# Ras activation in platelets after stimulation of the thrombin receptor, thromboxane $A_2$ receptor or protein kinase C

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Several reports have indicated that the small G-protein Ras is not present immunologically in platelets. However, here we report the identification of Ras in platelets by immunoprecipitation with the Ras-specific monoclonal antibodies Y13-259 or Y13-238, followed by Western blotting. The presence of Ras was not due to contamination of samples with erythrocytes or leucocytes. Immunofluorescence studies indicated that Ras was present in a peripheral rim pattern in fixed, permeabilized platelets, suggesting an intracellular, plasma membrane location. Activation of platelets with the thrombin receptor peptide$^{42–50}$, the prostaglandin $H_2$/thromboxane $A_2$ mimetic U46619 or phorbol 12-myristate 13-acetate induced a rapid increase in GTP-bound, activated Ras. In each case, this increase was inhibited by the protein kinase C (PKC) inhibitor bisindolylmaleimide GF 109203X, suggesting that Ras is activated downstream of PKC in platelets. Thus the activation of Ras in platelets by agonists will now allow consideration of multiple potential Ras-dependent signal transduction pathways in platelet activation processes.

## INTRODUCTION

Although Ras was discovered as an oncogene product, it is now known that Ras activation by conversion from a GDP- to GTP-bound state leads not only to proliferative responses but also to diverse events such as regulation of muscarinic receptor coupling to potassium channels [1] and activation events in T-lymphocytes [2], mast cells and NIH 3T3 cells (reviewed in [3]). Several proteins that interact with Ras or are downstream of Ras in other cells are present in platelets, including Ras GTPase-activating protein (GAP) [4,5], Raf-1 kinase [6], mitogen-activated protein kinase kinase [6], mitogen-activated protein kinase [7–9] and phosphoinositide-3-kinase [10]. Although some early reports with antibodies of unclear specificity suggested the presence of Ras in platelets [11,12], later studies with the Ras-specific monoclonal antibody (mAb) Y13-259 suggested its absence [13,14]. Therefore the activation of Ras in platelets has remained unexplored. Given current efforts to map signal transduction pathways in platelets leading to events such as integrin $\alpha$IIb$\beta$3 activation with subsequent platelet aggregation, as well as granule secretion, we re-evaluated the presence of Ras in platelets. Delineating the roles of small G-proteins in platelets might be critical to understanding normal platelet biology as well as to understanding events leading to platelet thrombotic disorders (reviewed in [15]).

In this study we identified Ras in platelets by immunoprecipitation followed by blotting, and by immunofluorescent microscopy. We also determined that Ras is rapidly activated in platelets upon stimulation of the thrombin receptor, prostaglandin $H_2$ ($PGH_2$)/thromboxane $A_2$ ($TXA_2$) receptor, or protein kinase C (PKC). Activation of Ras via the $PGH_2$/$TXA_2$ receptor has not previously been demonstrated in any cell type. Thus Ras-mediated pathways might contribute to various signal–response couplings in platelets.

## EXPERIMENTAL

### Materials

Carrier-free $H_3{}^{32}PO_4$ was purchased from ICN Radiochemicals (Irvine, CA, U.S.A.). Polyethyleneimine cellulose (PEI) plates were obtained from J. T. Baker (Phillipsburg, NJ, U.S.A.), Nycoprep (Nycodenz) medium from Accurate Chemical (Westbury, NY, U.S.A.), paraformaldehyde from Polysciences (Warrenton, PA, U.S.A.), poly(vinylidene difluoride) (PVDF) membranes from Novex (San Diego, CA, U.S.A.), Lymphocyte Separation Medium from Organon Technika (Durham, NC, U.S.A.) and bisindolylmaleimide GF 109203X from Calbiochem (San Diego, CA, U.S.A.). Phorbol 12-myristate 13-acetate (PMA), prostaglandin $E_1$, prostaglandin $I_2$, poly-L-lysine and lysophosphatidylcholine were purchased from Sigma Chemical (St. Louis, MO, U.S.A.). The thrombin receptor activating peptide$^{42–50}$, SFLLRNPND (TRP), was synthesized by the Protein Chemistry Laboratory of the University of North Carolina at Chapel Hill/NIEHS (Chapel Hill, NC, U.S.A.) and purified by HPLC. The enhanced chemiluminescence detection system was purchased from Amersham (Arlington Heights, IL, U.S.A.). Gammabind G Sepharose, Gammabind Plus Sepharose, Sepharose 2B, Sepharose CL-2B and Sephadex G-100 superfine were obtained from Pharmacia (Piscataway, NJ, U.S.A.). 9,11-Dideoxy-9$\alpha$,11$\alpha$-methanoepoxyprostaglandin $F_{2\alpha}$ (U46619) was purchased from Caymen Chemical (Ann Arbor, MI, U.S.A.).

