Regulation of platelet Rac1 and Cdc42 activation through interaction with calmodulin

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Abstract

Rac1 and Cdc42 are members of the Rho family of small GTPases and have been shown to induce lamellipodia and filopodia formation, respectively. This leads to changes in cytoskeleton organization and as a consequence affects cell migration. In the present work we demonstrate that endogenous Rac1 and Cdc42 interact with calmodulin (CaM) in a Ca^{2+}-dependent fashion. The interaction of Rac1 and Cdc42 with CaM was shown to be direct. This novel interaction was further confirmed in platelets using co-immunoprecipitation studies. Using CaM database analysis and *in vitro* peptide competition assays we have identified a 14 amino acid region in Rac1 that is essential for CaM binding. The scrambled form of the peptide did not bind CaM demonstrating specificity of the predicted CaM binding region in Rac1. A similar region capable of binding CaM exists in Cdc42. Furthermore, using the optimal activation time-point for each GTPase, the role of CaM in the function of Rac1 and Cdc42 was examined. Results demonstrate that in human platelets, thrombin caused maximal activation of Rac1 and Cdc42 at ~60 s and ~25 s respectively. The potent CaM antagonist W7 abolished thrombin-mediated activation of Rac1. However, addition of W7 resulted in the activation of Cdc42 over basal and W7 did not inhibit thrombin-mediated activation of Cdc42. The less potent CaM inhibitor, W5, did not have any effect on Rac1 and Cdc42 activation. The results demonstrate that in platelets, binding of CaM to Rac1 increases its activation while its binding to Cdc42 reduces the activation of this GTPase. This suggests an important role for CaM in coordinating Rac1 and Cdc42 activation and in the regulation of cytoskeleton remodeling.
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1. Introduction

Rac1 and Cdc42 are members of the Rho family of small GTP-binding proteins that have been found to play an essential role in cytoskeleton remodeling [1–3]. Rac1 induces membrane ruffling, chemotaxis, and lamellipodia formation whereas Cdc42 is involved in filopodia formation [4,5]. Rac1 and Cdc42 pro-teins cycle between the active GTP-bound form and inactive GDP-bound form [1,4,6]. This cycling is controlled by guanine nucleotide exchange factors (GEFs). Inactivation is mediated by GTPase-activating proteins (GAPs), which accelerate the rate of hydrolysis of bound GTP to GDP.

In addition to their role in cytoskeleton remodeling, Rac1 and Cdc42 are also involved in regulating gene expression via acti-vation of kinases leading to enhanced activity of stress-activated protein kinases (SPAKs), including JNK and p38 MAP kinases [7–11], Janus kinase (Jaks) and phosphorylation of signal trans-ducers and activators of transcription (STATs) [8,9,12]. The inactivation of Cdc42 using gene-targeted mutations in mouse embryonic stem (ES) and mouse germ line cells has de-monstrated that Cdc42-deficient ES cells exhibit normal proliferation and phosphorylation of mitogen- and stress-activated protein kinases. However, Cdc42 deficiency caused

early embryonic lethality and irregular actin cytoskeletal organization in ES cells [13].

Ca^{2+} ions are an important secondary messenger in a variety of cellular signaling pathways [14]. Majority of the effects of Ca^{2+} in the cell are mediated through proteins that bind calcium. Calmodulin (CaM), a major calcium (Ca^{2+}) sensor, is a highly conserved 17 kDa ubiquitous eukaryotic protein that in response to changes in intracellular Ca^{2+} levels binds to over 100 different targets in the cell including, serine/threonine kinases [15]. CaM has a unique structure consisting of two globular domains each containing a pair of EF-hand motifs that are connected by a central helix [15,16]. The α-helical structure within these two domains opens upon calcium binding resulting in the exposure of the hydrophobic binding sites which then allows other target proteins to bind to CaM [15]. Recently, it has been shown that CaM binds and regulates the function and activity of several small GTPases [17–20].

In the present study, we demonstrate that CaM associates with Rac1 and Cdc42 and this interaction was confirmed in platelets using co-immunoprecipitation studies. Treatment of human platelets with thrombin resulted in the activation of Rac1 and Cdc42. Incubation with W7 prior to the addition of thrombin resulted in inhibition of Rac1 activation. However, incubation of platelets with W7 or W7 plus thrombin resulted in the activation of Cdc42. The results demonstrate a role for CaM in the activation of Rac1 and Cdc42 and thus, in controlling cell morphology and cytoskeleton rearrangement.

2. Materials and methods

2.1. Reagents and plasmids

Sepharose 4B coupled CaM was purchased from Amersham Biosciences. Monoclonal Rac1 and Cdc42 antibodies were obtained from BD Transduction Laboratories. Monoclonal CaM antibody was obtained from Upstate Biotechnologies. Monoclonal Cdc42 immunoprecipitation-positive antibody and protein A/G PLUS agarose beads were purchased from Santa Cruz Biotechnology. Thrombin, glutathione–agarose and actin rabbit polyclonal antibody were purchased from Sigma. Bovine brain CaM, W7•HCl and W5•HCl were purchased from Calbiochem. Control mouse IgG_1 was purchased from MediCrop Inc. Triton X-100, BSA standard and Bio-Rad protein assay dye reagent were purchased from Bio-Rad Laboratories. The predicted Rac1 CaM-binding peptide (NH_2–AVKYLECSALTQRG–COOH) and the scrambled form of this peptide (NH_2–TEGAYACKSLQLVR–COOH) were custom synthesized by Sigma Genosys. All other reagents were from Sigma except where indicated. GST-Rac1 and GST-Cdc42, and GST-Pak1 bacterial expression plasmids were kindly provided by Dr. R. Weinberg and Dr. M. Hoshino respectively.

2.2. Isolation of GST fusion protein

GST and the recombinant GST fusion proteins, GST-Rac1, GST-Cdc42 and GST-Pak1, were expressed in *Escherichia coli* and purified using glutathione–agarose beads as described previously [21]. The purity of the final preparations was assessed using SDS-PAGE.

2.3. Preparation of human platelets

Blood was collected into acid citrate dextrose anti-coagulant (3.8 mM citric acid, 7.5 mM trisodium citrate, 125 mM dextrose; 3.6 mL anti-coagulant/20 mL whole blood) by venipuncture of human volunteers who had denied taking medication known to interfere with platelet function within the previous 2 weeks. Platelet-rich plasma was obtained by centrifugation at 600 ×g for 20 min at room

temperature. The supernatant containing the purified platelets was centrifuged at 1000 ×g for 15 min at room temperature, and the resultant platelet pellet was resuspended in HEPES/Tyrode buffer (10 mM HEPES, pH 7.4, 137 mM NaCl, 2.68 mM KCl, 0.42 mM NaH_2PO_4, 1.7 mM $MgCl_2$ and 5 mM Glucose). The platelets were allowed to equilibrate for 30 min at 37 °C prior to being used in experiment. Platelet studies were approved by the Human Research Ethics Board of the University of Manitoba and informed consent was obtained from all the volunteers.

