Platelets were used to study the activation of Rho and Rac through G-protein-coupled receptors and its regulation by cyclic nucleotides. The thromboxane A2 (TXA2) mimetic U46619 rapidly activated both small GTPases independently of integrin αIIbβ3 activation. U46619, which leads to the activation of Gq/G13 and Goq, did not induce Rac activation in Goq-deficient platelets but was able to activate Rho, to stimulate actin polymerization and phosphatidylinositol 4,5-bisphosphate formation, and to induce shape change. Rac activation by U46619 in wild-type platelets could be blocked by chelation of intracellular Ca2+ and was partially sensitive to apyrase and AR-C69931MX, an antagonist of the Gq-coupled ADP receptor. Cyclic AMP, which completely blocks platelet function, inhibited the U46619-induced activation of Goq and G12/G13 as well as of Rac and Rho. In contrast, cGMP, which has no effect on platelet shape change blocked only activation of Goq and Rac. These data demonstrate that Rho and Rac are differentially regulated through heterotrimeric G-proteins. The G12/G13-mediated Rho activation is involved in the shape change response, whereas Rac is activated through Goq and is not required for shape change. Cyclic AMP and cGMP differentially interfere with U46619-induced Rho and Rac activation at least in part by selective effects on the regulation of individual G-proteins through the TXA2 receptor.

The small GTPases Rho and Rac are central regulators of various cellular processes such as actin cytoskeleton dynamics, transcriptional regulation, cell cycle progression, and contractile processes (1). They are activated by a variety of receptors, including those coupled to heterotrimeric G-proteins (2). Various heterotrimeric G-proteins have been involved in linking receptors to the regulation of Rho and Rac. The α-subunits of the G12 family of heterotrimeric G-proteins, Go12 and Go13, are able to activate Rho (3, 4). Rho activation may be mediated by a group of Rho-specific guanine nucleotide exchange factors, which are able to interact with Go12/Go13 (5–7). However, in some cells Goq or βγ complexes of heterotrimeric G-proteins have been suggested to induce Rho activation (8–10). G-protein-coupled receptor-mediated activation of Rac has been shown to be mediated by Goi-type G-proteins via a mechanism, which in some cases appears to involve G-protein βγ subunits and activation of phosphoinositide 3-kinase (11–14).

Both Rho and Rac have been involved in early signaling processes underlying platelet activation. Platelets respond to various stimuli, which function through G-protein-coupled receptors with secretion of granule contents, aggregation, and a rapid change of their shape. Rac, which is rapidly activated after activation of the thrombin receptor PAR-1 (15), has been suggested to mediate thrombin receptor-induced actin assembly via stimulation of phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P2)3 production during platelets activation (16, 17), and evidence has been provided that Rho is involved in early processes underlying platelet activation by linking receptors to Rho-kinase and subsequent regulation of myosin light-chain phosphorylation (18–20). The mechanisms by which Rho and Rac become activated through G-protein-coupled receptors in platelets are unclear. Receptors activated by thrombin or thromboxane A2 (TXA2) couple to Goq and G12/G13. In addition, thrombin but not TXA2 is able to induce activation of Goi in platelet membrane fractions (18, 21). Platelets from Gαq-deficient mice have been instructive in delineating the initial mechanisms of platelet activation. TXA2 and thrombin are unable to induce phospholipase C activation as well as platelet aggregation and secretion in the absence of Goq, whereas induction of platelet shape change through the activation of G12/G13 appears to be basically unaffected (18, 22).

In this report we demonstrate by direct determination of Rho and Rac activation that both small GTPases become rapidly activated by TXA2 in an integrin αIIbβ3-independent manner. We used platelets from Goq-deficient mice to show that Rho and Rac are differentially regulated. Rho activation occurs through G12/G13 and is involved in processes underlying platelet shape change, including PtdIns(4,5)P2 formation. In contrast, Rac activation is mediated by Goq and is not required for shape change. Cyclic nucleotides cAMP and cGMP differentially interfere with Rac and Rho activation in platelets at least in part by affecting receptor-mediated activation of Goq and G12/G13.

