TRPM7, Calcium and the cytoskeleton

Langeslag, Michiel

Chapter V

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Calcium Signaling Regulates Translocation and Activation of Rac

Leo S. Price‡§, Michiel Langeslag‡, Jean Paul ten Klooster¶, Peter L. Hordijk¶, Kees Jalink‡, and John G. Collard‡

From the ‡Division of Cell Biology, The Netherlands Cancer Institute, Plesmanlaan 121, Amsterdam 1066 CX, The Netherlands and ¶Department of Experimental Immunohematology, Sanquin Research at CLB, Plesmanlaan 125, Amsterdam 1066 CX, The Netherlands

Rac is activated in response to various stimuli including growth factors and by adhesion to the extracellular matrix. However, how these stimuli ultimately result in Rac activation is poorly understood. The increase in intracellular calcium $[Ca^{2+}]_i$ represents a ubiquitous second messenger system in cells, linking receptor activation to downstream signaling pathways. Here we show that elevation of $[Ca^{2+}]_i$, either artificially or by thrombin receptor activation, potently induces Rac activation. Lamellipodia formation induced by artificial elevation of $[Ca^{2+}]_i$ is blocked by inhibition of Rac signaling, indicating that calcium-induced cytoskeletal changes are controlled by the activation of Rac. Calcium-dependent Rac activation was dependent on the activation of a conventional protein kinase C. Furthermore, both increased $[Ca^{2+}]_i$ and protein kinase C activation induce phosphorylation of RhoGDIα and induce the translocation of cytosolic Rac to the plasma membrane. Intracellular calcium signaling may thus contribute to the intracellular localization and activation of Rac to regulate the cytoskeletal changes in response to receptor stimulation.

Introduction

The Rho family of small GTPases, including Rho, Rac, and Cdc42 isoforms, regulates different aspects of cytoskeletal organization, which are coordinated in the process of cell migration (1). Of these, Rac is involved in the protrusion of lamellipodia, which occur principally at the leading edge of migrating cells but also emerge from around newly adherent cells to mediate cell spreading (2, 3). Rac also regulates gene transcription, cell cycle progression, and transformation *in vitro* (4–6) and is implicated in tumor initiation and progression *in vivo* (7). Rac is activated in response to various stimuli, including growth factors and adhesion to the extracellular matrix. However, how these stimuli ultimately result in Rac activation is poorly understood. The principal regulators of Rac activation are the guanine nucleotide exchange factors (GEFs)1 and GTPase activating proteins. GEFs induce activation by exchanging GDP for GTP, whereas GTPase activating proteins enhance the intrinsic rate of hydrolysis of bound GTP to GDP, resulting in inactivation. In cells, Rac exists predominantly in its inactive GDP-bound form in a complex with RhoGDI (8). RhoGDI binds and masks the hydrophobic C-terminal region of Rac, the same region that is responsible for targeting Rac to the plasma membrane (9). Thus RhoGDI maintains Rac in the cytoplasm and must dissociate to allow Rac to translocate to the membrane and interact with membrane-associated activators (10–12). It was shown recently (13, 14) that integrin signals disrupt the Rac-RhoGDI interaction, enabling Rac to target to regions of cell-matrix interaction and activate an adhesion-dependent signaling pathway. Thus appropriate localization, as well as activation, is necessary for Rac to carry out its functions. Increased intracellular calcium $[Ca^{2+}]_i$ represents a ubiquitous second messenger system in cells, linking receptor activation to downstream signaling pathways. Previous studies (15–18) have described relationships between intracellular calcium and the activation and function of Rho family GTPases in processes including muscle contraction and the exocytic response. Intracellular calcium is also required for thrombin- and collagen-induced Rac activation in platelets (19). In neutrophils, however, chemoattractant-induced Rac activation is independent of intracellular calcium (20), suggesting that the relationship between calcium and Rac signaling is dependent on the cell type and/or the growth factor receptor involved. Ras-GRF 1 and 2, exchange factors

specific for both Ras and Rac (21, 22), harbor a calcium-calmodulin binding site (23) whereas the Rac exchange factor, Tiam1, is phosphorylated by calcium-calmodulin-dependent protein kinase II, which leads to increased nucleotide exchange on Rac (24). These findings suggest that nucleotide exchange on Rac may be regulated by changes in intracellular calcium. Various studies have implicated protein kinase C (PKC) in the activation of Rac. In Swiss 3T3 cells, phorbol ester treatment induces membrane ruffling, which is indicative of Rac activation (2), whereas PKC is required for PDGF-induce Rac activation in NIH 3T3 cells (25). However, how PKC affects Rac activity is unclear. Here we have examined the effect of intracellular calcium transients on Rac signaling. We find that intracellular calcium transients induce the membrane translocation and activation of Rac. We propose an additional mechanism of regulation of Rac by calcium whereby calcium induces a PKC-dependent disruption of the Rac-Rho GDI complex. This promotes the translocation of Rac to the plasma membrane where it can be activated by membrane-associated or membrane-translocated guanine nucleotide exchange factors.

Experimental Procedures

Materials

To generate the biotinylated CRIB peptide the amino acid sequence n-KERPEISLPSDFEH-TIHVGFDAVTGEFTGMPEQWARLLQTSNIT-c was used and biotinylated during synthesis at the N-terminus. The TAT-CRIB peptide contained the additional N-terminal sequence GCGYGRKK-RRQRRR and was not biotinylated. Thrombin related peptide (TRP) was synthesized as described previously (26). Fura red, Oregon green, BAPTA-AM, and Alexa 568-phalloidin were from Molecular Probes. Thapsigargin, ionomycin, GF109203X (Gö 6850), U73122, calmidazolium chloride, and KN-93 were from Calbiochem. Streptavidin-agarose and PMA were from Sigma, and cytochalasin B was from Roche Applied Science.

