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**RAPID STIMULATION OF TYROSINE PHOSPHORYLATION SIGNALS  
DOWNSTREAM OF G PROTEIN-COUPLED RECEPTORS FOR THROMBOXANE  
A<sub>2</sub> IN HUMAN PLATELETS.**

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Short title: TXA<sub>2</sub> triggers platelet tyrosine phosphorylation

## SYNOPSIS

Signals ensuing from trimeric G protein-coupled receptors synergize to induce platelet activation. At low doses, the thromboxane A2 analogue U46619 does not activate the  $\alpha_{IIb}\beta_3$  integrin nor trigger platelet aggregation, but it induces shape changes. We addressed whether low U46619 doses trigger tyrosine phosphorylation signals independently of  $\alpha_{IIb}\beta_3$  integrin activation and ADP secretion, and synergize with epinephrine to induce aggregation in aspirin-treated platelets. Low U46619 doses triggered tyrosine phosphorylation of different proteins including the focal adhesion kinase, Src and Syk independently of signals ensuing from the  $\alpha_{IIb}\beta_3$  integrin or ADP receptors engaged by secreted ADP. The  $G_{12/13}$ -mediated Rho/Rho kinase pathway was also increased by low U46619 doses. However, this pathway was not upstream of tyrosine phosphorylation because this occurred in the presence of the Rho kinase inhibitor Y-27632. Whereas low U46619 doses or epinephrine alone were unable to trigger platelet aggregation and  $\alpha_{IIb}\beta_3$  activation, the combination of the two stimuli effectively induced these responses. PP2, a tyrosine kinase inhibitor, and Y-27632 inhibited platelet activation induced by low U46619 doses plus epinephrine and when used in combination totally suppressed this platelet response. However, the two inhibitors selectively blocked the tyrosine kinase or the Rho/Rho kinase pathway, respectively. These findings suggest that both tyrosine phosphorylation signals and the Rho/Rho kinase pathway are required to activate platelet aggregation via  $G_{12/13}$  plus  $G_z$  signaling.

**Key words:** Platelet / Thromboxane A2 / G proteins / Tyrosine phosphorylation / Rho/Rho kinase

## INTRODUCTION

Most platelet agonists interact with trimeric G protein-coupled receptors. Signaling pathways originating from different G proteins act in a highly integrated fashion to trigger platelet activation (reviewed in [1, 2]). For example, co-stimulation of G<sub>q</sub>-coupled P2Y<sub>1</sub> receptors and G<sub>i</sub>-coupled P2Y<sub>12</sub> receptors, [3], and combined signaling via G<sub>12/13</sub> and G<sub>i</sub> or G<sub>i</sub> and G<sub>z</sub> [4-6] are required to trigger full platelet aggregation.

Thromboxane A<sub>2</sub> (TXA<sub>2</sub>) is a platelet agonist that binds to receptors linked to both G<sub>q</sub> and G<sub>12/13</sub> proteins [7, 8]. At doses that stimulate full platelet activation, TXA<sub>2</sub> triggers G<sub>q</sub>-mediated responses [9, 10]. Besides activating the fibrinogen binding function of  $\alpha_{\text{IIb}}\beta_3$ , this signaling pathway is implicated in secretion of ADP, which amplifies the platelet response via the G<sub>i</sub>-coupled P2Y<sub>12</sub> receptor [2]. Signal transduction by G<sub>12/13</sub>-coupled TXA<sub>2</sub> receptors has been mainly addressed in the context of platelet shape change [7]. More recently, a G<sub>12/13</sub> signaling pathway was reported to act in concert with G<sub>i</sub>-mediated signaling to fully activate platelet aggregation and degranulation [4, 5], and G<sub>13</sub>-deficiency has been shown to result in a markedly reduced hemostatic response *in vivo* [11]. Signaling by G<sub>12/13</sub> implicates the small GTPase Rho and its target Rho-kinase/ROCK [7, 12, 13]. In addition, selective stimulation of G<sub>12/13</sub>-coupled TXA<sub>2</sub> receptors by low doses of the thromboxane analogue U46619 or the isoprostane 8-iso-PGF<sub>2 $\alpha$</sub>  was reported to activate cytoplasmic tyrosine kinases and the mitogen activated protein (MAP) kinase p38 [7, 12, 14, 15].

Signaling by trimeric G protein-coupled receptors implicate cytoplasmic tyrosine kinases in different cell types (see[16-23] and references contained therein). However, the role of tyrosine phosphorylation signals in activation of platelet responses via distinct G <sub>$\alpha$</sub>  protein families has been addressed only marginally [6, 7, 15, 24, 25], despite the first clear demonstration of an activation of the Src kinase by G protein-coupled receptors was provided by a study showing a rapid activation of Src in response to thrombin in platelets from patients with Glanzmann's thrombasthenia, which do not express  $\alpha_{\text{IIb}}\beta_3$  [24].

In order to elucidate the role of tyrosine phosphorylation in signal transduction by TXA<sub>2</sub> receptors we exploited the evidence that low doses of the TXA<sub>2</sub> analogue U46619 trigger some platelet responses, i.e. shape change and adhesion to surface bound fibrinogen, but no detectable aggregation and secretion [5, 14, 26, 27]. In this report, we show that U46619 at low doses triggers tyrosine phosphorylation signals and that this signaling pathway is not downstream of Rho/Rho-kinase activation. We also found that, whereas low doses of U46619 or epinephrine alone were unable to induce platelet activation, the two stimuli used in combination were potent agonists of platelet aggregation, providing evidence that both

tyrosine phosphorylation signals and the Rho/Rho kinase pathway are required to activate platelet aggregation resulting from combined  $G_{12/13}$  and  $G_z$  signaling.

