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Thrombopoietin Acts Synergistically on Ca^{2+} Mobilization in Platelets Caused by ADP or Thrombin Receptor Agonist Peptide

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Thrombopoietin (TPO) is the main regulator of megakaryopoiesis and influences also the function of mature platelets. TPO has been shown to synergize in multiple platelet activation processes induced by various agonists. Our aim was to elucidate whether TPO affects calcium signaling during platelet activation processes. TPO demonstrated a synergistic effect on the exocytosis induced by suboptimal doses of adenosine diphosphate (ADP) and the thrombin receptor agonist peptide (TRAP). We detected synergistic effects of TPO on the ADP or TRAP induced Ca^{2+} mobilization in a small range of very low agonist concentrations. The TPO synergism on Ca^{2+} mobilization and CD62P expression was measurable in different, nonoverlapping ranges of ADP or TRAP concentrations. Sustaining the agonist-induced calcium signal with thapsigargin led to a detectable TPO synergism in CD62P expression even in agonist concentrations in which the synergism only occurs in Ca^{2+} signaling without thapsigargin.

Thrombopoietin (TPO) is known to be the pivotal cytokine for regulation of megakaryopoiesis and thrombocytopoiesis *in vitro* and *in vivo* (for review see (1)). TPO is an important stimulator of proliferation and differentiation of megakaryocytic progenitors and influences the function of mature platelets. The receptor for TPO, the proto-oncogene product c-Mpl, is expressed on hematopoietic cells from early megakaryocytic progenitors up to platelets (2). It belongs to the cytokine receptor superfamily (3) that shows common signal transduction pathways by phosphorylation and dephosphorylation mainly of tyrosine residues (4).

Preincubation of human platelets with recombinant human (rh)TPO leads to an increased expression of the activation-dependent marker CD62P (P-selectin) (5) when platelets are stimulated with agonists like ADP or the thrombin receptor agonist peptide (TRAP). The mechanisms how TPO reacts synergistically with platelet agonists are poorly understood. An important step in platelet activation is a transient increase of intracellular Ca^{2+}. Contrary results have been published concerning the influence of TPO on Ca^{2+} mobilization (6, 7). Our aim was to elucidate the impact of TPO on Ca^{2+} mobilization in human platelets to obtain more insights into the role of TPO in platelet activation.

MATERIALS AND METHODS

Materials. rh-TPO was kindly provided by Dr. A. Shimosaka, Kirin Brewery (Tokyo, Japan). Acetylic salicylic acid (ASA), adenosine diphosphate (ADP), and probenecid were purchased by Sigma (Deisenhofen, Germany), thapsigargin by Calbiochem (Bad Soden, Germany), Fluo-3 acetoxymethyl ester (AM) by Molecular Probes (Leiden, The Netherlands). Human immunoglobulin (Ig) solution (Octagam) was obtained from Baxter (Langenfeld, Germany) and the thrombin receptor agonist peptide (SFLLRN-amid, TRAP) was purchased from Bachem (Heidelberg, Germany). Monoclonal antibodies against CD62P (clone CLB/Thromb/6) and CD41 (clone P2), as well as the IgG1 isotype controls were purchased from Coulter-Immunotech (Hamburg, Germany).

Platelet preparation. Blood from healthy volunteers was obtained from an antecubital vein through a 21-gauge needle into a plastic syringe containing trisodium citrate (10 mmol/L final concentration). Whole blood was centrifuged at 200*g* for 20 min and the supernatant platelet rich plasma (PRP) was removed.

Determination of platelet activation by flow cytometry. 5 μL of PRP were preincubated for 2 min without or with 150 ng/mL rh-TPO in a whole volume of 70 μL phosphate-buffered saline (PBS; 130 mmol/L NaCl, 10 mmol/L sodium phosphate, pH 7.5) before various concentrations of platelet activators ADP or TRAP were added. The incubation of the activators was terminated by adding 1 mL of a formaldehyde solution (1% in PBS). Unstimulated platelets serving as a control were fixed immediately after preparation of PRP. The platelets were stored on ice for 30 min before they were washed and stained for flow cytometric analysis. To avoid unspecific labeling the fixed platelets were resuspended in 20 μL of a human Ig solution (10 mg/mL) before staining. As a pan-platelet marker, a phycoerythrin

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(PE)-conjugated anti gpIIb/IIIa (CD41) and as an activation marker, a fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody anti P-selectin (CD62P) were used. FITC- and PE-labeled isotype control antibodies were used as controls. After staining, the platelets were analyzed in a FACScan flow cytometer (Becton Dickinson, San Jose, CA). Platelets were gated on the basis of CD41 expression. In experiments with thapsigargin, platelets were preincubated with ASA (2 mmol/L) for 20 min at room temperature to avoid indirect stimulation of platelets via thromboxane formation.

