Activation of G₁₂/G₁₃ Results in Shape Change and Rho/Rho-Kinase–mediated Myosin Light Chain Phosphorylation in Mouse Platelets

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Abstract. Platelets respond to various stimuli with rapid changes in shape followed by aggregation and secretion of their granule contents. Platelets lacking the α-subunit of the heterotrimeric G protein Gq do not aggregate and degranulate but still undergo shape change after activation through thromboxane-A₂ (TXA₂) or thrombin receptors. In contrast to thrombin, the TXA₂ mimetic U46619 led to the selective activation of G₁₂ and G₁₃ in Gq₂₁-deficient platelets indicating that these G proteins mediate TXA₂ receptor-induced shape change. TXA₂ receptor-mediated activation of G₁₂/G₁₃ resulted in tyrosine phosphorylation of pp72 and stimulation of pp60Syk as well as in phosphorylation of myosin light chain (MLC) in Gq₂₁-deficient platelets.

Both MLC phosphorylation and shape change induced through G₁₂/G₁₃ in the absence of Gq₂₁ were inhibited by the C₃ exoenzyme from Clostridium botulinum, by the Rho-kinase inhibitor Y-27632 and by cAMP-analogue Sp-5,6-DCl-cBIMPS. These data indicate that G₁₂/G₁₃ couple receptors to tyrosine kinases as well as to the Rho/Rho-kinase–mediated regulation of MLC phosphorylation. We provide evidence that G₁₂/G₁₃–mediated Rho/Rho-kinase–dependent regulation of MLC phosphorylation participates in receptor-induced platelet shape change.

Key words: platelet • platelet shape change • G protein • Rho-kinase • myosin light chain phosphorylation

The functional responses of platelets to various full platelet activators are well characterized and include secretion of granular contents, platelet aggregation, and platelet shape change. Platelets are discoid in their resting state and upon activation by most stimuli rapidly change into a spheroid shape and extrude pseudopodia. This shape change is one of the earliest effects detectable in response to various platelet stimuli. Platelet shape change is believed to be a prerequisite for full platelet activation including degranulation and aggregation. The activation of platelets is responsible for primary hemostasis and underlies various pathological situations such as unstable angina pectoris, myocardial infarction, or cerebrovascular diseases.

Platelet shape change results from a rapid reorganization of the cytoskeleton including formation of new actin filaments, disappearance of the marginal band of microtubules, and centralization of granules (Siess, 1989; Wurzinger, 1990; Fox, 1993; Morgenstern, 1997). Signal transduction mechanisms regulating platelet shape change are ill defined. An involvement of tyrosine phosphorylation events, myosin light chain phosphorylation and polyphosphoinositide-induced actin polymerization have been suggested (Daniel et al., 1984; Hartwig et al., 1995; Negrescu et al., 1995; Maeda et al., 1995). Several reports have demonstrated that, in contrast to full platelet activation, induction of platelet shape change does not require elevation of the free cytosolic Ca²⁺ concentration (Rink et al., 1982; Simpson et al., 1986; Ohkubo et al., 1996). Consistent with that, we recently demonstrated that incubation of Gq₂₁-deficient platelets with various stimuli failed to induce phospholipase C activation and [Ca²⁺]i elevation as well as aggregation and degranulation. Platelet shape change, however, could still be elicited (Offermanns et al., 1997b). Platelets do not contain Gα₁₁, a close homologue of Gq (Milligan et al., 1993; Johnson et al., 1996; Offermanns et al., 1997b).

The effect of full platelet stimuli like thromboxane A₂ (TXA₂)³ and thrombin are mediated through G protein-
coupled receptors which have been shown to activate Gaq, Gia, G12, and G13 (Shenker et al., 1991; Hung et al., 1992; Offermanns et al., 1994; Ushikubi et al., 1994). G proteins are heterotrimeric which are defined by their α-subunits. According to structural and functional similarities, G protein α-subunits are grouped into four families, Gαq, Gαi, Gα12, and Gα13 (Simon et al., 1991). Although Gq-mediated activation of phospholipase C β-isomers appears to play a central and essential role in agonist-induced platelet aggregation and secretion, Gq-type G proteins are involved in the inhibitory regulation of platelet adenyl cyclase (Brass et al., 1997, 1988; Offermanns et al., 1997b). The role of the Gq family members, G12, and G13, in the regulation of platelet function is unclear. The α-subunits of both G proteins appear to be involved in the regulation of cell growth and cell movement (Dhanasekaran and Dermott, 1996; Offermanns et al., 1997a). Since G12/G13-coupled receptors appear to also activate Gq family members it has been difficult to selectively study the cellular signaling processes regulated by receptor-mediated activation of G12/G13. Most knowledge about the signaling pathways influenced by G12/G13 results from the use of constitutively active forms of Gα12 and Gα13. Either mutant has been shown to cause Na+/H+ exchanger activation, stimulation of phospholipase D, cell transformation, and formation of actin stress fibers through the small molecular weight GTPase Rho (Buhl et al., 1995; Hooley et al., 1996; Fromm et al., 1997; Plonk et al., 1998). The effector directly regulated by Go12 and Go13 has been elusive. However, the guanine nucleotide exchange factor (GER) for Rho, p115RhoGER, has been shown to interact with Gα12 and Gα13 and represents a candidate effector (Kozasa et al., 1998; Hart et al., 1998).

