Activation of G_{12}/G_{13} 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 G_q do not aggregate and degranulate but still undergo shape change after activation through thromboxane-A_2 (TXA_2) or thrombin receptors. In contrast to thrombin, the TXA_2 mimetic U46619 led to the selective activation of G_{12} and G_{13} in $G\alpha_q$-deficient platelets indicating that these G proteins mediate TXA_2 receptor-induced shape change. TXA_2 receptor-mediated activation of G_{12}/G_{13} resulted in tyrosine phosphorylation of $pp72^{syk}$ and stimulation of $pp60^{c\text{-}src}$ as well as in phosphorylation of myosin light chain (MLC) in $G\alpha_q$-deficient platelets. Both MLC phosphorylation and shape change induced through G_{12}/G_{13} in the absence of $G\alpha_q$ 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 G_{12}/G_{13} couple receptors to tyrosine kinases as well as to the Rho/Rho-kinase–mediated regulation of MLC phosphorylation. We provide evidence that G_{12}/G_{13}-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 Ca^{2+} concentration (Rink et al., 1982; Simpson et al., 1986; Ohkubo et al., 1996). Consistent with that, we recently demonstrated that incubation of $G\alpha_q$-deficient platelets with various stimuli failed to induce phospholipase C activation and $[Ca^{2+}]_i$ elevation as well as aggregation and degranulation. Platelet shape change, however, could still be elicited (Offermanns et al., 1997b). Platelets do not contain $G\alpha_{11}$, a close homologue of $G\alpha_q$ (Milligan et al., 1993; Johnson et al., 1996; Offermanns et al., 1997b).

The effect of full platelet stimuli like thromboxane A_2 (TXA_2)[1] and thrombin are mediated through G protein–

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1. *Abbreviations used in this paper:* MLC, myosin light chain; MLCK, myosin light chain kinase; GEF, guanine nucleotide exchange factor; TXA_2, thromboxane A_2.

coupled receptors which have been shown to activate G_q, G_i, G_{12}, and G_{13} (Shenker et al., 1991; Hung et al., 1992; Offermanns et al., 1994; Ushikubi et al., 1994). G proteins are heterotrimers which are defined by their α-subunits. According to structural and functional similarities, G protein α-subunits are grouped into four families, $G\alpha_q$, $G\alpha_i$, $G\alpha_{12}$, and $G\alpha_s$ (Simon et al., 1991). Although G_q-mediated activation of phospholipase C β-isoforms appears to play a central and essential role in agonist-induced platelet aggregation and secretion, G_i-type G proteins are involved in the inhibitory regulation of platelet adenylyl cyclase (Brass et al., 1997, 1988; Offermanns et al., 1997b). The role of the G_{12} family members, G_{12} and G_{13}, 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 G_{12}/G_{13}-coupled receptors appear to also activate G_q family members it has been difficult to selectively study the cellular signaling processes regulated by receptor-mediated activation of G_{12}/G_{13}. Most knowledge about the signaling pathways influenced by G_{12}/G_{13} results from the use of constitutively active forms of $G\alpha_{12}$ and $G\alpha_{13}$. 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 $G\alpha_{12}$ and $G\alpha_{13}$ has been elusive. However, the guanine nucleotide exchange factor (GEF) for Rho, p115RhoGEF, has been shown to interact with $G\alpha_{12}$ and $G\alpha_{13}$ and represents a candidate effector (Kozasa et al., 1998; Hart et al., 1998).

To study the possible role of G_{12}/G_{13} in the platelet shape change response we took advantage of platelets from $G\alpha_q$-deficient mice. In this report, we demonstrate that selective activation of G_{12}/G_{13} is sufficient to induce the platelet shape change reaction, and we provide evidence that this involves Rho/Rho-kinase–mediated phosphorylation 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-DCl-cBIMPS 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 Biotechnologies, anti-$pp72^{syk}$ antibodies as well as anti-phosphotyrosine antibodies were from Santa Cruz Biotechnology, and anti-$pp60^{v\text{-}src}$ antibodies were from Oncogene. Antisera 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 $G\alpha_q$-deficient mice anesthetized with pentobarbital by puncturing the inferior vena cava with heparinized syringes at a final concentration of 25 U heparin/ml blood. The blood from three or four $G\alpha_q$-deficient mice and wild-type mice was pooled for each platelet aggregation experiment. Blood was diluted with half the volume of Hepes-Tyrode-buffer (134 mM NaCl, 0.34 mM Na_2HPO_4, 2.9 mM KCl, 12 mM $NaHCO_3$, 20 mM Hepes, 5 mM glucose, 1 mM $MgCl_2$, 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 *g* for 5 min. The platelet pellet was resuspended in Hepes-Tyrode buffer and incubated for 30 min at 37°C. Platelet suspension was adjusted to 300,000 platelets per microliter with Hepes-Tyrode buffer. Optical aggregation experiments were conducted in a four-channel aggregometer (model Aggrecorder II PA-3220; Kyoto Daiichi Kagaku). Preincubation in Hepes-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 Hepes-Tyrode buffer containing 1 mM $CaCl_2$.