2.4. CaM-Sepharose pull-down of Rac1 and Cdc42 from platelet lysate

Fresh isolated human platelets were lysed for 30 min at 4 °C in CaM-binding buffer containing 20 mM HEPES, pH 7.4, 200 mM KCl, 1 mM $MgCl_2$, 0.55% Triton X-100 and a protease inhibitor cocktail consisting of 200 mM benzamidine, 2 μg/μl aprotinin, 83.4 mM AEBSF, 5 μg/μl leupeptin, and 0.025 mM pepstatin A. The lysate was centrifuged at 14,000 ×g for 10 min at 4 °C. After centrifugation, platelet supernatant (∼2 mg) was incubated with 100 μl of CaM-Sepharose 4B beads (3:1 ratio of settled gel to buffer) that were previously equilibrated in CaM-binding buffer. Treatment conditions included: buffer, buffer plus 10 mM EGTA, buffer containing 5 mM Ca^{2+}, buffer containing 10 mM EGTA plus variable Ca^{2+} concentrations (1 mM Ca^{2+}, 3 mM Ca^{2+} or 5 mM Ca^{2+}). Blank Sepharose 4B beads (100 μl) were used as control. The reaction mixture was incubated for 2 h at 4 °C. Unbound proteins were removed by washing three times in CaM-binding buffer. Laemmli's sample buffer [22] was added to washed beads and heated at 100 °C for 5 min. Eluted proteins were subjected to 12% SDS-PAGE, electrophoretically transferred to PVDF membranes, and Western blotting was performed with anti-Rac1 or anti-Cdc42 (1 μg/ml) antibodies and horse-radish peroxidase (HRP)-conjugated secondary goat anti-mouse antibody (1:5000 dilution). The antigen–antibody complex was detected using enhanced chemiluminescence (ECL) reagents.

2.5. GST-Rac1 and GST-Cdc42 interaction with pure CaM

Purified GST or GST-Rac1 or GST-Cdc42 bound to GSH-agarose beads were washed with MOPS buffer consisting of 30 mM MOPS, pH 7.2, 1% NP-40 and 100 mM KCl. In addition to buffer alone, 10 mM EGTA, 5 mM Ca^{2+}, or 10 mM EGTA plus different Ca^{2+} concentrations (1 mM Ca^{2+}, 3 mM Ca^{2+}, 5 mM Ca^{2+}) plus 20 μg of pure CaM were added to tubes containing GST or GST-Rac1 or GST-Cdc42 (100 μl) and allowed to shake for 2 h at 4 °C. After incubation, the beads were washed three times with MOPS buffer containing the appropriate concentration of Ca^{2+} and/or EGTA. Laemmli's sample buffer was added to washed beads and heated at 100 °C for 5 min. Western blotting was performed using anti CaM (1 μg/ml) antibody and the antigen–antibody complex was visualized using ECL.

2.6. Coupling of Rac1 CaM-binding and scrambled peptides to CNBr-activated Sepharose beads

The potential CaM-binding and scrambled peptides from Rac1 were coupled to CNBr-activated Sepharose beads according to the manufacturer's instructions. Briefly, peptide (2.5 mg/ml) was dissolved in 1× PBS and mixed 0.5:1 v/v with 0.1–0.3 g of CNBr-activated Sepharose beads that had been washed in 1 mM HCl (15 times bead volume) and a final wash in PBS. The mixture was gently rocked overnight at 4 °C. The beads were pelleted, the supernatant decanted, 1.0 ml of 10 mM Tris–HCl, pH 7.5, was added and the mixture rocked gently at 4 °C for 3 h to block empty sites on the beads. The beads were pelleted and washed in 1.0 ml of 10 mM Tris–HCl, pH 8–9, followed by washing in 1.0 ml of 100 mM acetate buffer, pH 4.0. This washing process was repeated 5 times, after which the beads were recovered and stored in 20% ethanol at 4 °C until needed for experiment. Blocked CNBr-activated Sepharose beads were used as a control in these experiments.

2.7. In vitro binding assays

Rac1 CaM-binding peptide or scrambled version of the peptide coupled to CNBr-activated Sepharose beads was incubated with 20 μg of pure CaM or platelet lysate (∼2 mg) in Buffer B (50 mM Tris–HCl, pH7.5, 10% Glycerol,

200 mM NaCl, and 2 mM MgCl$_2$) and rocked gently for 2 or 24 h at 4 °C. Blocked CNBr-Sepharose beads were used to assess non-specific binding of CaM. After the incubation, beads were collected by centrifugation and unbound proteins removed by washing three times in Buffer B. Laemmli's sample buffer (30 µl) was added to beads and heated at 100 °C for 5 min. Western blotting was performed using anti-CaM (1 µg/ml) antibodies and the antigen–antibody complex was visualized using ECL.

2.8. In vitro competition assay

To assess if the peptide inhibits Rac1 binding to CaM, CaM-Sepharose beads (100 µl) were incubated with different concentrations of free peptide (0 µM, 250 µM, 500 µM, 750 µM, 1000 µM) and rocked gently for 2 h in CaM-binding buffer (20 mM HEPES, pH 7.4, 200 mM KCl, 1 mM MgCl$_2$, 0.55% Triton X-100 and a protease inhibitor cocktail consisting of 200 mM benzamidine, 2 µg/µl aprotinin, 83.4 mM AEBSF, 5 µg/µl leupeptin, and 0.025 mM pepstatin A). The supernatant was decanted and beads were washed once with CaM-binding buffer. Platelets were lysed in CaM-binding buffer containing a protease inhibitor cocktail. The lysate was centrifuged at 14,000 ×g for 10 min. The platelet supernatant (∼2 mg) was added to washed beads and rocked gently for 2 h at 4 °C. Blank Sepharose 4B beads (100 µl) were used as control. Unbound proteins were removed by washing three times in CaM-binding buffer. Laemmli's sample buffer (30 µl) was added to washed beads and heated at 100 °C for 5 min. Western blotting was performed using anti-Rac1 (1 µg/ml) antibody and the antigen–antibody complex was detected using ECL.