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** To whom correspondence should be addressed: Tel.: 49-6221-54-82467; Fax: 49-6221-54-8549; E-mail: stefan.offermanns@urz.uni-heidelberg.de.

1 The abbreviations used are: PtdIns(4,5)P2, phosphatidylinositol 4,5-bisphosphate; Gq/IIb/IIa, glycoprotein IIb/IIa (integrin αIIbβ3); TXA2, thromboxane A2; GST, glutathione S-transferase; PLC, phospholipase C; BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-N,N,N’,N”-tetraacetic acid tetrakis(acetoxyethyl ester); RIPA, radioimmune precipitation buffer; PAGE, polyacrylamide gel electrophoresis; Sp-5,6-DCI-bIMPS, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole-3’,5’- cyclic monophosphorothioate, Sp-isomer; 8-pCPT-cGMP, 8-(4-chlorophenylthio) guanosine-3’,5’-cyclic monophosphonate.
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EXPERIMENTAL PROCEDURES

Materials—U46619 was from Cayman Chemical (Ann Arbor, MI); thrombin, Sp-5,6-DCI-cBIMPS, and 8-pCPT-cGMP were from Biolog (Bremen, Germany); RO-31-8220, BAPTA-AM, A23187, and U-73122 were from Calbiochem. AR-C69931MX was a generous gift from Dr. J. Turner (ASTRA Charnwood, UK), whereas SR 121566A was obtained from Dr. P. Savi (Sanofi-Synthelabo, Toulouse, France). Anti-Rac monoclonal antibody was purchased from Upstate Biotechnology, Inc. Anti-RhoA monoclonal antibody was from Santa Cruz Biotechnology, Inc. Antiserum against G-protein α-subunits have been described previously (21, 23). All other reagents were obtained from Sigma Chemical Co. unless otherwise indicated. Y-27632 was kindly provided by Yoshitomi Pharmaceutical Industries, Ltd. (Saitama, Japan).

Platelet Preparation and Aggregation—Whole blood was collected from normal and Gq-deficient mice (129/Sv × C57BL/6) anesthetized with pentobarbital by puncturing the inferior vena cava with syringes containing acid citrate dextrose (1/9 volume). The blood from 3–4 Gq-deficient mice and wild-type mice was pooled for each platelet experiment. Blood was diluted with half the volume of Hepes-Tyrode buffer and finally suspended at a final concentration of 1×109 platelets/ml in the presence of 0.02 g/ml each of leupeptin and aprotinin, and 1 mM phenylmethylsulfonyl fluoride, and clarified cell lysates were incubated for 30 min at 37 °C. After centrifugation at 2500×g for 5 min, the supernatant was taken as a control. Pellets were washed four times with 50 mM Tris-HCl, pH 7.5, 10 mM MgCl2, 1 mM MgCl2, 0.35% human serum albumin, and platelet-rich plasma was obtained by centrifugation for 8 min at 2500×g at 37 °C. Thereafter, prostacyclin at a final concentration of 500 nM was added to the platelet-rich plasma, and platelets were pelleted twice by centrifugation at 1000×g for 5 min at 37 °C. The platelet pellet was resuspended in Hepes-Tyrode’s buffer at a density of 1×109 platelets per milliliter in the presence of 0.02 unit/ml of the ADP scavenger appyrase (adenosine-5′-triphosphate diphosphohydrolase) and incubated for 30 min at 37 °C.

For inositol lipid analysis, platelets were labeled with 0.5 mCi/ml [32P]orthophosphate during 50 min in a phosphate-free washing buffer (pH 6.5) at 37 °C. Labeled platelets were then washed once in the same buffer and finally suspended at a final concentration of 1×109 cells/ml (pH 7.3). Optical aggregation experiments were conducted in a two-channel aggregometer (Chronolog).

