Characterisation of Rac activation in thrombin- and collagen-stimulated human blood platelets

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Abstract In this study, we characterised the mechanisms of Rac GTPase activation in human platelets stimulated by two physiological agonists, either thrombin, acting through membrane receptors coupled to heterotrimeric G-proteins, or collagen which is known to mobilise a tyrosine kinase-dependent pathway. Both agonists induced a rapid activation of Rac that was not significantly affected by the inhibition of integrin $\alpha_{IIb}\beta_3$ engagement. Using pharmacological inhibitors, we found that phospholipase C activation and calcium mobilisation were essential for platelet Rac activation by either thrombin or collagen whereas protein kinase C inhibition was without effect. In contrast to Rac, Cdc42 activation was independent of phospholipase C activation, indicating that the two GTPases are differently regulated. We also found that phosphoinositide 3-kinase was not required for Rac activation in response to thrombin but was involved in its activation by collagen.

Key words: Rac activation; Human platelet; Thrombin; Collagen

1. Introduction

The actin cytoskeleton undergoes dramatic reorganisation during blood platelet activation and is essential for platelet shape change resulting in filopod formation, lamellar extension and cell spreading [1,2]. The temporal sequences of actin remodelling in platelets activated by various agonists make these cells useful for investigating the role and the regulation of the Rho family of small GTPases, which plays a critical role in the control of actin cytoskeleton dynamics [3–5]. RhoA, Rac and Cdc42 are strongly expressed in platelets [6] but their role in the different phases of platelet activation remains poorly characterised. Using permeabilised human platelets and exogenous active GTPases, it has been shown that Rac can play a major role in phosphatidylinositol 4,5-bisphosphate production and actin filament barbed-end uncapping leading to actin assembly [7,8]. Recently, Azim et al. [9] have clearly shown that both Rac and Cdc42 are indeed rapidly stimulated upon ligation of the thrombin receptor proteinase-activated receptor-1 (PAR1). However, the mechanisms by which Rho GTPases become activated through G-protein-coupled receptors in platelets are still unclear. In other cells, G-protein-coupled receptor-mediated activation of Rac has been shown to involve G_i-type G-proteins [10–12]. In endothelial cells, the G-protein $\beta\gamma$-subunits appear to be involved in this mechanism as well as phosphoinositide 3-kinase (PI 3-kinase) [11,12]. Receptors triggered by thrombin couple to G_i, G_q and G_{12}/G_{13} [13] and are able to rapidly recruit and/or activate several effectors such as phospholipase C (PLC), protein kinase C (PKC) or PI 3-kinase. In this study, we investigated the role of these key signalling enzymes in the process of GTP charging of Rac in response to thrombin. Moreover, since the recruitment of Rac downstream platelet receptors that directly stimulate tyrosine kinases remains poorly understood, we also characterised its activation by collagen, an agonist known to rapidly activate tyrosine kinases, mainly through its receptor GpVI [14].

By direct quantification of GTP-Rac, we found that this small GTPase became rapidly activated by both thrombin and collagen in an integrin $\alpha_{IIb}\beta_3$-independent manner. In both cases, PLC activity and an increase in intracellular Ca^{2+} concentration were required, whereas PKC inhibition was without effect. Interestingly, PLC was not required for Cdc42 activation. Finally, we showed that PI 3-kinase was involved in the activation process of Rac only by collagen.

2. Materials and methods

2.1. Materials

Human α-thrombin was purchased from Enzyme Research Laboratories and collagen from Nycomed Arzneimittel (Germany). RO 31-8220 and U-73122 were from Calbiochem. SR121566 and C7E3 Fab fragments (abciximab, ReoPro) were kindly provided by Dr P. Savi (Sanofi-Synthelabo, Toulouse, France). Anti-Rac monoclonal antibody was purchased from Upstate Biotechnology. Anti-Cdc42 antibody was from Santa Cruz Biotechnology. Enhanced chemiluminescence (ECL) immunoblotting reagents were from Amersham Pharmacia Biotech (Little Chalfont, UK). All other reagents were obtained from Sigma unless otherwise indicated.