Photolabeling of Membrane Proteins and Immunoprecipitation of Gα-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 $MgCl_2$, 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-^{32}P]GTP azidoanilide (130 kBq per tube). [α-^{32}P]GTP azidoanilide was synthesized and purified as described (Offermanns et al., 1991). For photolabeling of G_i α-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 predenatured in SDS. Solubilized membranes were preabsorbed 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 (10^8 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 agonists. The reaction was stopped by the addition of 300 μl of ice-cold 10% (wt/vol) trichloracetic acid. Samples were kept for 10 min on ice, and 180 μl of 1 M Tris, pH 9.8, was added to neutralize the sample. 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-^{3}H]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 × 10^7 platelets per tube) were incubated in 40 μl Hepes-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 Na_3VO_4. Heated samples were separated by SDS-PAGE on 10% gels. Immunoblotted proteins were analyzed for phosphotyrosine with an antiphosphotyrosine antibody.

Immunoprecipitation and Immune Complex Kinase Assay

For immunoprecipitation of tyrosine kinases $pp72^{syk}$ and $pp60^{c\text{-}src}$, platelet suspensions (0.4–1 × 10^9 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-pp72^{syk} IgG or 8 μl of agarose-conjugated mouse monoclonal anti-pp60$^{v\text{-}src}$ IgG_1 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 pp72^{syk} phosphorylation, precipitated proteins were eluted with 40 μl of 1× SDS sample buffer and separated by 10% polyacrylamide gels. Tyrosine phosphorylation of pp72^{syk} and pp72^{syk} protein were analyzed by immunoblotting. The anti-pp60$^{c\text{-}src}$ immunoprecipitates were divided into two aliquots; one was analyzed by anti-pp60$^{c\text{-}src}$ 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 $MnCl_2$, 1 μM ATP (7 μCi of [γ-^{32}P]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.

Scanning Electron Microscopy

Isolated platelets were preincubated under the indicated conditions. Thereafter, platelets were incubated in the absence or presence of 1 U/ml thrombin or 5 μM U46619 for 5 s at 37°C and then fixed for 10 min with 3% paraformaldehyde, 3.75% glutaraldehyde, 0.06 mM cacodylate buffer, and 3.4 mM $CaCl_2$. The fixed platelets were suction filtered onto polycarbonate filters (0.45 μm; Nucleopore) 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.

MLC Phosphorylation

MLC phosphorylation was determined as described (Daniel and Sellers, 1992). Isolated platelets (1–2 × 10^7 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 (10^8) 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 [^{32}P]NAD was performed as described (Morii et al., 1992), and ribosylated samples were separated on 12% polyacrylamide gels.

Results

We have recently shown that $G\alpha_q$-deficient platelets do not aggregate and secrete their granule contents in response to various stimuli indicating that G_q-mediated activation of phospholipase C represents the central early signal transduction process leading to full platelet activation. However, G_q-deficient platelets were still able to undergo ligand-induced platelet shape change. This suggests that G proteins other than G_q mediate the platelet shape change response. Shape change induced by the TXA_2 analogue U46619 could be observed in $G\alpha_q$-deficient platelets by scanning electron microscopy of single cells (Fig. 1, A–D)