Loading of platelets with fluo-3 AM and calcium measurement. 1 mL PRP was incubated for 30 min at room temperature with ASA (2 mmol/L) and with the organic-anion transport inhibitor probenecid (2.5 mmol/L) to prevent leakage of the dye (8). For loading with fluo-3 the PRP was incubated for 30 min at 37°C with fluo-3 AM (2 μmol/L). The platelets were centrifuged and resuspended at approx. 15×10^8 cells/mL in HEPES-Tyrode buffer (HTB: 129 mmol/L NaCl, 8.9 mmol/L $NaHCO_3$, 0.8 mmol/L KH_2PO_4, 0.8 mmol/L $MgCl_2$, 5.6 mmol/L Glucose, 10 mmol/L HEPES, pH 7.4) containing 0.2 mmol/L $CaCl_2$ and 2.5 mmol/L probenecid. The cell suspension was kept at room temperature until use. 20 μL-aliquots of the cell suspension were added to 430 μL HTB with or without rh-TPO (150 ng/mL) 1 min before flow cytometric analysis. The platelet activators ADP or TRAP were added with or without thapsigargin (final concentration 0.17μmol/L) by injection into the continuously stirred FACS tube 10 seconds after the measurement has started. Fluo-3 fluorescence was kinetically analyzed in a FACScan flow cytometer at 488 nm excitation and 530 nm emission. Light scatter and fluorescence data were obtained with gain settings in logarithmic mode. Platelets were distinguished from other cells, debris, and machine noise on the basis of there scatter profile. A threshold value for calculating the amount of platelets which demonstrate an increase in intracellular calcium was determined for each experiment in a time gate before the addition of the agonists. Significance was determined by a one-sided Mann-Whitney-Wilcoxon rank test.

RESULTS

Effects of TPO Preincubation on CD62P Expression of Platelets

If platelets are activated by ADP or TRAP, exocytosis of the alpha-granules leads to expression of P-selectin (CD62P) on the platelet surface which can be detected by flow cytometry.

Stimulation with increasing doses of ADP or TRAP alone led to increasing percentages of CD62P expressing platelets. The CD62P expression of TRAP stimulated platelets, however, was markedly higher than that of ADP stimulated platelets. rh-TPO synergizes with these agonists in platelet activation. Although TPO alone (150 ng/mL) did not lead to an increased CD62P expression (Fig. 1A), preincubation of platelets with rh-TPO led to a higher number of CD62P expressing platelets after stimulation with suboptimal doses of the agonists (Figs. 1B, 1D). Even the platelet activation achieved with optimal doses of ADP (>200 μmol/L) was increasable by preincubation with rh-TPO (Fig. 1C). In contrast, we found no synergistic effect of TPO with optimal doses of TRAP (>10 μmol/L) (Fig. 1E).

Effects of ADP and TRAP on Intracellular Ca^{2+} Mobilization in Platelets

Platelet agonists ADP and TRAP lead to a transient increase of intracellular free Ca^{2+} ($[Ca^{2+}]_i$). Using the fluorescent Ca^{2+} indicator dye fluo-3 we were able to measure the relative $[Ca^{2+}]_i$ in platelets by flow cytometry.

Ca^{2+} mobilization by ADP and TRAP was concentration dependent: At higher concentrations of the agonists (ADP >0.7 μmol/L, TRAP > 2 μmol/L) all platelets elevated $[Ca^{2+}]_i$ uniformly (Figs. 2C and 2E). Suboptimal concentrations led to a measurable Ca^{2+} flux only in subpopulations of platelets (Figs. 2B and 2D). We were able to measure elevated $[Ca^{2+}]_i$ in a small percentage of platelets even after stimulation with concentrations of 40 nmol/L ADP or 130 nmol/L TRAP, respectively (data not shown).

Incubation of the platelets with TPO alone (150 ng/mL) did not lead to a difference in platelets' $[Ca^{2+}]_i$ (Fig. 2F) compared4 to the unstimulated platelets (Fig. 2A).

Effect of TPO Preincubation on Agonist-Induced Ca^{2+} Flux in Platelets

We investigated the influence of TPO on the agonist-induced Ca^{2+} flux in platelets in order to examine whether the observed synergism of rhTPO with the platelet activators ADP or TRAP is due to a synergism in intracellular calcium signaling. Platelets were preincubated with or without rhTPO (150 ng/mL) at 37°C for one minute prior to flow cytometric analysis. Ten seconds after starting the kinetic measurement, the platelet agonists were injected into the sample tube.

We observed a small but marked increase in the number of activated platelets in the samples preincubated with TPO (Figs. 3A–3D). However, this synergistic effect could be detected only in a small range of suboptimal agonist concentration, which was 40–90 nmol/L for ADP and 125–250 nmol/L for TRAP, respectively (see also Fig. 4).

Comparison of the Synergistic Effect of TPO on Agonist Induced Ca^{2+} Flux and CD62P Expression on Platelets

Quantification of differences in Ca^{2+} mobilization after stimulation with various concentrations of activators in the presence or absence of TPO was done by determining the percentage of platelets over a fluorescence threshold in a time gate after stimulation (10–30 sec after addition of agonists).