## EXPERIMENTAL

### Platelet preparation and stimulation

Venous blood samples (40 ml) were collected from healthy adult human volunteers who had not been taking any drug in the previous 3 weeks and gave their informed consent. Acid-citrate-dextrose mixture (14 mmol/L sodium citrate, 11.8 mmol/L citric acid, 18 mmol/L dextrose, Merck, Darmstadt, Germany) was used as anticoagulant. Washed platelets were obtained by centrifugation at 200g for 10 min, followed by 700g for 15 min at room temperature, in the presence of 0.4 U/ml apyrase-V (Sigma, St. Louis, MO, USA). Platelets were then suspended at 37°C in 2-3 ml 10 mmol/L HEPES buffer pH 7.4 (145 mmol/L NaCl, 5 mmol/L KCl, 0.5 mmol/L  $Na_2HPO_4$  and 6 mmol/L glucose), according to Hallam [28]. 100  $\mu$ mol/L acetylsalicylic acid (ASA, Sigma) was added to platelet suspensions to inhibit the platelet COX-1 activity. Platelet suspensions were kept at room temperature and tested within 2 h. The stable thromboxane analogue U46619 (9,11-dideoxy-9,11-epoxymethanoprostanoic acid) (Sigma) was used at concentrations ranging between 0.05 and 1  $\mu$ mol/L [29, 30]. In some experiments, platelets were also co-stimulated with 10  $\mu$ mol/L epinephrine (A. Menarini diagnostics, FI, Italy) and 0.05  $\mu$ mol/L U46619 [5]. Platelet suspensions were pre-incubated with the following inhibitory compounds before the assays: Y-27632, [(R)-(+)-trans-N-(4-Pyridyl)-4-(1-aminoethyl)-cyclohexane-carboxamide, 2HCl; p160<sup>ROCK</sup> inhibitor] (Calbiochem) (final concentration, 30  $\mu$ mol/L, time of pre-incubation, 10 min at 37°C); PP2 (4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-d]pyrimidine) or its inactive analogue PP3 (4-Amino-7-phenylpyrazolo[3,4-d]pyrimidine) (Calbiochem) (final concentration, 10  $\mu$ mol/L, time of pre-incubation, 10 min at 37°C); Eptifibatid (Integrilin, Schering-Plough S.p.A., Bruxelles, Belgium), a synthetic RGD-mimetic (final concentration, 10  $\mu$ g/ml, time of pre-incubation, 30 min at 37°C); Apyrase-VII (Sigma) (final concentration, 10 U/ml, time of pre-incubation, 5 min at 37°C). To determine the contribution of ADP receptors, we used the P2Y antagonist MRS2179 (2'-Deoxy-N<sup>6</sup>-methyl adenosine 3',5'-diphosphate diammonium salt, Sigma) (final concentration 0.1 mmol/L, time of preincubation, 1 min) and the P2Y12 antagonist AR-C69931MX (final concentration 0.5  $\mu$ mol/L, time of preincubation, 1 min), a kind gift from AstraZeneca Charnwood, UK.

### **Platelet aggregation and cytofluorimetric assays**

Washed platelets were suspended at  $2 \times 10^8$  cells/ml in HEPES buffer (see above) supplemented with 1 mmol/L  $\text{CaCl}_2$  and 1 mmol /L  $\text{MgSO}_4$  and maintained at  $37^\circ\text{C}$  in the aggregometer cuvette under continuous stirring at 1000 rpm. After 1 min incubation, U46619 (0.05 or 1  $\mu\text{mol/L}$ ) was added to 0.5ml platelets and light transmission monitored for 3 minutes. Platelet aggregation was measured using a four channel aggregometer (Aggregorder II, PA-3220; Daiichi, Tokyo, Japan) and expressed as change in percentage of transmitted light (% T). In some experiments, 10  $\mu\text{mol/L}$  epinephrine and 50 nmol/L U46619 were added simultaneously and aggregation was monitored with platelet suspension pre-incubated either in the presence or absence of various inhibitors.

### **Cytofluorimetric assays**

Platelet suspensions ( $8 \times 10^4$  cells/ml, 180  $\mu\text{l}$ ) were pre-incubated at  $37^\circ\text{C}$  for 30 min with or without 10  $\mu\text{g/ml}$  eptifibatide and then incubated at  $37^\circ\text{C}$  for 3 min in the presence of U46619 (0.05-2  $\mu\text{mol/L}$ ) or thrombin (0.05-1 U/ml). FITC-labelled anti-human PAC-1 mAb (clone PAC-1, Becton Dickinson), a ligand mimetic antibody that specifically binds to the activated form of the  $\alpha\text{IIb}\beta_3$  complex, was added to platelet suspensions before the agonists. In all the experiments, an isotype-matched irrelevant mouse IgG1 (FITC/IgG1, Becton Dickinson) was included as negative control. After incubation at room temperature for 15 min samples were analyzed in duplicate by flow cytometry (FACScan, Becton Dickinson) using dual colour fluorescence. Platelets were identified on the basis of size and granularity.

### **Immunoblot analysis**

200  $\mu\text{l}$  washed platelets ( $17.5 \times 10^8$  cells/ml) were incubated at  $37^\circ\text{C}$ , under static conditions, in the presence or absence of U46619 (0.05  $\mu\text{mol/L}$ ), for the time indicated in the results. In these experiments the ADP scavenger apyrase VII, the inhibitor of the fibrinogen receptor eptifibatide (Integrilin) and ASA were pre-incubated with washed platelet preparations unless differently indicated. After different times (see Results), 4x Sample Buffer (SB: 100mM TRIS pH6.8, 200 mM  $\beta$ -mercaptoethanol, 4% SDS, 20% glycerol, 0.4% bromophenol blue) was added to the samples, which were then boiled for 3 min and, after centrifugation for 1 min at 13,000g, stored at  $-20^\circ\text{C}$ . To examine Syk tyrosine phosphorylation with phospho-specific Abs (see below and Results), samples were added with ice-cold 2x RIPA buffer

(Triton X-100 2%, Na-DOC 1%, NaCl 300 mmol/L, Tris 100 mmol/L pH 7.6, SDS 0.2%) supplemented with one tablet of protease inhibitors cocktail (Roche Molecular Biochemicals, Mannheim, Germany), 2 mM Na<sub>3</sub>VO<sub>4</sub>, 20 μM phenylarsine oxide (PAO, Sigma), 10 μg/ml pepstatin (Sigma) and 2 mM dithiothreitol (DTT, Sigma). After 30 min on ice, lysates were clarified by centrifugation at 4°C at 15,600×g for 10 min and stored at -20°C. An aliquot of each sample (5 μl) was utilized for assays of protein concentration. Samples were separated on 7.5% or 12% SDS-PAGE gel and transferred to nitrocellulose Hybond C (Amersham, Buckinghamshire, UK). After quenching for 1 hour at room temperature in 3% Bovine Serum Albumin (Sigma) in Tris-Saline Buffer (5 mM Tris pH 7.5, 170 mM NaCl, 0.02% Tween-20), blots were incubated at 4°C overnight with primary antibodies, followed by incubation with secondary, horseradish peroxidase-conjugated donkey anti-rabbit or goat anti-mouse IgG antibodies (Amersham) for 1 hour. Immunoreactivity was detected using the enhanced chemiluminescent Western Blotting detection reagent ECL (Amersham). After probing with anti-phosphotyrosine Abs, the membrane was stripped in 100 mmol/L 2-mercaptoethanol, 2% SDS, 62.5 mmol/L Tris-HCl, pH 6.7 at 50°C for 30 min, and then re-probed with specific anti-protein Abs. Anti-phospho Syk (Tyr352), anti-phospho-Src (Tyr416 of Src and equivalent Tyr residues of other Src family members) and anti-phospho Akt (Ser473) Abs were all from Cell Signaling Technology, Inc. (Beverly, MA, USA). Anti-phospho FAK (Tyr397) was from BioSource International (Camarillo, CA, USA). Anti-phospho MLC (Thr18/Ser19) was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-phospho-cortactin (Tyr486) was from Chemicon International Inc. (Temecula, CA, USA). Anti-phosphotyrosine Abs 4G10 and PY99 were from Upstate Biotechnology Inc. (Lake Placid, NY, USA) or Santa Cruz Biotechnology, Inc., respectively.

