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Sphingosylphosphorylcholine, a naturally occurring lipid mediator, inhibits human platelet function

[1]Christoph Altmann, [1]Dagmar Meyer zu Heringdorf, [2]Dilek Böyükbas, [2]Michael Haude, [1]Karl H. Jakobs & *,[2]Martin C. Michel

[1]Institut für Pharmakologie, Universitätsklinikum Essen, D-45122 Essen, Germany and [2]Medizinische Klinik, Universitätsklinikum Essen, D-45122 Essen, Germany

1 The lysophospholipids, lysophosphatidic acid and sphingosine 1-phosphate, have been reported to activate platelets. Here we examined effects of the naturally occurring related sphingosylphosphorylcholine (SPC) on human platelet function.

2 Platelet activation was determined as aggregation, elevation of intracellular Ca^{2+} concentrations, surface expression of P-selectin, GP 53, and GP IIb/IIIa neoepitope PAC-1, and of fibrinogen binding to the platelet surface.

3 Platelets were activated by ADP (5 and 20 μM), the thrombin receptor-activating peptide TRAP-6 (5 and 20 μM), the thromboxane A_2 mimetic U-46619 (1 μM) and collagen (20 and 50 μg ml^{-1}) but not by SPC (up to 20 μM).

4 SPC concentration-dependently (IC_{50} approximately 1–10 μM) inhibited activation of washed human platelets in response to all of the above agonists, with almost complete inhibition occurring at 20 μM SPC.

5 The SPC stereoisomers, D-*erythro* SPC and L-*threo* SPC, exhibited similar concentration–response curves in inhibiting 20 μM ADP-induced platelet aggregation, suggesting that SPC did not act *via* specific lysophospholipid receptors.

6 Although SPC slightly activated platelet protein kinase A (as assessed by VASP phosphorylation), this effect could not explain the marked platelet inhibition. Possible protein kinase C inhibition also did not explain the inhibition of platelet activation by SPC. On the other hand, SPC suppressed agonist-induced Ca^{2+} mobilization and phospholipase C stimulation.

7 These results indicate that the lysophospholipid SPC is an effective inhibitor of human platelet activation, apparently primarily by uncoupling agonist-activated receptors from their effectors.
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Abbreviations: GP, glycoprotein; $InsP_3$, inositol-1,4,5-trisphosphate; IBMX, 3-isobutyl-1-methylxanthine; LPA, lysophosphatidic acid; PBS, phosphate-buffered saline; PDE, phosphodiesterase; PGE_1, prostaglandin E_1; PK, protein kinase; PL, phospholipase; PMA, phorbol 12-myristate 13-acetate; SERCA, sarco-endoplasmic reticulum Ca^{2+}-ATPase; S1P, sphingosine 1-phosphate; SPC, sphingosylphosphorylcholine; TRAP, thrombin receptor-activating peptide; VASP, vasodilator-stimulated phosphoprotein

Introduction

The lysophospholipids, lysophosphatidic acid (LPA) and sphingosine-1-phosphate (S1P), serve as potent lipid mediators for a large variety of cell types. Recently, several G protein-coupled receptors have been identified, which can mediate cellular actions of LPA and S1P (Chun *et al*., 2002). Additionally, S1P has been implicated as an intracellular second messenger in the regulation of Ca^{2+} mobilization, cell growth, and programmed cell death (Moolenaar, 1999; Pyne & Pyne, 2000; Olivera & Spiegel, 2001; Spiegel & Milstien, 2000; Young & Nahorski, 2001). Similar to LPA and S1P, the related sphingosylphosphorylcholine (SPC) is endogenously found in blood plasma (Liliom *et al*., 2001). The structure, metabolism, biological functions and potential mechanisms of action of SPC have recently been reviewed in detail (Meyer zu Heringdorf *et al*., 2002). SPC has been reported to increase intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), to induce cytoskeletal re-arrangements, and to modulate cell growth and migration in various cell types. Specific G protein-coupled receptors for SPC have been proposed (Xu *et al*., 2000; Zhu *et al*., 2001) and SPC can additionally act as a low-affinity agonist at the S1P receptors (Pyne & Pyne, 2000; Spiegel & Milstien, 2000), but the exact targets of the multiple cellular actions of SPC are largely unknown (Meyer zu Heringdorf *et al*., 2002).

Platelets are a major source of endogenous LPA and S1P, from which these lipids can be released upon agonist activation (Eichholtz *et al*., 1993; Yatomi *et al*., 1995). In addition, platelets are apparently also targets of LPA and S1P. Exogenous LPA and S1P ($\geqslant$20 μM) have been reported to activate human platelets, as characterized by elevation of $[Ca^{2+}]_i$, shape change and aggregation (Yatomi *et al*., 1995; Yatomi *et al*., 1997; Gueguen *et al*., 1999). Platelet activation

*Author for correspondence at: Nephrologisches Labor, IG 1, Medizinische Klinik, Universitätsklinikum Essen, D-45122 Essen, Germany; E-mail: martin.michel@uni-essen.de

by LPA is apparently mediated by a specific surface receptor, for which S1P may serve as a low-affinity partial agonist. Recently, mRNAs of the lysophospholipid receptors, LPA_1, LPA_2, LPA_3 and $S1P_4$ (previously designated Edg-2, Edg-4, Edg-7 and Edg-6, respectively) were identified in human platelets by RT–PCR (Motohashi *et al*., 2000). In addition to S1P, the S1P precursor, sphingosine, has been reported to enhance platelet activation at low concentrations through an increase in phospholipase (PL) C activity, while high concentrations ($>50\ \mu$M) were found to cause inhibition, possibly *via* inhibiting protein kinase (PK) C activity (Hannun *et al*., 1987; Hashizume *et al*., 1992). Whether SPC affects platelet function and, if so, these effects resemble those of S1P and LPA, is unknown. Therefore, we have investigated the effects of SPC on human platelet function and attempted to identify underlying molecular mechanisms.

Methods

Materials

SPC was obtained from Biomol (Hamburg, Germany), dissolved in methanol, dried in a SpeedVac concentrator, and redissolved in a solution of bovine serum albumin (1 mg ml^{-1}). The L-*threo* and D-*erythro* SPC stereoisomers were obtained from Matreya (Pleasant Gap, PA, U.S.A.), ADP, apyrase, digitonin, inositol-1,4,5-trisphosphate ($InsP_3$), 3-isobutyl-1-methylxanthine (IBMX), phorbol 12-myristate 13-acetate (PMA), prostaglandin E_1 (PGE_1), Triton X-100 and human fibrinogen from Sigma (Deisenhofen, Germany), bisindolylmaleimide I, H-89 (N-[2-(p-bromocinnamylamino) ethyl]-5-isoquinolinesufonamide), thapsigargin and the thromboxane A_2 mimetic, U-46619 (9, 11-dideoxy-9α, 11α-methanoepoxyprostaglandin $F_{2\alpha}$), from Calbiochem (Bad Soden, Germany). Fura-2-AM was from Molecular Probes (Leiden, The Netherlands), calf skin collagen from NOBIS (Endingen, Germany), the thrombin receptor activating peptide, SFLLRN (TRAP-6), from Bachem (Heidelberg, Germany), and [^{3}H]-$InsP_3$ (22.0 Ci ml^{-1}) from NEN Life Science Products (Boston, U.S.A.). Fluorescence-conjugated monoclonal antibodies to the human platelet receptors, glycoprotein (GP) Ib (SZ2), P-selectin (CLB/Thromb6), GP 53 (CLB Gran/12) and the activated GP IIb/IIIa receptor (PAC-1) were purchased from Beckman Coulter (Krefeld, Germany) and Becton Dickinson (Heidelberg, Germany). Fluorescent polyclonal antibody to human fibrinogen was obtained from WAKChemie (Bad Soden, Germany) and monoclonal antibody to phosphorylated vasodilator-stimulated phosphoprotein (VASP, 5C6) from nanoTools (Teningen, Germany).