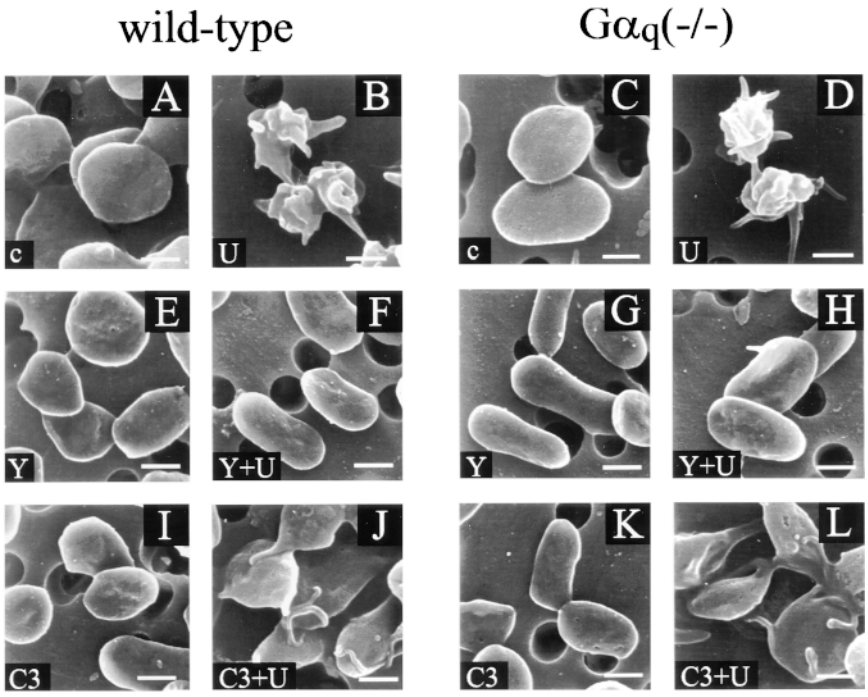

Figure 1. Scanning electron microscopy of inactivated and activated wild-type and $G\alpha_q$-deficient platelets. Wild-type platelets (A, B, E, F, I, and J) and $G\alpha_q$-deficient platelets (C, D, G, H, K, and L) were preincubated for 30 min in the absence (A–D) or presence of 10 μM Y-27632 (E–H) or were pretreated for 2 h with 50 μg/ml C3 exoenzyme (I–L). Thereafter, platelets were incubated for 5 s in the absence (A, C, E, G, I, and K) and presence of 5 μM U46619 (B, D, F, H, J, and L), fixed, and then analyzed as described in Materials and Methods. Platelets incubated for 2 h in C3-exoenzyme buffer alone showed normal shape change in response to U46619 (data not shown). Bar, 1 μm.

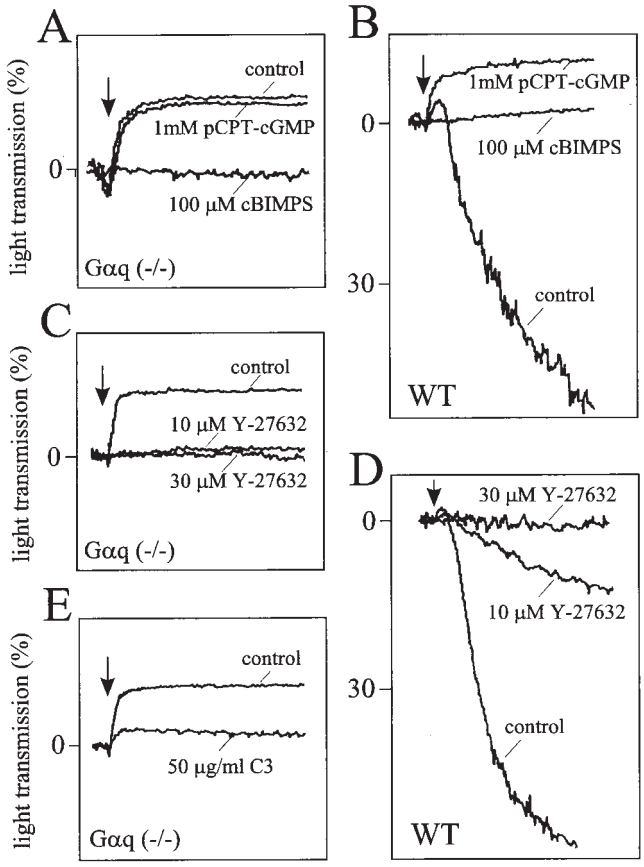

Figure 2. Aggregation response of wild-type and $G\alpha_q$-deficient platelets. Wild-type platelets (B and D) and platelets from $G\alpha_q$ (−/−) mice (A, C, and E) were preincubated for 20 min with the indicated concentrations of 8-pCPT-cGMP (pCPT-cGMP) or Sp-5,6-DCl-cBIMPS (cBIMPS) (A and B) or for 30 min with the indicated concentrations of Y-27632 (C and D), and $G\alpha_q$-deficient platelets were preincubated for 2 h in the absence or presence of C3 exoenzyme (E). Thereafter, incubation was started by the addition of 5 µM U46619. Shown is the relative light transmission through the platelet suspension. 0% represents transmission through the platelet suspension before addition of U46619, and 100% represents transmission through the buffer alone. Upward movements of the curve show decreases in light transmission indicating platelet shape change. Addition of stimuli is signified by the arrows, 3-min traces are shown.