## RESULTS

### **Low doses of U46619 are unable to induce $\alpha$ IIb $\beta$ 3 activation and $\alpha$ IIb $\beta$ 3-dependent aggregation in platelets.**

The TXA<sub>2</sub> analogue U46619 at low doses (below 0.05  $\mu$ mol/L), induces platelet shape change and adhesion to surface-bound fibrinogen, without triggering detectable platelet aggregation and secretion [4, 5, 7, 14, 15]. In order to exclude that, in our experimental conditions, other platelet signaling receptors, i.e. the fibrinogen receptor  $\alpha$ IIb $\beta$ 3 and receptors for ADP, were activated, we examined the effects of eptifibatide, a widely used synthetic antagonist of  $\alpha$ IIb $\beta$ 3-fibrinogen interaction [31, 32] and apyrase, an ADP scavenger, on platelet responses. U46619 triggered platelet aggregation at 1  $\mu$ mol/L, but not at 0.05  $\mu$ mol/L (data not shown, see Figure 4 and Table I). Because eptifibatide totally inhibited the platelet response to 1  $\mu$ mol/L U46619 (data not shown), we conclude that stimulating platelets with 0.05  $\mu$ mol/L U46619 in the presence of eptifibatide any possible contribution of signals ensuing from  $\alpha$ IIb $\beta$ 3 following its engagement by platelet-derived fibrinogen can be excluded. Low doses of U46619, even in the absence of apyrase, were also unable to induce expression of the activation-dependent  $\alpha$ IIb $\beta$ 3 epitope recognized by the PAC-1 Ab (data not shown). In contrast, both 1  $\mu$ mol/L U46619 (see Table II) and thrombin (data not shown) markedly increased PAC-1 binding. The addition of apyrase in the extracellular medium inhibited U46619-induced PAC-1 binding (data not shown), suggesting that U46619 effects are dependent on stimulation of ADP release and the consequent engagement of ADP receptors. We conclude that low U46619 doses are unable to induce conformational changes of  $\alpha$ IIb $\beta$ 3 detectable by an increase in PAC-1 binding, and  $\alpha$ IIb $\beta$ 3-dependent responses, and the use of eptifibatide and apyrase are useful tools to inhibit any possible participation of  $\alpha$ IIb $\beta$ 3 or ADP receptors in platelet signal transduction in response to low doses of U46619.

### **Low doses of U46619 trigger protein tyrosine phosphorylation independently of $\alpha$ IIb $\beta$ 3 outside-in or ADP receptor signalling.**

The capability of low (0.05  $\mu$ mol/L) U46619 doses to trigger protein tyrosine phosphorylation was examined in the presence of eptifibatide, apyrase and acetylsalicylic acid (see Materials and methods) to exclude any possible contribution of receptors for fibrinogen and ADP (see above), as well as for platelet-derived TXA<sub>2</sub>. As shown in Figure 1A, platelet stimulation with 0.05  $\mu$ mol/L U46619 resulted in a rapid increase in tyrosine phosphorylation of some proteins. Re-probing of the same blot with specific Abs indicated



that phospho-proteins of about 125, 80 and 70 kDa migrated at the level of FAK, cortactin and Syk, respectively; in contrast, the FAK-analogue Pyk2, whose migration in the gel was clearly distinct from that of FAK, was reproducibly found not to be phosphorylated (data not shown). To identify more clearly proteins whose tyrosine phosphorylation is increased by 0.05  $\mu\text{mol/L}$  U46619 we utilized phospho-specific Abs (Figure 1B). We found that 0.05  $\mu\text{mol/L}$  U46619 markedly increased tyrosine phosphorylation of FAK, Src, Syk (Fig. 1B) and cortactin (data not shown).

The presence of high concentrations of apyrase (10 U/ml) in our assays would safely exclude any potential contribution of secreted ADP to tyrosine phosphorylation pathway triggered by 0.05  $\mu\text{mol/L}$  U46619 (see above). The lack of involvement of secreted ADP was also confirmed using two different experimental approaches. As shown in Fig. 2A, 0.05  $\mu\text{mol/L}$  U46619 did not cause Akt phosphorylation. In contrast, 1  $\mu\text{mol/L}$  U46619 induced Akt phosphorylation, which was abolished by high concentrations of apyrase (10 U/ml). In addition, the ADP receptor antagonists AR-C69931MX and MRS 2179 which compete for ADP binding to P2Y<sub>12</sub> and P2Y<sub>1</sub> receptors, did not inhibit tyrosine phosphorylation signals triggered by 0.05  $\mu\text{mol/L}$  U46619 neither alone (data not shown), nor in combination (Figure 2B). Therefore, we can *bona fide* exclude that, upon platelet stimulation with 0.05  $\mu\text{mol/L}$  U46619 in the presence of apyrase, even trace amounts of ADP contribute to tyrosine phosphorylation.

### **Tyrosine phosphorylation signals triggered by low U46619 doses are distinct from those mediated by the Rho target Rho-kinase.**

At low doses, the TXA<sub>2</sub> analogue U46619 was shown to act primarily through G<sub>12/13</sub> [7, 11, 12, 14, 15]. The G<sub>12/13</sub>-dependent signaling pathways impinges on the small GTPase Rho and Rho-kinase represent an immediate Rho target which, upon activation by GTP-loaded Rho, increase either indirectly or directly MLC phosphorylation [33]. Tyrosine phosphorylation signals in response to 0.05  $\mu\text{mol/L}$  U46619 were not inhibited by Y27632, a specific inhibitor of Rho-kinase (Fig. 3A). In contrast, phosphorylation of MLC, a specific Rho-kinase target, was markedly inhibited by Y27632 (Figure 3B). Therefore, we conclude that U46619-induced tyrosine phosphorylation is not secondary to Rho-kinase activation.

