Synthetic lipopeptide $Pam_3CysSer(Lys)_4$ is an effective activator of human platelets

MICHAELA BERG, STEFAN OFFERMANNS, ROLAND SEIFERT, AND GÜNTER SCHULTZ
Institut für Pharmakologie, Freie Universität Berlin, 14195 Berlin, Germany

Berg, Michaela, Stefan Offermanns, Roland Seifert, and Günter Schultz. Synthetic lipopeptide $Pam_3CysSer(Lys)_4$ is an effective activator of human platelets. *Am. J. Physiol.* 266 (*Cell Physiol.* 35): C1684–C1691, 1994.—Lipopeptide analogues of the NH_2-terminus of bacterial lipoprotein are known to induce activation of macrophages, neutrophils, and lymphocytes. We studied the effect of the lipopeptide *N*-palmitoyl-*S*-[2,3-bis(palmitoyloxy)-(2*RS*)-propyl]-(*R*)-cysteinyl-(*S*)-seryl-(*S*)-lysyl-(*S*)-lysyl-(*S*)-lysyl-(*S*)-lysine [$Pam_3CysSer(Lys)_4$] on several functions of human platelets. $Pam_3CysSer(Lys)_4$ led to the aggregation of platelets and induced the secretion of serotonin with an effectiveness similar to thrombin. These cellular effects of $Pam_3CysSer(Lys)_4$ were concentration dependent, being half maximal at 2–3 μM and maximal at 10–30 μM. Another lipopeptide also induced platelet aggregation and serotonin secretion but was less potent and less effective than $Pam_3CysSer(Lys)_4$. The lipid moiety and the peptide moiety of $Pam_3CysSer(Lys)_4$ alone were without any effect. Lipopeptides also stimulated tyrosine phosphorylation of several proteins with molecular masses similar to those found to be tyrosine phosphorylated in response to thrombin, and $Pam_3CysSer(Lys)_4$ led to an increase in the cytosolic calcium concentration. All studied responses of platelets to lipopeptides were inhibited by the prostacyclin receptor agonist cicaprost. Taken together, our data show that lipopeptides are effective activators of human platelets and that this activation is susceptible to the action of physiological platelet inhibitors.

platelet aggregation; tyrosine phosphorylation; serotonin secretion; bacterial lipoprotein; lipopeptides; *N*-palmitoyl-*S*-[2,3-bis(palmitoyloxy)-(2*RS*)-propyl]-(*R*)-cysteinyl-(*S*)-seryl-(*S*)-lysyl-(*S*)-lysyl-(*S*)-lysyl-(*S*)-lysine

UNDER NORMAL CONDITIONS, human platelets circulate in the blood as resting cells. Platelets can be activated by a variety of stimuli. Activation of platelets ultimately leads to their aggregation and to the release of preformed mediators such as serotonin. Many of the biochemical events that accompany their activation process have been well described (for review see Refs. 16, 30). The initial response of platelets toward many activating stimuli involves the rapid hydrolysis of inositol phospholipids with the release of inositol 1,4,5-trisphosphate, a consecutive increase in cytosolic calcium concentration ($[Ca^{2+}]_i$), and activation of protein kinase C. More recently, rapid changes in tyrosine phosphorylation of platelet proteins have been recognized to be an important early mechanism during platelet activation (29).

The outer membrane of gram-negative bacteria contains lipoprotein as one of its major components (5). Bacterial lipoprotein carries a characteristic moiety at the NH_2-terminus, consisting of one amide-linked and two ester-linked fatty acids attached to *S*-(2,3-dihydroxypropyl)-cysteine. Synthetic lipopeptide analogues of the NH_2-terminus of bacterial lipoproteins are activators of lymphocytes (3), macrophages (12), and neutrophils (28). No cellular receptor for lipoprotein or synthetic lipopeptide analogues has so far been described, and the molecular mechanisms involved in the cellular activation by lipopeptides are not well understood. In macrophages, lipopeptides have been shown to lead to an increase in $[Ca^{2+}]_i$ (13), and activation of the membrane-bound form of protein kinase C was observed in B-cells (4). In myeloid cells, lipopeptides are effective stimulators of tyrosine phosphorylation (21).

To extend the knowledge about the biological actions of lipopeptides, we studied the effect of synthetic lipopeptides on several functions of human platelets and found them to be very effective platelet activators.

MATERIALS AND METHODS

Materials. Lipopeptides *N*-palmitoyl-*S*-[2,3-bis(palmitoyloxy)-(2*RS*)-propyl]-(*R*)-cysteinyl-(*S*)-seryl-(*S*)-lysyl-(*S*)-lysyl-(*S*)-lysyl-(*S*)-lysine [$Pam_3CysSer(Lys)_4$] and *N*-palmitoyl-*S*-[2,3-bis(palmitoyloxy)-(2*RS*)-propyl]-(*R*)-cysteinyl-(*S*)-alanyl-(*S*)-glycine [$Pam_3CysAlaGly$] as well as Pam_3Cys coupled to polyethylene glycol [$Pam_3CysPEG$] and the hexapeptide $CysSer(Lys)_4$ were from Rapp Polymere (Tübingen, Germany). Thrombin and genistein were from Sigma (Deisenhofen, Germany). [^{3}H]serotonin was from Du Pont-New England Nuclear (Bad Homburg, Germany). Cicaprost (ZK 96 480) was a gift of Drs. E. Schillinger and K. H. Thierauch (Schering, Berlin, Germany). Polyclonal antiphosphotyrosine antisera were generated and affinity purified according to Kamps and Sefton (14) with modifications (20).