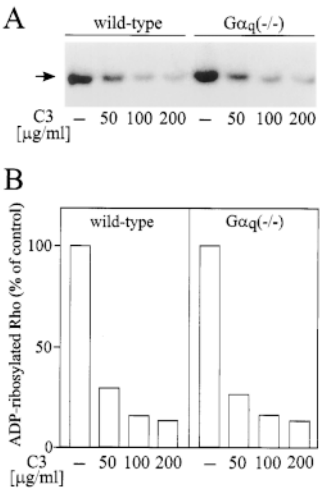

Figure 3. ADP ribosylation of Rho in C3-treated platelets. (A) Platelets from wild-type and $G\alpha_q$-deficient animals were preincubated for 2 h in the absence (−) or presence of the indicated concentrations of C3 exoenzyme. Thereafter, platelets were lysed and subjected to C3-exoenzyme catalyzed (^{32}P) ADP ribosylation as described under Materials and Methods. Shown is an autoradiogram with the position of the 21-kD standard protein on the left. (B) Densitometric evaluation of bands shown in A. Bars represent mean of two independent experiments as percent of control.

as well as by measuring the light transmission of a platelet suspension (Fig. 2). Shape change induced by U46619 in $G\alpha_q$-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 $G\alpha_q$-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 $G\alpha_q$-deficient platelets (Fig. 1, E–H and 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 (Morii 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 [^{32}P]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).

Since platelet shape change including protrusion of filopodia and lamellipodia is accompanied by actin polymerization (Siess, 1989; Wurzinger, 1990; Fox, 1993) we measured F-actin content in wild-type and $G\alpha_q$-deficient platelets (Fig. 4). U46619 induced an increase in F-actin content of both, wild-type and $G\alpha_q$-deficient platelets, which could be completely blocked by Y-27632. Reduction of the amount of functional Rho by pretreatment with 50 µg/ml C3 exoenzyme for 2 h markedly reduced the effect of U46619 in wild-type and $G\alpha_q$-deficient platelets.

To identify the G proteins mediating receptor-induced platelet shape change we studied the coupling of TXA_2 and thrombin receptors to heterotrimeric G proteins in wild-type and $G\alpha_q$-deficient mouse platelets. Receptors for both, thrombin and TXA_2, have been shown to be able to couple to members of the G_q, G_{12}, and G_i 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 G_q, G_{12}, G_{13}, and G_i, whereas TXA_2 receptors only activate G_q, G_{12}, and G_{13} (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 TXA_2 and thrombin receptors couple to G_q, G_{12}, and G_{13}, whereas G_i was only activated through the thrombin receptor (Fig. 5 A).

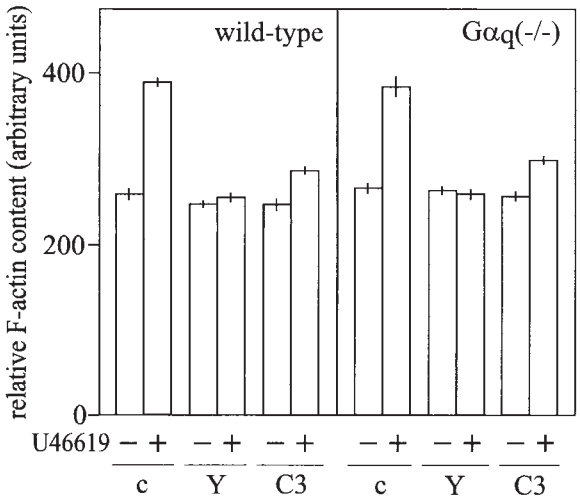

Figure 4. Determination of F-actin content. Wild-type and $G\alpha_q$-deficient platelets were preincubated for 30 min in the absence (c) or presence of 10 μM Y-27632 (Y) or were pretreated for 2 h with 50 μg/ml C3 exoenzyme (C3). Thereafter, platelets were incubated for 10 s in the absence (−) or presence of 5 μM U46619 (+), fixed, and then incubated with fluorescein isothiocyanate (FITC)-phalloidin. Platelets incubated for 2 h in C3 exoenzyme buffer alone showed full increase in F-actin content in response to U46619 (data not shown). F-actin content was determined as described in Materials and Methods. Shown are means ± SD of triplicates.