To study the possible role of G12/G13 in the platelet shape change response we took advantage of platelets from Gaq-deficient mice. In this report, we demonstrate that selective activation of G12/G13 is sufficient to induce the platelet shape change reaction, and we provide evidence that this involves Rho/Rho-kinase–mediated photophosphorylation of the myosin light chain.

**Materials and Methods**

**Materials**

U46619 was from Cayman Chemical, thrombin, histone (subgroup f2b), monoclonal anti-myosin light chain (MLC) antibody, and fluorescein isothiocyanate (FITC)-phalloidin were from Sigma. Sp-5,6-DCI-BIMPS and 8-PCPT-CGMP were from Biolog. Y-27632 was kindly provided by Yoshitomi Pharmaceutical Industries, *Clostridium botulinum* C3-exoenzyme was a donation from I. Just and K. Aktories (both from University of Freiburg, Freiburg, Germany) or was purchased from Upstate Biotechnology, anti-pTyr antibodies as well as anti-phosphotyrosine antibodies were from Santa Cruz Biotechnology, and anti-phosphotyrosine antibodies were from Oncogene. Antiseras against G protein α-subunits have been described (Offermanns et al., 1994; Laugwitz et al., 1994).

**Platelet Preparation and Aggregation**

Whole blood was collected from normal and Gaq-deficient mice anesthetized with pentobarbital by puncturing the inferior vena cava with hep- arinized syringes at a final concentration of 25 U heparin/ml blood. The blood from three or four Gaq-deficient mice and wild-type mice was pooled for each platelet aggregation experiment. Blood was diluted with half the volume of Heps-Tyrode-buffer (134 mM NaCl, 0.34 mM Na2HPO4, 2.9 mM KCl, 12 mM NaHCO3, 20 mM Heps, 5 mM glucose, 1 mM MgCl2, pH 7.3), and platelet rich plasma (PRP) was obtained by centrifugation for 7.5 min at 250 g. Thereafter, prostacyclin at a final concentration of 300 nM was added to the PRP, and platelets were pelleted by centrifugation at 1,200 for 5 min. The platelet pellet was resuspended in Heps-Tyrode buffer and incubated for 30 min at 37°C. Platelet suspension was adjusted to 300,000 platelets per microliter with Heps-Tyrode buffer. Optical aggregation experiments were conducted in a four-channel aggregometer (model Aggrecoader II PA-3220; Kyoto Daiichi Kagaku). Preincubation in Heps-Tyrode buffer without and with cGMP and cAMP analogues and Y-27632 was performed for 20 min at room temperature. Immediately before the aggregation experiments, platelets were preincubated for 1 min at 37°C in Heps-Tyrode buffer containing 1 mM CaCl2.

**Photolabeling of Membrane Proteins and Immunoprecipitation of Ga-subunits**

Platelet membranes were prepared and photolabeled as described (Offermanns et al., 1994). In brief, 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 Heps-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 (Offermanns et al., 1991). For photolabeling of Gα subunits, 5 μM GDP was present in the incubation buffer. Samples were washed, dissolved in labeling buffer, and then irradiated as described (Offermanns et al., 1994). Photolabeled membranes were pelleted and proteins were prenatrdated in SDS. Solubilized membranes were preadsorbed with protein A-Sepharose beads, and immunoprecipitation was done as described (Laugwitz et al., 1994).

**SDS-PAGE and Immunoblotting**

SDS-PAGE of photolabeled proteins was performed on 10% (wt/vol) acrylamide 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) has been described (Laugwitz et al., 1994).