2.9. Immunoprecipitation of CaM-Rac1 and CaM-Cdc42 complex from platelets

Fresh human platelets were lysed in Triton lysis buffer (50 mM Tris, pH 7.2, 1% Triton X-100, 10 mM EGTA, 1 mM Na$_2$VO$_4$) containing a protease inhibitor cocktail tablet (Roche Diagnostics). The lysates were clarified by centrifugation (14,000 ×g for 10 min), and the supernatants (∼1 mg) were pre-cleared by adding 30 µl of protein A/G Plus-agarose beads for 2 h at 4 °C. After centrifugation, the supernatant (∼2 mg) was pre-cleared with 4 µg/ml of control mouse IgG$_1$ prior to incubation for 3 h at 4 °C with 4.0 µg/ml of mouse anti-Rac1 or 4.0 µg/ml of mouse anti-Cdc42 monoclonal antibodies or 4 µg/ml of control mouse IgG$_1$. Protein A/G Plus-agarose beads (30 µl) were added to the mixture and rocked at 4 °C for 1 h. The beads were collected by centrifugation (16,000 ×g for 25 s) and washed four times in Triton lysis buffer minus detergent (50 mM Tris, pH 7.2, 10 mM EGTA, and 1 mM Na$_2$VO$_4$). The beads were resuspended in 20 µl of Laemmli's sample buffer and heated for 5 min at 100 °C. Western blotting was performed using anti-CaM (1 µg/ml) antibody and the antigen–antibody complex was detected using ECL.

2.10. Rac1 and Cdc42 activation assay in platelets

Platelets were purified as described previously and challenged with thrombin (1 U/ml) for different periods to determine optimal time-points for Rac1 and Cdc42 activation. Having determined the optimal time-point for Rac1 and Cdc42 activation, platelets were stimulated for the appropriate time using the following conditions: no addition, thrombin (1 U/ml), W7 (150 µM), W7 (150 µM) plus thrombin (1 U/ml), W5 (150 µM), W5 (150 µM) plus thrombin (1 U/ml). Platelets were incubated for 5 min with W7 or W5 prior to addition of thrombin. Platelets were lysed using RIPA buffer (50 mM Tris, pH 7.4, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 500 mM NaCl, 10 mM MgCl$_2$, 2.5 mM EGTA, and a protease inhibitor cocktail) and shaken for 30 min at 4 °C. The lysate was centrifuged at 14,000 ×g at 4 °C. After centrifugation, the supernatant (∼2 mg) was transferred into a separate tube and stored immediately on ice. The amount of activated Rac1 or Cdc42 in platelet lysate was determined using GST-Pak1. Thus, the centrifuged platelet lysate was incubated with GST-Pak1 coupled to GSH-agarose beads (100 µl) at 4 °C for 2 h. After incubation, the beads were washed three times with cold Rac1–Cdc42 washing buffer (50 mM Tris–HCl, pH 7.4, 10 mM MgCl$_2$, 150 mM NaCl, 1% Triton X-100, 5 mM EGTA, and a protease inhibitor cocktail). The final bead pellet was suspended in 30 µl of Laemmli's sample buffer and heated at 100 °C for 5 min. Western blotting was performed

using mouse anti-Rac1 or anti-Cdc42 monoclonal antibodies (1 µg/ml) and the antigen–antibody complex detected using ECL.

2.11. W7 and W5 competition assay

To assess if W7 can inhibit CaM interaction with Rac1 and Cdc42 we employed an *in vitro* assay. Platelets were purified as described above and lysed using RIPA buffer (50 mM Tris, pH 7.4, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 500 mM NaCl, 10 mM MgCl$_2$, 2.5 mM EGTA, and a protease inhibitor cocktail) and shaken for 30 min at 4 °C. The lysate was centrifuged at 14,000 ×g at 4 °C. After centrifugation, the supernatant (∼2 mg) was transferred into a separate tube and stored immediately on ice. The platelet cell lysate was then pre-incubated with W7 (150 µM) or W5 (150 µM) for 15 min. At the end of this incubation. the platelet lysate were further incubated with GST-Rac1 or GST-Cdc42 coupled to GSH-agarose beads (150 µl) at 4 °C for 2 h. Platelet cell lysate incubated with GST-Rac1 (150 µl) or GST-Cdc42 (150 µl) beads without the addition of W7 or W5 were used as control. After incubation, the beads were washed three times with cold RIPA buffer with the protease inhibitor cocktail. The final bead pellet was suspended in 30 µl of Laemmli's sample buffer and heated at 100 °C for 5 min. Western blotting was performed using anti-CaM antibodies (1 µg/ml) and the antigen–antibody complex was detected using ECL.

2.12. Calcium concentration determinations

Calcium concentration in the buffer containing no exogenous calcium or EGTA was determined by measuring total calcium ions using atomic absorption spectrophotometry [18]. Free calcium concentrations in buffers containing 10 mM EGTA and different amounts of exogenous calcium were calculated using the MAXCHELATOR software (www.stanford.edu/~cpatton/maxc.html).

2.13. Protein determination

Total protein concentration in lysates was determined using Bio-Rad dye reagent (Bio-Rad laboratories). The absorbance at 595 (A$_{595}$) was measured and compared to a standard curve of known protein BSA concentrations.

2.14. Statistical analysis

Where required the autoradiograph was scanned and bands quantified using Bio-Rad "Quantity One" program. The output was normalized and descriptive statistical analysis and *t*-tests were performed.

3. Results

3.1. Endogenous Rac1 and Cdc42 interact with CaM-Sepharose

To determine if Rac1/Cdc42 and CaM interact, and assess the Ca^{2+} dependence of such, CaM-Sepharose beads pull down assays were used. Results demonstrated that endogenous Rac1 (Fig. 1A) and Cdc42 (Fig. 1B) interact with CaM. There was no binding of endogenous Rac1 and Cdc42 to blank Sepharose beads (Fig. 1A and B). Quantification of the data confirmed that the interaction between Rac1 and CaM showed Ca^{2+}-dependency since addition of 10 mM EGTA almost eliminated binding and addition of increasing concentration of Ca^{2+} in the presence of 10 mM EGTA significantly enhanced the binding of CaM to Rac1 (Fig. 1A). A similar pattern was observed for the binding of Cdc42 to CaM (Fig. 1B). As indicated in the figure, the free calcium concentration in the various samples was within the physiological range. The lysates in various experimental samples contained equal amount of protein as determined using actin antibody (Fig. 1C).

