphate and 150 mM NaCl, pH 7.4, containing 0.3% (v/v) dimethyl sulfoxide (DMSO) and stored at 4 °C.

2.10. Peptido-precipitation studies using platelet lysates

Biotinylated synthetic peptides (10 μg/ml) were incubated with 1 ml of 15 000 × *g* Triton-solubilised platelet lysates and incubated overnight at 4 °C with constant mixing. 50 μl of a 50% suspension of streptavidin–agarose beads (Sigma) was added to all tubes and the peptide mixture incubated for a further hour at 4 °C with constant mixing. The beads were then washed five times with IP buffer [50 mM Tris, pH 7.4, containing 150 mM NaCl and 1% (v/v) Triton X-100] and centrifuged at 4000 rpm for 5 min. Bound proteins were eluted from the beads by addition of 30 μl SDS reducing buffer and samples were boiled for 10 min. Eluted proteins were resolved on 10% or 12.5% SDS–PAGE.

2.11. Peptido-precipitation studies using purified bovine calmodulin

Biotinylated synthetic PECAM-1 peptides (10 μg/ml) were incubated with 1 μg of purified bovine CaM dissolved in 1 ml PBS for 1 h at 4 °C with constant mixing. A 50% suspension of streptavidin–agarose beads (50 μl) was added to all tubes and incubated for a further hour at 4 °C with constant mixing. The beads were washed three times with a modified IP buffer (50 mM Tris, pH 7.4, containing 150 mM NaCl) and processed as described for immunoprecipitates.

3. Results

3.1. Constitutive association of calmodulin with PECAM-1 from platelet lysates

Examination of PECAM-1 cytoplasmic domain sequences revealed the presence of a cluster of positively charged amino acids in the juxtamembrane region analogous to CaM-binding sequences in other transmembrane receptors. In order to determine if CaM might be involved in binding to the cytoplasmic domain of PECAM-1, we performed reciprocal co-immunoprecipitation studies of Triton-soluble platelet lysates. In these experiments, PECAM-1 or CaM was immunoprecipitated from lysates of platelets that had been pretreated with iodoacetamide followed by (a) treatment with buffer (resting), (b) thrombin activated but not aggregated, (c) thrombin activated and fully aggregated and (d) treatment with integrin $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ blocker, c7E3 Fab (Reopro) prior to thrombin activation and stirring of platelets. Normal mouse IgG_1 was used as an isotype-matched control. Following IP, the presence of PECAM-1 and/or CaM in reciprocal precipitates was detected by immunoblot analysis. As shown in Fig. 1, PECAM-1 was constitutively associated with CaM under resting conditions and following platelet activation and aggregation (top and second panels). In contrast, CaM was not co-precipitated with the tetraspanin superfamily member, CD151, from resting and activated platelet lysates (Fig. 1, third and bottom panels). Together, these data provide strong evidence that the association of CaM with PECAM-1 occurs in vivo in primary cells such as platelets. This PECAM-1:CaM interaction was not only found in platelets but is also active in other cell types including T cells that express PECAM-1 (data not shown).

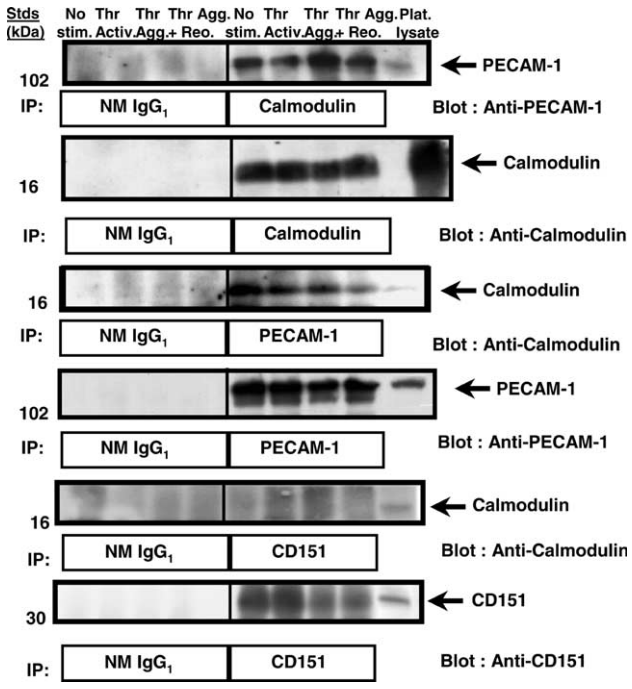

Fig. 1. Interaction of cytosolic CaM with full-length platelet PECAM-1. Platelets (1×10^9/ml) were incubated at 37 °C and stimulated with the following agonists in the presence of 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 100 μg/ml fibrinogen: (1) Buffer (stirred); (2) 1 U/ml thrombin for 5 min without stirring; (3) 1 U/ml thrombin for 5 min (stirred and aggregated); and (4) 1 U/ml thrombin (stirred) in the presence of 20 μg/ml c7E3 Fab (Reopro). Washed platelets were pre-incubated with 20 μg/ml c7E3 Fab (Reopro) for 10 min before thrombin stimulation for 5 min at 37 °C with stirring. Following detergent lysis, IPs were performed using either normal mouse IgG_1 (left top and bottom panels), PECAM-1.3 IgG_1 (right bottom panel) or CaM IgG_1 (right top panel). Bound proteins were resolved on 12.5% SDS–PAGE and analysed by immunoblotting using polyclonal anti-PECAM-1 (SEW16) (top panel), anti-CaM (second and third panel) or anti-CD151 (bottom panel). The migration and specificity of either CaM or PECAM-1 is confirmed by subjecting 5 μg platelet lysate to the immunoblot analysis. Stds, standards. Results are representatives of three separate experiments.