Similarly, in membranes from $G\alpha_q$-deficient platelets, only G_{12} and G_{13} were activated through the TXA_2 receptor, whereas activated thrombin receptors coupled to G_{12}, G_{13}, and G_i (Fig. 5 B and data not shown). Coupling of thrombin receptors to G_i in murine platelets corresponded with the ability of thrombin to decrease cAMP levels in wild-type as well as in $G\alpha_q$-deficient platelets, whereas activation of TXA_2 receptors had no effect on adenylyl cyclase activity in wild-type or $G\alpha_q$-deficient platelets (data not shown). These data clearly demonstrate that in $G\alpha_q$-deficient platelets thrombin-receptors couple to G_{12}, G_{13}, and G_i, whereas only G_{12} and G_{13} are activated through TXA_2 receptors. Consequently, effects which can still be induced by TXA_2 receptor agonists in $G\alpha_q$-deficient platelets like the shape change response are mediated by G_{12} and/or G_{13}. TXA_2-activated $G\alpha_q$-deficient platelets therefore represent a model to study of G_{12}/G_{13}-mediated signaling processes.

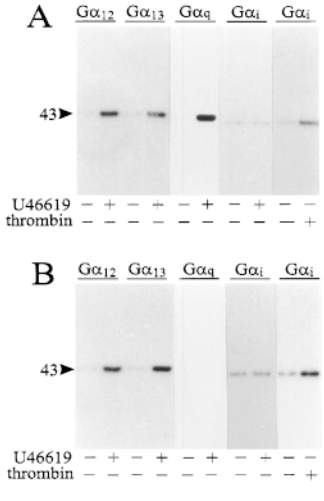

Figure 5. Receptor-dependent activation of G proteins in membranes of wild-type and $G\alpha_q$-deficient platelets. Membranes from wild-type (A) and $G\alpha_q$-deficient platelets (B) were photolabeled with [α-^{32}P]GTP azidoanilide in the absence (−) or presence of 5 μM U46619 or 5 U/ml thrombin (+). Membranes were solubilized and G protein α-subunits ($G\alpha_{12}$, $G\alpha_{13}$, $G\alpha_q$, and $G\alpha_i$) were immunoprecipitated as described under Materials and Methods. Anti-$G\alpha_{12}$, anti-$G\alpha_{13}$, anti-$G\alpha_{q/11}$ antisera, and an antiserum recognizing $G\alpha_{i1}$, $G\alpha_{i2}$, and $G\alpha_{i3}$ were used. Precipitated proteins were subjected to SDS-PAGE. Shown are autoradiograms of dried SDS gels with the position of the 43-kD standard protein shown on the left.

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 $\alpha_{IIb}\beta_3$-integrin (glycoprotein IIb-IIIa) (Clark et al., 1994b). In $G\alpha_q$-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 $G\alpha_q$-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 $G\alpha_q$-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 pp72^{syk} and pp60$^{c\text{-}src}$ 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 $G\alpha_q$-deficient platelets represented pp72^{syk}, we immunoprecipitated pp72^{syk} from lysates of platelets exposed to U46619. Anti-phosphotyrosine immunoblots of pp72^{syk} immunoprecipitates demonstrated increased tyrosine phosphorylation of pp72^{syk} in response to U46619 in $G\alpha_q$-deficient platelets as well as in wild-type platelets (Fig. 6 C). Autophosphorylation of pp72^{syk} 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 $G\alpha_q$-deficient platelets with U46619 also resulted in a rapid increase in the activity of pp60$^{c\text{-}src}$. 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-27632 or C3 exoenzyme (data not shown). These data indicate that TXA_2 receptor-mediated activation of G_{12}/G_{13} leads to rapid activation of the tyrosine kinases pp72^{syk} and pp60$^{c\text{-}src}$ in mouse platelets.

MLC phosphorylation has been suggested to be involved in early processes during platelet activation (Daniel et al., 1984). To test whether TXA_2 receptor-G_{12}/G_{13}–mediated signaling in $G\alpha_q$-deficient platelets resulted in