**Determination of Cellular cAMP Levels**

Platelets (106 per tube) were preincubated for 15 min with 300 μM 3-isobutyl-1-methylxanthine and 20 μM 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone (Ro 20-1724) and incubated for 20 min in the absence and presence of receptor agonist. The reaction was stopped by the addition of 300 μl of ice-cold 10% (wt/vol) trichloroacetic acid. Samples were kept for 10 min on ice, and 180 μl of 1 M Tris, pH 9.8, was added to neutralize the samples. Cyclic AMP was determined by the competitive-binding assay (Gilman and Murad, 1974). In brief, samples were incubated for 2 h with 2 pmol of [8-3H]cAMP (925 GBq/mmol; Amersham) and 62.5 μg of cAMP-dependent protein kinase purified from porcine heart (Sigma) in a final volume of 200 μl at 4°C. Then, 4% (wt/vol) charcoal in 5 mM EDTA and 50 mM Tris-HCl, pH 7.5, was added, and samples were immediately centrifuged for 2 min at 12,000 g. Supernatants were counted in a liquid scintillation counter, and the amount of cAMP in the test sample was calculated as described (Gilman and Murad, 1974).

**Determination of Tyrosine Phosphorylation**

Isolated platelets (1–2 × 1011 platelets per tube) were incubated in 40 μl Heps-Tyrode buffer at 37°C as indicated. Reactions were stopped by addition of 20 μl of 3× sample buffer containing a final concentration of 1 mM Na3VO4. Heated samples were separated by SDS-PAGE on 10% gels. Immunoblotted proteins were analyzed for phosphotyrosine with an antiphosphotyrosine antibody.

**Immunoprecipitation and Immunocomplex Kinase Assay**

For immunoprecipitation of tyrosine kinases pp72v-src and pp60v-src, platelet suspensions (0.4–1 × 1010 platelets) were incubated in the absence or presence of 5 μM U46619 for the indicated time periods, and platelets were...
lysed by addition of an equal volume of ice-cold 2× radioimmunoprecipitation assay (RIPA) buffer (final concentration: 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate, 150 mM NaCl, 50 mM Hepes/NaOH, pH 7.4, 3 mM EDTA, 3 mM EGTA, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml aprotinin, 20 μg/ml leupeptin). After incubation for 20 min on ice, samples were centrifuged for 20 min at 15,000 g at 4°C, and incubated with 5 μg agarose conjugates of rabbit polyclonal anti-pp72syk IgG or 8 μl of agarose-conjugated mouse monoclonal anti-pp60c-src IgG, for 2 h at 4°C. Immunoprecipitates were collected by centrifugation at 15,000 g for 10 min at 4°C and were washed twice with 1× RIPA buffer, once with 1% Triton X-100, 0.3% SDS, 600 mM NaCl, and 50 mM Tris-HCl, pH 7.4, and once with 300 mM NaCl, 10 mM EDTA, 100 mM Tris-HCl, pH 7.4. For detection of pp72syk phosphorylation, precipitated proteins were eluted with 40 μl of 1× SDS sample buffer and separated by 10% polyacrylamide gels. Tyrosine phosphorylation of pp72syk and pp72syk protein were analyzed by immunoblotting. The anti-pp60c-src immunoprecipitates were divided into two aliquots; one was analyzed by anti-pp60c-src immunoblotting, and the other was subjected to in vitro kinase assay. To examine in vitro kinase activity, precipitates were incubated for 5 min at 25°C in kinase buffer containing 25 mM HEPES/NaOH, pH 7.4, 10 mM MgCl2, 1 μM ATP (7 μCi of [γ-32P]ATP/tube), and 0.25 mg/ml histone. Reaction was terminated by addition of 2× sample buffer, and samples were subjected to SDS-PAGE. Phosphorylation of histone was analyzed by autoradiography of dried gels.

MLC Phosphorylation

MLC phosphorylation was determined as described (Daniel and Sellers, 1992). Isolated platelets (1–2 × 10^8 platelets per tube) were incubated in 30 μl HEPES-Tyrode buffer at 37°C as indicated. Reactions were stopped by addition of 30 μl of 40% (vol/vol) perchloric acid. Precipitated samples were kept on ice for 20–30 min. After centrifugation (10 min at 15,000 g at 4°C) pellets were washed twice with acetone containing 10 mM DTT. 30 μl of SDS sample buffer was added to dried samples, and proteins were solubilized by sonication for 30 min. Separation of proteins on urea/glycin gels was done as described (Daniel and Sellers, 1992), and MLC was detected after immunoblotting with an anti-MLC antibody.