Isolation of platelets. Venous blood was collected from healthy drug-free volunteers and anticoagulated with 6 mM citric acid-12 mM sodium citrate. Platelet isolation was carried out at room temperature. Platelet-rich plasma was obtained by centrifugation of whole blood for 20 min at 200 *g*. Platelet-rich plasma was adjusted with citrate buffer to pH 6.5 and centrifuged again two times for 15 min at 200 *g* to remove contaminating cells. Thereafter, platelets were pelleted by centrifugation for 10 min at 700 *g* and washed once in (in mM) 23.5 glucose, 133 NaCl, and 6 citric acid-12 sodium citrate. Washed platelets were carefully resuspended in Hanks' buffer devoid of calcium [in mM: 138 NaCl, 6 KCl, 1 $MgCl_2$, 1 Na_2HPO_4, 5 $NaHCO_3$, and 5.5 glucose, as well as 0.1% (wt/vol) bovine serum albumin and 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid-NaOH (pH 7.4)].

Measurement of platelet aggregation and serotonin release. For measurement of platelet aggregation, washed platelets were preincubated in Hanks' buffer containing 1 mM $CaCl_2$ and 0.1% (wt/vol) bovine serum albumin for 3 min, and incubation was started by addition of stimuli. Platelet aggregation was monitored using a four-channel lumiaggregometer (Aggrecorder II PA-3220; Kyoto Daiichi Kagaku, Kyoto, Japan).

For determination of serotonin release, anticoagulated platelet-rich plasma was incubated for 10 min at 37°C and for 30 min at room temperature in the presence of 50 nM [^{3}H]serotonin (35 kBq/ml). Thereafter, platelets were loaded on a gel filtration column (Sepharose 2B-CL; Pharmacia, Freiburg, Germany) equilibrated with Hanks' buffer without $CaCl_2$, and

gel-filtrated platelets were collected. Preincubation of gel-filtrated platelets for 3 min at 37°C was started by addition of $CaCl_2$ (1 mM final concentration). Immediately before addition of stimuli, imipramine was added at a final concentration of 2 μM. Incubation of samples (130 μl final vol) was conducted for the time periods indicated in the figure legends and was stopped by the addition of ice-cold 31 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA) and 633 mM formaldehyde (final concentrations). Samples were placed on ice and were centrifuged at 4°C for 15 min at 3,000 *g*. The amount of released [^{3}H]serotonin was determined by scintillation counting of the resulting supernatant fraction. Unstimulated platelets were lysed in 1% (vol/vol) Triton X-100, and cell-associated radioactivity was determined. The percent serotonin release is referred to as the total amount of [^{3}H]serotonin present in unstimulated cells.

Determination of $[Ca^{2+}]_i$. Washed platelets were incubated for 30 min at 37°C in the presence of 4 μM of the acetoxymethyl ester of fura 2. Thereafter, platelets were diluted with a 20-fold volume of Hanks' buffer without $CaCl_2$ containing citrate buffer (see above). After washing of platelets (700 *g* for 10 min), platelets were suspended in Hanks' buffer without $CaCl_2$, and preincubation for 3 min at 37°C was started by addition of $CaCl_2$ (1 mM final concentration). Fluorescence was determined at 37°C with constant stirring of the cells using a Ratio II spectrofluorometer (Aminco, Silver Spring, MD) as described in Ref. 27. The excitation and emission wavelengths were 340 and 500 nm, respectively.

Preparation of platelet lysates and immunoblotting. Washed platelets were preincubated for 3 min at 37°C in a final volume of 40 μl. Incubation was initiated by addition of stimuli. Incubation was stopped by adding 20 μl of lysis buffer [6% (wt/vol) sodium dodecyl sulfate (SDS), 18% (vol/vol) 2-mercaptoethanol, 30% (vol/vol) glycerol, 1 mM Na_3VO_4, and a trace amount of bromophenol blue dye in 200 mM tris(hydroxymethyl)aminomethane-HCl (pH 7.5)]. Samples were immediately incubated for 8 min at 100°C and subjected to SDS-polyacrylamide gel electrophoresis with separating gels containing 9% (wt/vol) acrylamide. Transfer of proteins onto nitrocellulose filters and detection of phosphotyrosine-containing proteins by the use of anti-phosphotyrosine antibodies and the chemiluminescence (ECL) Western blotting detection system (Amersham, Braunschweig, Germany) have been described previously (21).