3.2. Rac1 and Cdc42 bind to pure CaM

To establish if Rac1 and Cdc42 bind directly to CaM, we used GST-Rac1 and GST-Cdc42 fusion proteins and pure bovine brain CaM in *in vitro* binding assays. The expression of both GST-Rac1 and GST-Cdc42 in *E. coli* was assessed prior to performing experiments. The pure recombinant proteins demonstrated the expected molecular mass on SDS-PAGE gel. The recombinant proteins were then used to assess if Rac1 and Cdc42 interact directly with CaM. The results demonstrated
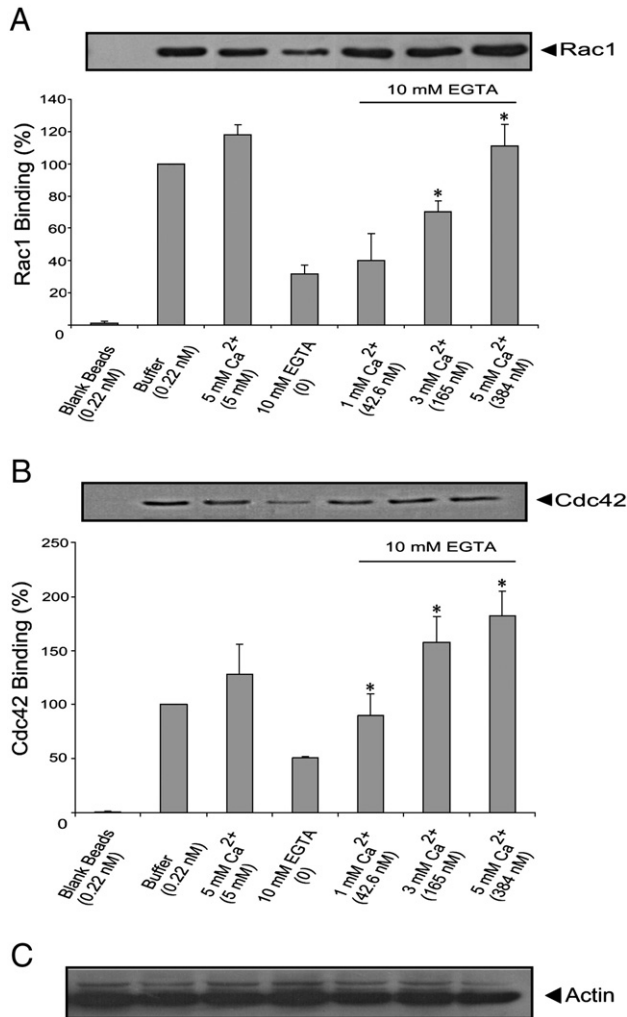

Fig. 1. Endogenous Rac1 and Cdc42 interact with CaM-Sepharose. To establish Rac1 and Cdc42 interaction with CaM and determine its Ca^{2+} dependence, CaM-Sepharose 4B beads (100 μl) were incubated with platelet lysate (∼2 mg) for 2 h at 4 °C in buffer alone, in buffer containing 10 mM EGTA, in buffer containing 5 mM Ca^{2+}, and in buffer containing 10 mM EGTA plus differing Ca^{2+} concentrations (1 mM, 3 mM and 5 mM). The calculated free Ca^{2+} concentration in various treatments are shown in brackets in the figure above. At the end of the incubation, beads were washed and bound proteins eluted using Laemmli's sample buffer. Western blot analysis was carried out using anti-Rac1 (A) or anti-Cdc42 (B) antibodies. Equal amount of protein in the starting platelet lysates was confirmed in (C) by probing for actin. Sepharose 4B beads (100 μl) were used to assess non-specific binding. Quantification was carried out using Bio-Rad "Quantity One" program and the values (mean ± S.E.M.) were considered significantly different when p values were <0.05 compared with the 10 mM EGTA samples (*) ($n \geq 3$).

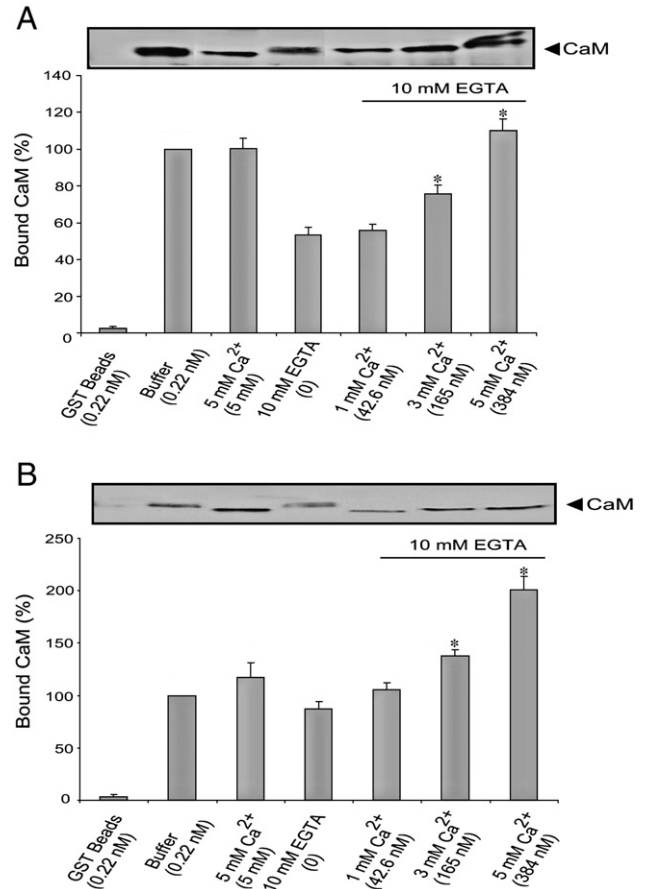Fig. 2. GST-Rac1 and GST-Cdc42 bind to purified bovine brain CaM. GST-Rac1 (A) or GST-Cdc42 (B) beads were incubated with purified CaM (20 μg) in MOPS buffer (30 mM MOPS pH 7.2, 1% NP-40, and 100 mM KCl) and allowed to shake for 2 h at 4 °C. GST beads were used as negative control. The incubation conditions included GST-Rac1 (A) or GST-Cdc42 (B) beads with buffer alone, buffer plus 10 mM EGTA, buffer plus 5 mM Ca^{2+}, buffer with 10 mM EGTA plus varying Ca^{2+} concentrations (1 mM, 3 mM and 5 mM). The calculated free Ca^{2+} concentration in various treatments are shown in brackets in the figure above. At the end of the incubation, beads were washed and bound proteins eluted using Laemmli's sample buffer. Western blot analysis was carried out using anti-CaM monoclonal antibodies. Quantification was carried out using Bio-Rad "Quantity One" program and the values (mean ± S.E.M.) were considered significantly different when p values were <0.05 compared with the 10 mM EGTA samples (*) ($n \geq 3$).

that pure CaM indeed binds specifically to Rac1 and Cdc42 (Fig. 2A and B). Quantification of the data confirmed that CaM binding demonstrated Ca^{2+}-dependency as an increase in free Ca^{2+} concentration (indicated in brackets in the figure) significantly enhanced the binding of CaM to both Rac1 (Fig. 2A) and Cdc42 (Fig. 2B). There was no binding of CaM observed to GSH-agarose beads containing bound GST (Fig. 2A and B). These results confirm that CaM directly interacts with Rac1 and Cdc42.