### Antibodies

The rat mAb Y13-259, against H-, N- and K-Ras [16,17], was provided by Dr. Channing Der and obtained from the American Type Culture Collection. Y13-259–agarose was obtained from two sources, Oncogene Science (Uniondale, NY, U.S.A.) and

Abbreviations used: mAb, monoclonal antibody; $PGH_2$, prostaglandin $H_2$; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PVDF, poly(vinylidene difluoride); RT, room temperature; TRP, thrombin receptor activating peptide$^{42–50}$; $TXA_2$, thromboxane $A_2$; U46619, 9,11-dideoxy-9$\alpha$,11$\alpha$-methanoepoxyprostaglandin $F_{2\alpha}$.

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Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). The H- and K-Ras-specific mAb Y13-238 [16,18] was obtained from the American Type Culture Collection. The Ras mAb F111-85 was from Santa Cruz Biotechnology. The H-Ras-specific mAb 146-3E4 [18] and the pantropic mAb 142-24E5 [19] were from Quality Biotech. (Camden, NJ, U.S.A.). A polyclonal antiserum raised against a synthetic peptide corresponding to amino acids 11–31 of mouse R-Ras was purchased from Pharmingen (San Diego, CA, U.S.A.). The polyclonal antiserum 1638 raised against human TC21/R-Ras2 [20] was a gift from Dr. Channing Der. Rat IgG–agarose and biotinylated goat anti-rat IgG were from Sigma. Control rat $IgG_{1\kappa}$ was from Pharmingen.

### Platelet preparation and $^{32}P$ labelling

#### Studies with PMA

Washed platelets were prepared as described [21], resuspended in buffer A [10 mM Hepes (pH 7.4)/138 mM NaCl/12 mM $NaHCO_3$/10 mM KCl/5.5 mM glucose], incubated with 2 mCi/ml $H_3{}^{32}PO_4$ for 2 h at 37 °C, washed and resuspended [$(5–6) \times 10^8$ platelets/ml] in buffer A containing 2 mM $CaCl_2$ and 0.5 mM $MgCl_2$.

#### Studies with TRP

Whole blood was obtained essentially as described above and centrifuged to obtain platelet-rich plasma. Prostaglandin $E_1$ (100 nM) was added and the platelets were pelleted and washed in buffer A containing 1 mM $CaCl_2$, 2 mM $MgCl_2$, 2 % (w/v) BSA and 100 nM prostaglandin $E_1$, and labelled as above in buffer A containing 1 mM $CaCl_2$, 2 mM $MgCl_2$ and 2 % (w/v) BSA. Unincorporated [$^{32}$P]$P_i$ was removed by gel-filtration [22]. Platelet integrity was tested by aggregation in response to TRP (10 $\mu$M) [23] or U46619 (1 $\mu$M).

### Activation of Ras

Unstirred, $^{32}$P-labelled platelets [500 $\mu$l at $(5–7) \times 10^8$/ml] were treated with agonist or buffer and frozen in liquid $N_2$. Platelets were pretreated with buffer or bisindolylmaleimide GF 109203X [24] for 1 min followed by agonist. Frozen platelets were thawed (1:1, v/v) into modified 2 × lysis buffer [2] [100 mM Hepes (pH 7.3)/1 M NaCl/10 mM $MgCl_2$/2 mM EGTA/2 % (w/v) Triton X-100/1 % (v/v) deoxycholate/0.1 % (v/v) SDS/20 $\mu$g/ml aprotonin/20 $\mu$g/ml leupeptin/20 $\mu$g/ml soyabean trypsin inhibitor/20 mM benzamidine/2 mM sodium phosphate/2 mM ATP]. Lysates were precleared with either Gammabind G or Gammabind Plus Sepharose and incubated overnight at 4 °C with 10 $\mu$g of Y13-259. Immunocomplexes were collected with either Gammabind G or Gammabind Plus Sepharose (2 h, 4 °C) and washed eight times with 50 mM Hepes (pH 7.3)/500 mM NaCl/5 mM $MgCl_2$/0.1 % (v/v) Triton X-100/0.005 % (v/v) SDS. Bound nucleotides were eluted with 2 mM EDTA/5 mM dithiothreitol/1 mM GTP/1 mM GDP/0.2 % (v/v) SDS (20 min, 68 °C) and resolved on polyethyleneimine cellulose plates in 1 M $KH_2PO_4$, pH 3.4. The plates were analysed with a Molecular Dynamics (Sunnyvale, CA) PhosphorImaging System with ImageQuant software (version 3.3).