The effects of various doses of ADP and TRAP and the preincubation with TPO on both CD62P expression and Ca^{2+} mobilization are demonstrated in Fig. 4. We detected an agonist-dependent effect on Ca^{2+} mobiliza-

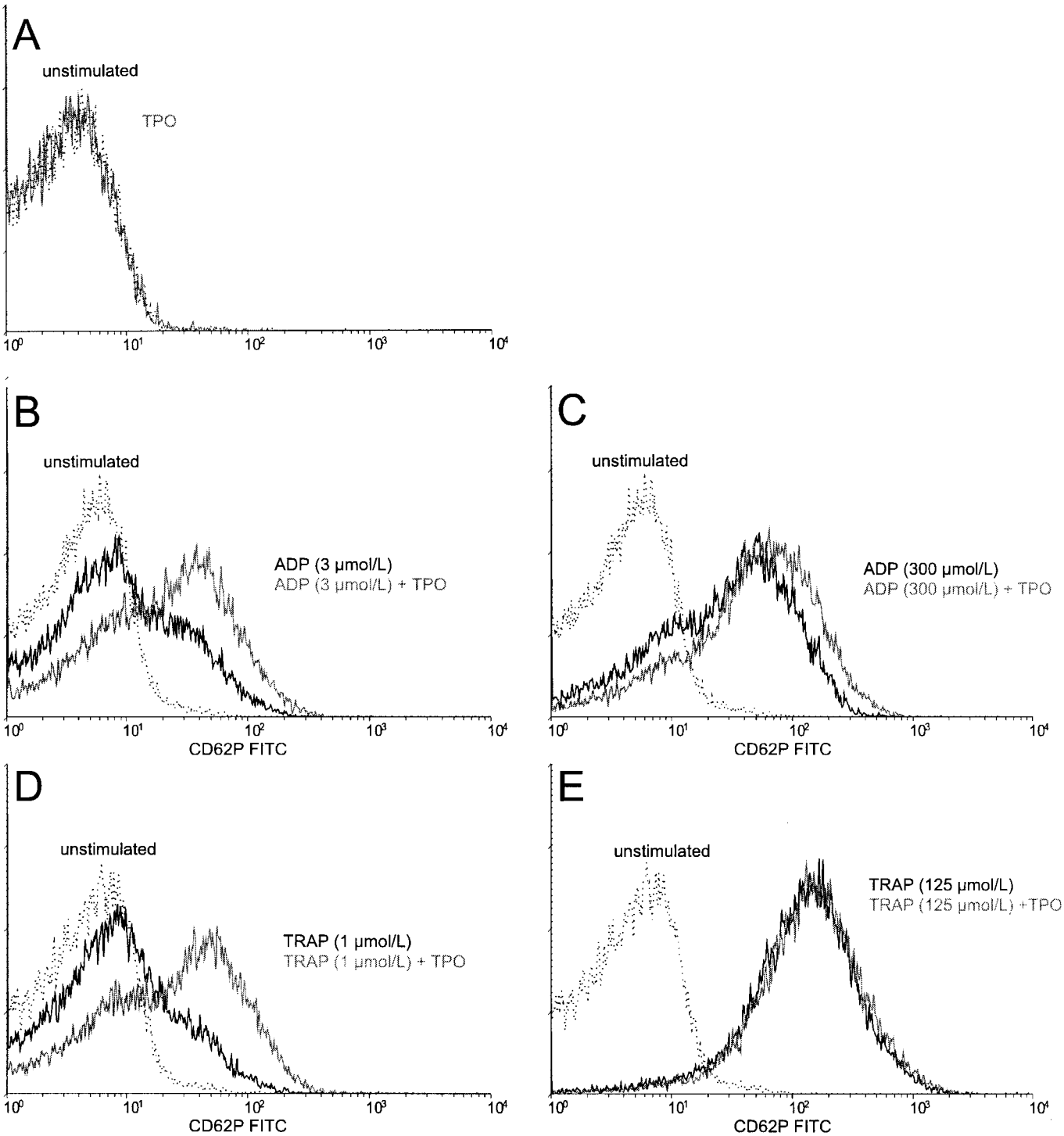

FIG. 1. Effect of TPO on CD62P expression on platelets. Platelets were stimulated with TPO alone (150 ng/mL, A) or with ADP (B, C) or TRAP (D, E) in the specified concentrations with or without preincubation with rh-TPO. Flow cytometric analysis of platelet activation was performed after fixation and staining with an FITC-labeled anti-CD62P mAb. CD62P expression of unstimulated platelets is shown in each histogram (dotted line).

tion in the presence of TPO in concentrations 16-fold lower for ADP and 4-fold lower for TRAP than those required for detectable exocytosis of α-granules.

Interestingly, the TPO synergism on Ca^{2+} mobilization and CD62P expression occurred in different, nonoverlapping ranges of agonist concentrations.