### **Tyrosine phosphorylation signals triggered by low U46619 doses synergize with those ensuing from G $\alpha$ -coupled receptors inducing platelet aggregation.**

$G_{12/13}$  was reported to synergize with  $G_i$  signaling pathways to activate platelets [4, 5]. We therefore wondered whether engagement of the  $G_{12/13}$ -coupled thromboxane receptors synergize with the  $G_z$ -coupled receptors for epinephrine. As illustrated in Figure 4 ( see Table I for mean results of several experiments), neither 10  $\mu\text{mol/L}$  epinephrine nor 0.05  $\mu\text{mol/L}$  U46619 alone were able to trigger platelet aggregation, but they were as effective as 1  $\mu\text{mol/L}$  U46619 when used in combination. As shown in Table I, apyrase inhibited the platelet response to 1  $\mu\text{M}$  U46619, but had no effects on the platelet response to the combination of 0.05  $\mu\text{M}$  U46619 and 10  $\mu\text{mol/L}$  epinephrine, indicating that this response is not dependent on secreted ADP.

In order to identify signals implicated in induction of platelet aggregation in response to epinephrine and U46619 in combination, we used different inhibitory drugs (Fig. 4 and Table I). PP2, a tyrosine kinase inhibitor displaying selectivity for Src family kinases [34, 35], but not its inactive analogue PP3 (Table I), strongly reduced platelet aggregation in response to epinephrine and U46619 in combination. Interestingly, also the Rho kinase inhibitor Y27632 markedly inhibited platelet aggregation in response to epinephrine and U46619 and had an additive effect with PP2. The two inhibitors, either alone or in combination, totally suppressed also PAC-1 binding (Table II) induced by epinephrine and U46619. We conclude that both tyrosine phosphorylation signals and Rho kinase activity are required to induce platelet aggregation and  $\alpha\text{IIb}\beta_3$  activation in response to low doses of thromboxane and epinephrine.

In order to elucidate whether PP2 and Y27632 displays selectivity in the inhibition of the tyrosine phosphorylation or the Rho/Rho kinase pathways we examined activation of these pathways by epinephrine and U46619. As shown in Fig. 5, 0.05  $\mu\text{mol/L}$  U46619 alone triggered both tyrosine phosphorylation and MLC phosphorylation, whereas 10  $\mu\text{mol/L}$  epinephrine failed to elicit any of these responses. In addition, a combination of the two stimuli did not consistently enhanced either protein tyrosine phosphorylation or MLC phosphorylation. PP2 suppressed the tyrosine phosphorylation signal triggered by 0.05  $\mu\text{mol/L}$  U46619 either alone or in combination with 10  $\mu\text{mol/L}$  epinephrine, but only slightly inhibited MLC phosphorylation. In contrast, Y27632 suppressed MLC phosphorylation, but had a negligible effect on protein tyrosine phosphorylation induced by U46619 and epinephrine. Despite PP2 had some inhibitory effect on MLC phosphorylation (see Discussion) and its use in combination with Y27632 had an even stronger inhibitory effect on tyrosine phosphorylation signals (compare the last three lanes of Fig. 5), these findings

suggest that protein tyrosine phosphorylation and MLC phosphorylation are two substantially distinct pathways triggered by  $G_{12/13}$ -coupled  $TXA_2$  receptors and are both required to trigger platelet aggregation in the presence of additional signals ensuing from  $G_z$ -coupled receptors.

## DISCUSSION

Our major findings can be summarized as follows. Low doses of the  $TXA_2$  analogue U46619 trigger tyrosine phosphorylation of FAK, Syk and Src which is independent of fibrinogen binding to  $\alpha IIb\beta 3$  integrin or the interaction of secreted ADP receptors with its platelet receptors P2Y1 and P2Y12. Moreover, this signaling pathway is independent of the Rho target Rho-kinase. Confirming previous findings [5, 14, 26, 27], this signaling pathway is insufficient to trigger full platelet activation, but it can synergize with the  $G_z$ -linked epinephrine receptor to trigger platelet aggregation.

Responses triggered by low doses of  $TXA_2$ , or other arachidonate-derived products, such as  $F_2$ -isoprostanes [5, 7, 14, 26, 27, 29] have been shown to depend on stimulation of  $G_{12/13}$ -coupled receptors [7, 11]. Thus, it is likely that the tyrosine kinase-based signaling pathway that we describe in this report depends on stimulation of the  $G_{12/13}$ -coupled  $TXA_2$  receptor; in line with this hypothesis is the observation that its stimulation resulted in activation of the Rho/Rho-kinase pathway (Figure 3 and 5), a classical  $G_{12/13}$  activated pathway [7, 36, 37]. Because studies in non haemopoietic cells showed that also  $G_{q/11}$  can impinge on Rho, via activation of the Rho GEFs LARG or p53RhoGEF [38, 39], we can not formally exclude that also  $G_q$ -coupled  $TXA_2$  receptors are implicated in the platelet response to low U46619 doses. However, it is important to note that other  $G_q$ -mediated signaling pathways, such as increase in intracellular free calcium [5, 26] or Akt phosphorylation [32, 40] (Figure 2) are not activated by low U46619 doses.

In analogy with studies in different cell types showing that G-protein-coupled receptors impinge on Src, as well as other families of tyrosine kinase (see Introduction), agonists of trimeric G protein-coupled receptors have been reported to activate tyrosine phosphorylation signals independently of the  $\alpha IIb\beta 3$  integrin in platelets [6, 7, 15, 24]. In this report we show that in these cells, a putative  $G_{12/13}$ -mediated signaling pathways enhances phosphorylation of members of three distinct cytoplasmic tyrosine kinase families, i.e. Src, Syk and FAK. This tyrosine kinase-based pathway was detected as early as 15 seconds after platelet stimulation and in conditions (aspirin treatment, presence of the ADP scavenger apyrase and ADP

receptor antagonists, presence of the  $\alpha$ IIb $\beta$ 3 integrin antagonist, eptifibatide) allowing us to *bona fide* exclude a contribution of signaling pathways triggered by platelet-derived TXA<sub>2</sub> and ADP, or by the binding of fibrinogen to the  $\alpha$ IIb $\beta$ 3 integrin. Our findings strengthen the notion [7, 15, 24, 25] that cytoplasmic tyrosine kinases are implicated in two different steps of platelet responses. G<sub>12/13</sub>-coupled, as well as G<sub>1/2</sub>-coupled [6], platelet receptors trigger an early wave of protein tyrosine phosphorylation. Following activation of the  $\alpha$ IIb $\beta$ 3 integrin binding to fibrinogen, a second, sustained wave of tyrosine phosphorylation ensues [41, 42].

Differently from Src and Syk [7, 15], tyrosine phosphorylation of FAK in response to G<sub>12/13</sub>-coupled TXA<sub>2</sub> receptors in platelets was not previously reported. However, thrombin was reported to induce phosphorylation of FAK in both platelets [43] and human embryonic kidney cells [16]. Differently from studies with HeLa cells expressing G $\alpha$ <sub>13</sub>, or G $\alpha$ <sub>q</sub> [17, 20], we did not find any evidence of an implication of Pyk2 in platelets stimulated with low U46619 doses.