2.2. Platelet preparation and aggregation

Human platelets were isolated from concentrates obtained from the local blood bank (Etablissement Français du Sang, Toulouse, France) as described previously [15]. Briefly, they were washed in a washing buffer (pH 6.8) containing 140 mM NaCl, 5 mM KCl, 5 mM KH_2PO_4, 1 mM $MgSO_4$, 10 mM HEPES, 5 mM glucose, 0.35%

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Abbreviations: PI 3-kinase, phosphoinositide 3-kinase; PLC, phospholipase C; PKC, protein kinase C; PAR, proteinase-activated receptor; PtdIns(3,4,5)P_3, phosphatidylinositol 3,4,5-trisphosphate

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bovine serum albumin (w/v). The same buffer plus 1 mM $CaCl_2$ was added to the final suspension and pH was adjusted to 7.4. In experiments concerning PI 3-kinase product measurements, platelets were labelled with 0.5 mCi/ml [^{32}P]orthophosphate during 60 min in phosphate-free washing buffer (pH 6.5) at 37°C. After stimulation lipids were extracted and analysed as described previously [15]. Aggregation was monitored by a turbidimetric method using a dual-channel Payton aggregometer (Payton Associates, Scarborough, ON, Canada) with stirring at 900 rpm at 37°C (7.5×10^8 platelets/ml).

2.3. Determination of activated cellular Rac and Cdc42

The amounts of activated cellular Rac and Cdc42 were determined by precipitation with a fusion protein consisting of GST and the Rac-binding domain from human PAK1 (amino acids 67–150) as described [10]. Platelets were lysed in RIPA buffer (50 mM Tris, pH 7.4, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecylsulphate (SDS), 500 mM NaCl, 10 mM $MgCl_2$, 2.5 mM ethylene glycol-bis(2-aminoethyl-ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 10 μg/ml each of leupeptin and aprotinin, and 1 mM phenylmethylsulphonyl fluoride (PMSF)) and clarified cell lysates were incubated with GST-PAK1 beads (20–30 μg) at 4°C for 60 min. The beads were washed four times with 50 mM Tris–HCl, pH 7.4, 10 mM $MgCl_2$, 150 mM NaCl, 1% Triton X-100, 1 mM PMSF, 5 mM EGTA and 5 μg/ml each of leupeptin and aprotinin. The bead pellet was finally suspended in 15 μl of Laemmli sample buffer, loaded on a 12% SDS–PAGE, transferred to nitrocellulose membrane, and blotted for the appropriate GTPase using specific antibody against Rac or Cdc42.

2.4. Gel electrophoresis and immunoblotting

SDS–PAGE and immunodetections were performed as previously described [15] using the relevant antibodies, peroxidase-conjugated secondary antibody, and the ECL system.

3. Results

The activation of Rac was investigated using the GST-PAK1 binding assay as previously described [10]. When human platelet lysate was incubated with GTPγS, Rac could massively bind to GST-PAK1-coated beads, whereas no binding was detected when the lysate was incubated with GDPβS, validating the technique in our model (Fig. 1A).

Triggering human platelets by thrombin or collagen led to a very rapid and sustained activation of Rac reaching a maximum 30 s after addition of thrombin and 1 min after addition of collagen and slowly decreasing thereafter (Fig. 1B,C). Quantification of precipitated Rac by densitometric analysis of immunoblots demonstrated that, under our experimental conditions, the amount of activated Rac was twice higher in thrombin-stimulated platelets compared to collagen-stimulated cells (data not shown).

The Arg-Gly-Asp-Ser (RGDS) peptide known to block the binding of fibrinogen on integrin $\alpha_{IIb}\beta_3$ was then used to check whether Rac activation was dependent on integrin $\alpha_{IIb}\beta_3$ engagement occurring during platelet aggregation. Rac activation by thrombin or by collagen was not affected by treatment of platelets by RGDS (Fig. 2A,B) which completely prevented platelet aggregation (Fig. 2, right panel). Since RGD peptides may be partial integrin agonists [16], we also used another $\alpha_{IIb}\beta_3$ inhibitor, the synthetic agent SR121566 [17]. Although this compound strongly inhibited platelet aggregation, it did not affect Rac activation upon thrombin or collagen stimulation (Fig. 2). Moreover, an unrelated $\alpha_{IIb}\beta_3$ blocking agent, antibody C7E3, had no significant effect on Rac activation upon thrombin stimulation (not shown). These data indicate that the rapid activation of Rac observed upon thrombin or collagen stimulation occurs independently of integrin engagement and aggregation.