### Quantification of Ras

In-dated human platelet concentrates (Virginia Blood Services) were centrifuged (250 ***g*** for 25 min). The resultant platelet-rich plasma was centrifuged (800 ***g*** for 20 min), platelets were resuspended in CGS buffer [129 mM sodium citrate/33.3 mM glucose/127 mM NaCl (pH 7.0)], and layered on a Nycodenz gradient. Gradients were centrifuged as described by the manufacturer, and a band containing purified platelets was harvested, washed twice with CGS buffer and resuspended in buffer A. To mimic any contamination in the final platelet preparation, erythrocyte-rich and leucocyte-rich fractions were prepared from whole blood: erythrocytes by centrifugation at 250 ***g*** followed by two washes in PBS, and leucocytes with Lymphocyte Separation Medium as described by the manufacturer. Preparations were analysed on a Technicon H-2 System (Bayer) to assess cellular contamination within each preparation. The preparations were lysed with 2 × lysis buffer and microcentrifuged (16000 ***g***, 15 min, 4 °C), and the protein concentration was determined with the bicinchoninic acid assay (Pierce).

Platelet lysate (2.5 mg of protein or approx. $1.15 \times 10^9$ cell equivalents) and a mixture of erythroctyte and leucocyte lysates ($5.4 \times 10^6$ and $2.7 \times 10^4$ cell equivalents respectively) were each precleared with Gammabind G Sepharose. Proteins were immunoprecipitated with either 10 $\mu$g of rat IgG–agarose or Y13-259–agarose for 1 h at 4 °C, subjected to reducing SDS/PAGE [25] and electrotransferred to a PVDF membrane (pore size 0.2 $\mu$m). The PVDF was immunostained with F111-85 or 146-3E4 and visualized with the enhanced chemiluminescence detection system. Exposures were performed with preflashed film to extend linearity and increase sensitivity.

### Immunofluorescence microscopy

Resting platelets were fixed with 1 % (w/v) paraformaldehyde as described [26]. Some samples were permeabilized for 5 min with 200 $\mu$g/ml lysophosphatidylcholine. Samples were rinsed with Tris-buffered saline containing 0.1 % BSA and incubated for 20 min with either Y13-259 or rat IgG1. Platelets were stained with biotinylated goat anti-rat IgG followed by rhodamine–avidin (Vector Laboratories), and viewed and photographed with a Jenval phase/fluorescence microscope as described [26].

## RESULTS AND DISCUSSION

Previous attempts to identify Ras from platelets on Western blots with the mAb Y13-259 against H-, K- and N-Ras [16,27,28] have been negative [13,14]; therefore the regulation of Ras in platelets has not been studied. However, Y13-259 is not particularly reactive on immunoblots [18]. We also used Y13-259 in an attempt to identify Ras in platelets, because it is considered a standard, widely used mAb in Ras-specific function studies [16,27,28]. To circumvent problems with the reactivity of the mAb on immunoblots, we instead used Y13-259 as an immunoprecipitating mAb, followed by the detection of Ras on Western blots by using the anti-Ras mAb F111-85, an efficient blotting mAb. A single band was observed (Figure 1, lanes 2–4) and its migration corresponded to a recombinant H-Ras standard (Figure 1, lanes 7–11). Immunoreactivity was not detected in immunoprecipitates from a species- and isotype-matched control mAb for Y13-259 (Figure 1, lane 5).

Because Ras is present in leucocytes [2] and erythrocytes [29], we determined whether these minimal cellular contaminants accounted for the observed immunoreactivity. (Maximal contamination was judged as described in the Experimental section to be 0.5 % erythrocytes and 0.002 % leucocytes.) However, Ras could not be immunoprecipitated from a mixture of erythrocyte and leucocyte lysates mimicking potential contamination within the platelet lysate (Figure 1, lane 1), demonstrating that the immunoreactivity is from platelets.