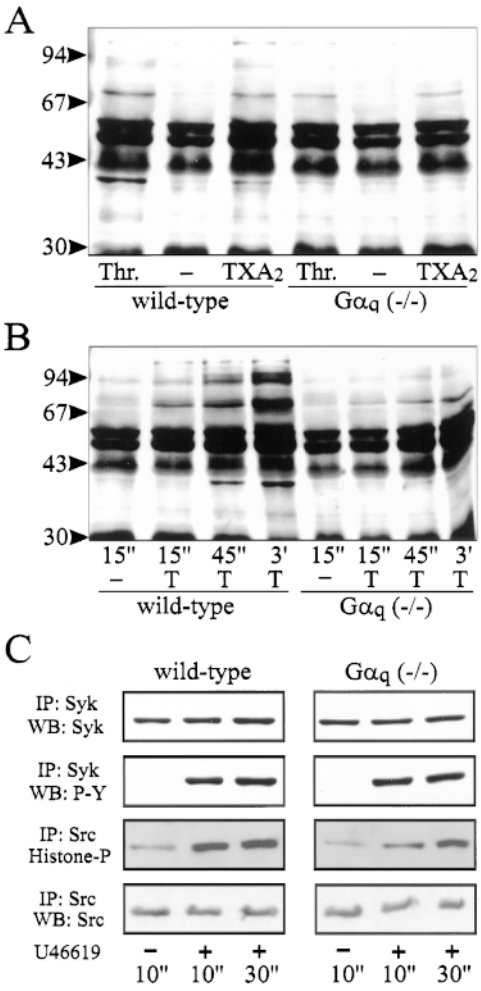

Figure 6. Effect of thrombin and U46619 on tyrosine phosphorylation and $pp72^{syk}$ and $pp60^{c\text{-}src}$-activity in wild-type and $G\alpha_q$-deficient platelets. (A) Wild-type and $G\alpha_q$-deficient platelets were incubated for 30 s in the absence (−) or presence of 5 U/ml thrombin (Thr.) or 5 μM U46619 (TXA_2). (B) Wild-type and $G\alpha_q$-deficient platelets were incubated for the indicated times in the absence (−) or presence of 5 μM of the thromboxane A_2 mimetic U46619 (T). Cells were lysed and cellular proteins were separated by SDS-PAGE and blotted on nitrocellulose filters. Phosphotyrosine was detected by an antiphosphotyrosine antibody. Shown are autoluminograms with the position of standard proteins shown on the left. (C) Wild-type (left) and $G\alpha_q$-deficient platelets (right) were incubated with buffer (−) or 5 μM U46619 (+) for the indicated times. Platelets were lysed and incubated with agarose conjugates of anti-$pp72^{syk}$ IgG (IP: Syk) or of anti-$pp60^{c\text{-}src}$ IgG (IP: Src), and immunoprecipitates were analyzed by immunoblotting with antiphosphotyrosine antibodies (WB: P-Y), anti-$pp72^{syk}$ antibodies (WB: Syk), anti-$pp60^{c\text{-}src}$ antibodies (WB: Src), or were subjected to in vitro kinase assays using histone as a substrate (Histone-P).

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 $G\alpha_q$-deficient platelets activated by U46619. Chelation of extracellular Ca^{2+} by EGTA or preincubation of platelets with various tyrosine kinase inhibitors had no effect on U46619-induced MLC phosphorylation in wild-type and $G\alpha_q$-deficient platelets (data not shown). Although the cAMP analogue Sp-5,6-DCl-cBIMPS completely inhibited MLC phosphorylation in wild-type and $G\alpha_q$-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 Ca^{2+}/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 $G\alpha_q$-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 $G\alpha_q$-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 $G\alpha_q$ (Fig. 7 F). These data indicate that activation of G_{12}/G_{13} through the TXA_2 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 TXA_2 and thrombin function through G protein–coupled receptors which activate G_q, G_{12}, G_{13}, and G_i type G proteins (Shenker et al., 1991; Hung et al., 1992; Ushikubi et al., 1994; Offermanns et al., 1994). $G\alpha_{11}$, a close homologue of $G\alpha_q$ and coexpressed with $G\alpha_q$ in most cells, is not present in platelets (Milligan et al., 1993; Johnson et al., 1996; Offermanns et al., 1997b). In $G\alpha_q$-deficient platelets, the TXA_2 mimetic U46619 and thrombin fail to induce platelet aggregation and degranulation. This is accompanied by a lack of phospholipase C activation and Ca^{2+} mobilization after TXA_2 and thrombin receptor activation supporting the concept that G_q-mediated phospholipase C activation represents the main signaling process leading to full platelet activation (Offermanns et al., 1997b). Lack of $G\alpha_q$-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 $G\alpha_q$-deficient platelets (see Fig. 1) and measurement of light transmission through a suspension of $G\alpha_q$-deficient platelets (see Fig. 2) (Offermanns et al., 1997b). Thus, induction of