Determination of Activated Cellular Rho and Rac—The amount of activated cellular Rho and Rac was determined by precipitation with a fusion protein consisting of GST and the Rho-binding domain of Rhotekin (GST-RBD) or the Rac-binding domain of PAK1 (PBD) as described previously (24). Platelets were lysed in RIPA buffer (50 mM Tris, pH 7.2, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 500 mM NaCl, 10 mM MgCl2, 10 μg/ml each of leupeptin and aprotinin, and 1 mM phenylmethylsulfonyl fluoride), and clarified cell lysates were incubated with GST-RBD or GST-PBD (20 μg of beads) at 4 °C for 45 min. The beads were washed four times with 50 mM Tris-HCl, pH 7.5, 10 mM MgCl2, 150 mM NaCl, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 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. RhoA and Rac were separated by 12% SDS-PAGE and transferred to nitrocellulose membrane, and GTPases were detected using a specific monoclonal antibody against RhoA and Rac.

SDS-PAGE and Immunoblotting—SDS-PAGE of photolabeled proteins was performed on 12% (w/v) polyacrylamide gels. Photolabeled membrane proteins were visualized by autoradiography of the dried gels. Blotting of membrane proteins separated by SDS-PAGE, processing of immunoblots, and detection of immunoreactive proteins by chemiluminescence procedure (Amersham Pharmacia Biotech, Braunschweig, Germany) have been described previously (23).

Isolation of Actin Cytoskeleton—Reactions were stopped, and the cytoskeleton was immediately extracted by adding one volume of ice-cold twice-concentrated cytoskeleton buffer containing 100 mM Tris-HCl, pH 7.4, 20 mM EGTA, 2 mM Na2VO4, 4 μg/ml each of aprotinin and leupeptin, 2 mM phenylmethylsulfonyl fluoride, and 2% (v/v) Triton X-100 as described previously (25, 26). After 10 min at 4 °C, the cytoskeleton was pelleted by centrifugation (12,000×g for 10 min at 4 °C), and a fraction of the supernatant was taken as a control. Pellets were washed once in cytoskeleton buffer with 0.5% Triton X-100 and once with the same buffer without Triton X-100. Cytoskeleton and a
fraction of the post-spin supernatants as well as of the initial lysate were then immediately prepared for SDS-PAGE.

**Lipid Extraction and Analysis**—Reactions were stopped by addition of chloroform/methanol (1:1, v/v) containing 0.4 N HCl, and lipids were immediately extracted following the modified procedure of Bligh and Dyer (27, 28). PtdIns(4,5)P₂ lipids were immediately deacylated by 20% methylamine and analyzed by high performance liquid chromatography on a Whatman Partisphere 5 SAX column (Whatman International Ltd., UK) as described previously (28).

**Scanning Electron Microscopy**—Isolated platelets were preincubated under the indicated conditions. Thereafter, platelets were incubated in the absence or presence of U46619 (5 μM) for 5 s at 37 °C and then fixed for 10 min with 5% paraformaldehyde, 3.75% glutaraldehyde, 0.06 M c cacodylate buffer, and 3.4 mM CaCl₂. The fixed platelets were suction-filtered onto Nucleopore polycarbonate filters (0.45 μm), which had been preincubated with 10 μg/ml polylysine. Filters were washed three times with 0.9% NaCl and dehydrated stepwise in aqueous ethanol. After exchange of ethanol for hexadimethyldisilazane, samples were air-dried and sputtered with gold. Scanning electron microscopy was carried out on a Zeiss-Gemini instrument using a beam voltage of 5 kV.

**Photolabeling of Membrane Proteins and Immunoprecipitation of Ga-subunits**—After a preincubation of platelets for 20 min with or without Sp-5,6-DCI-cBIMPS (cAMP) (100 μM) or 8-pCPT-cGMP (cGMP) (1 mM), platelet membranes were prepared and photolabeled as described (21). Briefly, cell membranes (50–100 μg of protein per assay tube) were incubated at 30 °C in a buffer containing 0.1 mM EDTA, 10 mM MgCl₂, 30 mM NaCl, 1 mM benzamidine, and 50 mM Hepes-NaOH (pH 7.4). After 3 min of preincubation in the absence and presence of receptor agonist, samples were incubated for another 15 min with 10–20 nM [α-³²P]GTP azidoanilide (130 kBq per tube). [α-³²P]GTP azidoanilide was synthesized and purified as described (29). Samples were washed, dissolved in labeling buffer, and irradiated as described (21). Photolabeled membranes were pelleted, and proteins were preadsorbed in SDS. Solubilized membranes were preabsorbed with Protein A-Sepharose beads, and immunoprecipitation was performed as described (23).