Preparation of human platelets

Washed platelets were used for all experiments. Platelet-rich plasma was prepared from citrate-anticoagulated blood samples obtained from healthy volunteers, by centrifugation at 150 *g* for 15 min. Platelets were then pelleted at 800 *g* for 10 min and resuspended in an acid citrate buffer, containing (mM): NaCl 120, NaH_2P0_4 4.26, sodium citrate 4.77 and citric acid 2.35, pH 6.5. After a second washing in acid citrate, the washed platelets were finally resuspended in a modified Tyrode's HEPES buffer, containing (mM): NaCl 138, KCl 2.9, $MgCl_2$ 1, $CaCl_2$ 2, NaH_2P0_4 3.3, glucose 5.5 and HEPES 20, pH 7.4. In order to prevent platelet activation during preparation, PGE_1 (1 μg ml^{-1}) and apyrase (0.5 U ml^{-1}) were added prior to centrifugation.

Platelet aggregation

Platelet aggregation was quantified at 37°C by the turbidimetric method in a dual channel platelet ionized calcium aggregometer (Chrono-Log, Haverton, U.S.A.), with stirring at 900 r.p.m.. The instrument was calibrated with the platelet suspension (2.0×10^8 ml^{-1}) for zero transmission and with the buffer for 100% transmission. Fibrinogen (0.5 mg ml^{-1}) was added just prior to experiments. Primary slope of increase in light transmission, maximal aggregation and occurrence of desaggregation were recorded for 6–10 min after stimulation. Measurements were performed in duplicate with the mean taken for further analyses.

Analysis of platelet activation by flow cytometry

Flow cytometric analyses were performed with an EPICS XL cytometer, using the System II software (Beckman Coulter). The day-to-day reproducibility of fluorescence intensity was controlled by beads of defined standard fluorescence (ImmunoCheck, Beckman Coulter). Platelet surface receptor expression was quantified in washed platelets (0.4×10^8 ml^{-1}). Fibrinogen (0.1 mg ml^{-1}) was added immediately prior to experiments. Following stimulation, fluorescence-conjugated antibodies were added at saturating concentrations and incubated for an additional 5 min in the dark at room temperature. Stimulation was stopped by addition of formaldehyde (1%) in phosphate-buffered saline (PBS).

Expression of the surface receptors, P-selectin (CD 62P), GP 53 (CD 63), GP Ib (CD 42b), and the activation-dependent GP IIb/IIIa receptor neoepitope (PAC-1), and fibrinogen binding were quantified by fluoresceine isothiocyanate (FITC)-labelled antibodies directed against the respective epitopes. IgG was used for isotype control. Fluorescence histograms were obtained for 10,000 cells gated per sample. Antibody binding to the cell surface was expressed as mean fluorescence intensity (MFI) of bound antibodies after subtraction of the respective isotype control. Duplicate measurements were performed with the mean taken for further analyses.

Intracellular VASP phosphorylation was determined as previously described (Schwarz *et al*., 1999) with minor modifications. In brief, platelets (0.4×10^8 ml^{-1}) were incubated with SPC or PGE_1 for 5 min, and incubations were stopped by addition of formaldehyde (3.7% in PBS). The platelet suspension was diluted with PBS and permeabilized with Triton X-100 (0.005%). Following a second dilution with PBS, cells were incubated with the FITC-conjugated antibody VASP–5C6, recognizing VASP when serine 157 is phosphorylated. The extent of VASP phosphorylation was quantified by MFI of antibody binding.

Measurement of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was determined fluorometrically with the fluorescent Ca^{2+} indicator Fura-2, using a F-2000 fluorescence spectro-

meter (Hitachi, Tokyo, Japan) as previously described (Meyer zu Heringdorf *et al*., 1998). Platelets in platelet-rich plasma were loaded with Fura-2-AM (4 μM), washed twice and resuspended in Tyrode's HEPES buffer as described above. Measurements were performed with a platelet concentration of 2.0×10^8 ml^{-1} at room temperature. For determination of $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+}, Fura-2-loaded platelets were resuspended in Ca^{2+}-free HEPES buffer, and EGTA (50 μM) was added immediately prior to experiments.

Determination of PLC activity

Phospholipase (PL) C activity was determined by $InsP_3$ mass measurements as previously described (Challiss *et al*., 1988; Schmidt *et al*., 1996). Briefly, platelets (0.8×10^9 cells ml^{-1}) supplemented with fibrinogen (0.5 mg ml^{-1}) were preincubated with SPC or vehicle, followed by stimulation with TRAP-6 for the indicated time points. After stop of the reaction with trichloroacetic acid, $InsP_3$ mass was determined in triplicates with a radioreceptor assay, using [^{3}H]-$InsP_3$ as ligand and $InsP_3$-binding protein from bovine adrenal cortex.

Data presentation and analysis

Results are presented as means$\pm$s.e.mean of 4–6 experiments. Comparisons between different groups were made by Student's *t*-test or ANOVA and Bonferroni *post-hoc* adjustment as indicated. Apparent pIC_{50} values for inhibition by SPC were calculated by fitting sigmoidal curves to the experimental data; due to the self-amplifying nature of platelet aggregation, however, it must be emphasized that these values only represent descriptive estimates.