**Determination of F-actin Content**

For actin filament content measurements, platelets (10%) were incubated as indicated and fixed in 2% paraformaldehyde for 30 min at 37°C. Fixed platelets were permeabilized with 0.1% Triton X-100, incubated with 10 μM fluorescein isothiocyanate (FITC)-phalloidin (Sigma) for 30 min at room temperature and were then washed. Bound FITC-phalloidin was quantified using a fluorescence spectrophotometer (Perkin-Elmer) (excitation at 495 nm; emission at 519 nm).

**ADP Ribosylation of Platelet Lysates by C3 Exoenzyme**

Washed platelets were incubated with the indicated concentrations of C3 exoenzyme in Hepes-Tyrode buffer. Platelets were lysed by addition of an equal volume of lysis buffer (1.5% Triton X-100, 0.8% DOC, 0.2% SDS, 145 mM NaCl, 20 mM Hepes, pH 7.4, 3 mM EGTA, 0.3 mM phenylmethylsulfonyl fluoride, 5 μM leupeptin, 5 μg/ml aprotinin). ADP-ribosylation using [3H]NAD was performed as described (Morii et al., 1992), and ribosylated samples were separated on 12% polyacrylamide gels.

**Results**

We have recently shown that Goq-deficient platelets do not aggregate and secrete their granule contents in response to various stimuli indicating that Gq-mediated activation of phospholipase C, which is a key enzyme in platelet activation, is required for full platelet activation. For further details, please refer to Materials and Methods. ADP-ribosylation using [3H]NAD could be observed in Goq-deficient platelets by scanning electron microscopy of single cells (Fig. 1, A–D).
as well as by measuring the light transmission of a platelet suspension (Fig. 2). Shape change induced by U46619 in Goq-deficient platelets and wild-type platelets was blocked by the cAMP analogue Sp-5,6-DCl-cBIMPS but not by the cGMP analogue 8-pCPT-cGMP, whereas both cyclic nucleotides blocked aggregation in wild-type platelets (Fig. 2, A and B). Similar results were observed with thrombin-activated wild-type and Goq-deficient platelets (data not shown). Preincubation of platelets with the recently described Rho-kinase inhibitor Y-27632 (Uehata et al., 1997) blocked U46619-induced shape change both in wild-type and Goq-deficient platelets (Fig. 2, C and D). To assess the role of Rho in agonist-induced platelet shape change we preincubated platelets for 2 h with 50 µg/ml C3 exoenzyme which ADP ribosylates and inactivates the small GTPase Rho (Mori et al., 1992). This C3 exoenzyme concentration and preincubation time resulted in ADP-ribosylation of 70–75% of endogenous Rho as determined by the inability of C3 exoenzyme to [32P]ADP-ribosylate Rho in subsequently prepared cell lysates (Fig. 3). Longer preincubation times and higher C3 exoenzyme concentrations further increased the ADP-ribosylated fraction of Rho (Fig. 3), but resulted in preactivation of platelets (data not shown). C3-pretreated platelets showed markedly reduced shape change in response to U46619 with only partial spheration and occasional filopodia formation (Fig. 1, I–L and Fig. 2 E).

To identify the G proteins mediating receptor-induced platelet shape change we studied the coupling of TXA2 and thrombin receptors to heterotrimeric G proteins in wild-type and Goq-deficient mouse platelets. Receptors for both, thrombin and TXA2, have been shown to couple to members of the Gq, G12, and Gi families (Shenker et al., 1991; Hung et al., 1992; Offermanns et al., 1994; Ushikubi et al., 1994). In membranes of human platelets, receptors activated by thrombin couple to Gq, G12, G13, and Gi, whereas TXA2 receptors only activate Gq, G12, and G13 (Offermanns et al., 1994; Brass et al., 1997). 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 and thrombin receptors couple to Giq, Gi12, and Gi13 whereas Giq was only activated through the thrombin receptor (Fig. 5 A).
activity in wild-type or Goq-deficient platelets (data not shown). These data clearly demonstrate that in Goq-deficient platelets thrombin-receptors couple to Gi2, Gi3, and Gq, whereas only Gi2 and Gi3 are activated through TXA2 receptors. Consequently, effects which can still be induced by TXA2 receptor agonists in Goq-deficient platelets like the shape change response are mediated by Gi2 and/or Gi3. TXA2-activated Goq-deficient platelets therefore represent a model to study of Gi2/Gi3-mediated signaling processes.