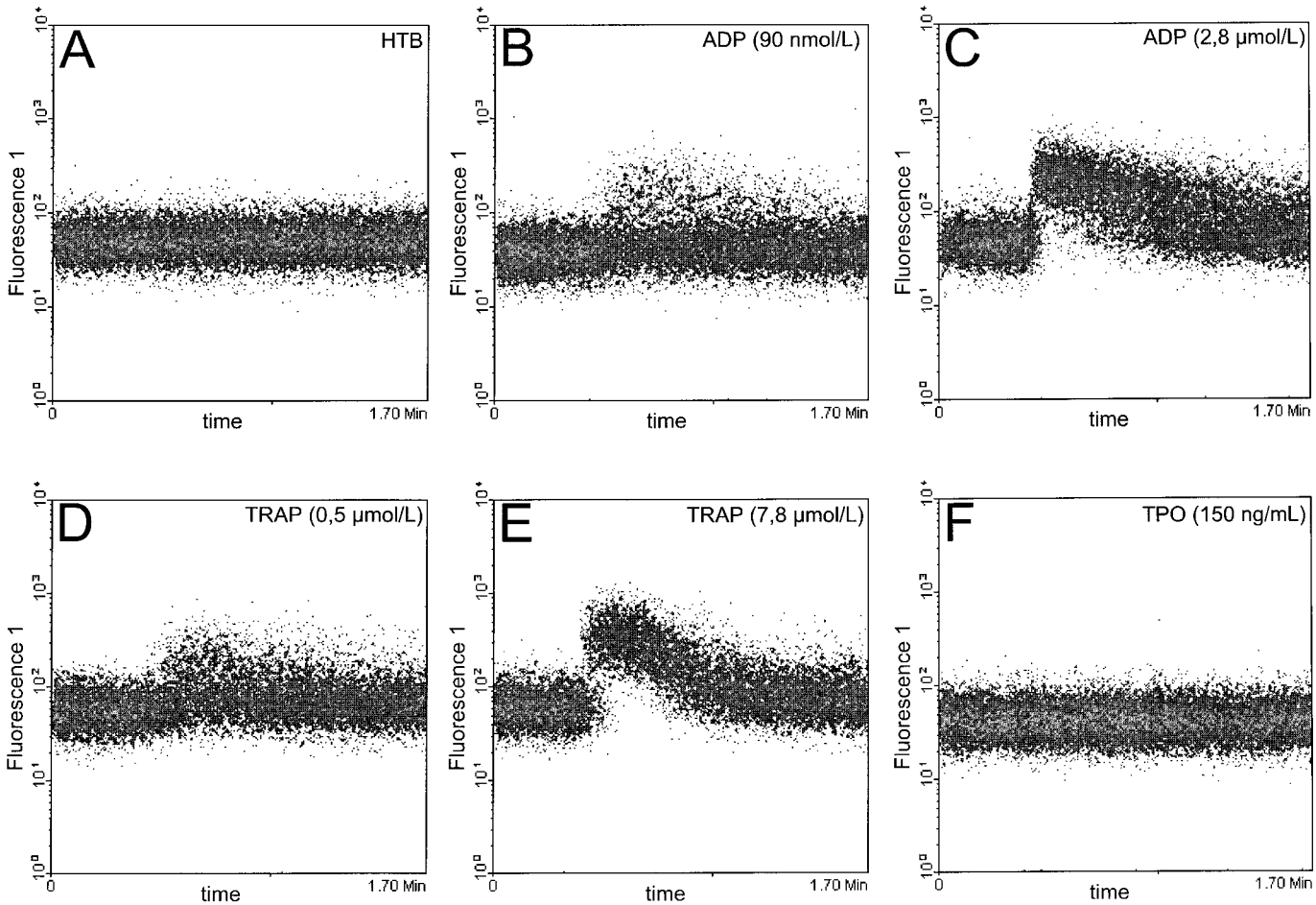

FIG. 2. Effects of ADP, TRAP, and TPO on intracellular Ca^{2+} mobilization in platelets. Platelets were loaded with fluo-3 AM and analyzed in a flow cytometer. The indicated agonists were added 10 seconds after starting the measurement. As a negative control Hepes-Tyrodes buffer (HTB) was added.

Effects of Thapsigargin on Agonist Induced Ca^{2+} Flux and CD62P Expression

Thapsigargin is a sesquiterpene lactone that specifically inhibits the Ca-ATPase function of the calcium pumps in the sarco-endoplasmatic reticulum (SERCA). Additional stimulating effects of thapsigargin depend on secondary production of thromboxane A_2 (TXA_2) and can be inhibited with acetylic salicylic acid (ASA). Incubation of platelets with 170 nmol/L thapsigargin alone led to a slow increase in intracellular calcium independent of the presence of TPO but not to exocytosis of the α-granules (Fig. 5A).

In order to elucidate whether a thapsigargin evoked elevation of the cytoplasmatic calcium concentration further enhances platelet activation, we added ADP to platelet suspensions which were preincubated with both thapsigargin and with or without TPO. Preincubation of platelets with thapsigargin led to detectable TPO synergism in CD62P expression even in ADP concentrations in which the synergism occurs in Ca^{2+} signaling without thapsigargin (Fig. 5B).