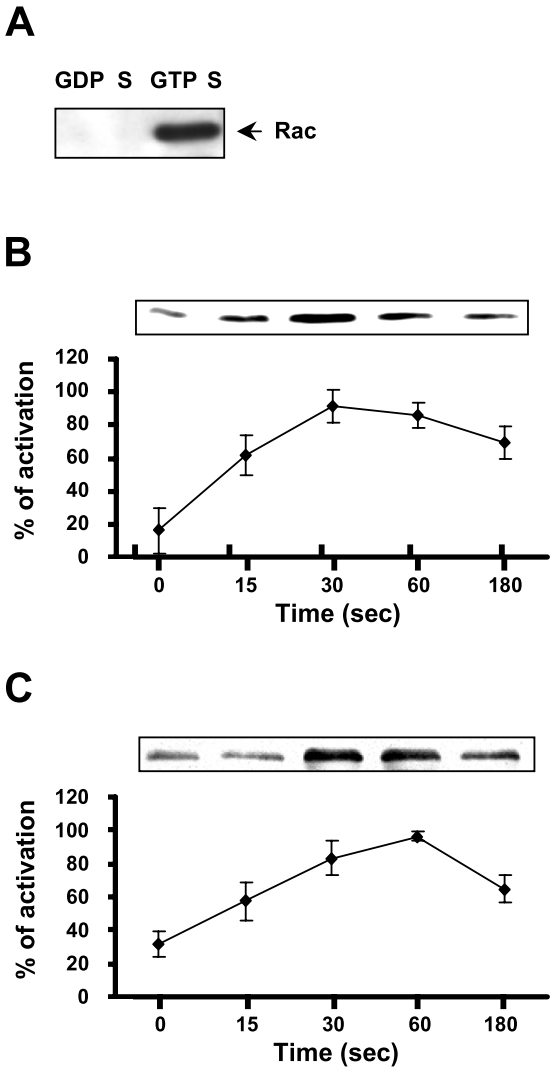

Fig. 1. Time course of Rac activation in human platelets stimulated either by thrombin or by collagen. A: Anti-Rac immunoblot showing the specificity of the interaction between Rac-GTP form and GST-PAK1. Platelet lysates were incubated with either 100 μM GDPβS or 100 μM GTPγS and the clarified lysates were used for the affinity precipitation assay as described in Section 2. B,C: Time course of Rac activation in thrombin- (B) or collagen- (C) stimulated platelets. Human platelets (7.5×10^8 cells/ml) were stimulated with thrombin (1 IU/ml) (B) or collagen (10 μg/ml) (C). At the indicated times, activation was stopped by addition of ice-cold 2× lysis buffer. The resulting cell lysate was clarified and used for the affinity precipitation assay for detection of activated Rac as described in Section 2. The amount of Rac-GTP was quantified by densitometric analysis and the results are expressed as percentage of maximal activation and are means ± S.E.M. of three to seven independent experiments using distinct donors. A representative immunoblot of Rac collected with the GST-PAK1 beads in thrombin- (B) or collagen- (C) stimulated platelets is shown in the inset.

The molecular mechanisms involved in the rapid activation of Rac were then investigated. Both thrombin and collagen are known to induce a rapid production of phosphoinositide-derived second messengers such as inositol 1,4,5-trisphosphate, diacylglycerol and D3-phosphoinositides, which are very important for platelet activation [15,18]. Interestingly, the PLC inhibitor U-73122 strongly decreased the GTP loading of Rac by thrombin or by collagen (Fig. 3A). The inactive analogue U-73343 was without effect (not shown). Con-