Cells and Generation of Stable Cell Lines by Retroviral Transduction

PC3 human prostate carcinoma cells (27) were cultured in DMEM supplemented with 10% fetal calf serum in a humidified incubator at 37 °C and with 5% CO2. NIH 3T3-Tiam1 cells (28) were cultured in DMEM + 10% bovine calf serum. Cells were seeded in tissue culture dishes 24 h before use to obtain a final density of ~70% confluence. For microscopy, cells were seeded at lower density on glass coverslips and grown for 24 h. PC3 cells expressing EGFP-Rac1 were generated by infection with a retrovirus containing EGFP-Rac1 (29, 30). Western blotting showed that EGFP-Rac1 was expressed at approximately the same level as endogenous Rac (not shown). EGFP-Rac1 was also functionally active as determined by Rac activation assays using thapsigargin as a stimulus (not shown).

GTPase Activity Assays

Rac activity was assayed essentially as described previously (28), with the exception that instead of GST-Pak-CRIB a biotinylated peptide corresponding to the CRIB domain of Pak (see above) was used to precipitate active Rac. Briefly, after treatment of cells with the relevant inhibitors and stimuli, cells were washed and then lysed with a 1% Nonidet P-40 buffer containing 2 μg/ml CRIB peptide. Cell lysates were cleared by centrifugation, and active Rac-CRIB complexes were precipitated with streptavidin-agarose and solubilized in SDS sample buffer. Rac was detected following Western blotting with anti-Rac antibodies (clone 23A8; Upstate Biotechnology, Inc.).

Live Cell Imaging of Calcium, PLC Activation, and GFP-tagged Rac

Changes in cytosolic Ca^{2+} in PC3 cells were monitored ratiometrically by simultaneous confocal imaging of the Ca^{2+} indicators Oregon green 488, BAPTA-1AM, and Fura red-AM (31). Cells were loaded for 30 min by incubation in DMEM with 10 μM of the AM-esters of these dyes in the presence of pluronic and incubated in fresh DMEM for 15 min prior to the experiment. Excitation was at 488 nm, and the emission was detected using a 522 ± 17-nm bandpass filter (green) and a 585-nm longpass filter (red). Measurements were performed at 37 °C in a buffer containing, in mM, 140 NaCl, 23 $NaHCO_3$, 5 KCl, 2 $MgCl_2$, 1$CaCl_2$, 10 HEPES, and 10 glucose under 5% CO_2. All measurements were calibrated using ionomycin. For simultaneous detection of phospholipase C activation and $[Ca^{2+}]_i$, PC3 cells

were transfected overnight with pcDNA3-EGFP-PH (32) and subsequently loaded with Fura red-AM and imaged as described above. PLC activation was visualized as translocation of the GFP-tagged, phosphatidylinositol 4,5-biphosphate-binding PH domain from the membrane to the cytosol and quantitated by taking the ratio of membrane to cytosolic fluorescence as described (32). To determine the membrane association of GFP-tagged Rac1, medial sections ~2 μm above the plane of the coverslip were imaged by confocal microscopy. These were captured at 10-s intervals and stored on disk for offline analysis. The mean fluorescence intensity at the membrane and at the cytosol was determined, and the ratio of those was plotted *versus* time, essentially as described (32).

Immunofluorescence Microscopy

PC3 cells or NIH 3T3 cells expressing Tiam1 were seeded on glass coverslips 24 h before use. Following treatment/stimulation, cells were fixed with 3.7% formaldehyde for 10 min and then permeabilized with 0.2% Triton X-100 for 5 min. Filamentous actin was labeled with 0.2 μM Alexa 568-phalloidin (Molecular Probes) for 30 min. Tiam1 was visualized with a polyclonal antibody (33). Where cells were treated with TAT-CRIB peptide, peptide (0.2 mg/ml) was added for 15 min prior to stimulation.

Calcium-dependent Phosphorylation by PKC

To visualize calcium-dependent phosphor-rylation of cellular proteins, PC3 cells were lysed in buffer containing 0.5% Nonidet P-40, and lysates cleared by centrifugation and resolved by SDS-PAGE. Cellular proteins were resolved by SDS-PAGE transferred to nitrocellulose and probed with Phospho(ser)-PKC substrate antibody (Cell Signaling Technology). GDI phosphorylation was detected as described previously (34). Briefly, PC3 cells were starved of phosphate for 2 h and then metabolically labeled with 0.5 mCi/ml [32P]orthophosphate for 2 h. Cells were treated with or without GF109203X and stimulated with thapsigargin (1 μM) or PMA (100 nM). Cells were washed in cold phosphate-buffered saline and lysed (0.5% Nonidet P-40, 20 mM Tris, pH 7.6, 250 mM NaCl, 5 mM EDTA, 3 mM EGTA, 20 mM $NaPO_4$, 3 mM β-glycerophosphate, 1 mM $NaVO_4$, 100 nM calyculin A, 10 mM NaF, and a protease inhibitor mixture). RhoGDI was precipitated with polyclonal anti-RhoGDIα (Santa Cruz Biotechnology, Inc.) and protein G-Sepharose. Precipitated RhoGDI and RhoGDI from total cell lysates was resolved by SDS-PAGE and Western blots probed with a monoclonal anti-RhoGDIα antibody (Transduction Laboratories).

Cell Fractionation

After treatment/stimulation, PC3 cells in 2-cm dishes were washed once with ice-cold phosphate-buffered saline and then washed again with permeabilization buffer (20 mM Na-PIPES, 137 mM NaCl, 2mM $MgCl_2$, 2.7mM KCl, 0.05% bovine serum albumin). Cells were then permeabilized with 25 μM digitonin in permeabilization buffer for 20 min to allow leakage of cytosolic proteins. Maximal leakage of Rac and complete leakage of the cytosolic markers mitogen-activated protein kinase and RhoGDIα was found to take place within 10 min. No E-cadherin, chosen as a representative transmembrane protein, was extracted by digitonin treatment (not shown). The cytosol-depleted cells were then washed twice with digitonin-containing buffer and lysed with radioimmune precipitation assay buffer. Lysates were cleared by centrifugation at 4 °C for 5 min at 15,000 × *g*, and 5× SDS sample buffer was added to the supernatants. Proteins were resolved on 4–20% gradient gels (Novex), and following Western blotting, membranes were cut and probed with antibodies against Rac, anti-PKCα (Transduction Laboratories), and anti-pan-cadherin (Sigma) for normalization of (membrane) protein loading.