Inhibition of the SERCA results in a slow, cumulative elevation of $[Ca^{2+}]_i$ (Fig. 5A, small figures), moreover, subthreshold Ca^{2+} signals induced by low agonist concentrations were sustained (Fig. 5B, small figures).

DISCUSSION

The synergistic effect of TPO on the activation of platelets has previously been reported by several groups (6, 7, 9–15). TPO affects aggregation, adhesion, exocytosis and TXA_2 production induced by multiple stimuli like thrombin, collagen, epinephrine, ADP, or shear stress. These synergisms have already been used as a diagnostic tool for the detection of defects in TPO reactivity (5, 16, 17). Most authors did not find any direct effect of TPO on platelet activation. However, Wun *et al.* demonstrated an increased CD62P expres-

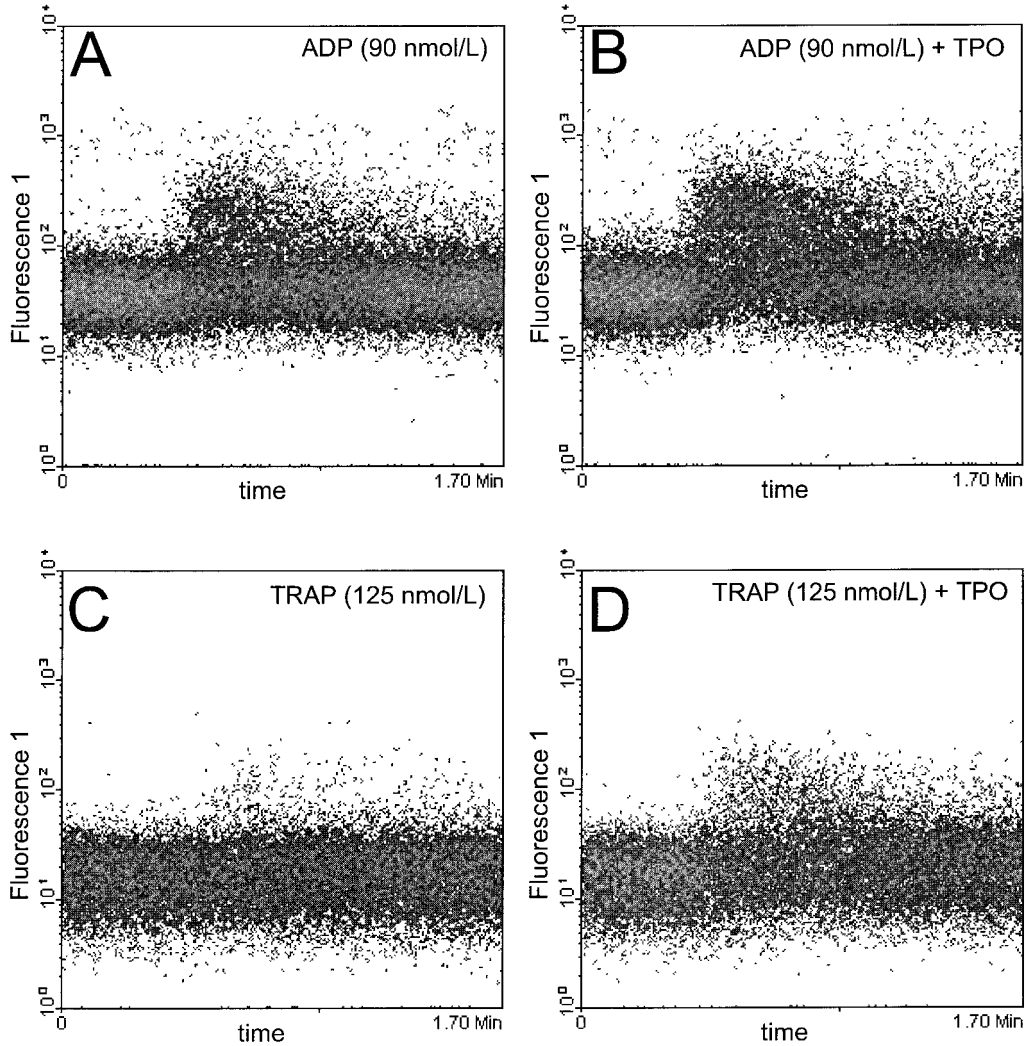

FIG. 3. Effect of TPO preincubation on agonist-induced Ca^{2+} flux in platelets. Platelets were loaded with fluo-3 AM. Samples were preincubated with (B, D) or without (A, C) TPO for 1 min before starting flow cytometric analysis. Agonists ADP or TRAP were added in the indicated concentrations 10 seconds after starting the measurement.

sion in response to high concentrations of TPO alone (100 ng/mL full-length TPO) (14). In contrast, we have never found an effect of TPO alone on CD62P expression of platelets even at higher TPO concentrations. Fontenay-Roupie *et al.* described an effect of TPO alone on thromboxane A2 formation and serotonin secretion of platelets (15). However, these experiments were done under continuous stirring, making more likely a synergistic effect of TPO on shear stress induced activation. In our experiments indirect effects on platelet activation via thromboxane formation were excluded by addition of ASA, a cyclooxygenase inhibitor.