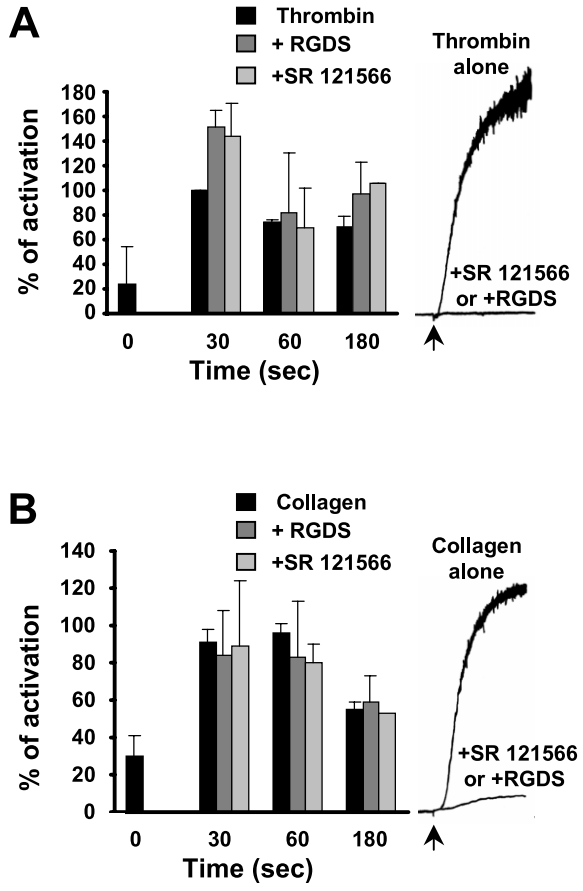

Fig. 2. Thrombin- or collagen-induced activation of Rac is independent of integrin engagement. Platelets were incubated either without or with RGDS (500 μM) or SR121566 (3.5 μM) for 1 min and were stimulated with thrombin (1 IU/ml) (A) or with collagen (10 μg/ml) (B). At the indicated times, Rac-GTP was precipitated with GST-PAK1-coated beads and analysed as in Fig. 1. Results are expressed as percentage of maximal activation and are means ± S.E.M. of three independent experiments when RGDS was used as an antagonist or means ± S.D. of two independent experiments when SR121566 was used. The effect of RGDS (500 μM) or SR121566 (3.5 μM) treatment on platelet aggregation induced by either thrombin or collagen is shown on the right.

versely, the activation of another member of the Rho family which also interacts with PAK1 [10], Cdc42, occurred very rapidly upon collagen addition (not shown), but did not require PLC activity (Fig. 3B). Indeed, the U-73122 compound, which efficiently inhibited the production of phosphatidic acid (a reflection of PLC activation in platelets [15]) as well as platelet aggregation (not shown), did not significantly affect Cdc42 activation induced by collagen under conditions where Rac was fully inhibited (Fig. 3B). Similar results were obtained when thrombin was used as an agonist (not shown). These results suggest a specific role of PLC upstream of Rac activation in human platelets and indicate that Cdc42 and Rac are differentially regulated in these cells.

The PKC inhibitor RO 31-8220 strongly inhibited platelet aggregation induced by thrombin or collagen (Fig. 3C) but did not affect the activation of Rac (Fig. 3A). Conversely, Rac activation by thrombin or collagen required an increase in the free cytosolic Ca^{2+} concentration. Indeed, EGTA, which chelates extracellular Ca^{2+}, only weakly affected Rac activation whereas addition of the intracellular Ca^{2+} chelator 1,2-bis-(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetrakis(acetoxymethyl ester) (BAPTA-AM) strongly impaired its activation by both agonists (Fig. 4A,B). It is noteworthy that GTP loading of Cdc42 was not significantly affected by BAPTA-AM treatment (not shown) suggesting that, in contrast to Rac, intracellular Ca^{2+} was not required for its activation.

Prostaglandin I_2 (PGI_2), a potent inducer of cAMP production through adenylate cyclase stimulation, is a strong negative regulator of platelet activation and is known to reduce the Ca^{2+} mobilisation induced by several agonists [18,19]. In agreement, PGI_2 strongly inhibited thrombin-induced activation of Rac (Fig. 4C). However, the fact that PGI_2 signifi-