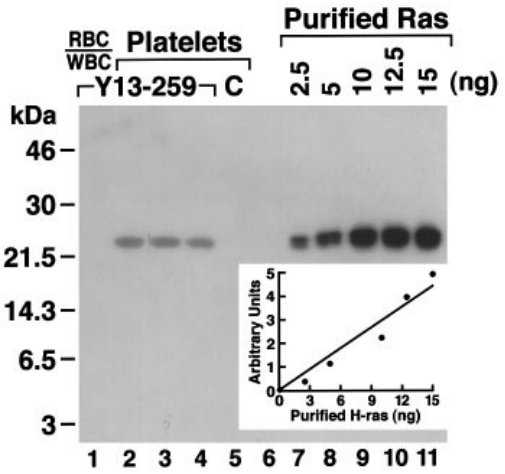


**Figure 1 Immunoprecipitation of Ras from platelets**

A Western blot of immunoprecipitates from platelet lysates and a mixture of erythrocyte and leucocyte lysates was prepared as described in the Experimental section and the blot was immunostained with mAb F111-85. Lane 1, Y13-259–agarose precipitate (10 $\mu$g of mAb) from erythrocyte plus leucocyte lysates corresponding to the amount of cross contamination within the platelet lysate; lanes 2–4, Y13-259–agarose precipitates of platelet lysate (triplicates); lane 5, control rat $IgG_1$–agarose precipitate of platelet lysate; lane 6, blank lane for densitometry background; lanes 7–11, recombinant H-Ras (Oncogene Science) for standard curve. Inset: linear fit of the recombinant H-Ras standard curve as determined by densitometric analysis ($r^2 = 0.93$).

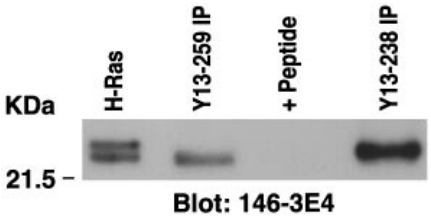


**Figure 2 Presence of H-Ras in platelets**

Platelet lysates were immunoprecipitated with the anti-H, N- and K-Ras mAb Y13-259–agarose or Y13-259–agarose neutralized with a peptide corresponding to residues 62–76 of H-Ras (50 $\mu$g of peptide). Lysates were also precipitated with the H- and K-Ras-specific mAb Y13-238 (5 $\mu$g). Precipitated proteins and a recombinant H-Ras standard (2 ng) were resolved on SDS/PAGE, electrotransferred to PVDF and immunostained with the H-Ras-specific mAb 146-3E4 to verify the presence of H-Ras [18].

Densitometric analysis of the immunoprecipitate from platelets (Figure 1, lanes 2–4) and from a standard curve of recombinant H-Ras (Figure 1, lanes 7–11 and inset) suggests the presence of approx. 0.5 pg of Ras/$\mu$g of platelet protein. Reprecipitation of lysates with Y13-259 did not result in significant amounts of detectable Ras. The accuracy of this determination could be limited by the fact that the immunoprecipitate from platelets might contain a mixture of Ras isoforms (H, N and/or K), in comparison with the purified H-Ras standard curve. The amount of Ras detected is small but similar to levels of endogenous Ras in some other cell types, e.g. 2.4–4.7 pg of Ras/$\mu$g of total protein in normal epithelial breast tissue and approx. 4.8 pg/$\mu$g in skeletal muscle, as determined with mAb Y13-259 [30].

To confirm the presence of Ras in platelets and to begin identifying isoforms, a platelet lysate was immunoprecipitated with either Y13-259 or the H- and K-Ras specific mAb Y13-238 [16,18]. Ras was detected in these immunoprecipitates on immunoblots stained with the H-Ras-specific mAb 146-3E4 [18], as shown in Figure 2. Moreover, when Y13-259–agarose was neutralized with a peptide corresponding to residues 62–76 of H-Ras, no immunoprecipitated protein was observed. These results demonstrate that one isoform of Ras in platelets is H-Ras. However, other Ras isoforms (N and K) might also be present.