The mechanisms, how TPO acts on platelet activation are still unclear. Tyrosine phosphorylation of a whole string of different molecules after stimulation with TPO has been demonstrated, like Jak2, Tyk2, Stat3, Stat5, Shc, Vav, Raf-1, Tec, CrkL, c-Cbl, PI3-K, and the c-Mpl receptor itself (7, 9, 10, 18–25). At least two distinct signaling pathways are activated by TPO, a specific Jak2/Stat1-Stat3-Stat5 signaling cascade, and the Ras/Raf-1/MAP-kinase cascade.

Changes in cytosolic $[Ca^{2+}]$ are a key "second messenger" in platelet activation. Platelet agonists such as ADP or TRAP stimulate a rise in $[Ca^{2+}]_i$ by promoting the entry of calcium ions into the cytoplasm from two sources: from the intracellular stores in the dense tubular system and from the extracellular medium across the plasma membrane. In the case of ADP and TRAP Ca^{2+} release from internal stores is mediated by activation of phospholipase (PL) C-β via G-proteins (26, 27). Activated PLC cleaves phosphatidylinositol-4,5-bisphosphate into diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3). The latter one opens a calcium channel by binding to its receptor in the dense

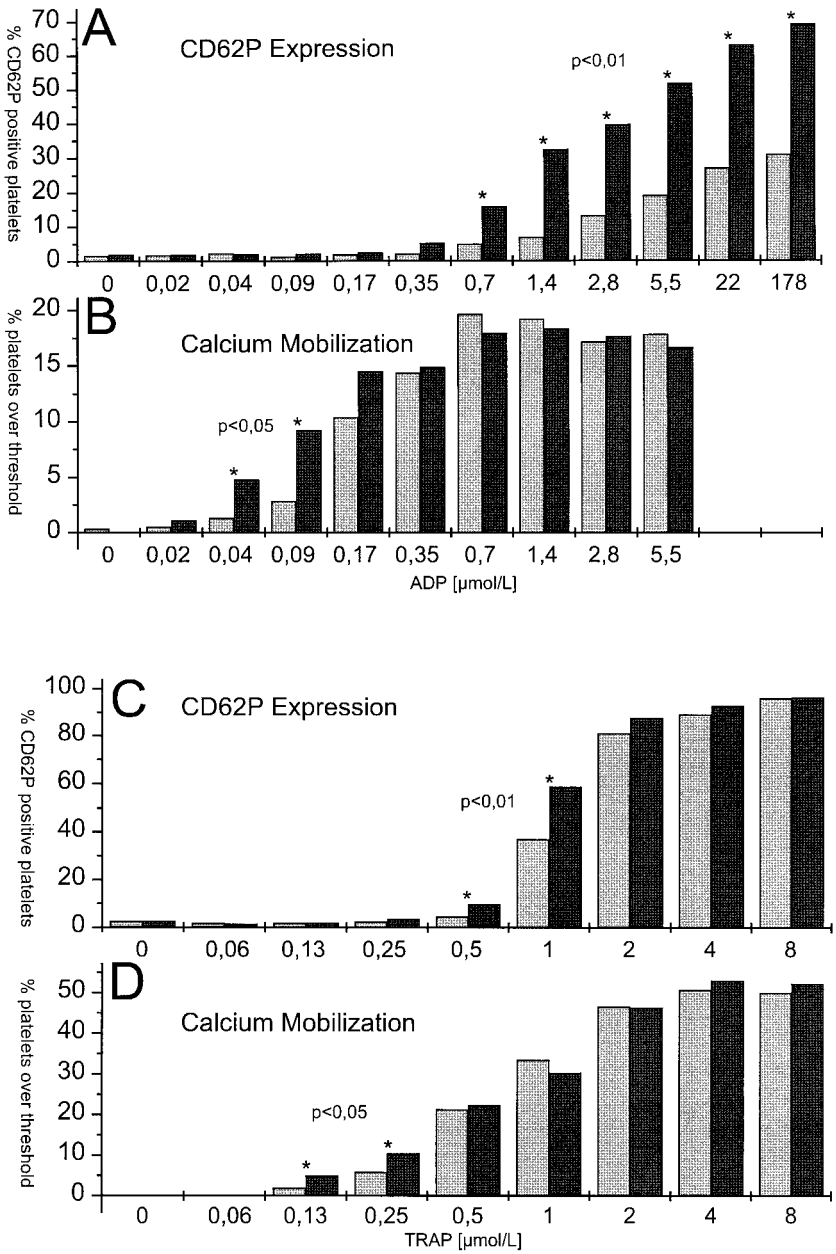

FIG. 4. Comparison of the synergistic effect of TPO on agonist induced Ca^{2+} flux and CD62P expression on platelets. Effects of TPO on agonist-induced CD62P expression (A, C) and Ca^{2+} flux (B, D) were compared for different concentrations of ADP (A, B, n = 6) and TRAP (C, D, n = 5). Quantification of differences in Ca^{2+} mobilization was done by determining the percentage of platelets over a fluorescence threshold in a time gate after stimulation (10–30 sec after addition of agonists). It is worth noting that the percentage of platelets over threshold does not represent the total number of activated platelets because of methodical reasons (time gate, weak increase in fluorescence intensity, etc.). (Black columns, activation in the presence of TPO; gray columns, activation in the absence of TPO; * = significant differences.)

tubular system. The calcium signal led to a subsequent activation of the α and β forms of the serine/threonine protein kinase C (PKC) and of several tyrosine protein kinases (28).