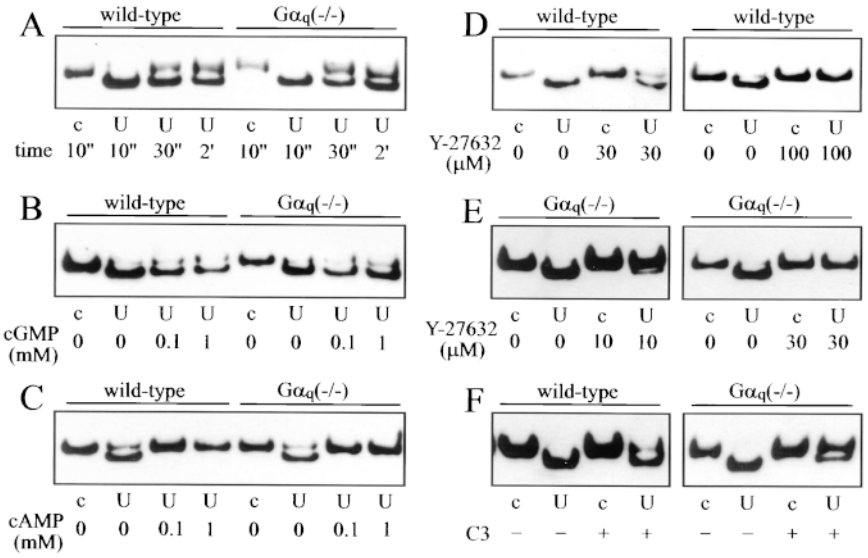

Figure 7. U46619-induced MLC phosphorylation in wild-type and $G\alpha_q$-deficient platelets. (A) Wild-type and $G\alpha_q$-deficient platelets were incubated for the indicated time periods with buffer (c) or 5 μM U46619 (U). (B and C) Platelets were preincubated for 20 min without or with the synthetic cyclic nucleotides 8-pCPT-cGMP (cGMP) or Sp-5,6-DCl-cBIMPS (cAMP) at the indicated concentrations. Thereafter, platelets were incubated for 10 s with buffer (c) or 5 μM U46619 (U). (D and E) Platelets were preincubated for 30 min without or with the Rho-kinase inhibitor Y-27632 at the indicated concentrations (30 or 100 μM for wild-type and 10 or 30 μM for $G\alpha_q$-deficient platelets). Incubation was conducted for 10 s with buffer (c) or 5 μM U46619 (U). (F) Wild-type and $G\alpha_q$-deficient platelets were preincubated for 120 min without (−) or with 50 μg/ml C3 exoenzyme (+). Thereafter, platelets were incubated for 10 s with buffer (c) or 5 μM U46619 (U). Reactions were stopped by addition of perchloric acid, and phosphorylation of MLC was determined using urea/glycin gels as described in Materials and Methods. Shown are autoluminograms of anti-MLC immunoblots. Phosphorylation of MLC results in a faster mobility (lower position) of MLC on urea/glycin gels.

platelet shape change through receptors of different platelet stimuli is mediated by G proteins other than G_q, and $G\alpha_q$-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 TXA_2 and thrombin receptors to G_{12} family members and G_i-type G proteins. Studies in human platelets have provided evidence that thrombin receptors but not TXA_2 receptors couple to G_i-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 $G\alpha_q$-deficient mouse platelets, thrombin increased incorporation of GTP-azidoanilide into G_i, whereas U46619 was without effect (see Fig. 5). Only thrombin was able to decrease cAMP-levels in wild-type and $G\alpha_q$-deficient platelets (data not shown). The fact that thrombin but not TXA_2-receptors couple to G_i in mouse platelets clearly demonstrates that G_i-mediated processes do not play a significant role in the regulation of platelet shape change. Both activated TXA_2 (see Fig. 5) and thrombin receptors (data not shown), coupled to G_{12} and G_{13} in wild-type and $G\alpha_q$-deficient platelets. Thus, in $G\alpha_q$-deficient platelets, the only G proteins found to be activated through TXA_2 receptors were G_{12} and G_{13}. We therefore conclude that G_{12} and/or G_{13} are the mediators of ligand-induced platelet shape change and that platelet shape change induced through TXA_2 receptors in $G\alpha_q$-deficient platelets can be regarded as a G_{12}/G_{13}-regulated physiological cellular function.

The signaling mechanisms regulating receptor-dependent platelet shape change are incompletely understood. Elevation of the cytosolic Ca^{2+} concentration is necessary for full platelet activation including granule secretion and aggregation. However, there is good evidence that elevation of $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ (Simpson et al., 1986; Negrescu et al., 1995; Ohkubo et al., 1996; Offermanns et al., 1997b). Tyrosine phosphorylation of various proteins has been associated with receptor-mediated induction of platelet shape change since this occurs rapidly in a Ca^{2+}- and $\alpha_{IIb}\beta_3$-integrin–independent manner (Clark et al., 1994b; Negrescu and Siess, 1996). The mechanism of early receptor-induced tyrosine phosphorylation is not known. Tyrosine kinases like $pp72^{syk}$ and $pp60^{c\text{-}src}$, which are rapidly activated in a partially $\alpha_{IIb}\beta_3$-integrin–independent manner, may be involved (Clark et al., 1994b; Presek and Martinson, 1997), and $pp72^{syk}$ has been implicated in the platelet shape change response in porcine platelets (Maeda et al., 1995). However, there is clear evidence that activation of $pp72^{syk}$ alone is not sufficient for induction of shape change (Negrescu and Siess, 1996). We show here that TXA_2-receptor–mediated activation of G_{12}/G_{13} leads to tyrosine phosphorylation of $pp72^{syk}$ and activation of $pp60^{c\text{-}src}$ (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 G_{12}-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 $[Ca^{2+}]_i$ activates the Ca^{2+}/calmodulin-dependent MLCK. MLC phosphorylation by MLCK leads to actin–myosin interaction resulting in actin-stimulated ATPase activity of smooth muscle and nonmuscle 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 (Majumdar et al., 1998), or endothelial cells (Essler et al., 1998). It is, however, unclear how the Rho-mediated pathway is regulated through receptors.