3.2. Direct binding of calmodulin to the cytoplasmic domain of PECAM-1

To address whether CaM binds directly to the cytoplasmic domain of PECAM-1, we synthesised a series of overlapping biotinylated 25-mer peptides corresponding to the entire cytoplasmic domain of human PECAM-1; the C-terminal peptides 684–711, is a 29-mer (Fig. 2A). These biotinylated peptides were mixed with purified bovine CaM and bound protein recovered using streptavidin–agarose beads. Immunoblot analysis using an anti-CaM monoclonal antibody demonstrated that CaM bound only to the PECAM-1-(594–619) C595A peptide (Fig. 2B). Using this assay, it appears that CaM is binding to a region encompassing PECAM-1-(594–

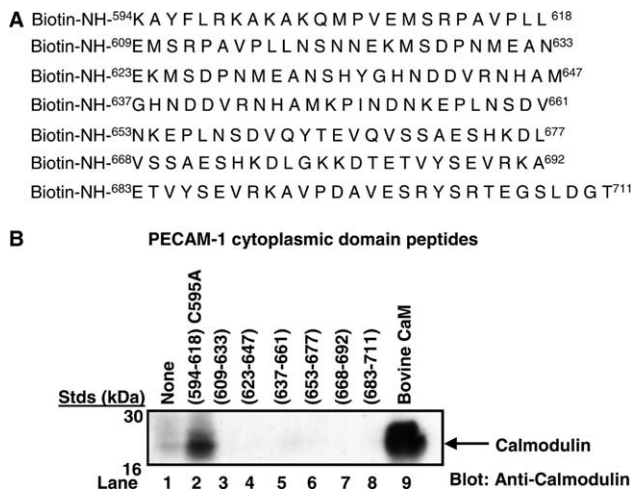

Fig. 2. Association of purified bovine CaM with overlapping PECAM-1 cytoplasmic domain peptides. A. Design of overlapping biotinylated PECAM-1 cytoplasmic domain peptides used in this study. The majority of the peptides were 25-mer in length. The only exception was PECAM-1$^{683–711}$, which was a 29-mer peptide. B. CaM immunoblot of proteins captured by PECAM-1 cytoplasmic domain peptides. 1 μg of purified bovine CaM was incubated with 10 μg of each biotinylated PECAM-1 cytoplasmic domain peptide suspended in PBS. Protein–peptide complexes were captured using streptavidin–agarose beads, separated by SDS–PAGE transferred to a polyvinylidene difluoride (PVDF) membrane, and subjected to immunoblot analysis using a monoclonal antibody directed to CaM. Note that PECAM-1-(594–619) C595A peptide (lane 2) bound CaM. The migration of CaM was also demonstrated by the loading of 0.5 μg purified bovine CaM (lane 9). Stds, standards.

608), as the corresponding PECAM-1-(609–634) peptide failed to bind CaM.

In order to narrow down the sequences required for CaM binding to PECAM-1, we synthesised a matched series of biotinylated 11-amino acid peptides corresponding to a specific region of the PECAM-1 cytoplasmic domain with and without phosphorylation of respective tyrosine residues (Fig. 3A). These biotinylated peptides were mixed with detergent lysates of resting human platelets and bound proteins were recovered with streptavidin–agarose beads. Immunoblot analysis using a specific CaM antibody revealed that CaM bound to PECAM-1-(594–604) C595A and PECAM-1-(594–604) C595A Y(P)596 peptides (Fig. 3B). These results indicate that the cysteine residue at position 595 and tyrosine residue at position 596 are not required for binding CaM with PECAM-1. In addition, the likely sequences involved in CaM binding to PECAM-1 involve residues 594–604.

In order to confirm whether this direct interaction involves a distinct CaM-binding motif between CaM and PECAM-1, we used these biotinylated 11-mer peptides in an in vitro assay. As shown in Fig. 3C, purified bovine CaM associated only with PECAM-1-(594–604) C595A, PECAM-1-(594–604) C595A Y(P)596 and GPVI peptides. In order to exclude the possibility that the interaction was mediated solely based on charged amino acids, we examined PECAM-1-(594–604) mutant RK → AA, KAK → AAA and a scrambled version of the peptide in a CaM-based ELISA assay. As shown in Fig. 4A, only the wild-type (594–604) PECAM-1 peptide showed binding to purified bovine CaM, while an RK → AA mutant, KAK → AAA mutant and a scrambled version of the PE-