**RESULTS**

Human as well as wild-type and Go₉-deficient mouse platelets were incubated with the TXA₂ mimetic U46619, and activation of Rho and Rac was investigated after different incubation times using the pull-down assays based on GST-Rho-binding domain or GST-Rac-binding domain fusion proteins (11, 24). Stimulation of human and mouse platelets by U46619 led to a very rapid and sustained activation of Rho and Rac, which reached a maximum a few seconds after addition of the stimulus (Fig. 1, A and B). In contrast, no activation of Rac was observed in Go₉-deficient platelets stimulated by U46619 (Fig. 1B). However, in the absence of Go₉, the TXA₂ analogue still induced a Rho activation with a time course indistinguishable from that observed in wild-type platelets (Fig. 1A). Quantification of precipitated Rho and of Rac in the lysates by densitometric analysis of immunoblots demonstrated that the amount of activated Rho in Go₉-deficient platelets exposed to U46619 was the same as that observed in wild-type platelets and amounted to about 10% of the total Rho (data not shown). These data indicate that the Go₉-mediated pathway is not required for Rho activation in platelets.

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**Fig. 3. Ca²⁺ and ADP are involved in U46619-mediated Rac activation.** Mouse platelets were preincubated for 15 min in the absence or in the presence of BAPTA-AM (30 μM), EGTA (2 mM), RO-31-8220 (10 μM), or 5 min with U-73122 (5 μM) and stimulated by U46619 (1 μM) (A). After 10 s, the reaction was stopped by ice-cold 2× RIPA buffer, and Rac activation was evaluated as described under “Experimental Procedures.” B, platelets were preincubated for 15 min with the indicated concentrations of BAPTA-AM before incubation in the absence or presence of 1 μM U46619 for 10 s was started. Shown is a densitometric evaluation of a blot representing the precipitated activated Rac. C, platelets were incubated with the indicated concentrations of the Ca²⁺ ionophore A23187, and Rac activation was determined as described. D, effect of ADP on Rac activation induced by U46619. Mouse platelet suspensions were incubated with apyrase (2 units/ml) or AR-C69931MX (1 μM) and stimulated with ADP (5 μM) or U46619 (1 μM) as indicated. For the Rac pull-down assay, aggregation was assessed at 37 °C with stirring, and after 10 s of stimulation the reaction was stopped and Rac activation was measured. E, platelets from Go₉-deficient mice were stimulated for 10 s with U46619 (1 μM), U46619 in the presence of ADP (10 μM), ADP alone, or with thrombin (5 IU/ml). Rac activation was assessed as described under “Experimental Procedures.” Data are representative of three independent experiments.
Gαq-deficient platelets do not show any aggregation and secretion in response to U46619 (22). Thus, the sustained Rho activation observed in Gαq-deficient platelets is obviously independent of integrin αIIbβ3 activation and feedback effects of secreted stimuli. To test whether Rac activation was dependent on integrin αIIbβ3-mediated aggregation, we pretreated platelets with the GP IIb/IIIa antagonist SR 121566A (30) as well as with the anti-GP IIb/IIIa antibody JON/A (31), which completely inhibited aggregation induced by U46619 (Fig. 2A). Rac activation by U46619 in mouse platelets was not affected by pretreatment of platelets with GP IIb/IIIa blocking agents (Fig. 2B). Similarly, blockade of integrin αIIbβ3 in human platelets by the peptide Arg-Gly-Asp-Ser (RGDS) had no effect on both Rho and Rac activation (Fig. 2C). These data demonstrate that the rapid activation of Rho and Rac after platelet activation occurs independently of integrin outside-in-signaling.