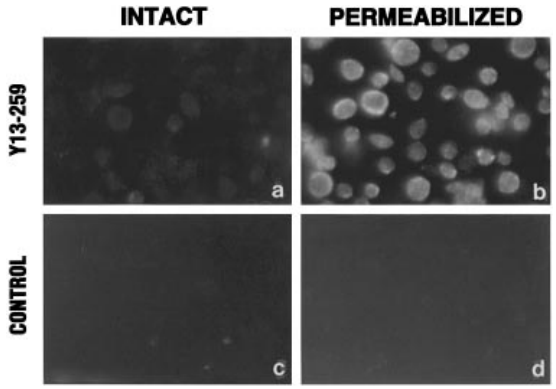


**Figure 3 Immunofluorescent localization of Ras in resting platelets**

Fixed platelets were sequentially stained with a primary mAb followed by biotinylated goat anti-rat IgG and rhodamine avidin as described in the Experimental section: intact (**a**) or permeabilized (**b**) platelets stained with Y13-259; intact (**c**) or permeabilized (**d**) platelets stained with control rat $IgG_1$. Magnification $\times 1500$.

Because the Y13-259 mAb has very recently been reported to cross-react weakly with TC-21 and R-Ras, the closest relatives of Ras, but not with other Ras-related proteins [18], experiments were performed to determine whether these molecules contributed significantly to the contents of the platelet Y13-259 immunoprecipitate. In one experiment, a Y13-259 immunoprecipitate from platelets was analysed by Western blotting with a specific polyclonal antiserum against TC21 [20]. No reactivity with the Y13-259 immunoprecipitate was observed (results not shown), suggesting that TC21 does not contribute significantly to the contents of the Y13-259 immunoprecipitate from platelets. To address the possible contamination of this immunoprecipitate with R-Ras, Y13-259-immunoprecipitated protein(s) were resolved on gels with low cross-linking under conditions that separated the 21 kDa Ras (H-, N- and/or K-isoforms) from the 23 kDa R-Ras. After electrotransfer and analysis by Western blotting with mAb 142-24E5, a broadly reactive Ras-family Ab [19], only one band of 21 kDa was observed, which was clearly separated from human R-Ras run in an adjacent lane (results not shown). Taken together, these results demonstrate that the major contents of the Y13-259 immunoprecipitate from platelets are indeed the major Ras isoforms (i.e. H, N and/or K).

We further tested the ability of several mAbs used above to detect Ras on Western blots of platelet lysate rather than of immunoprecipitate. We found, in agreement with previous reports [13,14], that Y13-259 did not detect Ras; neither did F111-85. However, 146-3E4 did detect a band in an appropriate position, demonstrating the increased sensitivity of this mAb (results not shown).

To localize Ras in platelets, intact and permeabilized platelets were stained with Y13-259 and viewed by fluorescence microscopy. No surface labelling of intact platelets was observed (Figure 3a). However, permeabilization of fixed platelets with lysophosphatidylcholine revealed a rim staining pattern,

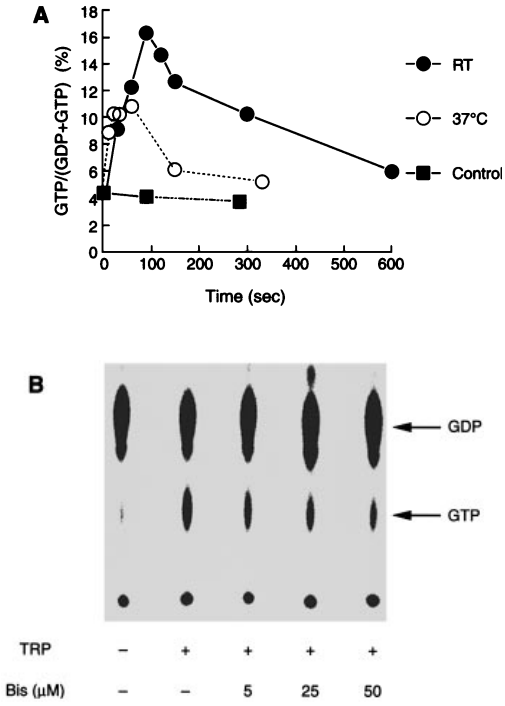


**Figure 4 Effects of TRP and bisindolylmaleimide GF 109203X on Ras activation in platelets**

(A) Time course of Ras activation in response to 50 $\mu$M TRP at RT (●) and 37 °C (○) or to Hepes-buffered saline (control, ■). Shown are results combined from one or two experiments, representing three or more similar experiments. (**B**) Inhibition of Ras activation by bisindolylmaleimide GF 109203X (Bis, HCl salt). Platelets were pretreated for 60 s at RT with Bis or doubly distilled water, followed by 50 $\mu$M TRP for 90 s. Shown is the phosphorimage of the separation of GTP from GDP by TLC. Note the decrease in GTP with increasing Bis concentrations. Numerical values from this set of experiments are provided in Table 1.