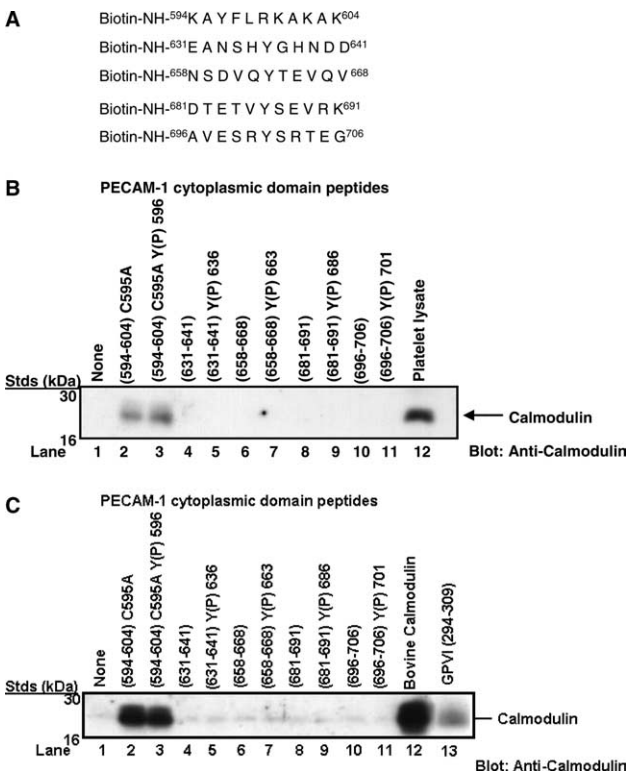

Fig. 3. Association of CaM with PECAM-1 cytoplasmic domain peptides. A. Design of biotinylated PECAM-1 cytoplasmic domain peptides used in this study. An identical set of biotinylated PECAM-1 cytoplasmic domain phosphopeptides (not shown) was prepared using a phosphorylated tyrosine amino acid in the appropriate position. B. CaM immunoblot of proteins captured by PECAM-1 cytoplasmic domain peptides. Protein (1.5 mg) derived from the 15 000 × *g* supernatant of Triton-solubilised resting platelets was incubated with each biotinylated PECAM-1 cytoplasmic domain peptide. Protein–peptide complexes were captured using streptavidin–agarose beads, separated by SDS–PAGE, transferred to PVDF membrane, and subjected to immunoblot analysis using a CaM antibody. Note that PECAM-1-(594–604) C595A and PECAM-1-(594–604) C595A 596 Y(P) peptides (lanes 2 and 3) bound to CaM, indicating that tyrosine phosphorylation of Y596 was not required for the interaction. The migration of CaM and specificity of the immunoblot was demonstrated by subjecting 5 μg of platelet lysate to CaM immunoblot analysis (lane 12). C. CaM immunoblot of proteins captured by PECAM-1 cytoplasmic domain and GPVI peptides. 1 μg of purified bovine CaM was incubated with 10 μg of each biotinylated PECAM-1 cytoplasmic domain peptide suspended in PBS. Protein–peptide complexes were captured using streptavidin–agarose beads, separated by SDS–PAGE, transferred to a PVDF membrane, and subjected to immunoblot analysis using a monoclonal antibody directed to CaM. Note that PECAM-1-(594–604) C595A and PECAM-1-(594–604) C595A 596 Y(P) peptides (lanes 2 and 3) bound to CaM. The migration of CaM was also demonstrated by the loading of 0.5 μg purified bovine CaM (lane 12). Stds, standards, Y(P), phosphotyrosine.

CAM-1 peptide failed to bind to bovine CaM. This interaction between CaM and wild-type (594–604) PECAM-1 peptide could be displaced by titration with a CaM inhibitor, trifluoperazine (Fig. 4B). These CaM inhibitors function by blocking the interaction of CaM with its substrates [30]. This CaM:PECAM-1 peptide interaction was also potentiated by the addition of calcium ions (Fig. 4C). In addition, in peptide-precipitation studies we have also confirmed that the wild-type (594–604) PECAM-1 peptide and GPVI peptide showed binding to purified bovine CaM, while a negative control