Results

Intracellular Calcium and Receptor-mediated Rac Activation

To study the relationship between intracellular calcium signaling and Rac function, we stimulated PC3 cells with the thrombin receptor agonist TRP and measured the effects on intracellular calcium concentration ($[Ca^{2+}]_i$) and on activation of Rac. Stimulation of the thrombin receptor with TRP results in release of calcium from inositol 1,4,5-trisphosphate-sensitive intracellular stores. As expected, stimulation of PC3 cells with TRP induced a rapid and transient increase in intracellular calcium $[Ca^{2+}]_i$ (Fig. 1*A*). To assay Rac activity, we used a modified Rac

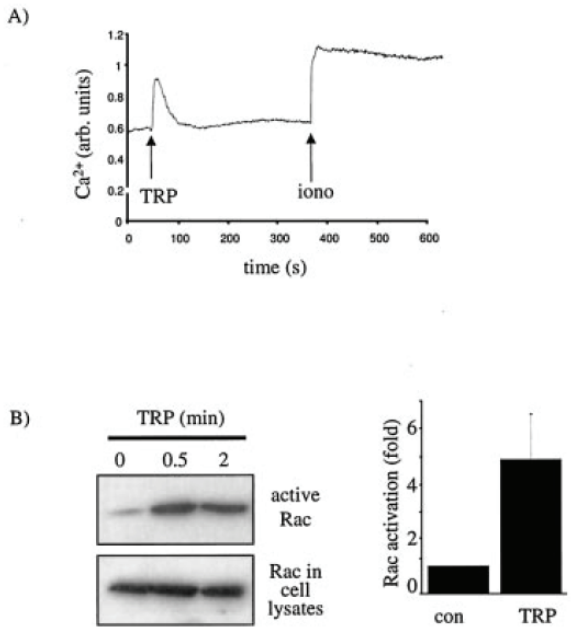

FIG. 1. **Thrombin-related peptide induces Rac activation and increased intracellular calcium.** *A*, TRP (12.5 μM) induces an increase in intracellular calcium in PC3 cells. Intracellular calcium levels were detected by ratiometric imaging of Oregon green and Fura red calcium indicators. The trace represents an average of six individual cells captured from one image field. A representative trace from a single cell is shown and is representative of at least 10 quantifications and three independent experiments. *iono*, ionomycin. *B*, Rac activation in PC3 cells following stimulation with 12.5 mM TRP. Active Rac was precipitated with biotin-CRIB peptide and detected following Western blotting with anti-Rac antibodies (as described under "Experimental Procedures"). Rac (both active and inactive) present in whole cell lysates are shown in the *lower panel* to demonstrate equal amounts of protein in samples. -Fold induction of Rac activation after 1 min of TRP stimulation is shown ($n = 6 \pm$ S.D.; $p = 0.002$). *con*, control.

pull-down assay, which utilizes a synthetic biotinylated peptide corresponding to a region of the Rac-interacting (CRIB) domain of the Rac effector, Pak (see "Experimental Procedures"). This region specifically binds to Rac when it is in its active conformation. The biotinylated CRIB peptide works equally efficiently as the GST-Pak fusion protein used in earlier studies (35, 36) but is more stable and less susceptible to batch-to-batch variation. Stimulating the thrombin receptor with TRP induced a rapid activation of Rac in PC3 cells (Fig. 1*B*). Rac activation was transient, returning to baseline levels after ~10 min (not shown). Treatment with TRP also induced extensive lamellipodia formation and membrane ruffling (Fig. 2*A*, *upper panels*), which is consistent with an increase in Rac-GTP levels.

To examine whether a causal relationship exists between increased $[Ca^{2+}]_i$ and activation of Rac and lamellipodia formation by TRP, we first examined the effects of calcium chelation on TRP-induced morphological changes. Pre-treatment of PC3 cells with the membrane-permeable calcium chelator, BAPTA-AM, strongly inhibited TRP-induced extension of lamellipodia (Fig. 2*A*), suggesting that lamellipodia formation was calcium-dependent. BAPTA-AM also inhibited TRP-induced Rac activation (Fig. 2*B*), suggesting that increased $[Ca^{2+}]_i$ was required for Rac activation. To ensure that the effects of BAPTA-AM were not because of disrupted receptor signaling, we examined the effect of BAPTA-AM on TRP-induced PLC activation. For this, PC3 cells were transfected with a GFP chimera of the PH domain of PLCδ1, which translocates from plasma membrane to cytosol upon activation with TRP (37). Confocal imaging of living cells showed that TRP induced the translocation of GFP-PH from membrane to cytosol, confirming previous findings that thrombin receptor activation leads to the activation of PLC. TRP induced GFP-PH translocation equally well in the presence or absence of BAPTA-AM (Fig. 2*C*, *lower traces*). However, concomitant measurement of $[Ca^{2+}]_i$ confirmed that BAPTA-AM treatment effectively blocked TRP-induced calcium transients (*upper traces*). This demonstrates that thrombin receptor-Gq-PLC signaling, which ultimately leads to release of intracellular calcium, is not disrupted by BAPTA-AM treatment. From these data we conclude that intracellular calcium is required for thrombin receptor-mediated Rac activation and lamellipodia formation.