The TXA_2 mimetic U46619 caused a rapid phosphorylation of MLC in wild-type and $G\alpha_q$-deficient platelets (see Fig. 7). Since U46619 does not lead to elevation of $[Ca^{2+}]_i$ in the absence of $G\alpha_q$ (Offermanns et al., 1997b) and since Rho-kinase inhibitor Y-27632 and C3 exoenzyme inhibited U46619-induced MLC phosphorylation in $G\alpha_q$-deficient platelets, we conclude that a Rho/Rho-kinase–mediated pathway regulating MLC phosphorylation operates in platelets. Consistent with this, the Rho-kinase p160ROCK has been shown to be phosphorylated upon activation of human platelets in an $\alpha_{IIb}\beta_3$-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 TXA_2-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 (Morii 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 $\alpha_{IIb}\beta_3$, 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 $[Ca^{2+}]_i$ and most likely leads to 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 $G\alpha_q$-deficient platelets (see Fig. 7, D–F). This suggests that both, 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 $G\alpha_q$-deficient platelets depends on the Ca^{2+}-independent, Rho-mediated pathway. Since shape change could be inhibited by the C3 exoenzyme as well as by Y-27632 in $G\alpha_q$-deficient platelets (see Figs. 1 and 2) we suggest that Rho/Rho-kinase–mediated MLC phosphorylation is involved in TXA_2 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 (Matsuoka et al., 1989; Menshikov et al., 1993). Similarly, we observed that the cAMP analogue Sp-5,6-DCl-cBIMPS but not the cGMP analogue 8-pCPT-cGMP inhibited TXA_2 receptor-G_{12}/G_{13}–mediated shape change and MLC phosphorylation in $G\alpha_q$-deficient platelets (see Figs. 2 and 7). Inhibition of MLC phosphorylation by Sp-5,6-DCl-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 α-subunits of G_{12} and G_{13} (Buhl et al., 1995; Gohla et al., 1998; Kozasa et al., 1998). Since G_{12} and G_{13} are the only G proteins activated through TXA_2 receptors in $G\alpha_q$-deficient platelets and since TXA_2 receptor-mediated MLC phosphorylation in $G\alpha_q$-deficient platelets was inhibited by C3 exoenzyme and Rho-kinase inhibitor Y-27632 we suggest that TXA_2 receptor-induced G_{12}/G_{13} 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 G_q-mediated activation of MLCK. The mechanism by which G_{12}/G_{13} activate Rho remains to be elucidated. Epidermal growth factor tyrosine kinase has recently been involved in the $G\alpha_{13}$-induced Rho-dependent actin stress fiber formation in fibroblasts (Gohla et al., 1998). However, various tyrosine kinase inhibitors were unable to block TXA_2 receptor-induced, G_{12}/G_{13}-mediated MLC phosphorylation in $G\alpha_q$-deficient platelets (data not shown). This suggests that in platelets, G_{12}/G_{13}-induced Rho activation is not mediated by receptor- or nonreceptor-tyrosine kinases. Another possibility is that regulation of Rho by G_{12}/G_{13} is mediated by a Rho-specific GEF. Genetic evidence in *Drosophila* showed that the *Drosophila* RhoGEF, DRhoGEF2, functions downstream of the *Drosophila* G_{12}/G_{13} homologue concertina (Barrett et al., 1997), and it has recently been shown that the related mammalian RhoGEF, p115 RhoGEF, can directly link $G\alpha_{13}$ to the regulation of Rho (Hart et al., 1998; Kozasa et al., 1998).

Using $G\alpha_q$-deficient platelets which do not aggregate and secrete but undergo shape change in response to various stimuli, we show that activation of G_{12}/G_{13} 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_q is necessary for full platelet activation including aggregation and secretion, activation of G_i may counteract anti-aggregatory influences through inhibition of adenylyl 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 MLC phosphorylation, and we provide evidence that the latter pathway participates in the receptor-mediated induction of platelet shape change.

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