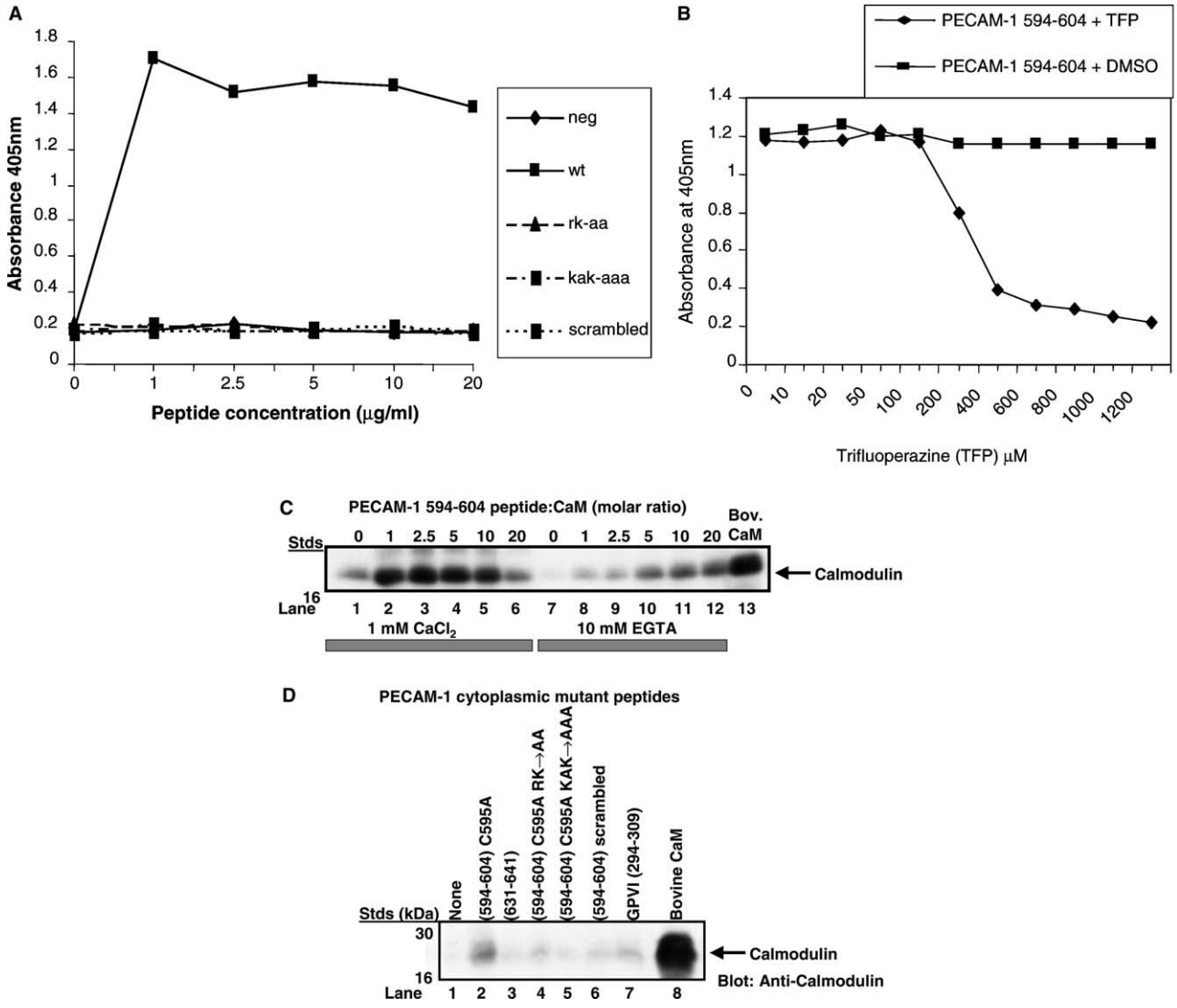

Fig. 4. CaM binds directly to a cytoplasmic-binding motif in PECAM-1. A. Purified bovine CaM was absorbed to the bottom of microtiter wells. Biotinylated 11-mer PECAM-1 peptides (594–604) either as wild-type, RK → AA, KAK → AAA and scrambled versions were titrated at various concentrations, as indicated. Binding of each peptide was visualised by the addition of streptavidin-alkaline phosphatase and *p*-nitrophenyl phosphate substrate. B. The binding of CaM to the PECAM-1 cytoplasmic domain peptide (594–604) is inhibited by trifluoperazine. The biotinylated PECAM-1-(594–604) peptide was incubated with immobilised CaM in the presence and absence of trifluoperazine and titrated at various concentrations, as indicated. C. Different molar ratios of biotinylated PECAM-1 594–604 peptide (0–20) with purified bovine CaM were incubated in the presence of 1 mM $CaCl_2$ or 10 mM EGTA. Protein–peptide complexes were captured using streptavidin–agarose beads, separated by SDS–PAGE, transferred to PVDF membrane, and subjected to CaM immunoblot analysis. The migration of CaM was also demonstrated by the loading of 0.5 μg purified bovine CaM (lane 13). Stds, standards. D. CaM immunoblot of proteins captured by PECAM-1 cytoplasmic domain mutant peptides and GPVI peptide. 1 μg of purified bovine CaM was incubated with 10 μg of each biotinylated PECAM-1 cytoplasmic domain peptide or GPVI peptide suspended in PBS. Protein–peptide complexes were processed as described in the legend for Fig. 3C.

PECAM-1-(631–641) peptide, an RK → AA mutant, KAK → AAA mutant and a scrambled version of the PECAM-1 peptide failed to bind to bovine CaM (Fig. 4D). Together, these data indicate that the PECAM-1 cytoplasmic domain can directly bind CaM that requires a distinct CaM-binding motif in the PECAM-1 cytoplasmic domain.