Increased $[Ca^{2+}]_i$ Is Sufficient to Activate Rac

To examine further the dependence of Rac activation on calcium signaling, we used pharmacological modulators of $[Ca^{2+}]_i$. Treatment of PC3 cells with thapsigargin, which liberates calcium from intracellular stores by blocking re-uptake from the cytosol by Ca^{2+}-ATPases, led to a transient elevation of $[Ca^{2+}]_i$ that remained somewhat above baseline levels (Fig. 3*A*). Analysis of the time course of Rac activation showed that thapsigargin treatment induced a strong activation of Rac that was maximal at ~5 min (Fig. 3*B*). Similarly, treatment of cells with

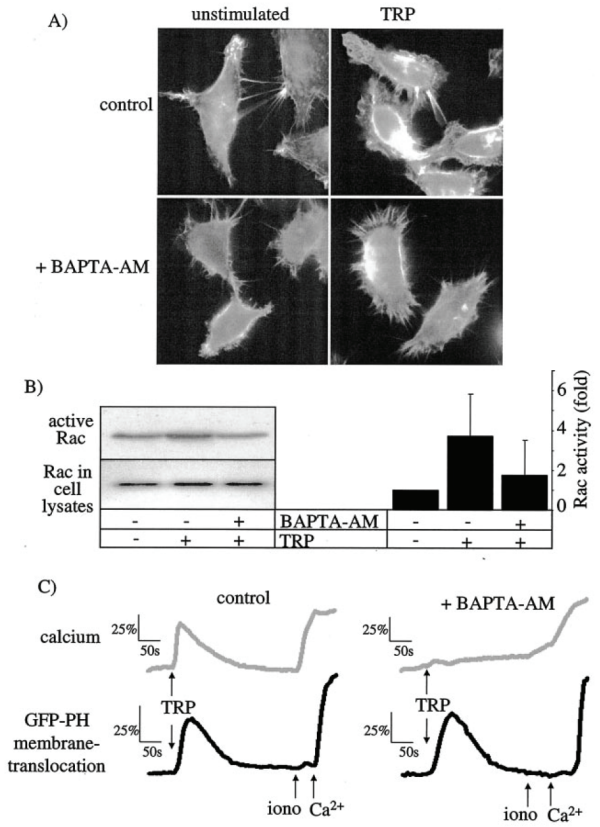

FIG. 2. **Intracellular calcium is required for thrombin receptor dependent Rac activation.** *A*, chelation of calcium with BAPTA-AM inhibits TRP-induced lamellipodia formation. BAPTA-AM (15 μM) was added to PC3 cells on glass coverslips for 10 min before stimulation with TRP for 5 min. Cells were then fixed, and F-actin was stained with rhodamine-phalloidin as described under "Experimental Procedures." *B*, calcium chelation inhibits thrombin peptide-induced Rac activity. To chelate intracellular calcium, cells were treated with 15 μM BAPTA-AM for 10 min. Cells were then stimulated with 12.5 μM TRP for 5 min, and Rac activity was analyzed by pull-down assays. Average -fold induction of Rac activation +S.D. is also shown (n = 4, BAPTA-AM significantly inhibited TRP-induced Rac activation; p = 0.04). *C*, calcium chelation does not disrupt receptor-phospholipase C signaling. Cells transiently expressing a GFP chimera of the PH domain of phospholipase Cδ were pre-treated with and without BAPTA-AM as above and stimulated with 12.5 μM TRP. Calcium traces (*upper graphs*) show the intracellular calcium concentration as reflected by changes in Fura-red intensity. GFP-PH relocalization from membrane to cytosol (*lower graphs*) reflects activation of PLC and was imaged simultaneously with calcium in the same cell. BAPTA-AM abolishes the TRP-induced calcium transient (*upper right trace*) but not translocation of GFP-PH (*lower right trace*). At the end of the experiment, ionomycin (*iono*) and excess extracellular calcium were added to the medium to give maximal (100%) responses. The *scale bar* indicates percent change of basal fluorescence.

$[Ca^{2+}]_i$ because of influx of extracellular calcium, also induced rapid activation of Rac (Fig. 3, *C* and *D*). Interestingly, we observed consistently that the kinetics of calcium elevation appeared to correlate with that of Rac activation; thus, the prolonged elevation of $[Ca^{2+}]_i$ induced by thapsigargin and ionomycin correlated with prolonged elevation of Rac activity, whereas TRP stimulation produced more transient increases in $[Ca^{2+}]_i$ and Rac activation. Activation of Rac was not because of calcium-induced changes in spreading or other actin-dependent changes, because thapsigargin-induced Rac activity was not inhibited by cytochalasin treatment (not shown). From these results we conclude that elevation of intracellular calcium is sufficient to activate Rac in the absence of receptor stimulation.

Our results also suggest that intracellular calcium mediates TRP-induced activation of Rac via the classical Gq-PLC-inositol 1,4,5-trisphosphate pathway. To test this hypothesis, we examined the effects of PLC inhibition, which is predicted to inhibit receptor-induced intracellular calcium increase but not calcium transients induced directly by thapsigargin. Pre-treatment of cells with the PLC inhibitor U73122 (38, 39) inhibited TRP-induced Rac activation but not thapsigargin-induced Rac activation (Fig. 3*E*). Although we can not exclude the possibility that U73122 inhibited TRP-induced Rac activation non-specifically, our results are consistent with the hypothesis that TRP-induced Rac activation is mediated by PLC-$[Ca^{2+}]_i$ signaling and that direct elevation of $[Ca^{2+}]_i$ can bypass the requirement for receptor-PLC signaling.

We also examined the effects of increased $[Ca^{2+}]_i$ on Rac activation in other cell types. In addition to PC3 cells, thapsigargin also induced Rac activation in T47D mammary carcinoma cells and Madin-Darby canine kidney cells but not in NIH 3T3 fibroblasts or lymphocytes (data not shown). This suggests that Ca^{2+}-dependent Rac activation is cell type-specific and may be restricted to cells of epithelial origin.