Mechanisms of activation of cytoplasmic tyrosine kinases by G-protein-coupled receptors have been elucidated only in part. In the case of Src-family kinases, several mechanisms have been described (see [23] for review). Among these, some are direct and involve binding of the kinase to the G-protein-coupled receptor [44], the G $\alpha$  subunits [45] or  $\beta$ -arrestins bound to the receptor itself [46, 47]. Among indirect mechanisms of tyrosine kinase activation by G-protein-coupled receptors the best characterized is certainly represented by the integrin-mediated assembly of cytoskeletal and signaling proteins in focal adhesion sites [16, 19, 23, 42]. A typical feature of this pathway is that it is blocked by inactivation of Rho with the C. botulinum C3 exoenzyme, the Rho-kinase inhibitor Y-27632 and disruption of the actin cytoskeleton with cytochalasin D [16, 19]. Notably, at doses that blocked MLC phosphorylation the Rho kinase inhibitor Y-27632 had a negligible effect on the tyrosine-kinase based signaling pathway induced by low doses of U46619 (Figure 3) or by U46619 and epinephrine in combination (Figure 5). Hence, it is tempting to speculate that tyrosine kinase activation by a putative G<sub>12/13</sub>-mediated pathway is direct and due to kinase interaction with the receptor or the G protein itself (see above). Certainly, the evidence that G $\alpha$ <sub>13</sub> binds to Pyk2, a FAK analogue, in HeLa cells over-expressing the two proteins [17] prompts studies to challenge the possibility that G $\alpha$ <sub>13</sub> and FAK interact in platelets.

The biological significance of the tyrosine kinase-based signaling pathway in response to low U46619 doses described in this report is highlighted by the evidence that this pathway synergizes with a G<sub>z</sub>-mediated pathway to induce platelet aggregation (Figure 4 and Table I). Previous findings demonstrated that combined signaling via G<sub>12/13</sub> and G<sub>i</sub> is required for full

platelet activation [4, 5]. Interestingly, both tyrosine kinase-dependent and Rho kinase-dependent signaling are required for the combined stimulation with low U46619 doses and epinephrine. In fact, both PP2 and Y-27632 significantly inhibited platelet aggregation induced by the combination of the two stimuli and, together, totally suppressed this response. Hence, G<sub>12/13</sub>-dependent Rho kinase- and tyrosine kinase-dependent signals converge in activation of the  $\alpha$ IIb $\beta$ 3 integrin provided additional signals ensuing from engagement of Gz-coupled receptors are generated. These two pathways are substantially independent (see for example Figure 3). However, we found that PP2, besides suppressing the tyrosine phosphorylation pathway, also partially inhibits MLC phosphorylation (Figure 5). These findings are in keeping with data obtained with smooth muscle and endothelial cells which established a link between Src family kinases activity and the pathway leading to MLC phosphorylation [48-50].

A part from their heuristic content, our findings may be of clinical relevance. Local release of TXA<sub>2</sub> (or other bioactive eicosanoids acting through the TP receptor) from activated platelet and monocytes/macrophages [51] may activate the tyrosine kinase-based signaling pathway. Full platelet activation may then be induced in the presence of a weak platelet agonists or sub-threshold concentrations of stronger agonists. Therefore, our findings suggest that tyrosine kinase activation may play an important role in platelet thrombus formation.

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342.



## FIGURE LEGENDS

**Figure 1.** Low doses of U46619 trigger tyrosine phosphorylation signals. ASA-treated platelets were preincubated with 10 U/ml apyrase and 10 µg/ml eptifibatide and then stimulated with 0,050 µmol/L U46619. At the indicated times, the reaction was stopped by the addition of 4× SDS sample buffer, proteins were subjected to electrophoresis and transferred to nitrocellulose. (A) Blots were probed with anti-phosphotyrosine antibodies. Results of one representative experiment of 3 performed are reported. (B) Blots were probed with phospho-specific antibodies recognizing the indicated tyrosine residues, followed by stripping and re-probing with anti-protein antibodies. (C) Densitometric analysis of blots probed with phospho-specific antibodies. Mean results ± SD of three independent experiments are reported.

**Figure 2.** Low doses of U46619 are unable to trigger Akt phosphorylation and their capability to trigger protein tyrosine phosphorylation is not inhibited by ADP receptor antagonists. (A) ASA-treated platelets, were stimulated with the indicated concentrations of U46619 in the presence or absence of apyrase (10 U/ml). After 5 min incubation, the reaction was stopped by the addition of 4× SDS Sample Buffer and equal amount of proteins (15 µg) were analyzed by western blot analysis with anti-Akt phospho-specific antibodies, followed by stripping and re-probing with anti-Akt antibody. Results of one representative experiment of 3 performed are reported. (B) ASA-treated platelets, preincubated with 10 U/ml apyrase and 10 µg/ml eptifibatide, were incubated with 0.5 µmol/L AR-C69931MX and 0.1 mmol/L MRS2179 prior to stimulation with 0,050 µmol/L U46619. At the indicated times, the reaction was stopped by the addition of 4× SDS sample buffer, proteins were subjected to electrophoresis and transferred to nitrocellulose. Blots were probed with anti-phosphotyrosine antibodies. Results of one representative experiment of the 2 performed are reported.

**Figure 3.** Tyrosine phosphorylation signals triggered by low doses of U46619 are independent of the Rho/Rho kinase pathway. ASA-treated platelets, preincubated with 10 U/ml apyrase and 10 µg/ml eptifibatide, were incubated with 30 µmol/L Y27632 prior to

stimulation with 0,050  $\mu\text{mol/L}$  U46619. At the indicated times, the reaction was stopped by the addition of 4 $\times$  SDS sample buffer, proteins were subjected to electrophoresis and transferred to nitrocellulose. (A) Blots were probed with anti-phosphotyrosine antibodies. (B) Blots were probed with phospho-specific antibodies recognizing the indicated Thr/Ser residues of MLC, followed by stripping and reprobing with anti-protein antibodies. Results of one representative experiment of 3 performed are reported.

**Figure 4.** Platelet aggregation induced by simultaneous stimulation of  $G_{12/13}$  and  $G_z$  pathways requires both tyrosine phosphorylation and Rho kinase-mediated signals. (A-C) ASA-treated platelets, were stimulated in the presence or absence of 10 U/ml apyrase with the indicated doses of U46619 and epinephrine, alone or in combination. (D) ASA-treated platelets were stimulated with the indicated doses of U46619 and epinephrine, in combination, following preincubation with 10  $\mu\text{mol/L}$  PP2 , 30  $\mu\text{mol/L}$  Y27632 or 10  $\mu\text{mol/L}$  PP2 and 30  $\mu\text{mol/L}$  Y27632.