Results

Inhibition of agonist-induced platelet aggregation by SPC

Activation of washed human platelets with ADP, the thrombin receptor-activating peptide, TRAP-6, the thromboxane A_2 mimetic, U-46619, or collagen caused marked aggregation. Maximal light transmission by 5 and 20 μM ADP amounted to 53.3$\pm$5.1% and 78.2$\pm$3.3%, respectively, by 5 and 20 μM TRAP-6 to 76.9$\pm$1.9% and 88.3$\pm$1.6%, respectively, by 1 μM U-46619 to 91.2$\pm$0.9%, and by 20 and 50 μg ml^{-1} collagen to 73.2$\pm$2.5% and 84.4$\pm$2.4%, respectively (n=4–6). In comparison, addition of SPC (up to 20 μM) did not induce aggregation (Figure 1A). On the contrary, preincubation of platelets for 2 min with SPC concentration-dependently inhibited platelet aggregation in response to ADP (5 or 20 μM) [pIC_{50}: 5.55$\pm$0.05, 5.20$\pm$0.03], TRAP-6 (5 or 20 μM) [pIC_{50}: 5.56$\pm$0.04, 4.98$\pm$0.02], U-46619 (1 μM) [pIC_{50}: 5.08$\pm$0.02], and collagen (20 or 50 μg ml^{-1}) [pIC_{50}: 5.77$\pm$0.05, 5.33$\pm$0.04] (Figure 1B–D). At 20 μM SPC completely suppressed agonist-induced aggregation. SPC also inhibited platelet aggregation without preincubation, i.e. upon addition during an ongoing aggregation response (data not shown). The potency of SPC in inhibiting aggregation was affected by the strength of receptor activation, i.e. shifted to the right at high agonist concentrations, as examined for two concentrations of ADP, TRAP-6 and collagen (Figure 1B–D). In contrast to SPC, addition of S1P (20 μM) to washed platelets had no or only a very minor inhibitory effect on agonist-induced aggregation (data not shown). The two SPC stereoisomers, D-*erythro* SPC and L-*threo* SPC, exhibited similar concentration-response curves in inhibiting 20 μM ADP-induced platelet aggregation (Figure 1E). In comparison to agonist-induced aggregation, platelet aggregation induced by the phorbol ester, PMA (50 nM), which was fully prevented by the PKC inhibitor, bisindolylmaleimide I (5 μM), was only slightly affected by SPC (Figure 1F). SPC (20 μM) reduced maximal PMA-induced aggregation, amounting to 92.8$\pm$1.4% increase in light transmission, by 14.2$\pm$0.7% (n=5).

Inhibition of platelet surface receptor expression by SPC

Platelet activation by ADP, TRAP-6, and U-46619 induced increased surface expression of the two platelet degranulation markers, P-selectin and GP 53, of the activation-dependent GP IIb/IIIa neoepitope PAC-1, and of fibrinogen binding to the platelet surface (Figure 2A–D). Furthermore, ADP and TRAP-6 caused internalization of GP Ib (Figure 2E). While SPC alone (up to 20 μM) did not significantly change expression of these activation-dependent surface epitopes, it concentration-dependently inhibited the effects of ADP (20 μM), TRAP-6 (10 μM), and U-46619 (1 μM), with a similar potency as observed for platelet aggregation (Figure 2A–E). At 20 μM SPC completely suppressed the agonist responses. Taken together, these data demonstrate that SPC strongly inhibits platelet activation by different receptor agonists, measured as aggregation and altered expression of surface proteins.

Role of protein kinase A in the inhibition of platelet function by SPC

As an increase in platelet cyclic AMP levels with subsequent activation of protein kinase (PK) A is the major inhibitory control of platelet activity (Schwarz *et al*., 2001), we examined whether SPC activates PKA in human platelets and whether inhibition of PKA can mitigate the inhibitory effects of SPC. Phosphorylation of VASP is regarded as a specific indicator of PKA activation in platelets (Schwarz *et al*., 1999; Burkhardt *et al*., 2000). As expected, VASP phosphorylation was strongly enhanced by treatment of platelets with PGE_1 (5 μM). SPC at 10 and 20 μM also increased VASP phosphorylation, but considerably less than PGE_1 (Figure 3A). If SPC's inhibitory action involves cyclic AMP, it should be potentiated by inhibition of cyclic AMP degradation. As shown in Figure 3B, preincubation of platelets with the phosphodiesterase (PDE) inhibitor, IBMX (10 μM), significantly enhanced the inhibitory effects of 1 and 5 μM SPC on 20 μM TRAP-6-induced aggregation, but less markedly than the inhibitory effect of 0.5 μM PGE_1. Preincubation of platelets with 10 μM IBMX alone only slightly reduced TRAP-6-induced aggregation (data not shown).

Pretreatment of platelets with the PKA inhibitor, H-89 (20 μM) (Davies *et al*., 2000), markedly attenuated the inhibitory effects of PGE_1 (1 μM) on TRAP-6-induced aggregation, surface expression of P-selectin, and fibrinogen binding (Figure 4C–E). In comparison, H-89 only slightly attenuated the inhibitory effect of SPC (at 5 and 10 μM) on