Calcium-induced Cytoskeletal Changes Are Mediated by Rac

Plasmid-based expression of the CRIB domain of Pak has been used previously (3, 40) to inhibit the signaling of Rac to endogenous CRIB-containing targets. To examine the role of Rac in cell responses, we inhibited Rac signaling using a peptide corresponding to the CRIB domain of Pak

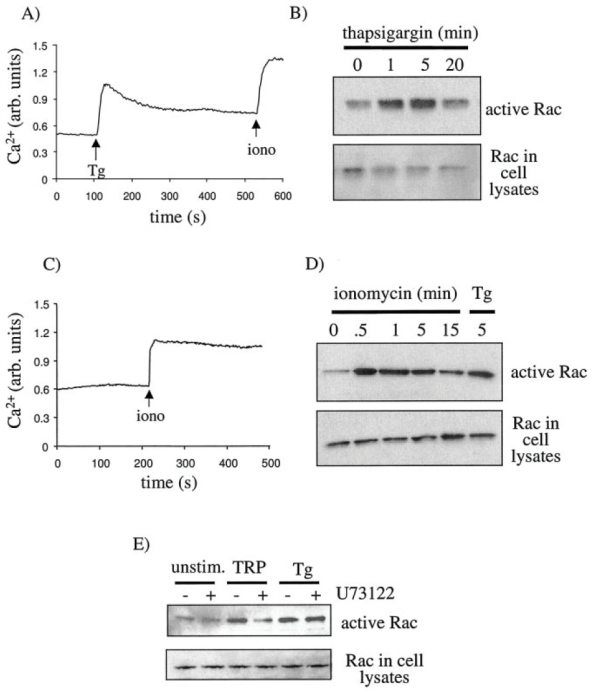

FIG. 3. **Increased intracellular calcium induces Rac activation.** The effect of thapsigargin treatment (100 nM) on intracellular calcium levels (*A*) and Rac activity (*B*) in PC3 cells measured as described for Fig. 1 is shown. The effect of ionomycin (*iono*) treatment (1 μM) on intracellular calcium levels (*C*) and Rac activity (*D*) in PC3 cells is shown. *D* also shows Rac activity in response to thapsigargin for comparison. *E*, effect of the PLC inhibitor U73122 (2.5 μM for 1 h) on Rac activation induced by activation of the thrombin receptor with TRP or by direct elevation of calcium with thapsigargin (*Tg*). PLC inhibition blocks TRP-induced Rac activation but not Rac activation induced by direct elevation of $[Ca^{2+}]_i$ by thapsigargin.

combined with a TAT sequence to confer membrane permeability (see Ref. 41 and "Experimental Procedures"). We first tested the ability of the TAT-CRIB peptide to inhibit Rac signaling using NIH 3T3 cells overexpressing the Rac exchange factor Tiam1, which shows high levels of activated Rac and as a consequence extensive membrane ruffling (28). Addition of TAT-CRIB peptide to the medium for 30 min dramatically attenuated Tiam1-mediated membrane ruffling in these cells, indicating that the peptide inhibits Rac downstream signaling (Fig. 4*A*). Control TAT peptides did not inhibit membrane ruffling (not shown). Treatment of cells with TAT-CRIB peptide for 30 min prior to thapsigargin stimulation also completely inhibited the subsequent detection of active Rac in cells (Fig. 4*B*), most likely because the TAT-CRIB blocked the binding of the biotinylated CRIB peptide used in the pull-down assay. The TAT-CRIB peptide can therefore be used as a tool to inhibit Rac function in living cells and avoids potential long-term effects of CRIB expression.

We then used the TAT-CRIB peptide to examine the role of Rac in calcium-induced effects in PC3 cells. Thapsigargin and TRP induced extensive membrane ruffling and lamellipodia formation at the cell cortex, which is indicative of Rac activation. This was strongly inhibited by pre-treatment with TATCRIB. From these results we conclude that the induction of lamellipodia by intracellular calcium is Rac-dependent.

PKC Mediates Calcium-dependent Rac Activation and Phosphorylation of RhoGDI

In considering the mechanism by which intracellular calcium transients could mediate Rac activation, we first examined the possible role of GEFs. The GEFs GRF and Tiam1 both promote nucleotide exchange on Rac and are potential targets for regulation by calmodulin and calmodulin-dependent protein kinase II. However, thapsigargin-induced Rac activity in PC3 cells was not blocked by calmidazolium chloride or KN-93, inhibitors of calmodulin and calmodulin-dependent protein kinase II, respectively (data not shown). Furthermore, thapsigargin failed to induce Rac activation in BW5146 T-lymphoma cells, despite the very high level of Tiam1 in these cells but did induce Rac activation in Madin-Darby canine kidney-f3 cells, which have undetectable levels of Tiam1 (not shown). Apparently, these particular exchange factors are most likely not directly involved in calcium-induced Rac activation, and we therefore investigated other potential mechanisms.

Various PKC isoforms have been implicated in the activation of Rho family GTPases. A subclass of these, the conventional PKCs, are also regulated by calcium (42). To determine whether PKC mediates calcium-induced Rac activation, we first inhibited PKC, both by long-term treatment of cells with the phorbol ester PMA, which down-regulates novel and conventional PKC proteins, and with the PKC inhibitor GF109203X, used at concentrations that preferentially inhibit conventional and novel PKCs (43). Both treatments potently inhibited thapsigargin-induced Rac activation (Fig. 5*A*). On the other hand, activation of PKC by short-term PMA treatment transiently activated Rac (Fig. 5*B*). To investigate

whether intracellular calcium transients are sufficient to induce PKC activation in PC3 cells, we used an antibody that recognizes phosphorylated substrates of PKC. Western blot analysis of cell lysates revealed multiple PKC-phosphorylated proteins in response to thapsigargin treatment, which were reduced by GF109203X treatment (Fig. 5*C*). TRP stimulation induced a similar profile of phosphorylation that was also blocked by the PKC inhibitor. These results suggest that intracellular calcium activates a calcium-dependent PKC in PC3 cells, which leads to activation of Rac. Although we cannot exclude the possibility that activation of PKC by calcium is indirect, our data suggest that a conventional PKC mediates calcium-induced Rac activation.

RhoGDIα possesses several potential PKC phosphorylation sites and has been shown to be a substrate for PKCα *in vitro*. Furthermore, it was shown that that phosphorylation of RhoGDI induces translocation of RhoA to the plasma membrane (34). We therefore examined whether calcium induced the phosphorylation of RhoGDI and if so, whether this was PKC-dependent. To do this, [32P] metabolically labeled PC3 cells were stimulated with thapsigargin or PMA in the presence and absence of PKC inhibitor, and RhoGDI phosphorylation was analyzed. We found that both PMA and thapsigargin treatments induced the phosphorylation of RhoGDI and that this phosphorylation was inhibited by GF109203X (Fig. 5*D*). These results demonstrate that elevation of intracellular calcium induces the PKC-dependent phosphorylation of RhoGDI.