**Figure 5.** PP2 inhibits tyrosine phosphorylation signals triggered by the combination of U46619 and epinephrine. ASA-treated platelets were stimulated in the presence of 10 U/ml apyrase with 0,050  $\mu\text{mol/L}$  U46619 and 10  $\mu\text{mol/L}$  epinephrine, alone or in combination, following preincubation with 10  $\mu\text{mol/L}$  PP2 , 30  $\mu\text{mol/L}$  Y27632 or 10  $\mu\text{mol/L}$  PP2 and 30  $\mu\text{mol/L}$  Y27632. After 40 seconds, the reaction was stopped by the addition of 4 $\times$  SDS sample buffer, proteins were subjected to electrophoresis and transferred to nitrocellulose. (A) Blots were probed with anti-phosphotyrosine antibodies. (B) Blots were probed with phospho-specific antibodies recognizing the indicated Thr/Ser residues of MLC, followed by stripping and reprobing with anti-protein antibodies. Results of one representative experiment of 3 performed are reported.

	<b>Aggregation (%)</b>
U46619 1 $\mu$ M	74.2 $\pm$ 4.8
U46619 1 $\mu$ M + apyrase	18.8 $\pm$ 6.1 <sup>+</sup>
U46619 50 nM	0.4 $\pm$ 0.4
Epinephrine 10 $\mu$ M	0.0 $\pm$ 0.0
U46619 50 nM + Epinephrine 10 $\mu$ M	72.5 $\pm$ 4.3
U46619 50 nM + Epinephrine 10 $\mu$ M + apyrase	67.6 $\pm$ 2.0
U46619 50 nM + Epinephrine 10 $\mu$ M + PP2 + apyrase	38.6 $\pm$ 6.5*
U46619 50 nM + Epinephrine 10 $\mu$ M + PP3 + apyrase	62.8 $\pm$ 6.8
U46619 50 nM + Epinephrine 10 $\mu$ M + Y27632 + apyrase	10.9 $\pm$ 5.8 *
U46619 50 nM + Epinephrine 10 $\mu$ M + Y27632 + PP2 + apyrase	1.0 $\pm$ 1.0 *

**Table I.** Platelet aggregation induced by simultaneous stimulation of G<sub>12/13</sub> and G<sub>z</sub> pathways is independent of ADP secretion and requires both tyrosine phosphorylation and Rho kinase-mediated signals.

ASA-treated platelets, were stimulated in the presence or absence of 10 U/ml apyrase with the indicated doses of U46619 and epinephrine, alone or in combination. Platelet aggregation was recorded for 3 minutes. For inhibitory studies, ASA-treated platelets were stimulated following preincubation with 10  $\mu$ mol/L PP2 or PP3, 30  $\mu$ mol/L Y27632 or 10  $\mu$ mol/L PP2 and 30  $\mu$ mol/L Y27632. Mean results of 5 independent experiments  $\pm$  SEM are reported.; \* P < 0.001 vs U46619 + Epinephrine without inhibitors; <sup>+</sup> P < 0.01 vs U46619 alone.

	<b>Active fibrinogen receptor (%)</b>
Resting	0.2 ± 0.1
U46619 1 μM	50.5 ± 22.6
U46619 50 nM + Epinephrine 10 μM	25.9 ± 2.7
U46619 50 nM + Epinephrine 10 μM + Y27632	2.57 ± 2.7 *
U46619 50 nM + Epinephrine 10 μM + PP2	2.5 ± 0.5 *
U46619 50 nM + Epinephrine 10 μM + Y27632 + PP2	2.0 ± 0.2*

**Table II.** Activation of the  $\alpha_{IIb}\beta_3$  fibrinogen receptor by simultaneous stimulation of  $G_{12/13}$  and  $G_z$  pathways requires both tyrosine phosphorylation and Rho kinase-mediated signals.

ASA-treated platelets, were stimulated in the presence of 10 U/ml apyrase with the indicated doses of U46619 and epinephrine, alone or in combination. Data are expressed as per cent of platelets binding the MAb PAC-1 (see Materials and Methods). Treatment with PP2 and Y27632 was as described in Table I. Mean results ± SEM of three independent experiments are reported. \*  $P < 0.001$  vs U46619 + Epinephrine without inhibitors.