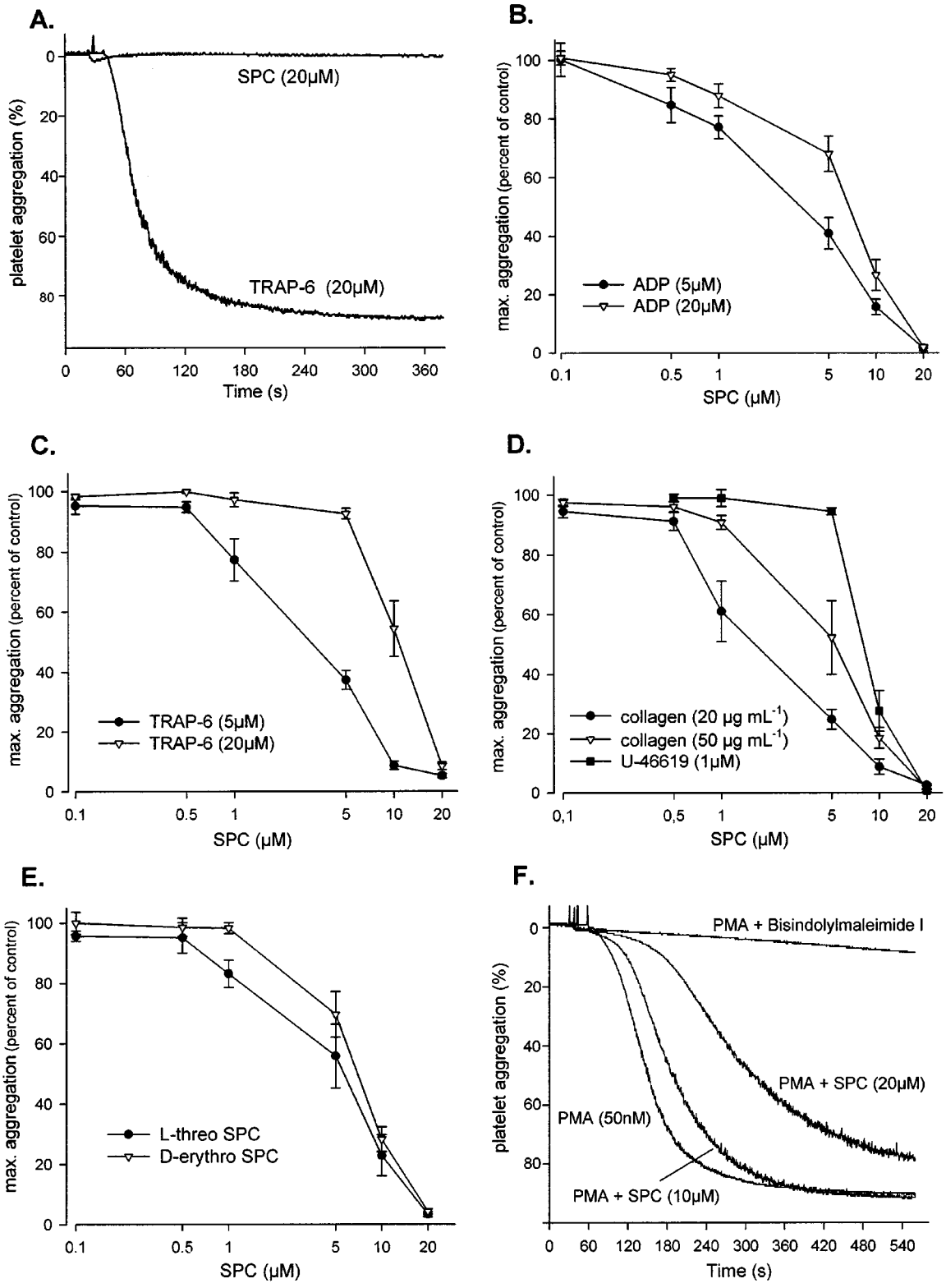

Figure 1 Inhibition of agonist-induced platelet aggregation by SPC. (A) Representative traces of aggregation responses upon addition of SPC (20 μM) or TRAP-6 (20 μM) to washed human platelets (2×10^8 ml^{-1}). (B–D) Maximal platelet aggregation induced by ADP (5 and 20 μM), TRAP-6 (5 and 20 μM), collagen (20 and 50 μg ml^{-1}), or U-46619 (1 μM) after 2 min preincubation of platelets with SPC (0.1–20 μM). (E) ADP (20 μM)-induced platelet aggregation after 2 min preincubation of platelets with L-*threo* or D-*erythro* SPC (0.1–20 μM). Data in B–E are expressed as per cent of controls. Means$\pm$s.e.mean, $n=4$. (F) Representative traces of PMA (50 nM)-induced aggregation after preincubation of platelets without and with SPC (10 and 20 μM) or 5 μM bisindolylmaleimide I ($n=5$).

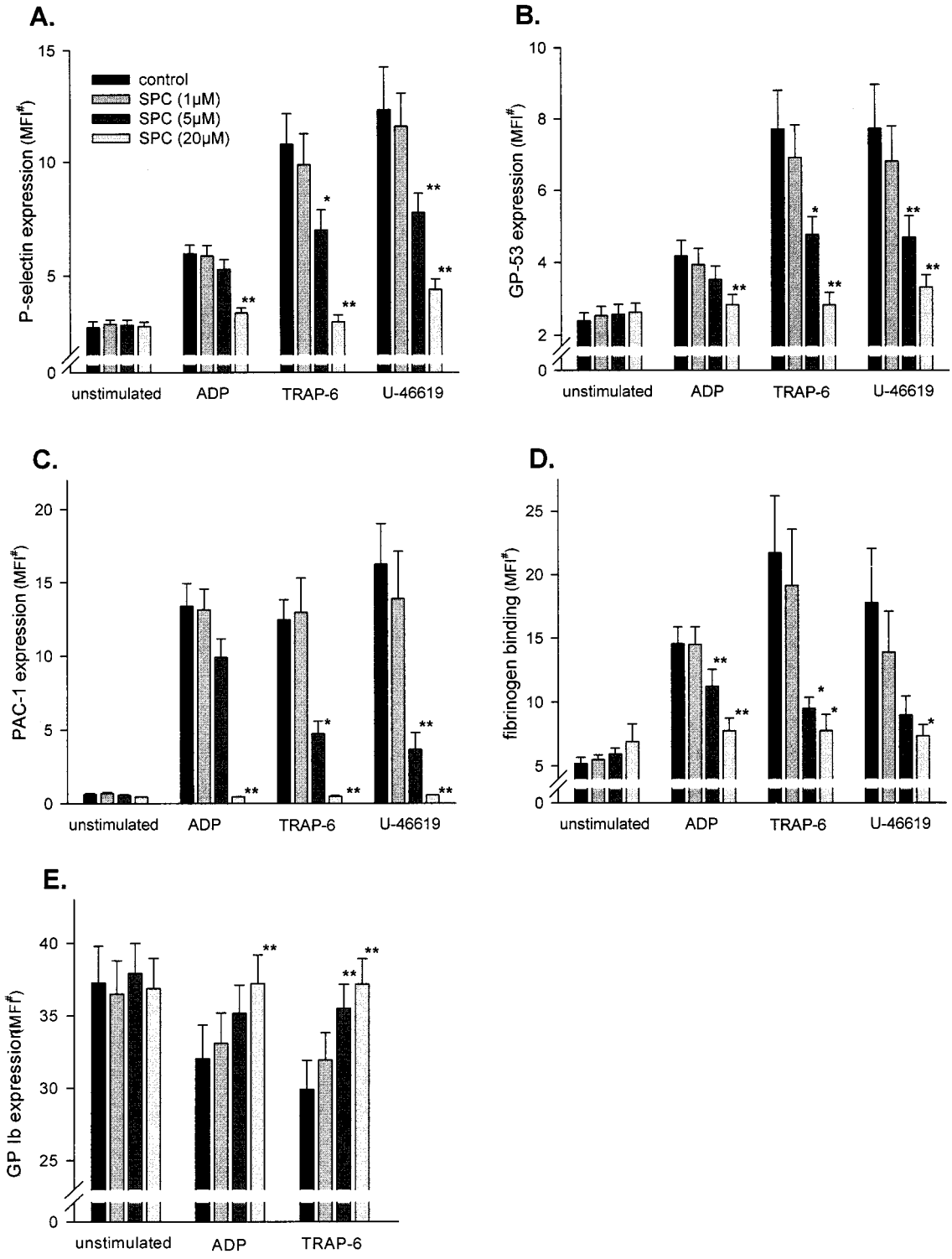

Figure 2 Inhibition of agonist-induced changes of platelet surface protein expression by SPC. Flow cytometric analysis of agonist-induced surface expression of (A) Pselectin, (B) GP 53, (C) the activated GP IIb/IIIa (PAC-1), (D) fibrinogen binding to the platelet surface, and (E) expression of GP Ib. Platelets were stimulated with 20 μM ADP, 10 μM TRAP-6, or 1 μM U-46619. Prior to stimulation, platelets were preincubated for 2 min with SPC (1–20 μM) or vehicle. #MFI of antibodies bound to the respective cell epitopes. Means ± s.e.mean, $n = 4-5$. $^{*}P < 0.05$, $^{**}P < 0.01$ (ANOVA with Bonferroni *post-hoc* adjustment).

TRAP-6-induced platelet aggregation and was without effect on SPC-induced inhibition of surface expression of P-selectin and fibrinogen binding (Figure 4C–E). These data suggested that, although SPC can apparently induce PKA activation, this activation contributes only to a minor extent to the effective inhibition of platelet function by the lysosphingolipid.