Our aim was to elucidate whether TPO affects the signaling pathways of the different platelet agonists upstream or downstream of the Ca^{2+} signal. There are very few reports referring to an influence of TPO on Ca^{2+} signaling. A direct effect of TPO on Ca^{2+} mobilization was observed only in the megakaryoblastic, TPO-dependent cell line UT-7/TPO (29): TPO induced the production of IP3 and the release of Ca^{2+} from

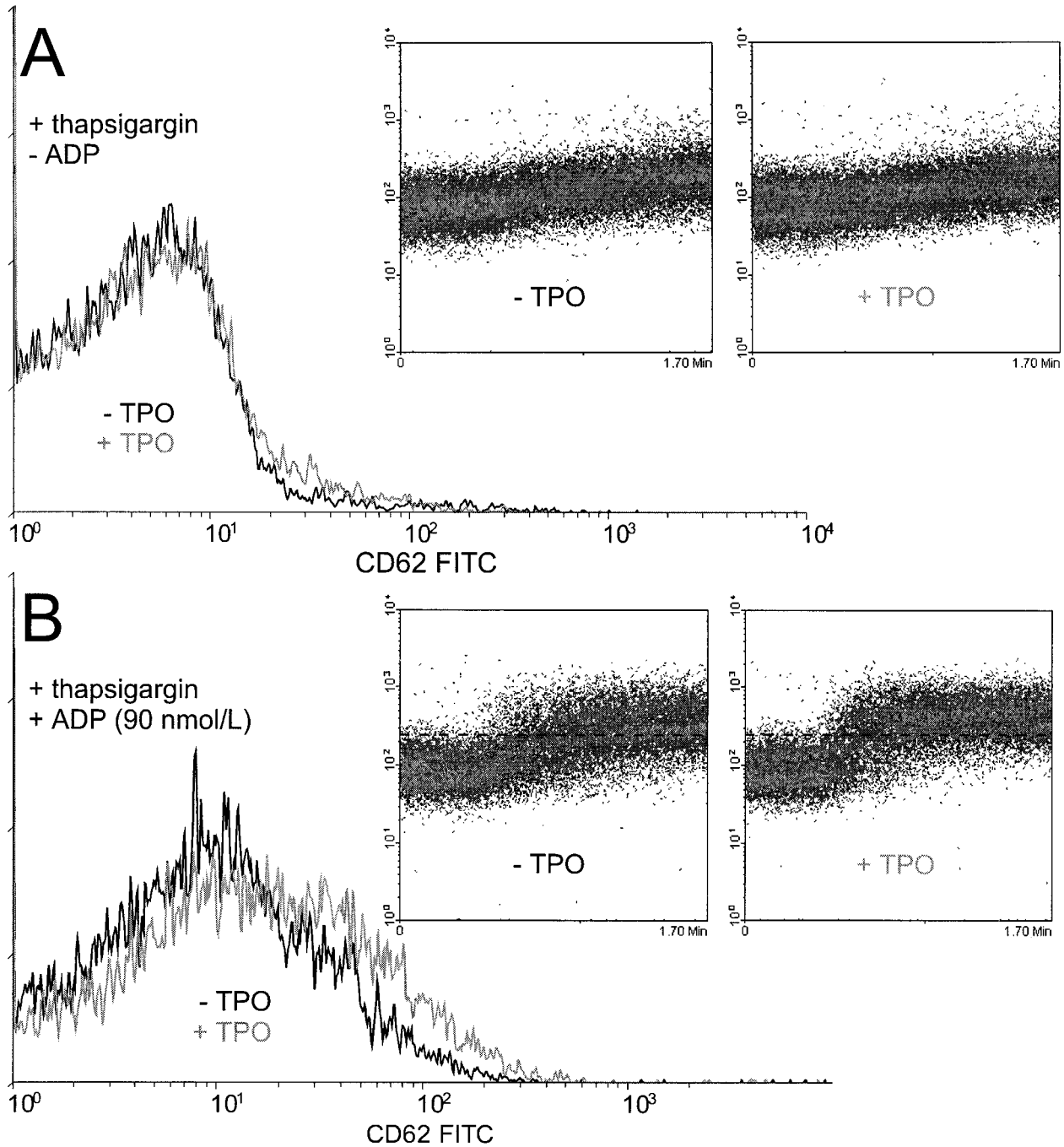

FIG. 5. Effects of thapsigargin on agonist-induced Ca^{2+} flux and CD62P expression. Platelets were preincubated with thapsigargin and with or without TPO (A). In a similar experiment a low concentration of ADP (90 nmol/L) was added (B). CD62P expression (large figures) and Ca^{2+} mobilization (small figures) were analyzed.