The lack of Rac activation in the absence of Gαq indicates that the Gα-mediated pathway is upstream of Rac. Rac activation by U46619 required an increase in the free cytosolic Ca2+ concentration, because it was strongly inhibited by pretreatment of platelets with the Ca2+ chelator BAPTA-AM and the phospholipase C inhibitor U-73122 but not by the protein kinase C inhibitor RO-31-8220 or the extracellular Ca2+ chelator EGTA (Fig. 3, A and B). To test whether an increase in the cytosolic calcium concentration would induce Rac activation, we incubated cells with A23187. Ionophore treatment resulted in a robust activation of Rac (Fig. 3C). The dependence of Rac activation on Gα, PLC, and Ca2+ suggests that a direct pathway mediated by phospholipase Cβ is involved in Rac activation or that Rac activation follows the Gα-mediated release of mediators, which in turn may activate Gβγ-coupled receptors. ADP, which is an important mediator of some TXA2 effects, can activate various receptors, including the Gβγ-coupled P2Y12 receptor (32). To exclude a possible contribution of Gβγ activated through U46619-induced ADP release, we degraded ADP with apyrase or blocked the Gβγ-coupled P2Y12 receptor with AR-C69931MX. Apyrase and AR-C69931MX completely blocked ADP-induced aggregation (Fig. 3D). Both apyrase and AR-C69931MX partially inhibited the U46619-induced Rac activation in wild-type platelets (Fig. 3D) indicating that ADP and the Gβγ-coupled P2Y12 receptor are involved in the effect of U46619 on Rac activation. To test whether Gβγ activation alone is sufficient to lead to activation of Rac in platelets, we tested the effect of thrombin and ADP on GTP loading of Rac in
**Differential Regulation of Rho and Rac**

Platelets have been extensively used as a model system to study rapid cell regulation through G-protein-coupled receptors. TXA₂ induces platelet shape change, aggregation, and granule secretion acting through receptors that couple to G_q and G₁₂/G₁₃ but not to G₁₃-type G-proteins (18, 21). Lack of G_q-mediated phospholipase C activation in platelets from Gα₉-deficient mice blocks TXA₂-induced platelet aggregation and response to U46619 in wild-type and Gα₉-deficient platelets. Although there was no Rac activation in Gα₉-deficient platelets in response to U46619, a significant increase in PtdIns(4,5)P₂ levels by about 30% could be observed shortly after addition of the TXA₂ analogue, which was about half of that seen in wild-type mouse platelets (Fig. 4C). At longer times of stimulation (3 min), the PtdIns(4,5)P₂ levels decreased in wild-type platelets probably due to the activation of phospholipase C, whereas in Gα₉-deficient platelets, which do not show any phospholipase C activation, PtdIns(4,5)P₂ levels remained elevated. These data show that platelets can form PtdIns(4,5)P₂ and undergo shape change in response to U46619 by a mechanism independent of Ca²⁺ and Rac activation. To test whether the Rho/Rho-kinase-mediated pathway is involved in the formation of PtdIns(4,5)P₂ found in Gα₉-deficient platelets, we preincubated wild-type platelets and platelets lacking Gα₉ with 10 μM of the Rho inhibitor Y-27632. Although PtdIns(4,5)P₂ formation in wild-type platelets was partially reduced, the U46619-dependent formation of PtdIns(4,5)P₂ in Gα₉-deficient platelets was completely blocked by Y27632 (Fig. 4D). This clearly suggests that PtdIns(4,5)P₂ formation in platelets is under dual control through a pathway mediated by G²/Rac and G₁₂/G₁₃/Rho/Rho-kinase.