Agonist-induced platelet activation results in tyrosine phosphorylation of multiple proteins (Ferrell and Martin, 1988; Nakamura and Yamamura, 1989; Golden and Brugge, 1989). Phosphorylation of these proteins occurs in three temporal phases which have been experimentally distinguished. Early tyrosine phosphorylation occurs by an integrin-independent mechanism, whereas the second and third wave of tyrosine phosphorylation depends on the aggregation of platelets through binding of fibrinogen to αIIbβ3-integrin (glycoprotein IIb-IIIa) (Clark et al., 1994b). In Goq-deficient platelets that do not aggregate in response to thrombin or U46619, only a subset of proteins became tyrosine phosphorylated upon exposure of platelets to both stimuli compared with wild-type platelets (Fig. 6, A and B). Most prominently, a rapid tyrosine phosphorylation of a protein of ~72 kD could be observed in Goq-deficient platelets activated with thrombin and U46619. In contrast, several proteins with relative molecular masses of 40 and 95–130 kD which were tyrosine phosphorylated in wild-type platelets did not show increased tyrosine phosphorylation in activated Goq-deficient platelets (Fig. 6, A and B).

Platelets contain several tyrosine kinases (Dhar and Shukla, 1993; Clark et al., 1994b; Jackson et al., 1996) among which pp72syk and pp60c-src are rapidly activated after stimulation of platelets in an aggregation-independent manner (Wong et al., 1992; Taniguchi et al., 1993; Maeda et al., 1993; Clark and Brugge, 1993; Clark et al., 1994a). To test whether the 72-kD protein that was tyrosine phosphorylated in response to U46619 and thrombin in Goq-deficient platelets represented pp72syk, we immunoprecipitated pp72syk from lysates of platelets exposed to U46619. Anti-phosphotyrosine immunoblots of pp72 syk immunoprecipitated demonstrated increased tyrosine phosphorylation of pp72syk in response to U46619 in Goq-deficient platelets as well as in wild-type platelets (Fig. 6 C). Auto-phosphorylation of pp72syk on tyrosine has been demonstrated to increase its enzymatic activity (Taniguchi et al., 1993; Clark et al., 1994a; Chacko et al., 1994; Fujii et al., 1994). Fig. 6 C shows that incubation of wild-type and Goq-deficient platelets with U46619 also resulted in a rapid increase in the activity of pp60c-src. Increases in tyrosine kinase activity could be observed within 10 s after addition of U46619 and were not affected by pretreatment of platelets with Y-27662 or C3 exoenzyme (data not shown). These data indicate that TXA2 receptor-mediated activation of Gi2/Gi3 leads to rapid activation of the tyrosine kinases pp72syk and pp60c-src in mouse platelets.

MLC phosphorylation has been suggested to be involved in early processes during platelet activation (Daniel et al., 1984). To test whether TXA2 receptor-Gi2/Gi3-mediated signaling in Goq-deficient platelets resulted in
MLC phosphorylation, we activated platelets with U46619 for different times and separated phosphorylated and unphosphorylated MLC on urea/glycin polyacrylamide gels. Separated proteins were blotted onto nitrocellulose filters and MLC was detected using a specific antiserum. Fig. 7 A shows that U46619 caused phosphorylation of the total detectable pool of MLC in wild-type platelets within 10 s. Interestingly, a rapid and apparently complete phosphorylation of MLC was also observed in Goq-deficient platelets activated by U46619. Chelation of extracellular Ca2+ by EGTA or preincubation of platelets with various tyrosine kinase inhibitors had no effect on U46619-induced MLC phosphorylation in wild-type and Goq-deficient platelets (data not shown). Although the cAMP analogue Sp,6-DCl-cBIMPS completely inhibited MLC phosphorylation in wild-type and Goq-deficient platelets the cGMP analogue 8-pCPT-cGMP was without effect (Fig. 7, B and C). In smooth muscle cells and fibroblasts, the phosphorylation state of MLC has been shown to be under dual control of the Ca2+/calmodulin-activated myosin light chain kinase (MLCK) as well as of myosin-phosphatase (Somlyo and Somlyo, 1994; Burridge and Chrzanowska-Wodnicka, 1996). Myosin-phosphatase has been demonstrated to be regulated by Rho/Rho-kinase (Kimura et al., 1996; Narumiya et al., 1997). Since U46619-induced platelet shape change was blocked by the Rho-kinase inhibitor Y-27632 and was greatly inhibited after reduction of the amount of active Rho by C3 exoenzyme (Figs. 1 and 2) we tested the effect of C3 exoenzyme and Y-27632 on U46619-induced phosphorylation of MLC. Fig. 7, D–F shows that Y-27632 blocked and C3 exoenzyme markedly inhibited U46619-induced MLC-phosphorylation in wild-type as well as in Goq-deficient platelets. Incomplete inhibition of MLC phosphorylation by C3 exoenzyme was most likely due to incomplete inactivation of Rho by C3 exoenzyme (see Fig. 3). Y-27632 exerted its inhibitory effect on receptor-induced MLC phosphorylation with higher potency in Goq-deficient platelets compared with wild-type platelets (Fig. 7, D and E). Similarly, the effect of C3 exoenzyme appeared to be more pronounced in the absence of Goq (Fig. 7 F). These data indicate that activation of G12/G13 through the TXA2 receptor results in MLC phosphorylation and that this process involves Rho/Rho-kinase. The data also provide further evidence for the concept that MLC phosphorylation underlies platelet shape change.