intracellular stores, although only a weak tyrosine phosphorylation of PLC-γ1 could be detected. There are other indications of a PLC activation by TPO in Mpl-transfected cell lines: TPO led only to a very weak phosphorylation of PLC-γ in the c-mpl transfected cell line BaF3 (30). A TPO dependent translocation of the Ca^{2+}/DAG-sensitive PKC isoforms α and β was observed in the Mpl-expressing cell line UT-7/mpl (31). Two contrary reports deal with the influence of TPO on calcium signaling in platelets. Rodriguez-Linares *et al.* demonstrated a synergistic effect of TPO on the Ca^{2+} mobilization induced by collagen, FcγRIIA cross-linker

or thrombin (7). They found that TPO potentiated the activation of PLC by thrombin and collagen, arguing for an influence of TPO on processes upstream of the Ca^{2+} mobilization. On the contrary, Oda *et al.* reported, that TPO does not modify the elevation of $[Ca^{2+}]_i$ induced by ADP or thrombin (6). However, the ADP concentrations used in this study for measurement of Ca^{2+}-fluxes (100–300 nmol/L) were in a range beyond that in which we could demonstrate a synergistic effect of TPO.

A synergistic effect of TPO on the ADP and TRAP induced Ca^{2+} mobilization could be observed in a small range of suboptimal agonist concentrations lower than those necessary for a measurable CD62P expression. We found no synergistic effect of TPO on Ca^{2+} mobilization in the concentration ranges which led to CD62P expression. There are two possible explanations for this discrepancy:

(1) The method of $[Ca^{2+}]_i$ measurement used in our study might not be optimal to detect differences in Ca^{2+} mobilization at higher agonist concentrations although present. The effect of the Ca^{2+} signal as a second messenger depends both on the magnitude and the duration of the Ca^{2+} response. Heemskerk *et al.* defined a Ca^{2+}-mobilizing potency (CMP) of agonists as the integral of the increase in $[Ca^{2+}]_i$ over time (32). $[Ca^{2+}]_i$ measurements in immobilized single platelets revealed that ADP and suboptimal doses of thrombin evoked repetitive spikes of $[Ca^{2+}]_i$ (33), the frequency of this spiking may also contribute to different activation of the platelets. The method of $[Ca^{2+}]_i$ measurement used in this study is not appropriate to measure $[Ca^{2+}]_i$ changes in single platelets or differences in CMP at agonist concentrations leading only to activation of subpopulations of platelets. Therefore we cannot exclude an effect of TPO on Ca^{2+} mobilization even at higher agonist concentrations.

(2) TPO affects platelet activation downstream of the Ca^{2+} signal or via Ca^{2+}-independent pathways, as can be concluded from the fact that TPO potentiates the phorbol ester induced, Ca^{2+}-independent, platelet aggregation (7) probably by activation of PKC isozymes or downstream molecules. PKC-δ, a Ca^{2+}-independent PKC isoform which is expressed in human platelets, can be activated by tyrosine phosphorylation (34) and contributes to the exocytosis of antigen-stimulated rat basophilic rbl-2h3 cells (35). The SNARE hypothesis is a current model for the exocytosis of granules in neurons (36) as well as in platelets (37). Some of the proteins that are involved in the 7S docking complex or the 20S fusion complex of granule exocytosis can be regulated by phosphorylation of PKC isozymes (38, 39). The TPO-dependent regulation of the PKC pathway could thus be responsible for the synergism of ADP and TRAP with TPO in CD62P expression of human platelets.

To document the importance of Ca^{2+} mobilization for the platelet activation we used the Ca^{2+}-ATPase inhibitor thapsigargin which blocks the Ca^{2+} uptake into internal stores in the dense tubules of platelets (40). Indirect effects of thapsigargin treatment of platelets include the activation of eicosanoid biosynthesis which leads to a fast platelet activation (41). These indirect effects can be avoided by using cyclooxygenase inhibitors such as ASA. The synergistic effect of TPO in agonist concentrations which were not high enough to induce an exocytosis of platelets without thapsigargin seems to be only due to the influence of TPO on the Ca^{2+} mobilization.

In parallel with stimulatory pathways triggered by agonists such as ADP or TRAP, also inhibitory pathways are activated in platelets after stimulation of adenyl or guanyl cyclase (42). It is likely that intracellular messengers linked to inhibitory or stimulating signals are generated simultaneously in platelets and the balance of these processes may determine the reactivity to different agonists. TPO appears to affect several elements of this complex network of positive and negative regulators. The influence of TPO on the ADP- or TRAP-induced Ca^{2+} mobilization may play an important and physiological role in synergizing especially with suboptimal agonist concentrations.

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