The two main intracellular mediators of platelet inhibition, cAMP and cGMP strongly inhibit platelet aggregation but have been shown to differentially affect platelet shape change. While cAMP blocks the platelet shape change response, cGMP has no effect on shape change of human or mouse platelets (Fig. 5, Ref. 18). Preincubation of mouse platelets with prostacyclin which induces cAMP formation through the prostacyclin receptor or with the cAMP analogue Sp-5,6-DCl-cBIMPS strongly suppressed U46619-induced Rac activation in wild-type (Fig. 6A) and Gα₉-deficient platelets (data not shown). In contrast, the cGMP analogue 8-pCPT-cGMP was without effect (Fig. 6A). Activation of Rac by U46619 was, however, strongly inhibited by the cGMP analogue as well as by prostacyclin and the cAMP analogue. Very similar effects of cyclic nucleotides on Rho and Rac activation were observed in human platelets (Fig. 6B). Thus, cAMP inhibits both Rac and Rho activation while cGMP blocks only receptor-mediated Rac activation.

Because Rac activation by U46619 appears to be mediated by G₁₂/G₁₃, whereas Rac involves Gα₉, we tested whether cAMP and cGMP interfere with TXA₂ receptor-mediated activation of Gα₉ and/or G₁₂/G₁₃. Photolabeling of receptor-activated G-proteins in mouse platelet membranes and subsequent immunoprecipitation of individual G-protein α-subunits showed that, in wild-type mouse platelets, activated TXA₂ receptors couple to Gα₉, G₁₂, and G₁₃ (18). Preincubation of human platelet membranes with the cGMP analogue 8-pCPT-cGMP almost completely blocked U46619-induced activation of Gα₉ but had no effect on the activation of G₁₂ and G₁₃ (Fig. 7). In contrast, preincubation with the cAMP analogue Sp-5,6-DCl-cBIMPS resulted in the inhibition of U46619-induced activation of both Gα₉ and G₁₂/G₁₃ (Fig. 7). The effects of cAMP were, however, less pronounced than those of cGMP. These data indicate that cAMP and cGMP differentially interfere with coupling of TXA₂ receptors to Gα₉ and G₁₂/G₁₃. The selective effects of cGMP and cAMP on receptor-G-protein coupling may contribute to inhibition of G-protein-mediated Rho and Rac activation in platelets.

**DISCUSSION**

Platelets have been extensively used as a model system to study rapid cell regulation through G-protein-coupled receptors. TXA₂ induces platelet shape change, aggregation, and granule secretion acting through receptors that couple to G_q and G₁₂/G₁₃ but not to G₁₃-type G-proteins (18, 21). Lack of G_q-mediated phospholipase C activation in platelets from Gα₉-deficient mice blocks TXA₂-induced platelet aggregation and...
Differential Regulation of Rho and Rac

secretion but does not interfere with the ability of the TXA2 mimetic U46619 to induce platelet shape change, which appears to be mediated by G12/G13 (18, 22). Here we used human and mouse platelets to study the regulation of the small GTPases Rho and Rac.

Rho became rapidly activated in response to U46619 in wild-type platelets as well as in platelets lacking Gαq. This indicates that G-proteins of the G12 family are involved in the activation of Rho. In contrast, Rac activation in response to U46619 could only be observed in wild-type platelets and was absent in Gαq-deficient platelets, suggesting that Gαi mediates this effect and that G12/G13 are not involved. In neuronal cells and cardiomyocytes, it has been shown that Rac activation can be induced by stimuli that are believed to function through Gq/PLCγ. This indicates that it is mediated by Gq or G12/G13 (40, 41). On the other hand, in neutrophils, activation of Rac through the G-protein-coupled receptor of the chemotactant N-formyl-L-methionyl-L-leucyl-L-phenylalanine has been shown to occur in a pertussis toxin-sensitive manner indicating that it is mediated by Gq, type G-proteins (11, 13, 42).

TXA2 effects in platelets have been shown to be at least partially mediated through the release of mediators like ADP-Ribosylation of about 90% of Rho in human platelets

FIG. 6. Effect of cyclic nucleotides on RhoA and Rac activation. Platelets from wild-type mice (A) or human (B) were preincubated for 20 min with Sp-5,6-DCI-cBIMPS (cAMP) (100 μM) or 8-pCPT-cGMP (cGMP) (1 mM) or for 1 min with prostacyclin (PGI2, 500 nM). Thereafter, stimulation was started by the addition of U46619 (1 μM). After 10 s, activation was stopped by addition of ice-cold 2× RIPA buffer. The amount of activated Rho and Rac was determined as described under “Experimental Procedures.” Data are representative of three independent experiments. Shown are blots of the precipitates (Rho/Rac pull-down) as well as of 40% of the lysates (500 μl).