**Discussion**

Full platelet activators like TXA2 and thrombin function through G protein–coupled receptors which activate Goq, Go11, Go12, Go13, and G1 type G proteins (Shenker et al., 1991; Hung et al., 1992; Ushikubi et al., 1994; Offermanns et al., 1994). Go11, a close homologue of Goq and coexpressed with Goq in most cells, is not present in platelets (Milligan et al., 1993; Johnson et al., 1996; Offermanns et al., 1997b). In Goq-deficient platelets, the TXA2 mimetic U46619 and thrombin fail to induce platelet aggregation and degranulation. This is accompanied by a lack of phospholipase C activation and Ca2+ mobilization after TXA2 and thrombin receptor activation supporting the concept that Goq-mediated phospholipase C activation represents the main signaling process leading to full platelet activation (Offermanns et al., 1997b). Lack of Goq-mediated phospholipase C activation did not interfere with the ability of U46619 and thrombin to induce platelet shape change as shown by scanning electron microscopy of activated Goq-deficient platelets (see Fig. 1) and measurement of light transmission through a suspension of Goq-deficient platelets (see Fig. 2) (Offermanns et al., 1997b). Thus, induction of...
platelet shape change through receptors of different platelet stimuli is mediated by G proteins other than Gαq, and Gαq-deficient platelets provide a good model to study the mechanisms underlying receptor-induced shape change independently of secondary processes involving secretion and aggregation.

To identify the G proteins mediating platelet shape change, we studied the coupling of TXA2 and thrombin receptors to G12 family members and Gαq-type G proteins. Studies in human platelets have provided evidence that thrombin receptors but not TXA2 receptors couple to Gα1q-type G proteins resulting in an inhibition of adenylyl cyclase (Aktories and Jakobs, 1984; Houslay et al., 1986; Brass et al., 1988; Offermanns et al., 1994). Similarly, in membranes from wild-type and Gαq-deficient mouse platelets, thrombin increased incorporation of GTP-azidoanilide into Gα, whereas U46619 was without effect (see Fig. 5). Only thrombin was able to decrease cAMP levels in wild-type and Gαq-deficient platelets (data not shown). The fact that thrombin, but not TXA2 receptors, couple to Gα1q in mouse platelets clearly demonstrates that Gα1q-mediated processes do not play a significant role in the regulation of platelet shape change. Both activated TXA2 (see Fig. 5) and thrombin receptors (data not shown), coupled to Gα12 and Gα13 in wild-type and Gαq-deficient platelets. Thus, in Gαq-deficient platelets, the only G proteins found to be activated through TXA2 receptors were Gα12 and Gα13. Therefore, we conclude that Gα12 and/or Gα13 are the mediators of ligand-induced platelet shape change and that platelet shape change induced through TXA2 receptors in Gαq-deficient platelets can be regarded as a Gα12/Gα13-regulated physiological cellular function.

The signaling mechanisms regulating receptor-dependent platelet shape change are incompletely understood. Elevation of the cytosolic Ca2+ concentration is necessary for full platelet activation including granule secretion and aggregation. However, there is good evidence that elevation of [Ca2+]i alone is not sufficient to induce platelet shape change and that agonists can induce shape change without an increase in phospholipase C activity and without an increase in [Ca2+]i (Simpson et al., 1986; Negrescu and Siess, 1996). The mechanism of early receptor-induced tyrosine phosphorylation is not known. Tyrosine kinases like pp72sk and pp60src, which are rapidly activated in a partially αιβ3-integrin-independent manner, may be involved (Clark et al., 1994b; Negrescu and Siess, 1996). The mechanism of early receptor-mediated induction of platelet shape change since this occurs rapidly in a Ca2+- and αιβ3-integrin-independent manner (Clark et al., 1994b; Negrescu and Siess, 1996). We show here that TXA2-receptor-mediated activation of Gα12/Gα13 leads to tyrosine phosphorylation of pp72sk and activation of pp60src (see Fig. 6) supporting the concept that these tyrosine kinases are involved in early platelet activation. These data also indicate that G proteins of the Gα12-family can regulate tyrosine kinases. The mechanism of this regulation remains unknown.