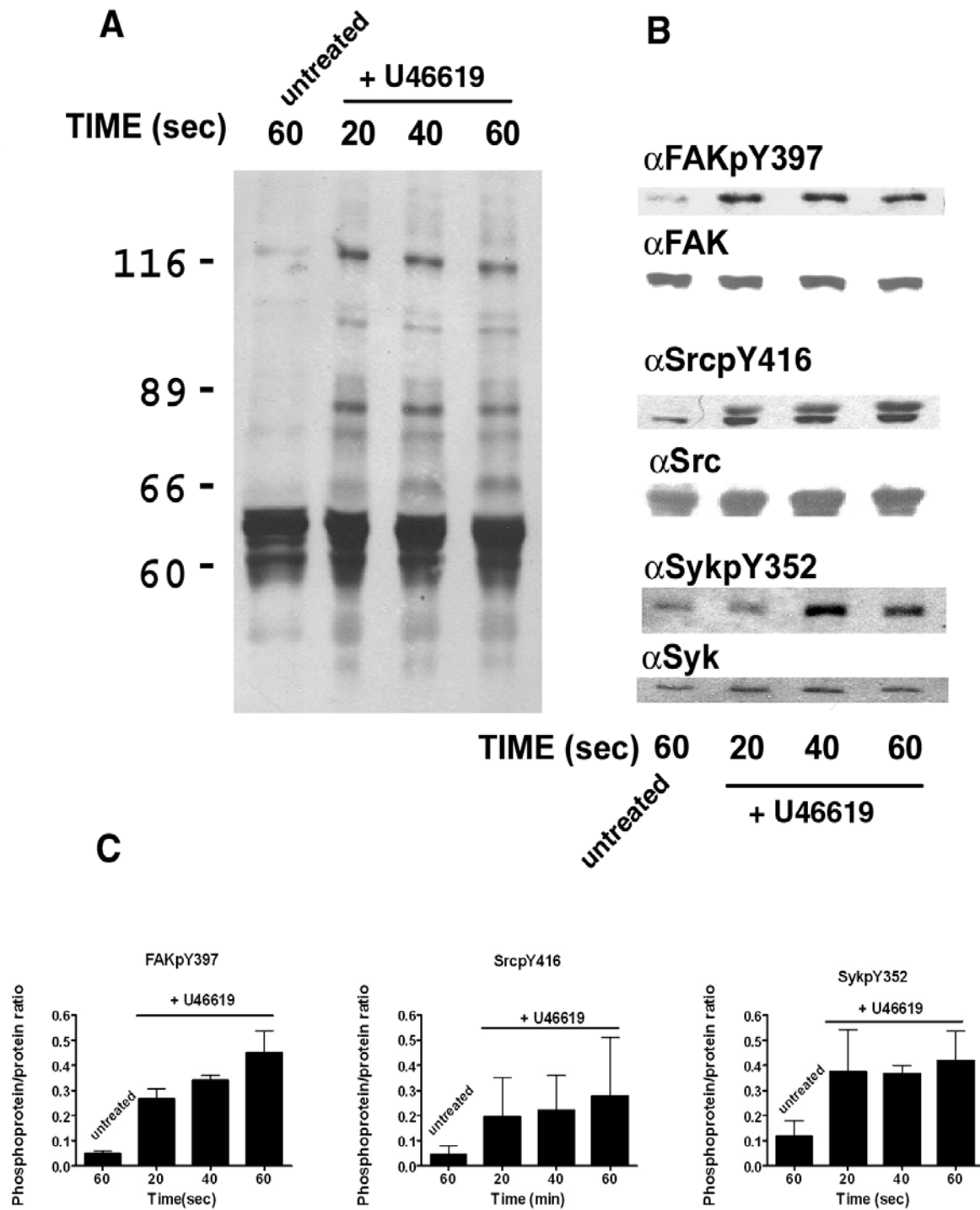


FIGURE 1

**A**

U46619 (nM)	-	50	50	1000	1000
Apyrase	-	-	+	-	+



**B**

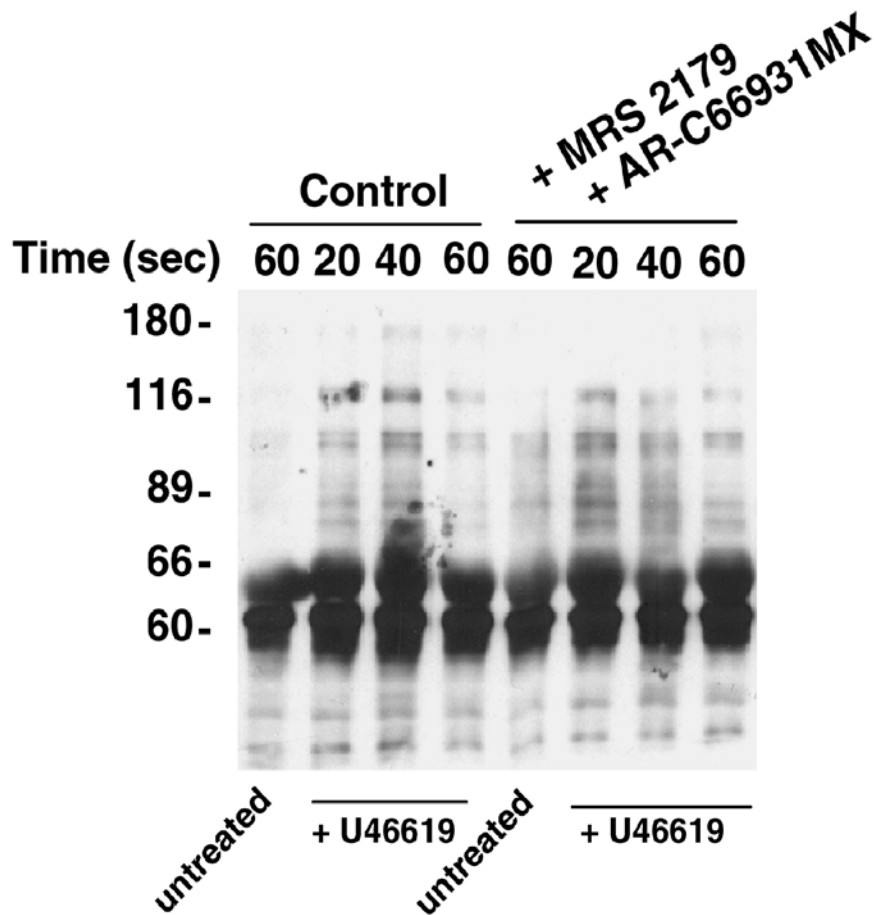