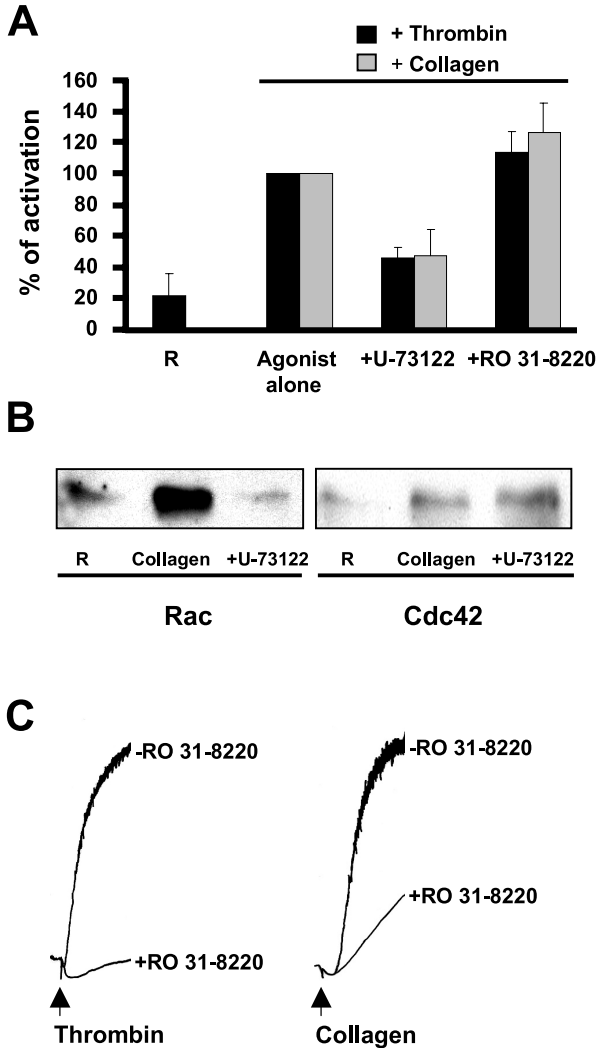

Fig. 3. Role of PLC activity but not of PKC activity in human platelet Rac activation. A: Platelets were preincubated either without or with U-73122 (10 μM) for 5 min or RO 31-8220 (5 μM) for 10 min and stimulated with thrombin (1 IU/ml) or with collagen (10 μg/ml). After 1 min, Rac-GTP was precipitated with GST-PAK1-coated beads and analysed as indicated in Fig. 1. Results are expressed as percentage of maximal activation and are means ± S.E.M. of three independent experiments. B: On the left, anti-Rac immunoblot showing the involvement of PLC activity on Rac activation induced by collagen (10 μg/ml, 1 min). On the right, anti-Cdc42 immunoblot of the same experiment showing that PLC activity is not required for Cdc42 activation. Results shown are representative of three independent experiments. C: The effect of RO 31-8220 (5 μM) treatment on platelet aggregation induced by either thrombin or collagen is shown.

cantly affected the activation of Rac induced upon ionophore A23187 addition (Fig. 4C, right panel) suggests that cAMP can also regulate a factor downstream of calcium in the activation of Rac.

PI 3-kinase has been shown to play a role in the activation of Rac in several models [20,21]. To study the role of PI 3-kinase in the activation of Rac by thrombin or collagen, we inhibited this enzyme by 50 nM wortmannin. Under these conditions the production of phosphatidylinositol 3,4-bisphosphate and phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P_3) induced either by thrombin or by collagen was inhibited by 93 ± 3% and 97 ± 5% ($n = 3$), respectively. Fig. 5 shows that PI 3-kinase was not required for Rac activation in response to thrombin activation but was involved in its activation through collagen receptors. Indeed, the extent of GTP-Rac recovered with the GST-PAK1-coated beads was significantly reduced in wortmannin-treated platelets (35% inhibition at 30 s, $P < 0.05$, $n = 3$ and 63% inhibition at 180 s, $P < 0.01$, $n = 3$). The effect of PI 3-kinase inhibition of GTP charging of Rac was always stronger at the longest time of activation suggesting that this lipid kinase is important for the sustained activation of Rac. Similar results were obtained with 25 μM LY294002, another unrelated PI 3-kinase inhibitor (not shown).