MLC phosphorylation has been implicated in the regulation of cytoskeletal reorganization during platelet shape change (Daniel et al., 1984; Nachmias et al., 1985). Phosphorylated myosin interacts mainly with central actin filaments in platelets, and the forming myosin–actin complex has been suggested to be involved in the granule centralization process (Fox and Phillips, 1982; Stark et al., 1991; Fox, 1993). The phosphorylation state of MLC is under dual control of MLCK and myosin-phosphatase. It is well...
established that increase in \([\text{Ca}^{2+}]\) activates the \(\text{Ca}^{2+}\)/calmodulin-dependent MLCK. MLC phosphorylation by MLCK leads to actin–myosin interaction resulting in actin-stimulated ATPase activity of smooth muscle and non-muscle myosin (Somlyo and Somlyo, 1994; Kohama et al., 1996). Recently, it has been shown that upstream regulation of myosin phosphatase occurs independently of the cytosolic free calcium concentration through phosphorylation and inactivation of its regulatory subunit by Rho-kinase, a specific target of the small GTPase Rho (Kimura et al., 1996; Narumiya et al., 1997). In addition, Rho-kinase can directly phosphorylate MLC in vitro (Amano et al., 1996). There is increasing evidence that Rho/Rho-kinase–mediated MLC phosphorylation is involved in contractile responses in various cell types like vascular smooth muscle cells (Uehata et al., 1997), fibroblasts (Chihara et al., 1997), neuroblastoma cells (Amano et al., 1998; Hirose et al., 1998), astrocytoma cells (Majorum et al., 1998), or endothelial cells (Essler et al., 1998). It is, however, unclear how the Rho-mediated pathway is regulated through receptors.

The TXA2 mimetic U46619 caused a rapid phosphorylation of MLC in wild-type and \(\text{G}_{\alpha}\)-deficient platelets (see Fig. 7). Since U46619 does not lead to elevation of \([\text{Ca}^{2+}]\), in the absence of \(\text{G}_{\alpha}\) (Offermanns et al., 1997b) and since Rho-kinase inhibitor Y-27632 and C3 exoenzyme inhibited U46619-induced MLC phosphorylation in \(\text{G}_{\alpha}\)-deficient platelets, we conclude that a Rho/Rho-kinase–mediated pathway regulating MLC phosphorylation operates in platelets. Consistent with this, the Rho-kinase p60ROCK has been shown to be phosphorylated upon activation of human platelets in an \(\alpha_{\text{IIb}}\beta_{3}\)-integrin–independent way (Fujita et al., 1997). In addition, Rho and Rho-kinase can be coimmunoprecipitated with the myosin-binding subunit of myosin phosphatase from human platelets, and treatment of platelets with a TXA2-mimetic leads to rapid phosphorylation and inactivation of myosin phosphatase (Nakai et al., 1997). Conflicting data exist with regard to the role of Rho in early platelet activation as determined by C3 exoenzyme treatment. This is most likely due to the difficulties associated with the length of incubation and the high concentration of C3 exoenzyme required to inactivate a sufficient fraction of Rho. Although partial inactivation of the RhoA pool in human platelets by C3 exoenzyme has been shown to inhibit platelet activation (Mori et al., 1992), a recent report showed that ADP ribosylation of \(-90\%\) of Rho in human platelets did not affect inside-out signaling of integrin \(\alpha_{\text{IIb}}\beta_{3}\), ligand-induced aggregation and F-actin content (Leng et al., 1998). Our data clearly support a role of Rho in early platelet activation.