3.3. Calmodulin inhibitors induce cleavage of PECAM-1 in platelets

Previous studies have shown that CaM inhibitors can induce cleavage of several membrane proteins, including L-selectin, the membrane-bound growth factor precursors pro-transforming growth factor-α, the receptor tyrosine kinase, TrkA and the β-amyloid precursor protein [30]. To investigate whether an analogous mechanism regulates the cleavage of PECAM-1 on the surface of platelets, we first analysed the effects of CaM inhibitors on the cleavage of PECAM-1. In these experiments, resting platelets were stimulated with different CaM inhibitors, W7 and trifluoperazine (TFP) either as a dose–response or time course, then platelets were lysed, electrophoresed and blotted for PECAM-1 antigen. As shown in Fig. 5A–C, the CaM inhibitors, W7 and TFP induced a rapid cleavage of PECAM-1 from a 120 kDa form to a 100 kDa form in both a time- and dose-dependent manner. This 100 kDa form of PECAM-1 lacks its cytoplasmic domain as demonstrated by loss of reactivity with a polyclonal anti-PE-

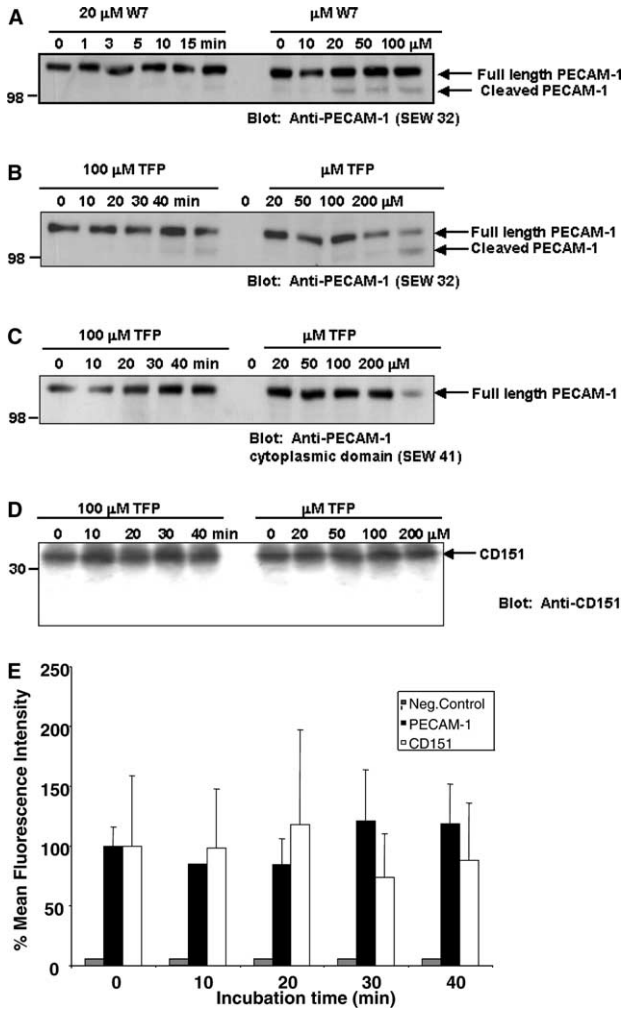

Fig. 5. CaM inhibitors trigger the cleavage of PECAM-1 in platelets. A. Washed human platelets (1×10^9/l) were treated with CaM inhibitor, W7 (20 μM), for a time course (0–15 min) or over a dose-dependent range 0–100 μM W7 for 30 min at 37 °C. W7-treated platelets were lysed with Triton-lysis buffer and 200 μg of lysates was subjected to SDS–PAGE and Western blotting with polyclonal anti-PECAM-1 antibody (SEW32). B. Washed human platelets (1×10^9/l) were treated with CaM inhibitor, TFP (100 μM), for a time course of 0–40 min or over a dose-dependent range 0–200 μM TFP for 30 min at 37 °C. TFP-treated platelets were lysed with Triton X-100 lysis buffer and 200 μg of lysates was subjected to SDS–PAGE and Western blotting with polyclonal anti-PECAM-1 antibody (SEW32). C. Same experimental conditions as A, except the Western blot was probed with a polyclonal anti-PECAM-1 cytoplasmic domain antibody (SEW41). D. Washed human platelets (1×10^9/l) were treated with CaM inhibitor, TFP (100 μM), for a time course (0–40 min) or over a dose-dependent range 0–200 μM TFP for 30 min at 37 °C. TFP-treated platelets were lysed with Triton-lysis buffer and 200 μg of lysates was subjected to SDS–PAGE and Western blotting with monoclonal anti-CD151 antibody (11B1). E. Trifluoperazine does not induce PECAM-1 shedding from the surface of human platelets. 1 ml aliquots of washed human platelets (1×10^9 platelets/ml) were treated with 200 μM TFP at 37 °C over time. PECAM-1 and CD151 expressions on the surface of platelets were monitored over time by flow cytometry. Data are represented as means ± S.E.M. and are representatives of at least three similar experiments.

CAM-1 cytoplasmic domain antibody (Fig. 5C). As we had used relatively high concentrations of CaM inhibitors to induce PECAM-1 cleavage, we wanted to exclude the possibility of non-specific degradation of platelet membrane glycoproteins. Using similar conditions, we were able to show that another platelet membrane glycoprotein, CD151, remains intact in platelets exposed to high concentrations of CaM inhibitors (Fig. 5D). In order to exclude the possibility that cleavage of PECAM-1 leads to receptor shedding from the surface of platelets, we labelled human platelets with FITC-conjugated anti-human PECAM-1 mAbs and then performed a time course (0–4 h) of 400 μM TFP treatment. Treated platelets were then washed and analysed by flow cytometry to monitor PECAM-1 expression over time. Over this time course, we did not observe PECAM-1 receptor shedding from the surface of platelets indicating the possibility that the 100 kDa form is still attached to the platelet membrane and that displacement of CaM:PECAM-1 interaction exposes an intracellular cleavage site (Fig. 5E).