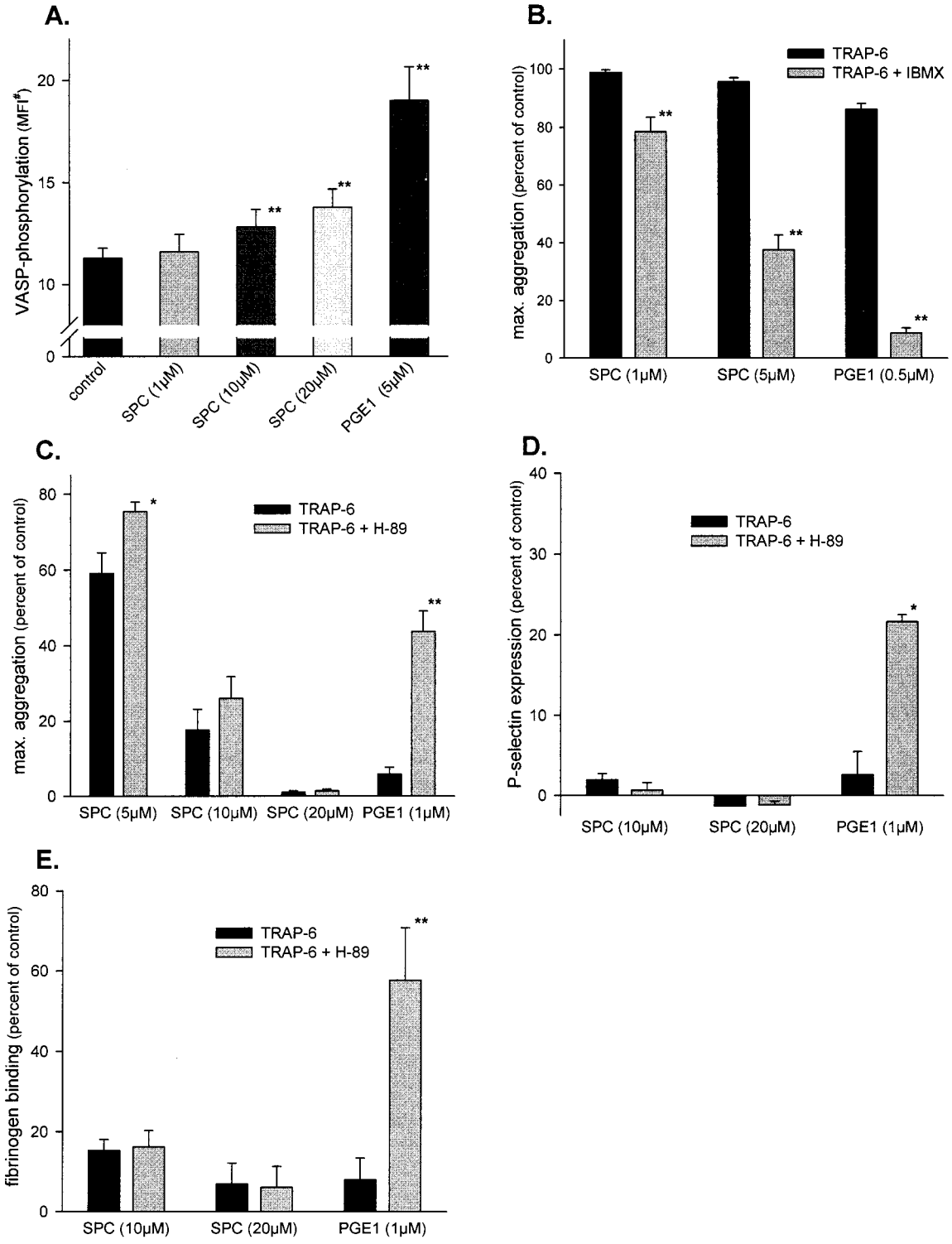

Figure 3 Role of PKA in the inhibitory effects of SPC on platelet function. (A) Flow cytometric analysis of intracellular VASP phosphorylation after incubation of platelets for 5 min without and with SPC (1–20 μM) or PGE_1 (5 μM). #MFI of the antibody bound to phosphorylated VASP. (B) Effects of a preincubation of platelets with the PDE inhibitor, IBMX (10 μM), on the inhibitory effects of SPC (1 and 5 μM) or PGE_1 (0.5 μM) on TRAP-6 (20 μM)-induced platelet aggregation. Data are expressed as per cent of controls corrected for inhibitory effects of IBMX alone on TRAP-6-induced aggregation. (C–E) Influence of a preincubation of platelets with the PKA inhibitor, H-89 (20 μM), on the inhibitory effects of SPC (5–20 μM) and PGE_1 (1 μM) on (C) platelet aggregation, (D) P-selectin expression, and (E) fibrinogen binding induced by 10 μM TRAP-6. Data are expressed as per cent of controls. Means$\pm$s.e.mean, $n=4-6$. $^*P<0.05$, $^{**}P<0.01$ (ANOVA with Bonferroni *post-hoc* adjustment for SPC; Student's *t*-test for PGE_1 *vs.* control).