FIGURE 2

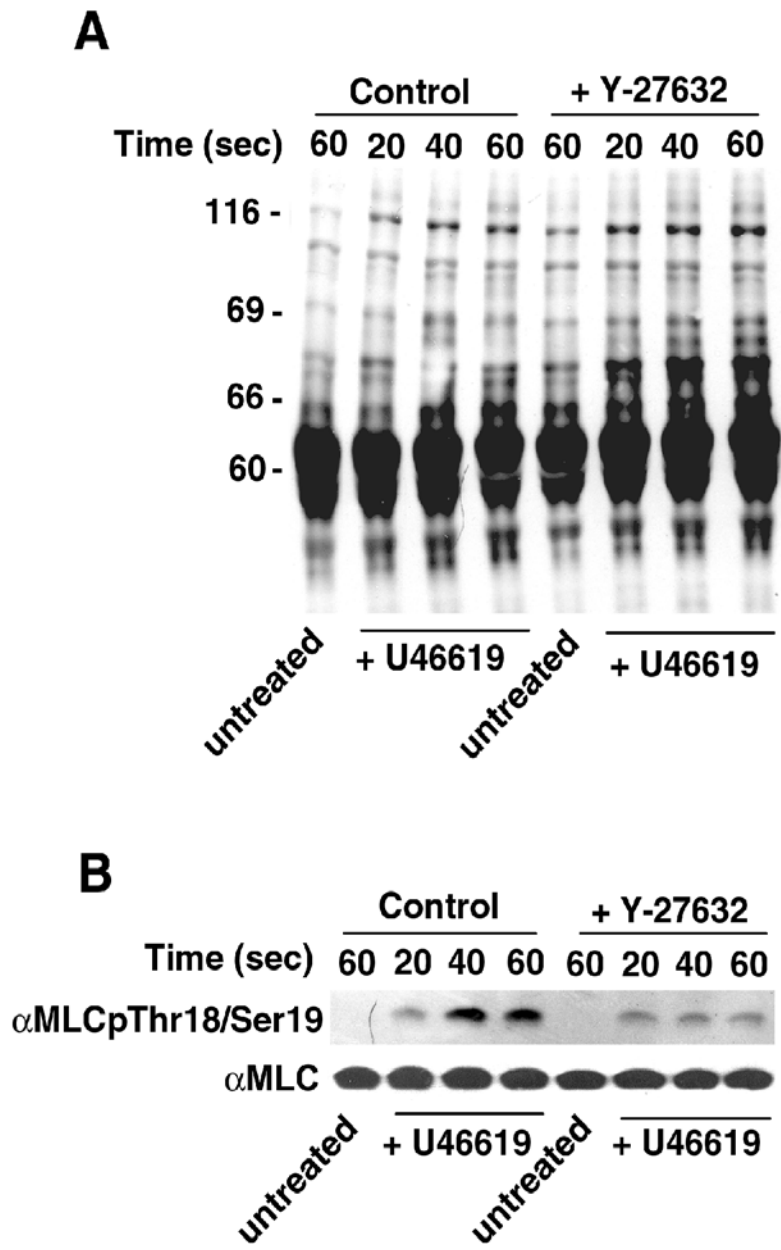


FIGURE 3

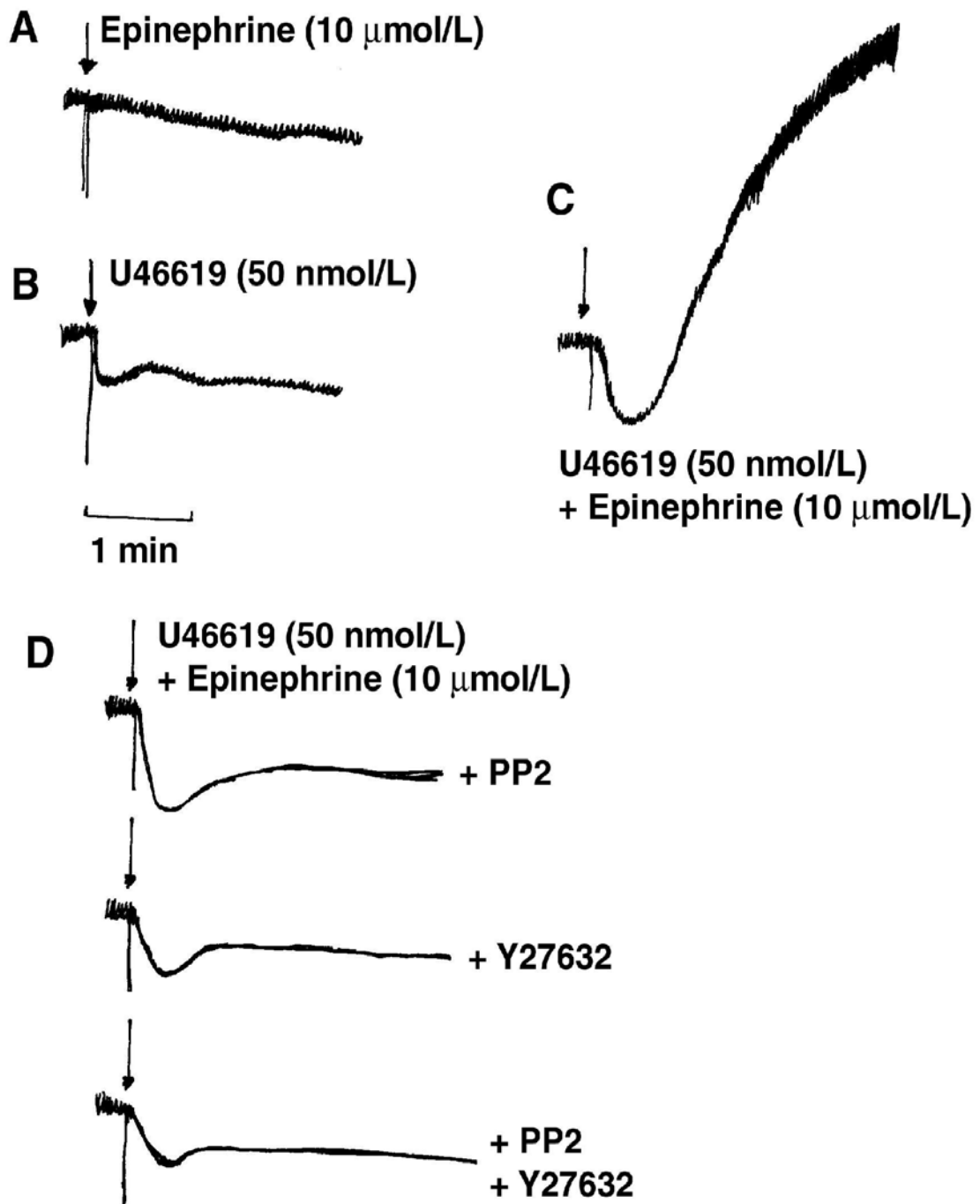


FIGURE 4



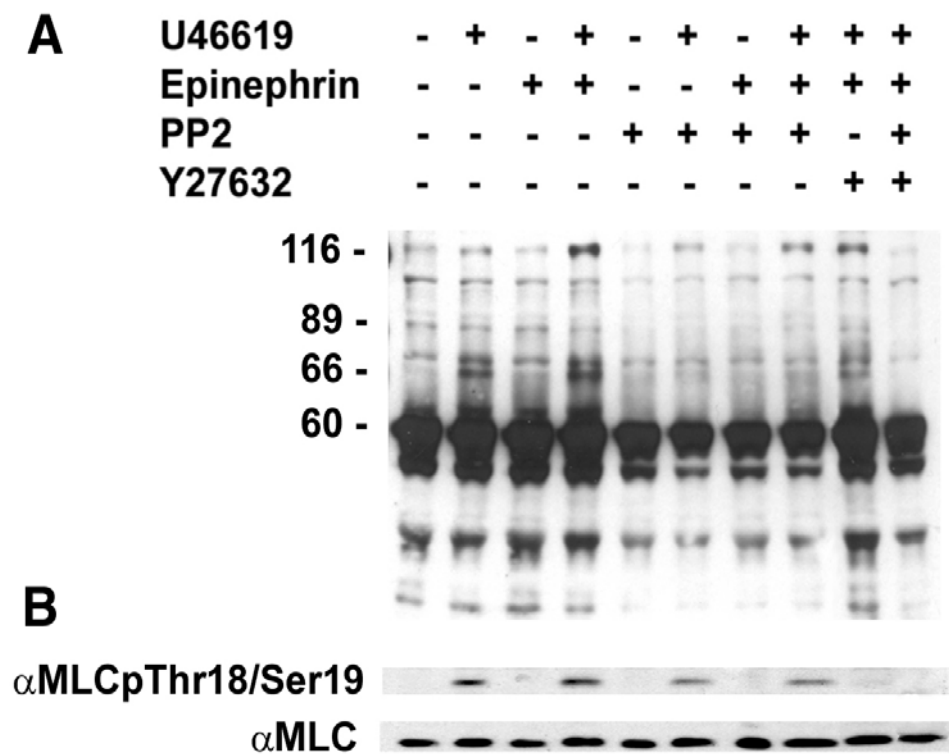


FIGURE 5