3.3. Determination of potential CaM-binding site in Rac1

Since we demonstrated that CaM binds directly to Rac1, we next screened the Rac1 sequence for potential CaM-binding domains using the CaM target database [23]. The database revealed a
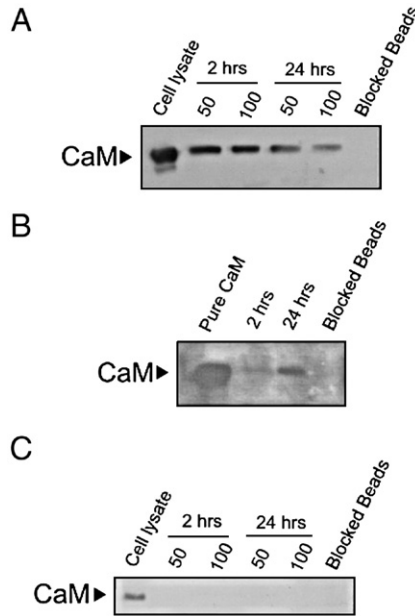
Fig. 3. CaM-binding peptide from Rac1 binds endogenous and pure CaM. CaM-binding peptide (AVKYLECSALTQRG) from Rac1 or scrambled version of the peptide (TEGAYACKSLQLVR) were covalently coupled to CNBr-activated Sepharose beads as described in "Materials and methods". The beads (50 µl or 100 µl) containing Rac1 CaM-binding peptide were incubated with platelet lysate (A) or pure CaM (B) and the scrambled peptide (50 µl or 100 µl) coupled to beads was incubated with platelet lysate (C) for 2 h or 24 h at 4 °C. After washing, proteins bound to beads were separated by SDS-PAGE and Western blotting performed using antibodies against CaM. Blocked empty Sepharose beads (100 µl) were used for determining non-specific binding. A representative autoradiograph is shown above but the experiment was repeated a minimum of three times with consistent results.

3.4. Rac1 CaM-binding peptide interacts with endogenous and pure CaM

To assess whether the synthesized Rac1 peptide [AVKYLEC-SALTQRG] binds CaM, we coupled it to CNBr-activated Sepharose beads. We used a direct approach in which the coupled peptide was added to platelet cell lysate to examine binding to endogenous CaM. The results demonstrate that there was no binding observed when blocked CNBr-Sepharose beads (100 µl)

were used with platelet lysate (Fig. 3A). But binding was observed when coupled peptide was used (Fig. 3A). Higher binding was observed in platelet lysate incubated for 2 h with the peptide (Fig. 3A). The opposite approach in which coupled peptide was
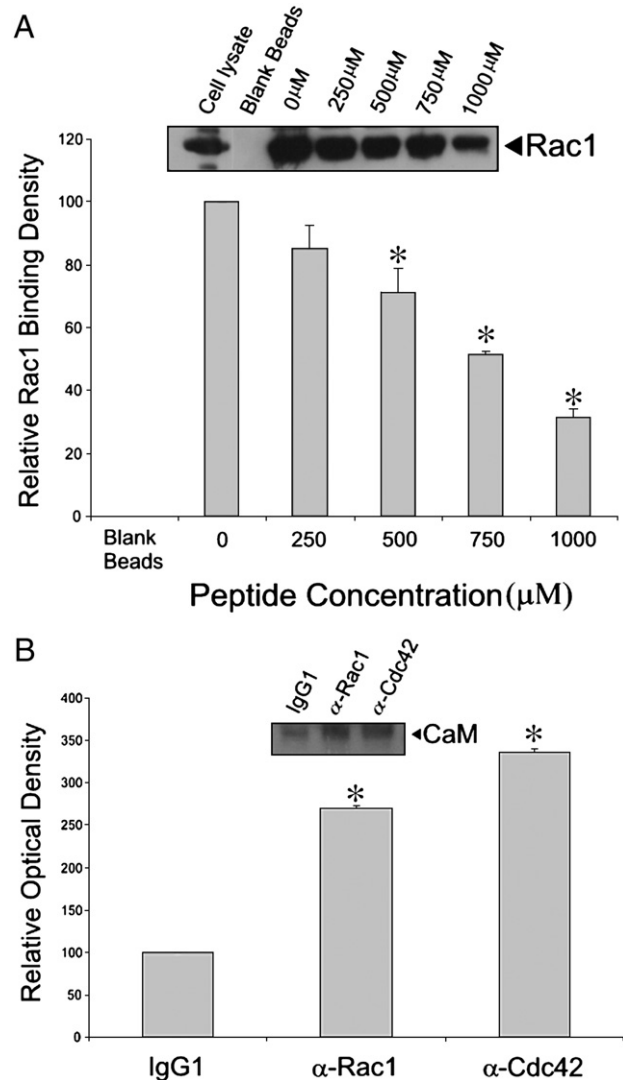

Fig. 4. Free peptide competes with endogenous Rac1 for binding to CaM-Sepharose beads and, CaM-Rac1 and CaM-Cdc42 coprecipitate from platelet cell lysate. CaM-Sepharose beads were used to establish whether Rac1 binding to these beads is inhibited by the Rac1 CaM-binding peptide. (A) CaM-Sepharose beads were incubated with 0, 250, 500, 750, or 1000 µM of peptide for 2 h, washed to remove free peptide and incubated with platelet lysates for 2 h at 4 °C. After washing, proteins bound to beads were separated by SDS-PAGE and Western blotting was performed using antibodies against Rac1. (B) For immunoprecipitation, platelets were lysed using Triton X-100 buffer and prepared for Co-IP as described under "Materials and methods". Briefly, platelet lysates were incubated with anti-Rac1 or anti-Cdc42 monoclonal antibodies or with mouse IgG$_1$ antibody. The antigen–antibody complex was isolated using protein A/G Plus-Agarose beads. The immunoprecipitated proteins were separated by 12% SDS-PAGE and probed with anti-CaM antibodies. In the case of (A) and (B), quantification was carried out using Bio-Rad "Quantity Onee" program. The values (mean±S.E.M.) were considered significantly different when p values were <0.05 compared with (A) Rac1 binding in the absence of exogenous peptide and (B) non-specific CaM in IgG$_1$ immunoprecipitate samples (*) ($n \geq 3$).

region (amino acids 151–164) with CaM-binding potential located in the C-terminal of Rac1. CaM binding to target proteins can be calcium-dependent or -independent [15]. The typical CaM-binding regions vary in length between 16–30 amino acids. They also have tendency to form amphipathic α-helices with a hydrophobic and a basic face [24,25]. The proposed 14 amino acid Rac1 CaM-binding sequence [AVKYLECSALTQRG] is composed of 57.14% hydrophobic and 42.86% hydrophilic residues [25]. This region of Rac1 has an alanine and a glycine residue at each end, which are weak hydrophobic amino acids and are characteristic of CaM-binding motifs. It is not a CaM-binding IQ motif since it does not comply with the IQ motif sequence [IVL]Qxxx[RK]xxxxx[RK] where X is any amino acid. A similar CaM-binding site in Cdc42 [AVKYVECSALTQK] that is composed of 53.85% hydrophobic and 46.15% hydrophilic residues was also identified.