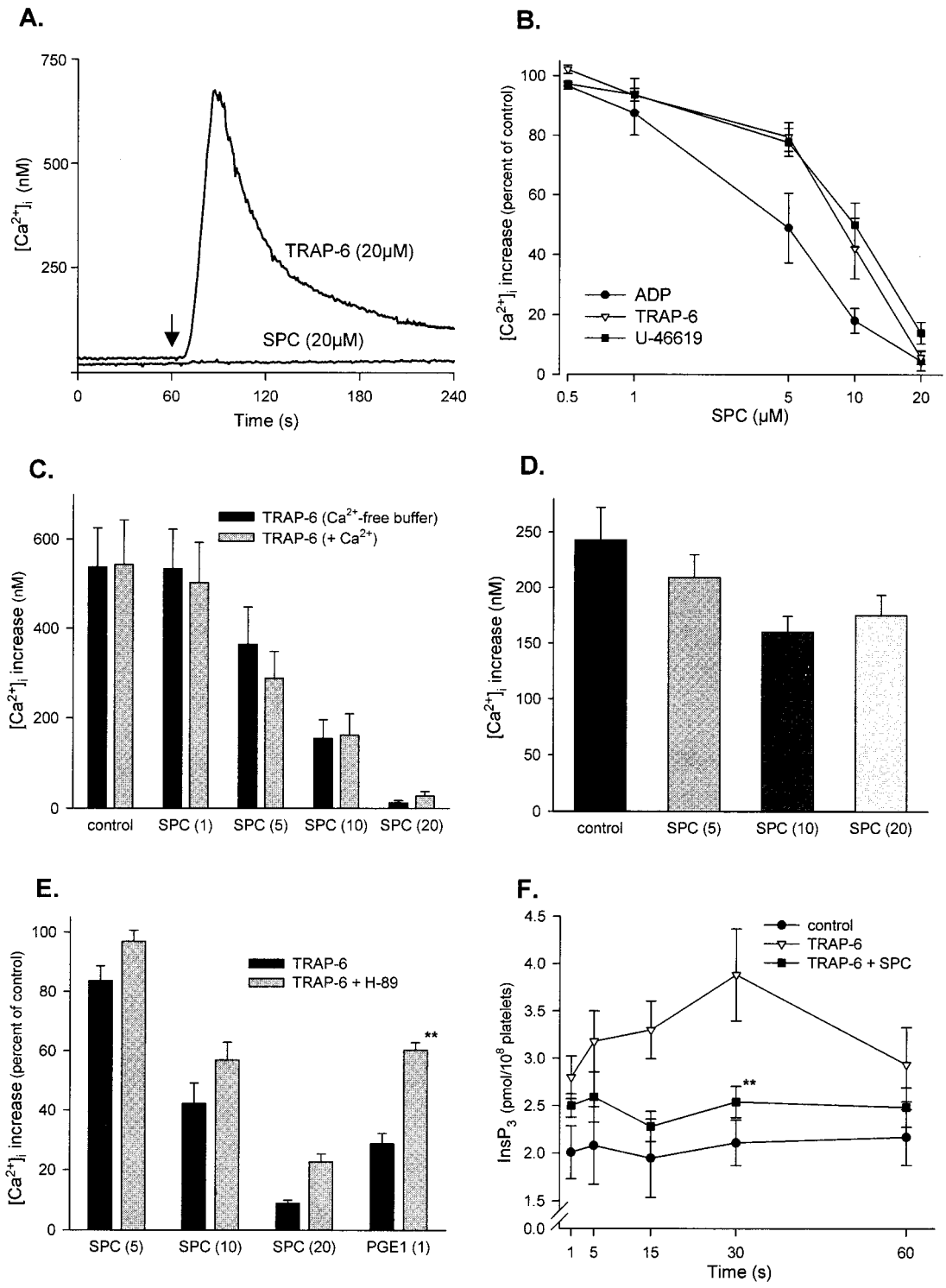

Figure 4 Influence of SPC on $[Ca^{2+}]_i$ changes and PLC stimulation in human platelets. (A) Representative traces of $[Ca^{2+}]_i$ changes after addition of SPC (20 μM) or TRAP-6 (20 μM) to Fura-2-loaded platelets (2×10^8 ml^{-1}). (B) Maximal increases of $[Ca^{2+}]_i$ induced by 20 μM ADP, 20 μM TRAP-6, or 1 μM U-46619 after 2 min preincubation of platelets with SPC (0.5–20 μM) or vehicle. (C) Influence of SPC (1–20 μM) on the maximal $[Ca^{2+}]_i$ increase induced by TRAP-6 (20 μM) measured in the presence (2 mM) or absence (Ca^{2+}-free buffer supplemented with 50 μM EGTA) of extracellular Ca^{2+}. (D) Effects of SPC (5–20 μM) on thapsigargin-induced intracellular Ca^{2+} release, determined by maximal $[Ca^{2+}]_i$ increase after stimulation of Fura-2- loaded platelets with 0.5 μM thapsigargin in the absence of extracellular Ca^{2+} (Ca^{2+}-free buffer supplemented with 50 μM EGTA). Data are expressed as percent of controls. ANOVA with Bonferroni *post-hoc* adjustment for (C) comparison between $[Ca^{2+}]_i$ increase in the presence or absence of extracellular Ca^{2+} and (D) SPC *vs.* control, respectively. (E) Influence of a preincubation of platelets with H-89 (20 μM) on the inhibitory effects of SPC (5–20 μM) and PGE_1 (1 μM) on TRAP-6 (10 μM)- induced $[Ca^{2+}]_i$ increase. (E) Effect of a preincubation of platelets with 20 μM SPC or vehicle on TRAP-6 (20 μM)-induced $InsP_3$ formation measured for the indicated periods of time. Means$\pm$s.e.mean, $n=4-5$. $^{**}P<0.01$ for comparison between TRAP-6- induced $InsP_3$ formation with or without SPC preincubation (ANOVA with Bonferroni *post-hoc* adjustment).

Inhibition of agonist-induced Ca^{2+} mobilization and PLC stimulation by SPC

The strong inhibitory effect of SPC on platelet activation by different receptor agonists suggested that this lysosphingolipid interferes with an essential signalling process of platelet activation. Therefore, we examined whether SPC inhibits agonist-induced Ca^{2+} mobilization. Stimulation of washed platelets with ADP (20 μM), U-46619 (1 μM), and TRAP-6 (20 μM) caused a rapid increase in $[Ca^{2+}]_i$, by maximally 239 ± 27 nM, 423 ± 37 nM, and 796 ± 71 nM, respectively ($n=4$). In contrast, SPC (up to 20 μM) by itself caused only minor if any $[Ca^{2+}]_i$ increase (Figure 4A). However, pretreatment of platelets with SPC concentration-dependently and nearly completely inhibited elevations of $[Ca^{2+}]_i$ induced by ADP (20 μM), U-46619 (1 μM) or TRAP-6 (20 μM) with pIC_{50} values of 5.38 ± 0.05, 5.03 ± 0.03, and 5.11 ± 0.03, respectively (Figure 4B).

Maximal $[Ca^{2+}]_i$ increase induced by the thrombin receptor-activating peptide, TRAP-6 (20 μM), was not significantly affected by removal of extracellular Ca^{2+} (Figure 4C), indicating that the agonist-induced $[Ca^{2+}]_i$ increase was mainly due to mobilization of intracellular Ca^{2+}. Accordingly, the inhibitory effect of SPC on TRAP-6- induced $[Ca^{2+}]_i$ increase was not affected by the presence or absence of extracellular Ca^{2+} (Figure 4C). The inhibitory effect of SPC on agonist-induced Ca^{2+} mobilization, however, was apparently not due to emptying of intracellular Ca^{2+} stores. Treatment of platelets with SPC (up to 20 μM) only slightly reduced Ca^{2+} release induced by the sarco-endoplasmic reticulum Ca^{2+}-ATPase (SERCA) inhibitor, thapsigargin (0.5 μM) (Figure 4D). Furthermore, the SPC-induced inhibition of agonist-induced Ca^{2+} release was apparently not due to PKA activation. Treatment of platelets with H-89 (20 μM) had only a minor effect on SPC-induced inhibition of TRAP-6-induced $[Ca^{2+}]_i$ increase, whereas the inhibitory effect of PGE_1 (1 μM) was markedly attenuated by the PKA inhibitor (Figure 4E). These data suggested that SPC interferes with receptor signalling processes leading to Ca^{2+} mobilization. Therefore, we studied the effect of SPC on TRAP-6-induced PLC stimulation, by measuring $InsP_3$ formation. Stimulation of platelets with 20 μM TRAP-6 rapidly increased $InsP_3$ levels, reaching a maximum after 30 s and then gradually declining to basal unstimulated levels (Figure 4F). Preincubation of platelets with 20 μM SPC, which completely inhibited the TRAP-6- induced $[Ca^{2+}]_i$ increase, also completely prevented the TRAP-6-induced $InsP_3$ formation (Figure 4F).

Discussion

Human platelets positively respond to several lipid mediators, including thromboxane A_2, platelet-activating factor, LPA, and the lysosphingolipid S1P. The latter is a very weak agonist, activating platelets only at rather high concentrations ($\geqslant 20$ μM) and apparently acting as low-affinity partial agonist at platelet LPA receptors (Gueguen *et al*., 1999; Motohashi *et al*., 2000). Here we report that the related lysosphingolipid SPC, which is endogenously found in human blood plasma and serum in concentrations of 50 and 130 nM, respectively (Liliom *et al*., 2001), effectively inhibits human platelet activation *in vitro*. In concentrations up to 20 μM, SPC did not show signs of platelet activation, but rather concentration-dependently inhibited platelet activation by diverse receptor agonists. At the highest concentration used here (20 μM), inhibition of platelet activation was almost complete. While it can be argued that this concentration is markedly higher than that found in plasma (Liliom *et al*., 2001), SPC is unlikely to act as a hormone but rather as a paracrine mediator. The SPC concentrations in local microenvironments, however, are unknown, and SPC action on several other cell types also was reported in the range of up to 30 μM (Bischoff *et al*., 2000; Meyer zu Heringdorf *et al*., 2002; Shirao *et al*., 2002).