Membrane Translocation of Rac

To examine whether calcium and PKC regulate membrane translocation of Rac, we examined the localization of Rac in single living cells. For this we used real-time confocal imaging of PC3 cells stably expressing low levels of wild type eGFP-Rac1. Prior to stimulation, GFP-Rac was predominantly cytoplasmic, with some regions of enrichment at membrane protrusions. Following stimulation with thapsigargin, fluorescence images of medial sections and corresponding line scan analysis showed that GFP-Rac levels increased in most parts of the plasma membrane (Fig. 6*A*). Enrichment of GFP-Rac occurred in particular at regions of the membrane that were often sites of subsequent membrane protrusion. Quantitative analysis of membrane and cytosolic fluorescence over time showed that GFP-Rac translocation

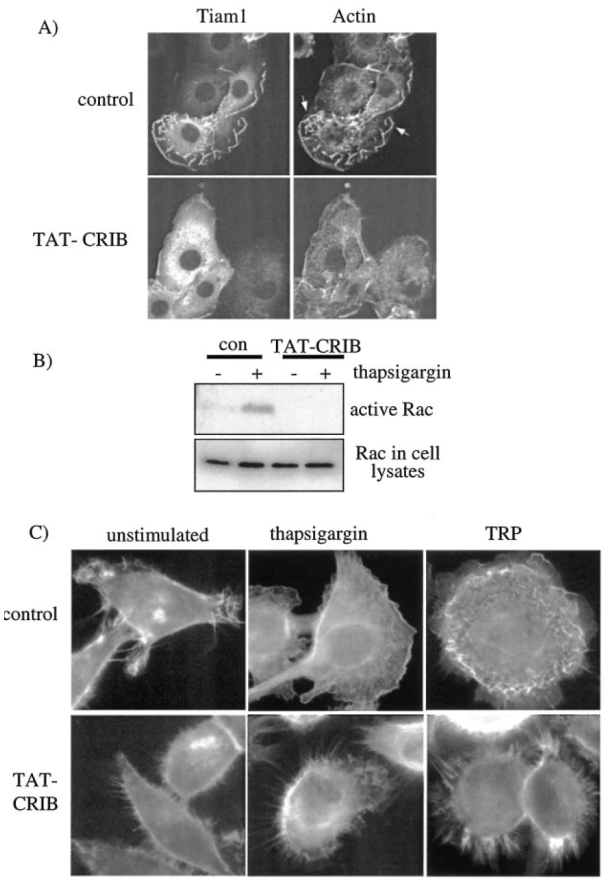

FIG. 4. **Calcium-induced lamellipodia formation is Rac-dependent.** *A*, treatment of NIH 3T3 cells stably overexpressing Tiam1 with or without 0.2 mg/ml TAT-CRIB peptide for 30 min. Cells were stained for Tiam1 expression and F-actin distribution. Note, control cells expressing Tiam1 show extensive actin-rich membrane ruffles (*arrows*), which are absent in TAT-CRIB-treated cells. *B*, treatment of PC3 cells with TAT-CRIB peptide (0.2 mg/ml) inhibits thapsigargin-induced detection of active Rac. *C*, fluorescence micrograph of actin staining of PC3 cells pre-treated with or without TAT-CRIB peptide and stimulated with thapsigargin or TRP. Unlike control cells, TAT-CRIBtreated cells do not extend broad lamellipodia upon stimulation.

peaked at ~2 min and gradually returned to a baseline distribution over 10–30 min (Fig. 6*B*). In addition to an increase of GFP-Rac in the plasma membrane, following stimulation we observed a consistent decrease in cytosolic fluorescence of 10 ± 3% (+S.E.). Although increased labeling in membrane ruffles can sometimes be attributed to increased amounts of membrane (44), simultaneous imaging of GFP-Rac with the membrane dye DiI showed that there is very little increase in membrane in these medial sections (data not shown). Furthermore, a decrease in cytosolic fluorescence support the finding that

translocation to the membrane occurs. Our findings therefore indicate that calcium transients induce a temporary increase in translocation of Rac to the plasma membrane, which precedes the formation of Rac-dependent membrane protrusions. A translocation of 10% of total Rac from cytoplasm to plasma membrane is highly significant in view of the fact that at most 5–10% of total Rac is activated in response to extracellular stimulation as determined by pull-down assays[2].

To further support the live-cell imaging results, we quantified the cytosolic and membrane-bound Rac using a rapid cell fractionation procedure based on cell permeabilization (see "Experimental Procedures"). We found that thapsigargin treatment increased the amount of Rac in the membrane-containing fraction (Fig. 6*C*, compare *lanes 1* and *3*). Densitometry analysis of films from several experiments showed that this represented a mean increase of 2.4-fold ± 0.8 (n = 4, p = 0.05). To examine the requirement for PKC in calcium-induced translocation of Rac, we inhibited PKC by long-term PMA treatment prior to thapsigargin stimulation. This resulted in inhibition of thapsigargin-induced translocation and also of that induced by TRP (Fig. 6*C*, compare *lanes 3–6*). Moreover, activation of PKC by brief PMA treatment enhanced membrane translocation of Rac (Fig. 6*C*, *lanes 7* and *8*). These results confirm that calcium induces the translocation of Rac to the plasma membrane and further support our conclusion that PKC mediates the membrane translocation and activation of Rac by phosphorylation of RhoGDI.