incubated with pure CaM demonstrated that the CaM-binding peptide binds pure CaM (Fig. 3B). The scrambled version of the Rac1 CaM-binding peptide [TEGAYACKSLQLVR] failed to demonstrate any interaction with CaM (Fig. 3C). These results
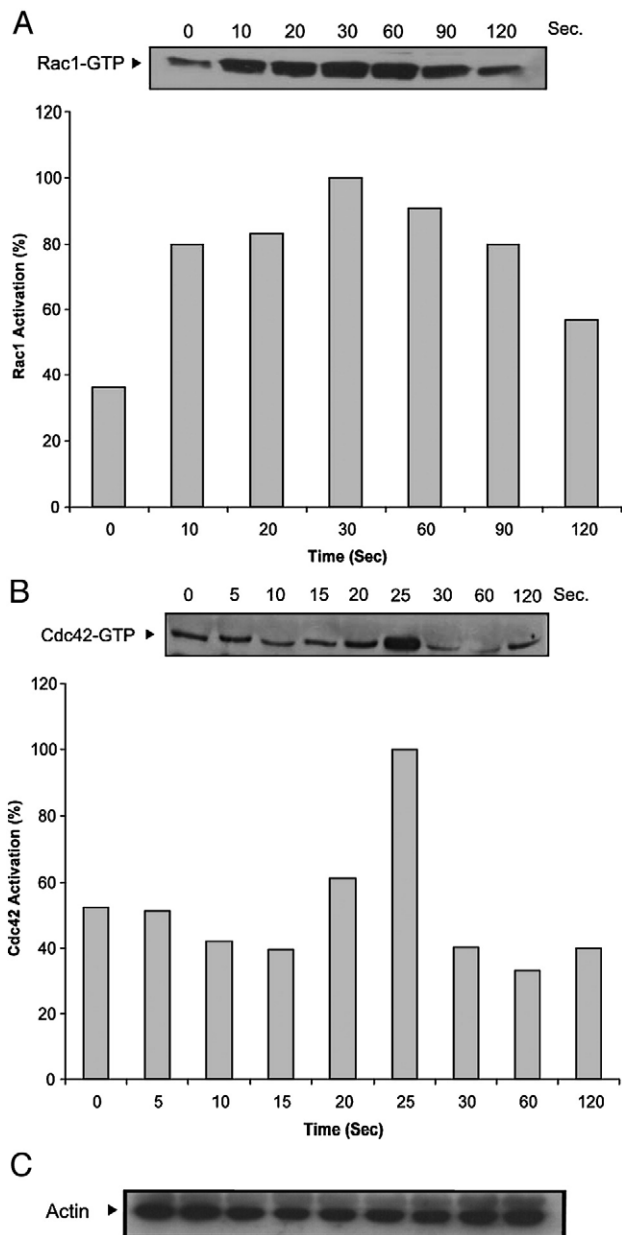

Fig. 5. Time-course for activation of platelet Rac1 and Cdc42. Freshly drawn human platelets were prepared as described under "Materials and methods", divided into 500 μl aliquots and incubated for 30 min at 37 °C prior to thrombin treatment (1 U/ml) for the indicated times. After thrombin treatment, the platelets were lysed in RIPA buffer and incubated with 100 μl of GST-Pak1 beads for 2 h at 4 °C to recover GTP-bound Rac1 or GTP-bound Cdc42. The beads were washed three times with Rac1 or Cdc42 washing buffer. Bound proteins were eluted using Laemmli's sample buffer and Rac1-GTP or Cdc42-GTP was detected by Western blotting using a monoclonal antibody against Rac1 (A) or Cdc42 (B). Part (C) represents confirmation of equal amount of protein in the starting lysates as demonstrated by probing for actin. The blots shown are a representative autoradiograph from experiments repeated two times with consistent results. The data was quantified using Bio-Rad "Quantity One" program. The time-point of maximal activation was taken as 100%.
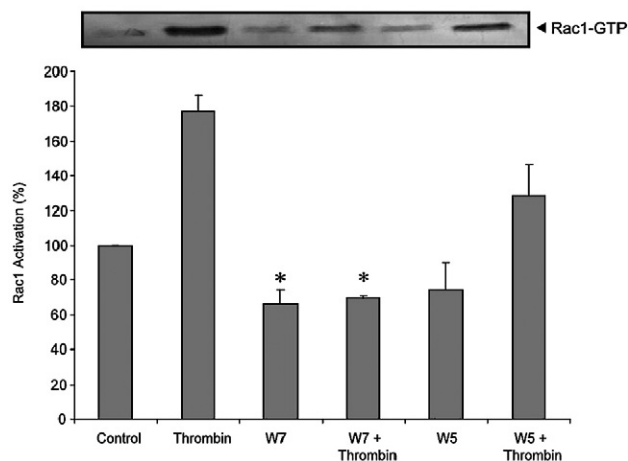

Fig. 6. CaM is required for thrombin-induced activation of platelet Rac1. Freshly drawn human platelets were prepared as described under "Materials and methods", divided into 500 μl aliquots and incubated for 30 min at 37 °C prior to thrombin treatment (1 U/ml). Five minutes before thrombin addition, the indicated aliquots were treated with 150 μM of CaM inhibitor W7 or 150 μM of W5. After thrombin treatment for 1 min, the platelets were lysed in RIPA buffer and incubated with 100 μl of GST-Pak1 beads for 2 h at 4 °C to recover GTP-bound Rac1. After washing, proteins bound to beads were eluted using Laemmli's sample buffer and Rac1-GTP detected by Western blotting using a monoclonal antibody against Rac1. Quantification was carried out using Bio-Rad "Quantity One" program and the values (mean±S.E.M.) were considered significantly different when p values were <0.05 compared with the control or thrombin samples (*) ($n \geq 3$). The experiment was repeated a minimum of three times using blood from three different donors.

confirm that Rac1 CaM-binding peptide interacts directly and specifically with CaM.

3.5. Rac1 CaM-binding peptide competes with Rac1 for binding to CaM-Sepharose

To determine whether the proposed Rac1 CaM-binding peptide competes for binding to CaM, we used CaM-Sepharose beads as a tool. CaM-Sepharose beads were incubated with different concentrations of free peptide for 2 h prior to incubation with platelet lysate (Fig. 4A). Quantification of results demonstrated a significant decrease in Rac1 binding to CaM-Sepharose beads as the concentration of free CaM-binding peptide added was increased (Fig. 4A). Blank Sepharose beads did not interact with Rac1 (Fig. 4A). This confirms that the exogenous Rac1 CaM-binding peptide is interacting with CaM bound to Sepharose beads thus making CaM unavailable for binding to Rac1 from platelet lysate.