SPC effectively inhibited platelet aggregation and altered expression of surface receptors induced by agonists at various G protein-coupled receptors (for ADP, thrombin, and thromboxane A_2) as well as by an agonist at a non-G protein-coupled receptor (for collagen). This broad inhibitory action suggested that SPC does not inactivate a single specific receptor or its signalling mechanisms, but somehow generally interferes with platelet activation by these diverse agonists. Therefore, we have investigated three potentially relevant pathways of platelet inhibition, i.e., inhibition of PKC, activation of PKA, and blockade of Ca^{2+} signalling.

Activation of PKC is a necessary and common event in platelet activation, and inhibition of PKC has been suggested to underlie platelet inhibition by sphingosine (Hannun *et al*., 1987). Therefore, we examined whether SPC attenuates platelet activation by the PKC-stimulating phorbol ester PMA. While PMA-induced platelet aggregation was fully suppressed by the PKC inhibitor, bisindolylmaleimide I, SPC exerted only a slight inhibition. Most important, the inhibitory effect of SPC on PMA-induced platelet aggregation was much smaller than that against the agonists acting at platelet surface receptors. Thus, inhibition of PKC is unlikely to explain the marked SPC-induced inhibition of platelet activation by receptor agonists.

An increase in platelet cyclic AMP levels, e.g. by PGE_1, with subsequent activation of PKA is the most powerful means to inhibit platelet activation (Schwarz *et al*., 2001). Therefore, we have tested a potential role of cyclic AMP and PKA activation in the inhibitory effects of SPC, in comparison to PGE_1 as a positive control. We demonstrate that SPC increases VASP serine 157 phosphorylation, an established indicator of platelet PKA activation (Schwarz *et al*., 2001), but considerably less than PGE_1. Furthermore, treatment of platelets with the PDE inhibitor IBMX enhanced the inhibitory effect of SPC on TRAP-6-induced aggregation. These data suggested that cyclic AMP/PKA may be involved in the platelet inhibitory action of SPC. To address this problem directly, we examined whether inhibition of PKA by H-89 (Davies *et al*., 2000) mitigates the inhibitory effects of SPC. Treatment of platelets with H-89 markedly attenuated the inhibitory effects of PGE_1, but had little effect on SPC-induced inhibition of aggregation and $[Ca^{2+}]_i$ increase by TRAP-6 and was without effect on SPC-induced inhibition of P-selectin expression and fibrinogen binding. These data demonstrated that, although SPC can induce PKA activation, this effect is apparently not a major contributor to the strong inhibitory effects of SPC on platelet function.

Elevation of platelet $[Ca^{2+}]_i$ is a central mechanism for platelet activation by receptor agonists. We demonstrate that

SPC inhibits this platelet response with a similar potency as the final steps of platelet activation, i.e. altered expression of surface proteins and aggregation. As studied in detail with TRAP-6, this thrombin receptor-activating peptide increased platelet $[Ca^{2+}]_i$ mainly by mobilization of Ca^{2+} from intracellular stores, and SPC inhibited this Ca^{2+} mobilization equally well as total $[Ca^{2+}]_i$ increase. As intracellular Ca^{2+} release by the SERCA inhibitor, thapsigargin, was only slightly impaired by SPC, the data suggested that the lysosphingolipid interferes with the major signalling process leading to agonist-induced Ca^{2+} release, i.e. PLC stimulation. In fact, we demonstrate that SPC suppresses not only Ca^{2+} mobilization but also $InsP_3$ formation stimulated by the thrombin receptor-activating peptide. Overall, our findings suggest that SPC inhibits platelet activation by TRAP-6, and probably also by the other receptor agonists, by uncoupling the receptors from PLC, the major effector enzyme for platelet activation. Such an uncoupling may also explain the finding that the inhibitory potency of SPC was dependent on the strength of receptor activation, i.e. decreased at high platelet agonist concentration, partially overcoming the inhibitory effect of SPC.

The exact target and mechanism of inhibitory action of SPC remain to be identified. Several lines of evidence, however, suggest that inhibition of platelet function by SPC does not involve specific lysophospholipid receptors (Chun *et al*., 2002). First, for activation of the proposed specific SPC receptors lower concentrations of SPC are required than for inhibition of platelet function by SPC (Xu *et al*., 2000; Zhu *et al*., 2001). Second, high SPC concentrations can activate S1P receptors (Pyne & Pyne, 2000; Spiegel & Milstien, 2000) but in the present study the platelet inhibitory action of SPC was not mimicked by S1P. Third, receptor-mediated effects of SPC are stereospecific, with the naturally occurring stereoisomer, D-*erythro* SPC, acting as agonist (Meyer zu Heringdorf *et al*., 1998; 2002). However, as demonstrated for ADP-induced aggregation, the two SPC stereoisomers, D-*erythro* and L-*threo* SPC, were similarly potent and effective in inhibiting platelet activation. Thus, although a receptor-mediated action of SPC cannot be firmly excluded, we rather suggest that the lysosphingolipid interferes with protein–protein interactions in the platelet plasma membrane, finally resulting in receptor-effector uncoupling.

Apart from these mechanistic considerations, the question remains whether the present findings are pathophysiologically relevant and/or point towards a potential target in antithrombotic therapy. At first glance the relationship between micromolar IC_{50} values in the present study and SPC plasma and serum concentrations of 50 and 130 nM (Liliom *et al*., 2001) does not support a pathophysiological relevance. However, SPC is likely to act in a local paracrine rather than a systemic manner (Meyer zu Heringdorf *et al*., 2002), and hence active concentrations in local microenvironments may considerably exceed plasma or serum concentrations. Nevertheless, further experiments are needed to analyse the potential antithrombotic action of SPC *in vivo*. With regard to the development of antithrombotic SPC mimetics it has to be kept in mind that the targets of platelet inhibition by SPC have not yet been identified at the molecular level. Moreover, development of a useful antithrombotic SPC-mimetic will require to dissociate this action from the SPC induced vasoconstriction mediated by G protein-coupled SPC receptors (Bischoff *et al*., 2000). Therefore, further studies, preferably involving selective inhibitors, are necessary to clarify a potential pathophysiological and/or therapeutic relevance of the present findings.

In summary, our data demonstrate that, in contrast to other lipid mediators, the naturally occurring lysosphingolipid SPC is not an activator but an effective inhibitor of human platelet activation by diverse receptor agonists. While activation of PKA apparently makes only a minor contribution to the inhibitory effect of SPC, the marked platelet inhibition caused by the lysosphingolipid is most likely due to uncoupling of platelet receptors from PLC stimulation and subsequent Ca^{2+} mobilization.

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References

BISCHOFF, A., CZYBORRA, P., FETSCHER, C., MEYER ZU HERINGDORF, D., JAKOBS, K.H. & MICHEL, M.C. (2000). Sphingosine-1-phosphate and sphingosylphosphorylcholine constrict renal and mesenteric microvessels in vitro. *Br. J. Pharmacol.*, **130,** 1871–1877.