In wild-type platelets in which U46619 induces an elevation of \([\text{Ca}^{2+}]\), and most likely leads to \(\text{Ca}^{2+}\)/calmodulin-MLCK–mediated MLC phosphorylation, Rho-kinase blocker Y-27632 and C3 exoenzyme also inhibited MLC phosphorylation induced by U46619. Interestingly, both agents appeared to be less potent in wild-type platelets than in \(\text{G}_{\alpha}\)-deficient platelets (see Fig. 7, D–F). This suggests that both, \(\text{Ca}^{2+}\)-mediated activation of MLCK and inhibition of myosin phosphatase through Rho/Rho-kinase may synergistically increase MLC phosphorylation in activated wild-type platelets. In contrast, receptor-mediated MLC phosphorylation in \(\text{G}_{\alpha}\)-deficient platelets depends on the \(\text{Ca}^{2+}\)-independent, Rho-mediated pathway. Since shape change could be inhibited by the C3 exoenzyme as well as by Y-27632 in \(\text{G}_{\alpha}\)-deficient platelets (see Figs. 1 and 2) we suggest that Rho/Rho-kinase–mediated MLC phosphorylation is involved in TXA2 receptor-induced platelet shape change.

Cyclic nucleotides like cAMP and cGMP mediate physiological inhibition of platelet activation through activation of cAMP- and cGMP-dependent kinases. Although analogues of both cyclic nucleotides can block full platelet activation, only cAMP analogues inhibit platelet shape change (Matsuka et al., 1989; Menshikov et al., 1993). Similarly, we observed that the cAMP analogue Sp-5,6-DCI-cBIMPS but not the cGMP analogue 8-pCPT-cGMP inhibited TXA2 receptor-G12/G13–mediated shape change and MLC phosphorylation in \(\text{G}_{\alpha}\)-deficient platelets (see Figs. 2 and 7). Inhibition of MLC phosphorylation by Sp-5,6-DCI-cBIMPS but not by 8-pCPT-cGMP suggests that the Rho/Rho-kinase–mediated signaling cascade may be inhibited by the cAMP-dependent pathway. A similar role of cAMP was suggested for the inhibition of Rho/Rho-kinase–mediated neurite remodeling and morphology change in epithelial-like cells (Hirose et al., 1998; Dong et al., 1998).

Rho has been shown to be regulated by the activated \(\alpha\)-subunits of G12 and G13 (Buhl et al., 1995; Gohla et al., 1998; Kozasa et al., 1998). Since G12 and G13 are the only G proteins activated through TXA2 receptors in \(\text{G}_{\alpha}\)-deficient platelets and since TXA2 receptor-mediated MLC phosphorylation in \(\text{G}_{\alpha}\)-deficient platelets was inhibited by C3 exoenzyme and Rho-kinase inhibitor Y-27632 we suggest that TXA2 receptor-induced G12/G13 activation results in MLC phosphorylation through Rho-mediated activation of Rho-kinase. Activated Rho-kinase may phosphorylate MLC directly or act through phosphorylation and inhibition of myosin phosphatase. Additional, synergistic regulation of MLC phosphorylation in wild-type platelets occurs through G13-mediated activation of MLCK. The mechanism by which G12/G13 activate Rho remains to be elucidated. Epidermal growth factor tyrosine kinase has recently been involved in the G13-mediated Rho-dependent actin stress fiber formation in fibroblasts (Gohla et al., 1998). However, various tyrosine kinase inhibitors were unable to block TXA2 receptor-induced, G12/G13-mediated MLC phosphorylation in \(\text{G}_{\alpha}\)-deficient platelets (data not shown). This suggests that in platelets, G12/G13-induced Rho activation is not mediated by receptor- or nonreceptor-tyrosine kinases. Another possibility is that regulation of Rho by G12/G13 is mediated by a Rho-specific GEF. Genetic evidence in Drosophila showed that the Drosophila RhoGEF, DRhoGEF2, functions downstream of the Drosophila G12/G13 homologue concertina (Barrett et al., 1997), and it has recently been shown that the related mammalian RhoGEF, p115 RhoGEF, can directly link G13 to the regulation of Rho (Hart et al., 1998; Kozasa et al., 1998).

Using \(\text{G}_{\alpha}\)-deficient platelets which do not aggregate and secrete but undergo shape change in response to various stimuli, we show that activation of G12/G13 is sufficient to induce platelet shape change. Thus, different G protein–mediated signaling pathways appear to be specifically involved in the regulation of distinct processes during re-
ceptor-induced platelet activation. Although $G_\alpha$ is necessary for full platelet activation including aggregation and secretion, activation of $G_\alpha$ may counteract anti-aggregatory influences through inhibition of adenyl cyclase, and $G_{12}/G_{13}$ appear to be centrally involved in the platelet shape change response. Our data also indicate that $G_{12}/G_{13}$ can link receptors to tyrosine kinases as well as to Rho/Rho-kinase-mediated regulation of PLC phosphorylation, and we provide evidence that the latter pathway participates in the receptor-mediated induction of platelet shape change.

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