3.4. Calmodulin-binding motif in PECAM-1

CaM-binding domains are characteristically amphipathic helices that are rich in basic residues. The PECAM-1 cytoplasmic domain has a cluster of highly charged basic residues, ^{599}**RKAKAK**^{604} (basic residues are shown in boldface) of which these six amino acid residues are completely conserved across species including human, murine, rat, bovine, porcine and canine (Fig. 6A). The CaM-binding sequence in the cytoplasmic domain of PECAM-1 in the juxtamembrane region (Fig. 6B) is analogous to CaM-binding sites in other receptors including L-selectin, GPIbβ and GPVI (Fig. 7A). A helical model of the PECAM-1 cytoplasmic juxtamembrane region shows a basic face similar to those seen in other CaM-binding proteins including L-selectin, GPIbβ and GPVI receptors (Fig. 7B-C). CBCP (CaM-binding control peptide) is a non-physiological control peptide, which forms an amphipathic helix typical of CaM recognition sequences in other proteins [31].

Predictive algorithms for identifying putative CaM recruitment sites are now available to subclassify CaM-binding domains in receptors and proteins [32,33]. When PECAM-1 594–604 sequence was submitted into these computer

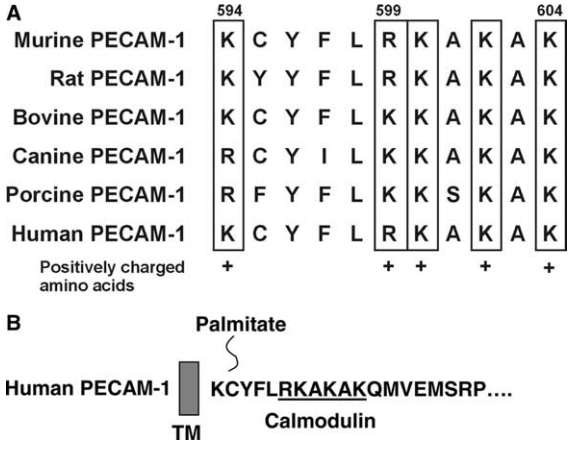

Fig. 6. Evolutionary conservation of the CaM-binding motif in PECAM-1. A. The region of the cytoplasmic domain of PECAM-1 corresponding to amino acids 594–604. Positively charged amino acid residues of human PECAM-1 involved in CaM binding and conserved in other species are boxed. B. Cytoplasmic domain sequence of human PECAM-1 showing sequences that interact with CaM.

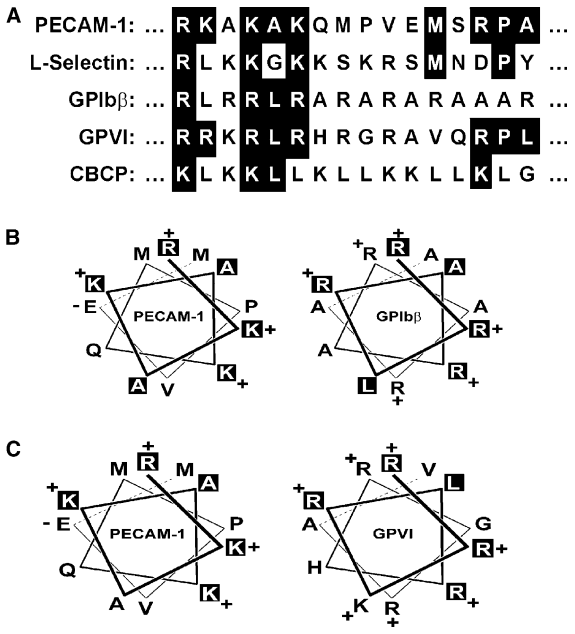

Fig. 7. Sequences within PECAM-1 compared to other CaM-recognition sequences. A. Alignment of the CaM-binding cytoplasmic sequence of PECAM-1 (Arg^{599}–Ala^{614}) with sequences spanning CaM-binding sites in L-selectin (Arg^{356}–Tyr^{372}), GPIbβ (Arg^{149}–Arg^{164}), and GPVI (Arg^{294}–Pro^{309}). Identical residues or conserved substitutions are highlighted. Positively charged lysine and arginine amino acids are in bold. CBCP (CaM-binding control peptide) is a non-physiological control peptide which forms an amphipathic helix typical of CaM recognition sequences in other proteins [31]. B and C. Helical wheel representation of the PECAM-1 sequence (Arg^{599}–Met^{610}) compared with other CaM-binding protein sequences, GPIbβ (Arg^{149}–Leu^{167}) and GPVI (Arg^{294}–Pro^{309}).