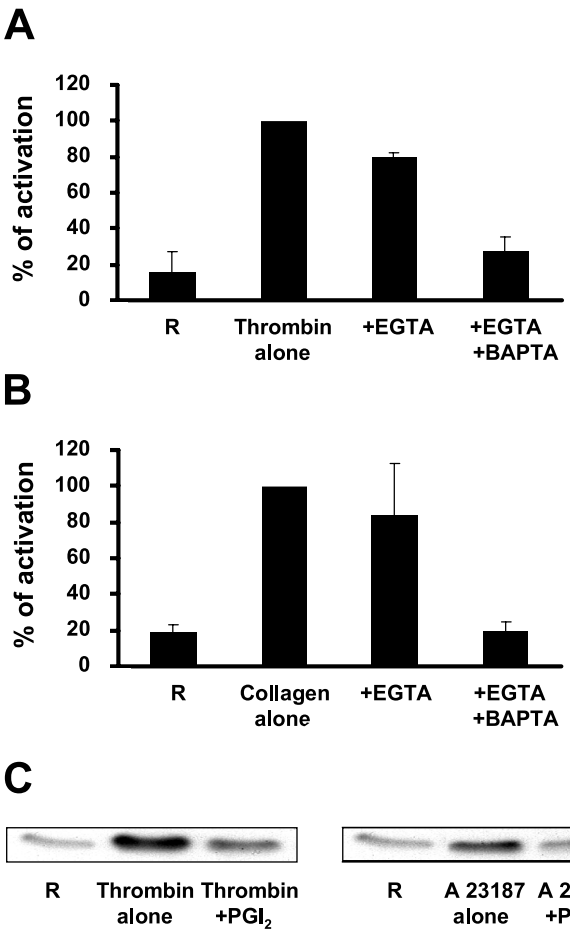

Fig. 4. Intracellular Ca^{2+} is required for thrombin- and collagen-mediated Rac activation. A,B: Platelets were preincubated either with Ca^{2+} (1 mM) or without Ca^{2+} plus EGTA (5 mM) (+EGTA) and stimulated for 1 min with 0.5 IU/ml thrombin (A) or with 10 μg/ml collagen (B). They were also preincubated with BAPTA-AM (30 μM) for 20 min in a buffer without Ca^{2+} plus EGTA (5 mM) (+EGTA+BAPTA) and were stimulated for 1 min with 0.5 IU/ml thrombin (A) or with 10 μg/ml collagen (B). Rac-GTP was precipitated with GST-PAK1-coated beads and analysed as indicated in Fig. 1. Results are expressed as percentage of maximal activation and are means ± S.E.M. of three to five independent experiments. C: Effect of prostaglandin I_2 treatment of platelets on Rac activation. Platelets were preincubated either without or with 10 ng/ml prostaglandin I_2 (+PGI_2) for 2 min and were stimulated with 1 IU/ml thrombin during 1 min (left panel) or with 2 μM ionophore A23187 during 30 s (right panel). Rac-GTP was precipitated with GST-PAK1-coated beads and analysed as indicated in the legend to Fig. 1.

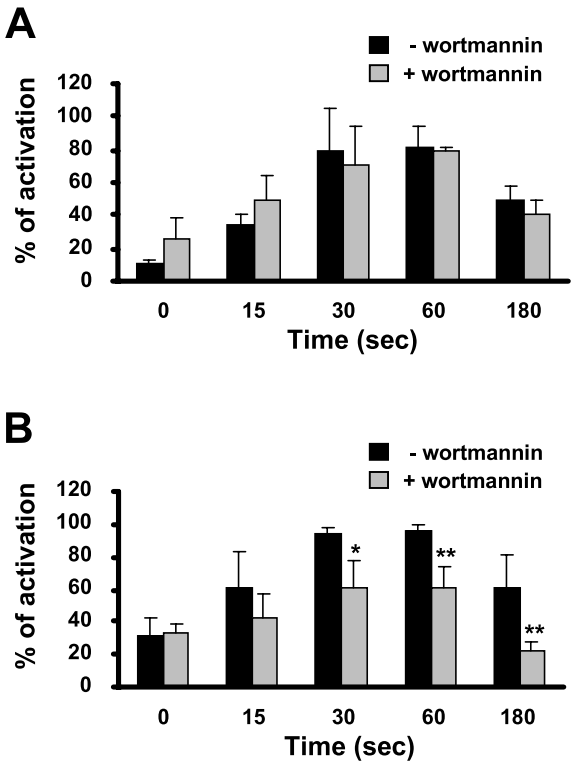

Fig. 5. Effects of the PI 3-kinase inhibitor wortmannin on Rac activation in thrombin- and collagen-stimulated platelets. Platelets were preincubated either without or with wortmannin (50 nM) for 15 min and stimulated with thrombin (1 IU/ml) (A) or with collagen (10 μg/ml) (B). At indicated times, Rac-GTP was precipitated with GST-PAK1-coated beads and analysed as indicated in Fig. 1. Results are expressed as percentage of maximal activation and are means ± S.E.M. of three independent experiments. *$P \leq 0.05$, **$P \leq 0.01$.

4. Discussion

Several lines of indirect evidence implicate the Rho family GTPases in platelet activation pathways [6,7]. Recently Azim et al. [9] provided the first direct measurement of platelet Rac and Cdc42 activation in response to the thrombin receptor PAR1 activating peptide. Their results confirm the temporal link between GTPase activation, actin assembly and physiological platelet responses. In the present study, we investigated the mechanisms of Rac activation in response to thrombin, an agonist acting through membrane receptors coupled to heterotrimeric G-proteins, and to collagen which is known to mobilise a tyrosine kinase-dependent pathway. Both agonists induced a very rapid activation of Rac that was dependent on PLC activity. Interestingly, Cdc42, another GTPase of this

family also precipitated by the GST-PAK1 binding assay [10], was not activated through PLC. This result is consistent with the fact that overexpression of Rac or Cdc42 induces different cell morphologies [4]. Moreover, their different cellular location [9] suggests that they probably function in different ways in human platelets. In contrast to Cdc42, Rac does not form tight complexes with the actin cytoskeleton but rather interacts with the plasma membrane where it may modulate phosphoinositide kinase activities [9]. Cdc42 can physically interact with WASp family members to organise ARP2/3 complex-mediated actin nucleation.