BURKHARDT, M., GLAZOVA, M., GAMBARYAN, S., VOLLKOMMER, T., BUTT, E., BADER, B., HEERMEIER, K., LINCOLN, T.M., WALTER, U. & PALMETSHOFER, A. (2000). KT5823 inhibits cGMP-dependent protein kinase activity in vitro but not in intact human platelets and rat mesangial cells. *J. Biol. Chem.*, **275,** 33536–33541.

CHALLISS, R.A., BATTY, H. & NAHORSKI, S.R. (1988). Mass measurements of inositol 1,4,5-trisphosphate in rat cerebral cortex slices using a radioreceptor assay: effects of neurotransmitters and depolarization. *Biochem. Biophys. Res. Commun.*, **157,** 684–691.

CHUN, J., GOETZL, E.J., HLA, T., IGARASHI, Y., LYNCH, K.R., MOOLENAAR, W., PYNE, S. & TIGYI. G. (2002). International Union of Pharmacology. XXXIV. Lysophospholipid receptor nomenclature. *Pharmacol. Rev.*, **54,** 265–269.

DAVIES, S.P., REDDY, H., CAIVANO, M. & COHEN, P. (2000). Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochem. J.*, **351,** 95–105.

EICHHOLTZ, T., JALINK, K., FAHRENFORT, I. & MOOLENAAR, W.H. (1993). The bioactive phospholipid lysophosphatidic acid is released from activated platelets. *Biochem. J.*, **291,** 677–680.

GUEGUEN, G., GAIGE, B., GREVY, J.M., ROGALLE, P., BELLAN, J., WILSON, M., KLABE, A., PONT, F., SIMON, M.F. & CHAP, H. (1999). Structure-activity analysis of the effects of lysophosphatidic acid on platelet aggregation. *Biochemistry*, **38,** 8440–8450.

HANNUN, Y.A., GREENBERG, C.S. & BELL, R.M. (1987). Sphingosine inhibition of agonist-dependent secretion and activation of human platelets implies that protein kinase C is a necessary and common event in the signal transduction pathways. *J. Biol. Chem.*, **262,** 13620–13626.

HASHIZUME, T., SATO, T. & FUJII, T. (1992). Sphingosine enhances platelet aggregation through an increase in phospholipase C activity by a protein kinase C-independent mechanism. *Biochem. J.*, **282,** 243–247.

LILIOM, K., SUN, G., BÜNEMANN, M., VIRAG, T., NUSSR, N., BAKER, D.L, WANG, D., FABIAN, M.J., BRANDTS, B., BENDER, K., EICKEL, A., MALIK, K., MILLER, D.P., DESIDERIO, D.M., TIGYI, G. & POTT, L. (2001). Sphingosylphosphocholine is a naturally occurring lipid mediator in blood plasma: a possible role in regulating cardiac function via sphingolipid receptors. *Biochem. J.,* **355,** 189 – 197.

MEYER ZU HERINGDORF, D., NIEDERDRÄING, N., NEUMANN, E., FRÖDE, R., LASS, H., VAN KOPPEN, C.J. & JAKOBS, K.H. (1998). Discrimination between plasma membrane and intracellular target sites of sphingosylphosphorylcholine. *Eur. J. Pharmacol.,* **354,** 113 – 122.

MEYER ZU HERINGDORF, D., HIMMEL, H.M. & JAKOBS, K.H. (2002). Sphingosylphosphorylcholine – biological functions and mechanisms of action. *Biochim. Biophys. Acta.,* **1582,** 178 – 189.

MOOLENAAR, W.H. (1999). Bioactive lysophospholipids and their G protein-coupled receptors. *Exp. Cell. Res.,* **253,** 230 – 238.

MOTOHASHI, K., SHIBATA, S., OZAKI, Y, YATOMI, Y. & IGARASHI, Y. (2000). Identification of lysophospholipid receptors in human platelets: the relation of two agonists, lysophosphatidic acid and sphingosine 1-phosphate. *FEBS Lett.,* **468,** 189 – 193.

OLIVERA, A. & SPIEGEL, S. (2001). Sphingosine kinase: a mediator of vital cellular functions. *Prostaglandins,* **64,** 123 – 134.

PYNE, S. & PYNE, N.J. (2000). Sphingosine 1-phosphate signaling in mammalian cells. *Biochem. J.,* **349,** 385 – 402.

SCHMIDT, M., NEHLS, C., RÜMENAPP, U. & JAKOBS, K.H. (1996). M3 Muscarinic receptor-induced and G_i-mediated heterologous potentiation of phospholipase C stimulation: role of phosphoinositide synthesis. *Mol. Pharmacol.,* **50,** 1038 – 1046.

SCHWARZ, U.R., GEIGER, J., WALTER, U. & EIGENTHALER, M. (1999). Flow cytometry analysis of intracellular VASP phosphorylation for the assessment of activating and inhibitory signal transduction pathways in human platelets - definition and detection of ticlopidine/clopidogrel effects. *Thromb. Haemost.,* **82,** 1145 – 1152.

SCHWARZ, U.R., WALTER, U. & EIGENTHALER, M. (2001). Taming platelets with cyclic nucleotides. *Biochem. Pharmacol.,* **62,** 1153 – 1161.

SHIRAO, S., KASHIWAGI, S., SATO, M., MIWA, S., NAKAO, F., KUROKAWA, T., TODOROKI-IKEDA, N., MOGAMI, Y., KURIYAMA, S., HAZE, K., SUSUKI, M. & KOBAYASHI, S. (2002). Sphingosylphosphotylcholine is a novel messenger for rho-kinase-mediated Ca^{2+} sensitization in the bovine cerebral artery. *Circ. Res.,* **91,** 112 – 119.

SPIEGEL, S. & MILSTIEN, S. (2000). Functions of a new family of sphingosine-1- phosphate receptors. *Biochim. Biophys. Acta.,* **1484,** 107 – 116.

XU, Y., ZHU, K., HONG, G. WU, W., BAUDHUIN, L.M., XIAO, Y. & DAMRON, D.S. (2000). Sphingosylphosphorylcholine is a ligand for ovarian cancer G-proteincoupled receptolr 1. *Nat. Cell. Biol.,* **2,** 261 – 267.

YATOMI, Y, RUAN, F., HAOMORI, S. & IGARASHI, Y. (1995). Sphingosine-1- phosphate: a platelet-activating sphingolipid released from agonist-stimulated human platelets. *Blood,* **86,** 193 – 202.

YATOMI, Y., YAMAMURA, S., RUAN, F. & IGARASHI, Y. (1997). Sphingosine 1- phosphate induces platelet activation through an extracellular action and shares a platelet surface receptor with lysophosphatidic acid. *J. Biol. Chem.,* **272,** 5291 – 5297.

YOUNG, K.W. & NAHORSKI, S.R. (2001). Intracellular sphingosine 1-phosphate production: a novel pathway for Ca^{2+} release. Semin. *Cell. Dev. Biol.,* **12,** 19 – 25.

ZHU, K., BAUDHUIN, L.M., HONG, G., WILLIAMS, F.S., CRISTINA, K.L., KABAROWSKI, J.H., WITTE, O.N. & XU, Y. (2001). Sphingosylphosphorylcholine and lysophosphatidylcholine are ligands for the G protein-coupled receptor GPR4. *J. Biol. Chem.,* **276,** 41325 – 41335.

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