ADP+U46619 were able to activate Rac in Gαq-deficient platelets. Thus, full Rac activation appears to require both a direct Gq-dependent signal as well as the Gαi-mediated release of mediators acting through G12-coupled receptors.

Induction of Rho and Rac activation by U46619 occurred very rapidly. The fact that activation of Rho could also be observed in Gαq-deficient platelets, which do not secrete their granule content and aggregate in response to U46619, shows that Rho activation was independent of the Gq/PLCγ-mediated pathway resulting in degranulation and integrin αIIbβ3 activation. Blockade of integrin αIIbβ3 in wild-type platelets did not interfere with U46619-induced Rac activation, indicating that Rac activation also occurred independent of integrin signaling. Although Rho and Rac become activated in an integrin αIIbβ3-independent manner early during platelet activation, integrin activation may contribute to Rho and Rac activation at later stages of platelet activation in a manner similar to that occurring during integrin-mediated cell adhesion (23, 47–49).

Gαq-deficient platelets still undergo shape changes, including actin polymerization and phosphorylation of the myosin light chain (16, 50). Rho has been suggested to link receptor-activated G-proteins of the G12 family to the stimulation of Rho-kinase, which via inhibition of myosin phosphatase results in an increased phosphorylation of the myosin light chain (18–20). The fact that U46619-induced Rho activation was not affected by the loss of Gαq supports the concept that Rho is involved in the induction of platelet shape change by transducing the Gq/G12-mediated signal. Whether Rho is involved in regulation of integrin αIIbβ3 in platelets is not clear. Although partial activation of the RhoA pool in human platelets by C3 exoenzyme has been shown to inhibit platelet activation (51, 52), ADP-ribosylation of about 90% of Rho in human platelets...
mediators of platelet inhibition through activation of cAMP and cGMP-dependent kinases. Although analogues of both cyclic nucleotides can block platelet aggregation, only cAMP analogues inhibit platelet shape change (18, 56, 57). Similarly, we observed that the cAMP analogue Sp-5,6-DCl-cBIMPS but not the cGMP analogue 8-pCPT-cGMP inhibited TXA2 receptor-mediated shape change in wild-type and Gαq-deficient platelets (Fig. 5). The inhibition of Rho activation by Sp-5,6-DCl-cBIMPS but not by 8-pCPT-cGMP in human and mouse platelets as well as in Gαq-deficient platelets suggests that the Rho/Rho-kinase-mediated signaling cascade is inhibited by the cAMP-dependent pathway (Fig. 6). A similar role of cAMP was suggested for the inhibition of Rho/Rho-kinase-mediated neurite remodeling and morphology change in epithelial-like cells (58, 59). In contrast to platelets, cGMP appears to inhibit the Rho/Rho-kinase-mediated pathway in smooth muscle cells probably at a site downstream of Rho (60, 61). Both cGMP and cAMP inhibit Ca2+ mobilization through TXA2 receptors in platelets (62), which is mediated by the Gq/phospholipase Cβ pathway (22). Consistent with that, we found that both cyclic nucleotides blocked U46619-induced Rac activation in mouse and human platelets (Fig. 6). Because cGMP in contrast to cAMP does not interfere with platelet shape change, these data provide additional evidence that Rac activation is not required for platelet shape change. These findings also indicate that cAMP and cGMP exert their inhibitory effects on platelet activation at least in part by interfering with upstream mechanisms of receptor-induced signaling cascades.