It has been shown that G_i-protein-coupled receptors, such as chemoattractant fMLP receptor, can activate Rac [10–12] but G_q/G_{11}-protein-coupled receptors, such as bradykinin or endothelin-1 receptor, are also able to stimulate this GTPase [21–23]. The activation of G_q/PLCβ by the thrombin receptor PAR1 is a key event in platelet activation as $G\alpha_q$-deficient platelets no longer respond to thrombin [24]. This suggests that G_q/PLCβ activation is required for Rac activation induced by this agonist. In the case of collagen, PLCγ2 has been shown to be activated by a mechanism involving the tyrosine kinase Syk, downstream of GpVI, one of the major collagen receptors at the platelet surface [14]. Besides the recruitment of different PLC isoforms, one common denominator for these two agonists is the increase in intracellular Ca^{2+} concentration by mobilisation of Ca^{2+} from internal stores and by influx of extracellular Ca^{2+}. We found that EGTA, which chelates extracellular Ca^{2+}, had a weak inhibitory effect on Rac activation. Conversely, addition of BAPTA-AM, which chelates intracellular Ca^{2+}, inhibited both thrombin- and collagen-induced Rac activation. This is consistent with the fact that PGI_2, a potent activator of cAMP production, inhibited the GTP loading of Rac. Indeed, cAMP, which is a physiological mediator of platelet inhibition through activation of cAMP-dependent kinases, is known to inhibit Ca^{2+} mobilisation in stimulated platelets [19,25]. However, the fact that PGI_2 significantly affects Rac activation induced by a rise in intracellular Ca^{2+} in ionophore-treated platelets strongly suggests that there are also cAMP-regulated intermediate factors downstream of calcium in the activation of Rac. The mechanism by which the intracellular Ca^{2+} increase results in Rac activation is unclear and is currently under investigation. PLC activation and Ca^{2+} mobilisation are important for platelet granule secretion [18]. A defect in mediator release through inhibition of granule secretion could explain, at least in part, the lack of Rac activation upon PLC blockade or upon chelation of intracellular Ca^{2+}. However, PKC, which is also involved in granule secretion, is not required for Rac activation. The release of thromboxane A2, production of which requires the action of a cytosolic phospholipase A2 regulated through increased cytosolic Ca^{2+} [26], may amplify the activation of Rac by thrombin, collagen or even ionophore. Nevertheless, the very rapid activation of Rac by thrombin or collagen suggests that the primary agonist directly activates the GTPase. Although a regulation of an exchange factor for Rac by Ca^{2+} has not been described, one cannot exclude such a possibility. Another hypothesis involves calpain, a Ca^{2+}-dependent thiol protease present in the cytosol of platelets. It was recently suggested that calpain might be implicated in the regulation of Rac activation [27]. Although some evidence suggests that calpain may be activated directly by thrombin, its activation in platelets was originally shown to mainly occur after engagement of integrins [28]. However, our results show that integrin engagement was not required for thrombin- or collagen-induced Rac activation indicating that the early stimulation of Rac occurred independently of integrin signalling. Although this result does not exclude activation of Rac by integrin engagement at later stages of platelet activation as in other cells during integrin-mediated cell adhesion [27,29,30], it is not in favour of a role of calpain in the early activation of Rac.

In various cell types, inhibition of PI 3-kinase was shown to block GTP charging of Rac [20,21]. The Rac GTPase exchange factor VAV1 has a pleckstrin homology domain able to interact with PtdIns(3,4,5)P_3 and may account for this PI 3-kinase-dependent Rac activation. We also previously showed that PtdIns(3,4,5)P_3 can directly interact with Rac [31] in vitro, a mechanism that may participate in the adequate location of this GTPase. Here, we show that PI 3-kinase is not required for Rac activation by thrombin but is involved in the activation of Rac by collagen. GpVI, one of the major collagen receptors, requires a PI 3-kinase for full activation of PLCγ2 and calcium mobilisation [32]. Therefore, it is likely that PI 3-kinase inhibition affects the efficiency of PLC activation and the subsequent Ca^{2+} mobilisation in collagen-stimulated platelets. Alternatively, an exchange factor of Rac such as VAV1, activation of which involves PI 3-kinase, may be implicated in collagen-dependent Rac activation. Direct interaction of Rac1 with PtdIns(3,4,5)P_3 may also account for the adequate location of activated Rac and possibly for its sustained activation [31].

Taken together, our data show that human platelet Rac is rapidly activated by thrombin and collagen through a PLC and Ca^{2+} mobilisation-mediated mechanism. Conversely, the rapid activation of Cdc42 is not sensitive to PLC inhibition. This early activation of Rac is dependent neither on PKC activity nor on integrin engagement and is inhibited by the cAMP-producing physiologic mediator PGI_2. Although the molecular mechanism supporting the role of Ca^{2+} in the activation of platelet Rac is still unknown, our data add new information about the regulation of this GTPase in human platelets.