Table 1
Equilibrium dissociation constants for the binding of bovine CaM with human PECAM-1 and GPVI peptides

Immobilised biotinylated peptide	Binding protein	K_D values (nM) (Mean ± S.D.)
PECAM-1-(594–604) (^{594}KAYFLRKAKAK^{604})	Bovine CaM	39.93 ± 13.63
GPVI-(294-309) (^{294}RRKRLRHRGRAVQRPL^{309})	Bovine CaM	154.80 ± 63.78

K_D values were obtained from linear regression analysis of equilibrium sensorgrams over a 131–2630 nM concentration range of purified bovine CaM, where saturable binding was observed by surface plasmon resonance analysis. R_{eq} values were derived at the point where equilibrium had occurred after binding of bovine CaM. The mean ± S.D. was derived from three independent experiments.

programmes, it did not conform to a classical CaM-binding domain indicating that this is a novel CaM-binding sequence. In order to test differences in affinity for the PECAM-1 CaM-binding domain with GPVI CaM-binding domain [basic 1-8-(14)], surface plasmon resonance studies were carried out to compare the capacity of PECAM-1 CaM-binding peptide versus GPVI CaM-binding domain with purified bovine CaM. As shown in Table 1, PECAM-1 594–604 sequence showed a threefold higher affinity than GPVI 294–309 sequence for binding purified bovine CaM (40 versus 150 nM). Therefore, based upon these results, PECAM-1 594–604 sequence would appear to represent a new high-affinity CaM-binding domain.

4. Discussion

In this study, we provide several lines of evidence to demonstrate a novel direct interaction between basic/hydrophobic residues in the juxtamembrane region of PECAM-1 with cytosolic CaM in platelets. First, CaM co-immunoprecipitated with PECAM-1 from the Triton-soluble fraction of resting platelets, thrombin-stimulated platelets and aggregated platelets (Fig. 1). Second, peptido-precipitation studies revealed that CaM-binding sites are localised to the juxtamembrane region of PECAM-1 involving a highly charged cluster of basic/hydrophobic amino acids, ^{599}RKAKAK^{604} (Figs. 2 and 3). The PECAM-1 cytoplasmic domain peptides bound CaM from platelet cytosol and this interaction is potentiated by the presence of calcium ions. This interaction could be ablated by mutation of ^{599}RK^{600} to AA, ^{602}KAK^{604} to AAA, or scrambling the CaM-binding motif in PECAM-1 and by displacement using a CaM inhibitor, trifluoperazine (Figs. 4A–D). In contrast, the intact PECAM-1 protein was co-immunoprecipitated with CaM from platelet extracts that contained EGTA (Figs. 1 and 4C). This finding is consistent with previous reports for other CaM-binding peptides such as those derived from glycoprotein GPVI [34]. Treatment of platelets with CaM inhibitors, W7 and TFP-induced cleavage of PECAM-1 but not CD151 in a time- and dose-dependent manner (Fig. 5), indicating that displacement of CaM from the PECAM-1 cytoplasmic domain may expose a cleavage site to allow proteolysis to occur.

The membrane proximal region of PECAM-1 contains a 12-amino acid sequence that is similar to other reported CaM-binding sequences, including L-selectin, glycoprotein GPIbβ, GPV and GPVI [34]. These CaM-binding sequences comprise a basic/hydrophobic composition that has the capacity to form an amphipathic α-helix, that is typically observed in CaM recognition sites (Fig. 7A–C). In PECAM-1, the sequence 599–614 contains a proline residue at position 607 which would not normally favour α-helix formation, although the structure of this region of PECAM-1 has not been determined. A feature observed in these transmembrane receptors with GPIbβ is that the CaM recognition sequence is flanked at the N-terminus by a palmitoylation site and at the C-terminus by a serine residue that is phosphorylated by either protein kinase A (PKA) or protein kinase C (PKC). PECAM-1 contains an unpaired cysteine residue at position 595 that constitutes a potential site for palmitoylation. It also contains a serine residue at amino acid position 611 that is a potential site for phosphorylation by either PKA or PKC. Further studies will be required to more definitively define these sites in PECAM-1. A common mechanism of negatively regulating CaM binding is the induction of serine phosphorylation at sites in or adjacent to the CaM-binding site. There is now accumulating evidence that Ca^{2+}–CaM and PKCs obstruct each others actions by the embedding of PKC phosphorylation sites in CaM crypts or Ca^{2+}–CaM-binding domains of its substrates [35]. These substrates have been shown to contain critical serine residues which upon phosphorylation by phorbol esters or with chemoattractants disrupt CaM binding. These include L-selectin, GPIbβ, MARCKS, neuromodulin and neurogranin [36–41]. Studies in resting human neutrophils have demonstrated that the constitutive association of CaM with the cytoplasmic domain of L-selectin is important for the topography of the extracellular membrane cleavage domain of L-selectin, that normally ren-

ders it resistant to cleavage. L-selectin shedding can be induced by stimulation by phorbol ester, PMA or CaM inhibitors that release CaM from the L-selectin cytoplasmic domain and alter the topography of the cleavage domain to allow proteolytic cleavage to occur.