By studying the effect of cGMP and cAMP analogues on coupling of TXA2 receptors to Gq and G12/G13, we found that cGMP strongly inhibited TXA2 receptor-mediated Gα activation, whereas cAMP partially inhibited activation of all three G-proteins through the activated receptors (Fig. 7). Thus, cGMP appears to selectively interfere with activation of Gα but not of G12/G13, which may explain the lack of cGMP effects on U46619-induced Rac activation. The TXA2 receptor has been shown to be a substrate for cAMP-dependent kinase (63, 64) as well as for the cGMP-dependent kinase (65), and cGMP inhibits TXA2 receptor-mediated stimulation of high affinity GTPase in platelet membranes (65). In addition, cAMP leads to the phosphorylation of TXA2 receptor-coupled Gα13 (66). These cyclic nucleotide-dependent kinase effects on the level of TXA2 receptors and their interaction with G-proteins may contribute to the observed inhibition of Gα-mediated Rac activation by cGMP and cAMP as well as of the G12/G13-mediated Rac activation by cAMP. Other mechanisms mediated by cyclic nucleotide-dependent kinases are likely to be also involved in cGMP/cAMP effects on Rho and Rac activation (67–70).

Taken together, our data show that Rho and Rac are differentially regulated by G12/G13 and through Gα-mediated mechanisms. Both processes occur rapidly upon receptor activation and are not dependent on integrin activation. Activation of Rac is not required for platelet shape change involving actin polymerization and myosin light-chain phosphorylation. The differential upstream regulation of Rho and Rac activity explains their different sensitivity toward the effects of cyclic nucleotides. Cyclic AMP partially inhibits receptor-mediated activation of Gq/G13 and Gα whereas cGMP only inhibits activation of Gq. Analogously, Rho and Rac activation via heterotrimeric G-proteins is subject to inhibition by cAMP whereas only the Rac activation can be blocked by cGMP.

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did not affect inside-out signaling of integrin α10β3, ligand-induced aggregation, and F-actin content (53).

The lack of Rac activation in the absence of Gαq demonstrates that activation of Rac is not required for the platelet shape change response in Gαq-deficient platelets that still show increased F-actin formation in response to U46619 (see Fig. 4, A and B (18)). Although Rac has been involved in PtdIns(4,5)P2-mediated actin polymerization (16), there appear to be alternative Rac-independent mechanisms that lead to actin polymerization in the absence of Rac activation. In Gαq-deficient platelets U46619 still increased the formation of PtdIns(4,5)P2. However, stimulation of PtdIns(4,5)P2 formation was about 50% lower than in wild-type platelets. This residual formation of PtdIns(4,5)P2 in the absence of Gαq could be completely blocked by the Rho-kinase inhibitor Y-27632 indicating that this Gαq- and Rac-independent formation is mediated through a Rho/Rho-kinase-dependent pathway. Both Rho and Rho-kinase have been shown to induce activation of phosphatidylinositol-4-phosphate 5-kinase in other cellular systems (35, 54). Collectively, these data show that two independent pathways involving Gα/Rac and G12/G13/Rho/Rho-kinase appear to mediate the receptor-dependent formation of PtdIns(4,5)P2. This regulation is different from a recently reported model based on a cotransfected Cos-7 cell system in which PtdIns(4,5)P2 formation through the G-protein-coupled receptor PAR1 was shown to involve the sequential activation of Gαq, Rac, Rho, and phosphatidylinositol-4-phosphate 5-kinase Iα (55).

The cyclic nucleotides cAMP and cGMP are physiological

![Fig. 7. Effects of cAMP and cGMP on receptor-dependent activation of G-proteins in membranes of human platelets. Human platelets were preincubated for 20 min with Sp-5,6-DCl-cBIMPS (cAMP) (100 μM) or 8-pCPT-cGMP (cGMP) (1 μM). Membranes were photolabeled with [γ-32P]GTP γ-sulfate in the absence or presence of U46619 (1 μM) and solubilized, and G-protein α-subunits (Gαq, Gα12, and Gα13) were immunoprecipitated as described under “Experimental Procedures.” Anti-Gαq, anti-Gα12, and anti-Gα13 antisera were used. Immunoprecipitated proteins were subjected to SDS-PAGE. Shown are autoradiograms of dried SDS gels. Data are representative of four independent experiments.](image-url)