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References

[1] Barkalow, K., Witke, W., Kwiatkowski, D.J. and Hartwig, J.H. (1996) J. Cell Biol. 134, 389–399.
[2] Hartwig, J.H. (1992) J. Cell Biol. 118, 1421–1442.
[3] Kjoller, L. and Hall, A. (1999) Exp. Cell Res. 253, 166–179.
[4] Nobes, C. and Hall, A. (1995) Cell 81, 53–62.
[5] Bishop, A.L. and Hall, A. (2000) Biochem. J. 348, 241–255.
[6] Dash, D., Aepfelbacher, M. and Siess, W. (1995) J. Biol. Chem. 270, 17321–17326.
[7] Hartwig, J.H., Bokoch, G.M., Carpenter, C.L., Janmey, P.A., Taylor, L.A., Toker, A. and Stossel, T.P. (1995) Cell 82, 643–653.
[8] Tolias, K.F., Hartwig, J.H., Ishihara, H., Shibasaki, Y., Cantley, L.C. and Carpenter, C.L. (2000) Curr. Biol. 10, 153–156.
[9] Azim, A.C., Barkalow, K., Chou, J. and Hartwig, J.H. (2000) Blood 95, 959–964.
[10] Benard, V., Bohl, B.P. and Bokoch, G.M. (1999) J. Biol. Chem. 274, 13198–13204.
[11] Belisle, B. and Abo, A. (2000) J. Biol. Chem. 275, 26225–26232.

[12] Geijsen, N., van Delft, S., Raaijmakers, J.A., Lammers, J.W., Collard, J.G. and Koenderman, L. (1999) Blood 94, 1121–1130.
[13] Brass, L.F. and Molino, M. (1997) Thromb. Haemost. 78, 234–241.
[14] Watson, S.P. and Gibbins, J. (1998) Immunol. Today 19, 260–264.
[15] Gratacap, M.P., Hérault, J.P., Viala, C., Ragab, A., Savi, P., Herbert, J.M., Chap, H., Plantavid, M. and Payrastre, B. (2000) Blood 96, 3439–3446.
[16] Du, X.P., Plow, E.F., Frelinger, A.L., O'Toole, T.E., Loftus, J.C. and Ginsberg, M. (1991) Cell 65, 409–416.
[17] Savi, P., Zamboni, G., Rescanieres, O. and Herbert, J.M. (2001) Thromb. Haemost. 85, 702–709.
[18] Siess, W. (1989) Physiol. Rev. 69, 58–178.
[19] Matsuoka, I., Nakahata, N. and Nakanishi, H. (1989) Jpn. J. Pharmacol. 49, 142P.
[20] Parker, P.J. (1995) Curr. Biol. 5, 577–579.
[21] Akasaki, T., Koga, H. and Sumimoto, H. (1999) J. Biol. Chem. 274, 18055–18059.
[22] van Leeuwen, F.N., van Delft, S., Kain, H.E., van der Kammen, R.A. and Collard, J.G. (1999) Nature Cell Biol. 1, 242–248.
[23] Clerk, A., Pham, F.H., Fuller, S.J., Sahai, E., Aktories, K., Marais, R., Marshall, C. and Sugden, P.H. (2001) Mol. Cell. Biol. 21, 1173–1184.
[24] Offermanns, S., Toombs, C.F., Hu, Y.H. and Simon, M.I. (1997) Nature 389, 183–186.
[25] Geiger, J., Nolte, C. and Walter, U. (1994) Am. J. Physiol. 267, 236–244.
[26] Borsch-Haubold, A.G., Kramer, R.M. and Watson, S.P. (1995) J. Biol. Chem. 270, 25885–25892.
[27] Bialkowska, K., Kulkarni, S., Du, X., Goll, D.E., Saido, T.S. and Fox, J.E.B. (2000) J. Cell Biol. 151, 685–695.
[28] Fox, J.E.B. (1993) Thromb. Haemost. 70, 884–893.
[29] del Pozo, M.A., Price, L.S., Alderson, N.B., Ren, X.D. and Schwartz, M.A. (2000) EMBO J. 19, 2008–2014.
[30] Schwartz, M.A. and Shattil, S.J. (2000) Trends Biochem. Sci. 25, 388–391.
[31] Missy, K., Van Poucke, V., Raynal, P., Viala, C., Mauco, G., Plantavid, M., Chap, H. and Payrastre, B. (1998) J. Biol. Chem. 273, 30279–30286.
[32] Pasquet, J.M., Bobe, R., Gross, B., Gratacap, M.P., Tomlinson, M.G., Payrastre, B. and Watson, S.P. (1999) Biochem. J. 342, 171–177.