3.6. Rac1 and Cdc42 are associated with CaM in platelets

Co-immunoprecipitation experiments were performed to establish whether endogenous Rac1 and Cdc42 are associated with CaM in platelets. CaM coprecipitated from platelet extracts with anti-Rac1 and anti-Cdc42 (Fig. 4B). Quantification of data confirmed that significantly higher level of CaM was immunoprecipitated with anti-Rac1 or anti-Cdc42 antibodies than with control IgG$_1$ (Fig. 4B). The co-immunoprecipitation data provides further evidence that Rac1 and Cdc42 interact with CaM in platelets.
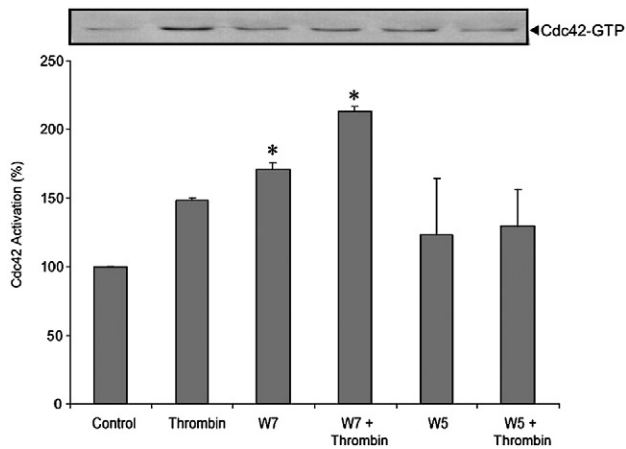
Fig. 7. CaM inhibits platelet Cdc42 GTPase activity. Freshly drawn human platelets were prepared as described under "Materials and methods", divided into 500 µl aliquots and incubated for 30 min at 37 °C prior to thrombin treatment (1 U/ml). Five minutes before thrombin treatment, the indicated aliquots were treated with 150 µM of CaM inhibitor W7 or 150 µM of W5. After thrombin treatment for 25 s, the platelets were lysed in RIPA buffer and incubated with 100 µl of GST-Pak1 beads for 2 h at 4 °C to recover GTP-bound Cdc42. The beads were washed three times with Cdc42 washing buffer. Bound proteins were eluted using Laemmli's sample buffer and Cdc42-GTP detected by Western blotting using a monoclonal antibody against Cdc42. Quantification was carried out using Bio-Rad "Quantity One" program and the values (mean ± S.E.M.) were considered significantly different when p values were <0.05 compared with the control or thrombin samples (*) ($n \geq 3$). The experiment was repeated a minimum of three times using blood from three different donors.

3.7. Role of CaM in Rac1 and Cdc42 function

We have previously shown that CaM is required for the activation of RalA and RalB in human platelets [17]. Thus, to assess a potential role of CaM in the activation of Rac1 and Cdc42, we used human platelets as the model cell system. We used the p-21 activated kinase (Pak) to determine the level of Rac1-GTP and Cdc42-GTP in cells. The p-21 activated kinase (Pak) is a downstream target of Rac1 and Cdc42, and interacts specifically with the GTP-bound form of both Rac1 and Cdc42 [26–28]. This feature of Pak has been exploited to determine the level of active Rac1 and Cdc42 in cells [27–29]. Thus, initially we used GST–Pak1 (expressing the Rac1 and Cdc42 binding domain of Pak1 coupled to the GST protein) fusion protein to confirm that it only interacts with the Rac1-GTP and Cdc42-GTP forms in platelets (results not shown). Expression and purification of recombinant GST–Pak1 in *E. coli* was assessed using SDS-PAGE.

To determine if CaM is involved in activation of Rac1 and Cdc42, we initially confirmed the optimal time-point for Rac1 and Cdc42 activation using the known agonist, thrombin. Thrombin has been shown to activate platelet Rac1 and Cdc42 [27,28]. We used GST–Pak1 fusion protein to pull down the active form of Rac1 and Cdc42 from platelets at different time-points. The optimal time-point for Rac1 and Cdc42 was determined to be 60 s and 25 s, respectively (Fig. 5A and B), and this is consistent with data previously reported by others [27,28]. Equal amounts of protein were present in the various samples (Fig. 5C).

3.8. CaM activates Rac1 and inhibits Cdc42 activation in platelets

Having established the optimal time-point for Rac1 and Cdc42 activation, we next investigated the role of CaM in Rac1 and Cdc42 activation in platelets. Thrombin caused an approximately two fold increase in the activation of Rac1 (Fig. 6). This thrombin-induced activation was abolished in the presence of the CaM inhibitor, W7, but was not affected to the same extent in the presence of the less potent CaM inhibitor, W5 (Fig. 6). In case of Cdc42, thrombin caused a 1.5-fold increase in Cdc42 activation in human platelets (Fig. 7). Addition of W7 alone to platelets resulted in a significant increase in the GTP-bound form of Cdc42 and this was further enhanced in the presence of thrombin plus W7 (Fig. 7). W5 did not result in activation of Cdc42 (Fig. 7). These results suggest that CaM has opposing effects on the activation process of Rac1 and Cdc42. In case of Rac1, CaM is
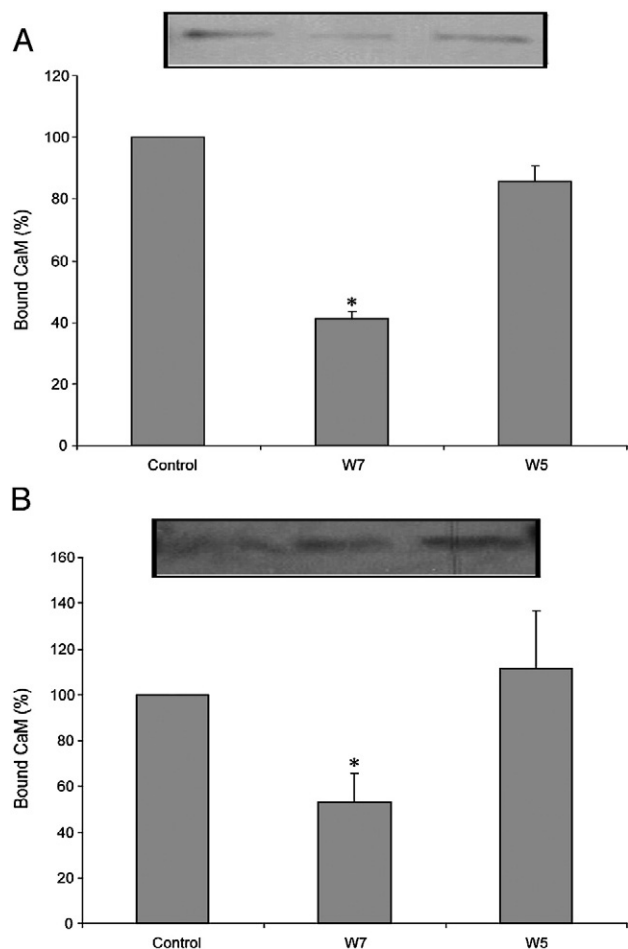

Fig. 8. W7 inhibits CaM binding to Rac1 and Cdc42. Freshly drawn human platelets were prepared and lysed in RIPA buffer as described under "Materials and methods". 500 µl aliquots of the lysate were incubated with 150 µM of CaM inhibitor W7 or 150 µM of W5 for 15 min. After the incubation, (A) GST-Rac1 (150 µl) or (B) GST-Cdc42 beads (150 µl) were added and incubated for 2 h at 4 °C. After centrifugation, the beads were washed three times with RIPA buffer and CaM detected by Western blotting using a monoclonal antibody against CaM. Quantification was carried out using Bio-Rad "Quantity One" program and the values (mean ± S.E.M.) were considered significantly different when p values were <0.05 compared with the control sample (*) ($n \geq 3$).

necessary for the activation. However, CaM is inhibitory to Cdc42 activation since addition of W7 resulted in activation of Cdc42.