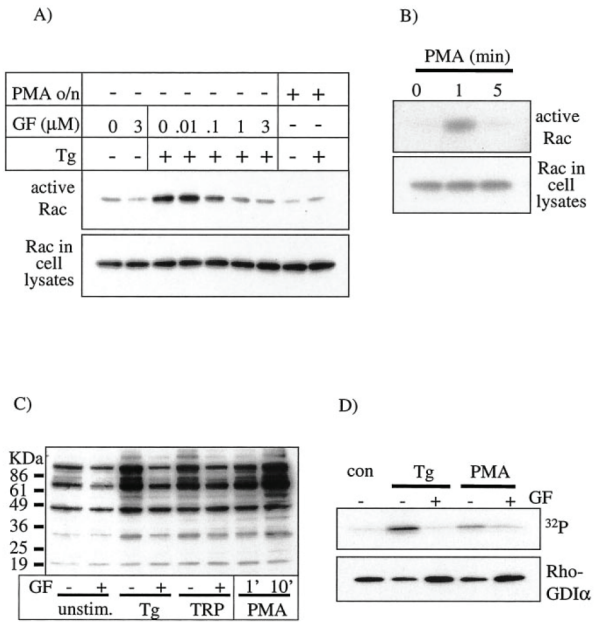

FIG. 5. **PKC-dependent Rac activation and RhoGDI phosphorylation.** *A*, thapsigargin-induced Rac activation is inhibited in a dose-dependent manner by inhibition of PKC with either GF109203X (*GF*) or overnight (16 h overnight (*o/n*)) treatment with PMA (100 nm). The *upper panel* shows active Rac; the *lower panel* shows both active and inactive Rac in original cell lysates. Down-regulation of PKCα was confirmed by Western blotting (not shown). *B*, Rac activation in response to 1- and 5-min treatments with PMA (100 nM). *C*, phosphorylation of cellular proteins by PKC. Cells were stimulated with thapsigargin (5 min; *Tg*) or thrombin peptide (2 min) with or without treatment with GF109203X (*GF*). As a positive control for PKC-induced phosphorylation, cells were also stimulated with PMA for 1 or 10 min. A Western blot of total cell lysates probed with an antibody that recognizes proteins phosphorylated on serine by PKC is shown. Longer exposure of the film revealed multiple additional bands (not shown). *D*, PKC-dependent phosphorylation of RhoGDI. 32-P-labeled PC3 cells were stimulated with thapsigargin (*Tg*) or PMA in the presence or absence of GF109203X (*GF*). Endogenous RhoGDIα was immunoprecipitated, and both 32-Pphosphorylated RhoGDIα and total RhoGDIα were visualized (see "Experimental Procedures"). Note that the PKC inhibitor blocks thapsigargin-induced phosphorylation. *con*, control.

DISCUSSION

We report here that intracellular calcium transients regulate the activation of Rac. Rac activation could be induced artificially either by releasing calcium from intracellular stores or by inducing the influx of extracellular calcium and was thus not a consequence of store emptying *per se*. Rac activation induced by thrombin receptor stimulation was also calcium-dependent, because it could be blocked by chelation of intracellular calcium. Furthermore, thrombin receptor-mediated Rac activation required phospholipase C activity, whereas thapsigargin-induced Rac activity did not. These findings suggest that calcium transients induced by receptor activation of the canonical Gq-PLCinositol 1,4,5-trisphosphate pathway are sufficient and necessary to activate Rac. Increased $[Ca^{2+}]_i$ was sufficient to induce Rac activation in several epithelial cell lines, including Madin-Darby canine kidney cells and T47D mammary carcinoma cells, but did not activate Rac in NIH 3T3 fibroblasts or lymphocytes (data not shown). It was reported previously (45, 46) that cell-cell adhesion can regulate Rac activity. However, the differences that we observed between cell types

[2] L. S. Price and J. G. Collard, unpublished results.

are unlikely to be because of the formation of adherens junctions in epithelial cells, because PC3 cells do not form cadherin-mediated adhesions. In neutrophils, chemoattractant-induced Rac activation is completely independent of intracellular calcium (20). Cells in which Rac is not activated directly by increased $[Ca^{2+}]_i$ may therefore utilize a calcium-independent mechanism to modulate Rac-RhoGDI interaction or may instead be critically dependent on other receptor-mediated signals, such as those that regulate the activation of specific GEFs.

Intracellular calcium transients also led to increased cell spreading and the formation of lamellipodia, a hallmark of Rac activation. Lamellipodia were inhibited by calcium chelation and also by TAT-CRIB peptide, a membrane-permeable Rac inhibitor, demonstrating that calcium-induced lamellipodia were mediated by Rac. Lamellipodia formation at the leading edge is an important component of the coordinated cytoskeletal reorganization that occurs during cell migration. Oscillations in $[Ca^{2+}]_i$ have been observed during the migration of various cell types and are either essential for or contribute toward migration of cells (47, 48). Rac activation may therefore be coordinated by oscillations in $[Ca^{2+}]_i$ that occur during the migratory process. Previous studies have demonstrated targeting of active Rac to membrane ruffles and the leading edge of migrating cells in response to growth factor and integrin signalling (14, 49). Increases in intracellular calcium can also be highly localized to sites of receptor activation and may therefore be an important factor in the spatial regulation of Rac activation.

In NIH 3T3 cells, PDGF-induced membrane translocation and phosphorylation of the GEF Tiam1 is regulated by calcium-calmodulin kinase II but is independent of PKC (25). However, the Rac activation that we observed in PC3 cells was calmodulin-calmodulin kinase II-independent and strongly dependent on PKC. Furthermore, the capacity of intracellular calcium to regulate Rac activity did not correlate with cellular Tiam1 levels. We conclude therefore that the signaling pathway described in the present study represents an additional mechanism of activation of Rac that is independent of Tiam1 or other calmodulin-regulated Rac exchange factors. Calcium-dependent Rac activation was strongly inhibited by treatments that preferentially inhibit conventional and non-conventional PKCs, whereas stimulation of PKC by transient PMA treatment induced the activation of Rac. Thapsigargin treatment also