**Table 1 Bisindolylmaleimide GF 109203X (Bis) inhibition of Ras activation (% of control activation)**

Platelets were pretreated for 60 s at RT with Bis at the concentrations indicated or vehicle [doubly distilled water or 0.01% (v/v) DMSO] followed by agonist treatment for times necessary to reach maximal stimulation for each agonist (i.e. 1.5 min for TRP, 2.5 min for U46619, and 3.5 min for PMA). Experiments shown are averages or are means ± S.D., representative of two or more experiments. Abbreviation: n.d., not determined.

| | Ras activity (% of control) | | | |
|---|---|---|---|---|
| Agonist | [Bis] ($\mu$M) ... 0 | 5 | 25 | 50 |
| TRP | 100 ± 17 | 56.0 ± 12.3 | 33.4 ± 12.7 | 23.6 ± 9.8 |
| U46619 | 100 ± 14.8 | 13.8 ± 13.2 | n.d. | n.d. |
| PMA | 100 | 11.5 | n.d. | n.d. |

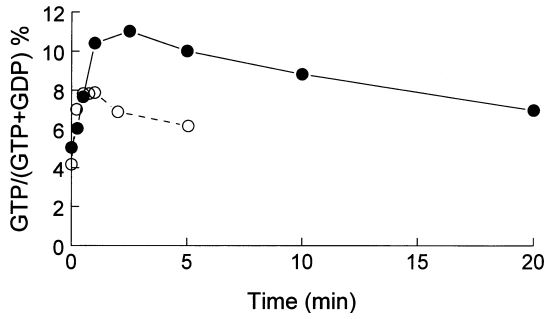


**Figure 5 Time course of Ras activation in response to the $PGH_2/TXA_2$ mimetic, U46619**

Platelets were pretreated with 1 $\mu$M U46619 at RT (●) and 37 °C (○) for the times indicated. The results shown are combined from two separate experiments.

suggesting that Ras epitopes are localized to the inner face of the plasma membrane (Figure 3b) as observed in other cell types [31]. Moreover, neither intact nor permeabilized platelets stained with control rat $IgG_1$ (Figures 3c and 3d). These results confirm that Ras is in platelets as opposed to other potentially contaminating cell types, and is localized to the inner plasma membrane as in other cells.

To determine whether Ras becomes activated in platelets, unstirred platelets were exposed to a variety of agonists at different times and temperatures. One agonist, TRP, mimics thrombin by binding to and activating the thrombin receptor, resulting in the induction of both arachidonic acid-dependent and independent pathways [32]. Treatment of platelets with TRP induced a rapid, transient activation of Ras: a 2.5-fold increase in Ras-associated GTP within 20 s at 37 °C and a 3.6-fold increase within 90 s at room temperature (RT) (Figure 4A). We speculate that the extent of Ras activation might be less at 37 °C than at RT owing to an increased effectiveness of Ras-GAP, which would facilitate the return of Ras to an inactive state. RT conditions were used in some subsequent experiments with inhibitors so that the time of peak Ras activation could be readily obtained. To determine whether $PGH_2$ and $TXA_2$, potent products of the cyclo-oxygenase-dependent arachidonic acid pathway, contributed significantly to TRP-induced activation of Ras, platelets were pretreated for 1 min with the cyclo-oxygenase inhibitor indomethacin. Indomethacin at 10 and 100 $\mu$M inhibited TRP-induced Ras activation by 14.9% and 29.3% respectively, suggesting that part of the observed Ras activation occurs through this pathway. In addition, because both TRP and $PGH_2/TXA_2$ activate PKC in platelets [33,34], we examined whether Ras activation occurred independently of or downstream of PKC. Platelets were pretreated with the specific PKC inhibitor bisindolylmaleimide GF 109203X [24]. Concentrations of this inhibitor that inhibited phosphorylation of the PKC substrate pleckstrin (results not shown) also inhibited TRP-induced Ras activation (maximal inhibition 76.4%; Figure 4B and Table 1), suggesting that Ras activation by TRP is largely PKC-dependent. However, a PKC-independent component might also be involved.