To confirm that W7 inhibits interaction of CaM with Rac1 and Cdc42, we used an *in vitro* binding assay. The results demonstrated that W7 significantly inhibits the interaction between CaM and Rac1 (Fig. 8A) and CaM and Cdc42 (Fig. 8B). The less potent CaM inhibitor, W5, did not affect these interactions (Fig. 8A and B).

5. Discussion

In response to an increase in intracellular Ca^{2+} concentration, CaM undergoes a conformational change that allows it to interact with its target proteins and modify their function. Thus, CaM and CaM-binding proteins play an important role in intracellular Ca^{2+} signaling and in various physiological functions including glycogen metabolism, secretion, muscle contraction, actin/cytoskeletal organization and cell division [30]. It has been shown that epidermal growth factor induced Ca^{2+} influx is mediated by Rac proteins [31] and that CaM antagonists block the activation of small GTPases Rac1 and Ras in neutrophils [32]. However, the mechanism by which CaM regulates Rac1 GTPase activity is not understood. In addition, it is not known if other members of the Rho family of small GTPases (for example, Cdc42) are also regulated by CaM. The fact that CaM has been shown to interact and regulate the activation of several small GTPases [17–20] led us to test the hypothesis that CaM interacts and regulates the activity of GTPases belonging to the Rho family.

We have found a novel interaction between the small GTPases Rac1, Cdc42 and CaM. This interaction was found to be direct and occurs at low levels of calcium and is increased at higher calcium concentrations. The free calcium concentration used in our *in vitro* study is in the physiological range suggesting that this interaction is likely to occur in cells. The interaction of CaM with Rac1 or Cdc42 was further confirmed in platelets using co-immunoprecipitation. The results from co-immunoprecipitation studies confirmed that CaM is associated with these GTPases in platelets (Fig. 4B).

CaM has been shown to interact with a variety of small GTPases [17–20]. Thus, CaM binds to RalA and RalB and is necessary for the thrombin-mediated activation of these GTPases in platelets [17]. It has also been shown that CaM can promote GTP-GDP exchange on RalA [33]. CaM also causes dissociation of RalA from synaptic vesicle membranes in a Ca^{2+}-dependent fashion [33]. Similar regulatory mechanisms have been reported for K-RasB [35], Rab3A [34] and Rab3B [18]. The interaction of CaM with its target proteins is predominantly hydrophobic and strong electrostatic interactions [20]. The proposed Rac1 CaM-binding peptide identified by us is located within amino acids 151–164 in the C-terminal of Rac1. This region is composed mostly of hydrophobic amino acid residues and can potentially serve as the CaM-binding site in Rac1. The Rac1 CaM-binding peptide was indeed able to bind endogenous (Fig. 3A) and pure CaM (Fig. 3B) while the scrambled version of the peptide failed to interact with CaM (Fig. 3C). Moreover, the peptide was able to

inhibit binding of Rac1 to CaM (Fig. 4A). Further studies are required to confirm that in the full-length version of Rac1 this region is responsible for binding CaM. A similar CaM-binding region is present in Cdc42 but its ability to interact with CaM has not yet been confirmed.

In platelets, Rac1 and Cdc42 activation occurs after stimulation of G-coupled PAR-1 by thrombin [27]. The optimal time-points for Rac1 and Cdc42 activation by thrombin were re-examined in platelets and determined to be 60 s and 25 s, respectively (Fig. 5A and B). These results are similar to those reported by other investigators previously for optimal platelet Rac1 [27] and Cdc42 [28] activation in response to thrombin. The role of CaM in Rac1 and Cdc42 function was examined using platelet as our model system at the optimal time-point for activation of Rac1 and Cdc42. Previously it has been shown that the CaM inhibitor W7 inhibits the activation of Rac1 in neutrophils [32]. Similar results were obtained in our studies with platelets (Fig. 6). The addition of thrombin after pretreatment with W7 did not reverse the inhibitory effects of W7 on Rac1 activation. This suggests a role for CaM in Rac1 activation. In contrast, amount of active Cdc42 was significantly increased after the addition of W7 and a further increase was observed upon the addition of W7 plus thrombin (Fig. 7). The less potent CaM inhibitor, W5, had little effect on Rac1 and Cdc42 activation (Fig. 6 and 7). This implies that CaM in platelets acts to maintain Cdc42 in the inactive state. A similar type of inhibitory role for CaM has been described in the case of K-RasB [35]. Furthermore, we have shown that in comparison to W5, W7 is much more effective in disrupting CaM interaction with Rac1 and Cdc42 (Fig. 8). The present results suggest that CaM is important in coordinating the activation of Rac1 and Cdc42 in platelets. The reason for the opposite effect of CaM on Rac1 and Cdc42 function is not clear and it is unknown at the present time if a similar mechanism exists in cell types other than platelets. The real-time visualization of Rac1 and Cdc42 activation in platelets or another cell type may help in confirming the role CaM in the activation step of these GTPases.

Rac1 is known to regulate membrane ruffles (lamellapodia) through formation of cortical actin fibers [1,2,5] and, Cdc42 is implicated in filopodia formation and in cell migration [4,5,36]. This type of cytoskeletal rearrangement is the hallmark during platelet activation step. How CaM participates in these pathways through its ability to regulate Rac1 and Cdc42 activation is not clear. However, CaM has been reported to act as a GEF for the Ral GTPase [37]. Thus, it is possible that CaM may play a similar role in controlling Rac1 and Cdc42 activation. It will be important to confirm the precise region within Rac1, Cdc42 that interacts with CaM and, how these novel interactions impact platelet physiology.

In conclusion, we have shown in the present study that the calcium sensor protein, CaM, interacts with and regulates the activity of small GTPases Rac1 and Cdc42. This interaction was found to occur within the physiological range of intracellular Ca^{2+}-concentration. The binding of CaM to Rac1 increases its activation in cells while its binding to Cdc42 inhibits the activation of this GTPase. Further, using a synthetic peptide representing the CaM-binding region in Rac1, we have

identified the site for Rac1–CaM interaction. We have also confirmed this novel interaction in cells using co-immunoprecipitation. The opposing effects of Ca^{2+}/CaM on the activation of Rac1 and Cdc42 suggests a complex mechanism for the coordination of the function of these GTPases in the cell and provides a link between Ca^{2+}/CaM and changes in cell morphology and cytoskeleton dynamics.

Acknowledgements

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