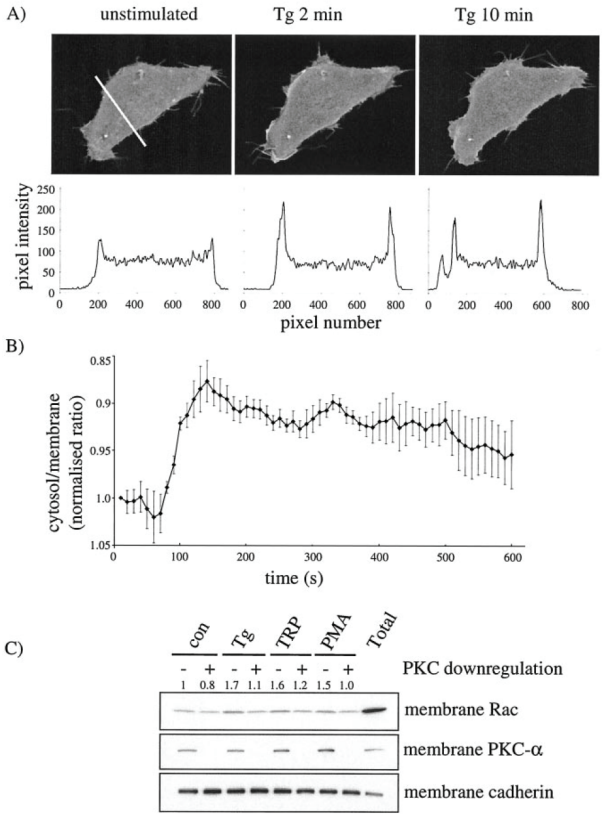

FIG. 6. **Calcium- and PKC-dependent translocation of Rac.** *A*, real-time confocal imaging of GFP-Rac localization (*upper panel*). Cells were stimulated with thapsigargin (*Tg*). Representative images 2 μM above the plane of the coverslip from the same cell were taken at different times after stimulation. The *lower panels* show GFP-Rac fluorescence intensity across the cell at the *line* depicted in the *upper left panel* and demonstrate an increase in cortical fluorescence after stimulation. *B*, time course of the ratio of total cytosol/membrane fluorescence averaged over four representative cells ± S.D. The cytosol/membrane ratio for each cell at $t = 0$ was normalized to 1. *C*, Rac levels in membrane fractions of PC3 cells transiently stimulated with thapsigargin (*Tg*), TRP, or PMA. PKC down-regulation was by overnight PMA treatment. After stimulation, cells were permeabilized to remove cytosolic proteins (see "Experimental Procedures") and then solubilized in radioimmune precipitation assay buffer. These lysates, which contain membraneassociated proteins, were then analyzed for the presence of Rac. -Fold induction of membrane Rac (determined by densitometry) is shown *above* the corresponding *band*. Total Rac content is also shown. Cadherin levels in the membrane fraction, determined using pan-cadherin antibodies, demonstrate equal gel loading. PKCα levels are shown to demonstrate efficient degradation of PKC by overnight PMA treatment. Results are representative of four separate experiments that gave essentially the same results. *con*, control.

resulted in increased PKC kinase activity. From these results we conclude that PKC mediates calcium-induced Rac activation and suggest the involvement of a calcium-dependent conventional PKC isoform. Interestingly, the study by Buchanan *et al.* (25) concluded that PKC was involved in PDGF-induced activation of Rac although this was in a Tiam1-independent manner, which is consistent with our findings. However, increased $[Ca^{2+}]_i$ was not sufficient to induce Rac activation in NIH 3T3 cells. Furthermore, BAPTA-AM did not inhibit PDGF-induced circular ruffle formation, structures that are also associated with Rac activation (data not shown) (50), suggesting that PDGF can induce Rac activation via a pathway that is not dependent on intracellular calcium. Both calcium-dependent and calcium-independent PKCs have been implicated in signaling by Rho family GTPases (25, 43, 51, 52). It is tempting to speculate that calcium-independent PKCs may perform an equivalent function in regulating Rac where intracellular calcium is not critical for activation.

Both increased intracellular calcium and PKC activity induced the translocation of Rac to the plasma membrane. Translocation of Rac has been shown to correlate with activation (53, 54). However, these two events can be uncoupled. Thus, the membrane translocation of a constitutively active Rac mutant (V12Rac) is still regulated by an extracellular stimulus (14), whereas membrane translocation and activation of Tiam1 is not sufficient to activate Rac (25). These findings are consistent with the view that the translocation and activation of Rac are independently regulated events. Our findings show that intracellular calcium- and PKC-dependent targeting of Rac to the plasma membrane may be sufficient to lead to activation of Rac by membrane-associated or membrane translocated exchange factors.

Elevation of $[Ca^{2+}]_i$ resulted in the PKC-dependent phosphorylation of numerous cellular proteins. One of these was RhoGDIα, a protein that binds to the hydrophobic C terminus of Rho family GTPases and maintains them in the cytoplasm (8, 55). These results suggest that calcium- and PKC-dependent phosphorylation of RhoGDI may promote the release of bound Rac and subsequent translocation to the plasma membrane. This hypothesis is supported by previous studies (34, 56), which demonstrated that a conventional PKC, PKCα, induces the phosphorylation of RhoGDI and induces the membrane translocation and activation of RhoA. RhoGDI preferentially associates with the inactive GDP-bound form of endogenous Rho family GTPases (8, 55, 57). Furthermore, it has been reported that the binding of RhoGDI and Tiam1 to Rac are mutually exclusive (11), a phenomenon that might also hold true for other exchange factors. Together these results suggest that release of Rac from RhoGDI is a prerequisite for the activation of Rac by exchange factors. It was shown recently (14) that integrin signals act on the Rac-RhoGDI interaction inducing release of Rac to sites of cell adhesion. Reduced affinity of RhoGDI for Rac may therefore be a common feature of receptor-mediated Rac activation.

In conclusion, intracellular calcium transients modulate Rac activity through different mechanisms. In addition to regulating guanine nucleotide exchange factors, we show here that calcium also regulates the membrane translocation of Rac. This is mediated by PKC, which we propose acts on the Rac-RhoGDI complex, resulting in translocation of Rac to the plasma membrane, where it is activated by exchange factors, which could be membrane-associated or recruited to the plasma membrane by receptor signaling.

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