Thrombin receptor-mediated activation of Ras has also been observed in astrocytoma cells [27], potentially linked to PKC, and in fibroblasts [28] independent of PKC. In fibroblasts, thrombin receptor-mediated Ras activation was inhibited by pertussis toxin, implicating signalling through the $G_i$ family of G-proteins [28]. The associated $G\beta\gamma$ subunits might also activate Ras by a PKC-independent pathway [35]. In platelets and other cells, the thrombin receptor seems to couple to either pertussis toxin-sensitive $G_i$, which inhibits adenylate cyclase, or a pertussis toxin-insensitive $G_q$, which induces phosphoinositide hydrolysis [36] with resultant diacylglycerol generation and PKC activation. Perhaps a $G_q$-mediated pathway contributes to the PKC-dependent portion of TRP-induced Ras activation in platelets,

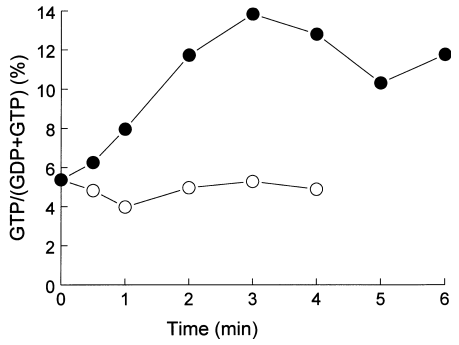


**Figure 6 Time course of Ras activation in response to PMA**

Platelets were pretreated with 1 $\mu$M PMA (●) or 0.1% DMSO (control, ○) at RT. Results are combined from two separate experiments.

although additional studies will be required to delineate the contributions of various heterotrimeric G-proteins to this event.

Because the results with indomethacin described above suggested that arachidonic acid metabolites induce Ras activation, we examined this directly by treating platelets with the stable $PGH_2/TXA_2$ analogue, U46619. This agonist induced a significant activation of Ras at RT or 37 °C (Figure 5), which did not decrease as rapidly as with TRP. However, as above, U46619-induced Ras activation was inhibited by pretreating platelets with the PKC inhibitor bisindolylmaleimide GF 109203X (Table 1), indicating the PKC-dependence of this event. U46619 or its naturally occurring homologues have not been previously described as activators of Ras. Thus we note a new pathway of Ras activation. Recent evidence suggests that the $TXA_2$ receptor in platelets couples to a member of the $G_q$ family [37], which, as with the thrombin receptor, might lead to Ras activation.

To confirm PKC-dependent Ras activation, platelets were treated with PMA, to activate PKC directly. PMA induced a 2.5-fold increase in Ras activation within 3 min at RT (Figure 6). This increase was almost completely inhibited by bisindolylmaleimide GF 109203X (Table 1), confirming that Ras activation can occur in a PKC-dependent manner in platelets. As with platelets, Ras activation downstream of PKC has been observed in some cells [2,38,39] but not in others [26,40,41]. Further studies will be required to verify whether a PKC-independent pathway of Ras activation also exists in platelets. Finally, neither the PMA- nor the TRP-induced activation of Ras was inhibited by pretreating platelets with 50 $\mu$M RGDW peptide (results not shown), which inhibits ligand binding to RGD-sensitive integrin adhesion molecules. This suggests that outside-in signalling through integrins does not contribute to Ras activation under the present conditions.

How Ras activation might affect platelet function is unknown. Phosphoinositide-3-kinase [41,42] and Rho [43] each seem to be critical signalling molecules in events leading to the activation of integrin $\alpha$IIb$\beta$3 and subsequent platelet aggregation. Some intriguing possibilities are that Ras activation might lie either upstream [44] or downstream [45] of phosphoinositide-3-kinase, or upstream of Rho [46,47]. Moreover, agonist-stimulated platelets also activate mitogen-activated protein kinase [7–9], which probably involves Ras activation. It will therefore be interesting to determine the relation of platelet Ras activation to these and other potential effector molecules and signalling pathways in platelets. Finally, it has recently been demonstrated that when integrin $\alpha$IIb$\beta$3 is expressed in CHO cells along with constitutively active R-Ras, the integrin adopts an active, ligand-binding, conformation [48]. These results suggest that this or other closely related small G-proteins might play a role in the process of inside-out signalling leading to integrin activation.

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