There is growing evidence that the ectodomains of a number of transmembrane proteins can be shed by proteolytic cleavage through the action of cell surface proteases. Substrates include growth factor receptors, cell adhesion molecules and extracellular matrix proteins [42–46]. Recent studies examining the shedding of an adhesion molecule, L-selectin, have shown that it is regulated by the interaction of CaM with the cytoplasmic domain of L-selectin. Prevention of the CaM:L-selectin interaction by CaM inhibitors or mutation of the CaM-binding site on L-selectin induced L-selectin ectodomain shedding [47]. Recent studies have reported that CaM inhibitors trigger shedding of transmembrane proteins by a novel PKC- and Ca^{2+}-independent mechanism [30]. These studies have indicated that there are different mechanisms for regulated receptor shedding induced by CaM inhibitors, that is independent of CaM:substrate recognition. Further studies will be required to resolve these issues. PECAM-1 receptor shedding has been observed in several clinical settings in vivo. For example, induction of apoptosis results in endothelial cell shedding of the PECAM-1 ectodomain from their surface [48]. PECAM-1 shedding from the neutrophil surface has been reported in viral infections such as respiratory syncytial virus in vivo [49]. Neutrophils may also shed PECAM-1 before or during extravasation into inflamed tissues, as workers have observed that the expression of neutrophil PECAM-1 is downregulated after transendothelial migration into inflamed tissues [50]. However, in contrast to L-selectin, we did not observe PECAM-1 ectodomain shedding following prevention of CaM:PECAM-1 interaction in platelets. An exciting novel finding in this study is the feature of intracellular cleavage of PECAM-1 as a result of displacement of the CaM:PECAM-1 interaction by CaM inhibitors. This cleavage of PECAM-1 appears to produce a cytoplasmic domainless form of PECAM-1. As the cleavage site is only exposed upon displacement of the CaM:PECAM-1 interaction, it will be interesting to examine physiological conditions where this protein:protein interaction is disrupted.

Ca^{2+}–CaM provides a ubiquitous cellular sensor that acts as a co-factor for regulating many enzymes involved in numerous signalling pathways and plays a role in intracellular membrane transport. These include Ral GTPases, RalA and RalB, activating the function of kinases (CaM kinase I and II, myosin light chain kinase), phosphatases (calcineurin), transcription factors, motor proteins, ion channels (plasma membrane Ca^{2+} pump), metabolic enzymes and cytoskeletal components (myosin). Ca^{2+}–CaM has been shown to play a critical role in calcium-dependent exocytosis [51]. CaM also acts as an ion channel subunit for a variety of ion channels, including voltage-gated Ca^{2+} channels, non-selective cation channels, Ca^{2+} or ligand-gated channels, Trp family channels and Ca^{2+}-induced Ca^{2+} release channels from organelles [52]. Recent reports indicate that PECAM-1 regulates a hydrogen peroxide-activated non-selective cation channel in endothelial cells [53]. However, these workers reported that an intact ITIM in PECAM-1 was important for conferring cation activity. Therefore, further studies will be required to resolve the functional importance of the Ca^{2+}–CaM:PECAM-1 interaction in regulating voltage-gated channel activity.

The basic/hydrophobic amino acids involved in CaM binding in the juxtamembrane region of PECAM-1 have been shown to be sufficient to direct efficient localisation of the PECAM-1 molecule to cell–cell borders [29]. This feature suggests that the PECAM-1:CaM interaction may be important in linking this receptor with cytoskeletal components. This hypothesis is further supported by the observation that cytokine stimulation by either IFN-γ or TNF-α leads to redistribution of PECAM-1 away from cell–cell borders [54]. Studies using temperature-sensitive CaM mutations in yeast *Saccharomyces cerevisiae* have demonstrated that CaM is an essential protein for organisation of the actin cytoskeleton, endocytosis, nuclear division and bud emergence [55]. CaM positively regulates phosphatidylinositol (4,5)-bisphosphate synthesis in *S. cerevisiae*, which in turn regulates the interactions between actin and the actin-binding proteins [55]. Rearrangement of the actin cytoskeleton is an essential component that contributes to cell motility and cell shape change that occurs during activation of platelets or neutrophils.

Previous studies have confirmed that another Ig-ITIM superfamily member, CEACAM-1 and CEACAM2, has the capacity to bind CaM involving basic/hydrophobic amino acids in the juxtamembrane region of these molecules [56]. Using dot-blot binding experiments, these workers showed that the binding of CaM to the membrane proximal region of CEACAM caused downregulation of homophilic self-self association of CEACAM. These studies suggested that the CaM:CEACAM interaction was sufficient to induce inside-out signalling events that modulated the homophilic adhesive properties of CEACAM [56].

In summary, this study has identified a direct physical interaction between CaM and PECAM-1 that is detectable in the presence and absence of detergent solubilised platelet membranes and the specific recognition sequences in PECAM-1 required for binding. In addition, release of CaM from the PECAM-1 cytoplasmic domain by CaM inhibitors was associated with exposure of a cleavage domain that allowed proteolytic cleavage to occur. It will be of future interest to further define other mechanisms that regulate PECAM-1 receptor cleavage.

Acknowledgements: We thank Dr. Peter Newman for supply of PECAM-1.3 IgG monoclonal antibody and polyclonal anti-PECAM-1 SEW16 and SEW32 antibodies.

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