Miscellaneous and reproducibility. Protein was determined using the bicinchoninic acid Protein Assay System (Pierce, Rockford, IL). Experiments shown are representative for at least three independently performed experiments using platelets from different donors.

RESULTS

Incubation of human platelets with 30 μM $Pam_3CysSer(Lys)_4$ led to the rapid aggregation of platelets (Fig. 1*A*). $Pam_3CysSer(Lys)_4$ was as effective as was 1.5 U/ml thrombin. Although $Pam_3CysSer(Lys)_4$-induced aggregation was maximal after ~3–4 min, aggregation induced by thrombin did not reach a maximum until 10 min. The lipopeptide $Pam_3CysAlaGly$ (30 μM), which differs in its peptide moiety from $Pam_3CysSer(Lys)_4$, also induced platelet aggregation. However, aggregation of platelets in response to $Pam_3CysAlaGly$ occurred more slowly than $Pam_3CysSer(Lys)_4$-induced aggregation, and $Pam_3CysAlaGly$ was less effective.

Figure 1*B* shows a concentration-response relationship of the effect of $Pam_3CysSer(Lys)_4$ on platelet aggregation. The effectiveness and the velocity of the lipopeptide-induced platelet aggregation increased with increasing concentrations of $Pam_3CysSer(Lys)_4$. Platelet aggregation in response to $Pam_3CysSer(Lys)_4$ and thrombin was markedly reduced in the absence of calcium, indicating that aggregation induced by these stimuli is dependent on the presence of extracellular calcium (Fig. 1*C*).

To test the effect of lipopeptides on the release of mediators from platelets, platelets were loaded with [^{3}H]serotonin, and [^{3}H]serotonin release was measured after addition of stimuli (Fig. 2*A*). $Pam_3CysSer(Lys)_4$ (30 μM), like thrombin (1.5 U/ml), led to a nearly maximal release of [^{3}H]serotonin. The release of [^{3}H]serotonin in response to $Pam_3CysSer(Lys)_4$ occurred very rapidly, being half maximal after <0.5 min. Half-maximal release induced by thrombin was observed after ~1 min. Lipopeptide $Pam_3CysAlaGly$ was less

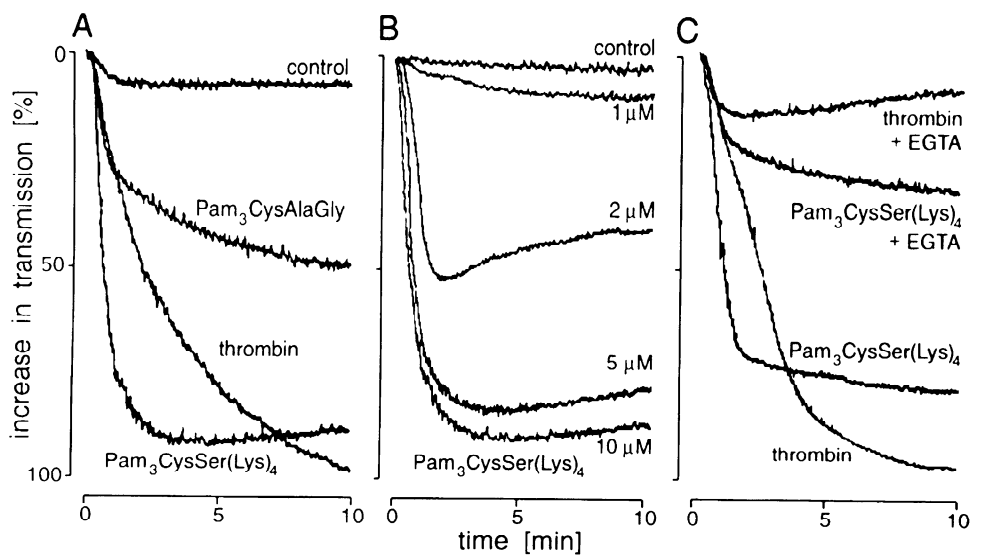

Fig. 1. Effects of lipopeptides on platelet aggregation. About 1×10^7 platelets were incubated under indicated conditions, and platelet aggregation was measured as described in MATERIALS AND METHODS. *A*: platelets were incubated with 30 μM $Pam_3CysSer(Lys)_4$ and $Pam_3CysAlaGly$ or with 1.5 U/ml thrombin. *B*: effect of increasing concentrations of $Pam_3CysSer(Lys)_4$ on platelet aggregation. *C*: platelets were either incubated in presence of extracellular $CaCl_2$ with 1.5 U/ml thrombin or 10 μM $Pam_3CysSer(Lys)_4$ or in buffer devoid of $CaCl_2$ and supplemented with 3 mM EGTA [thrombin + EGTA, $Pam_3CysSer(Lys)_4$ + EGTA]. Control: traces recorded in absence of any agonist. Shown is increase in light transmission of platelet suspension in relation to time after addition of stimuli. See text for definition of synthetic lipopeptides.

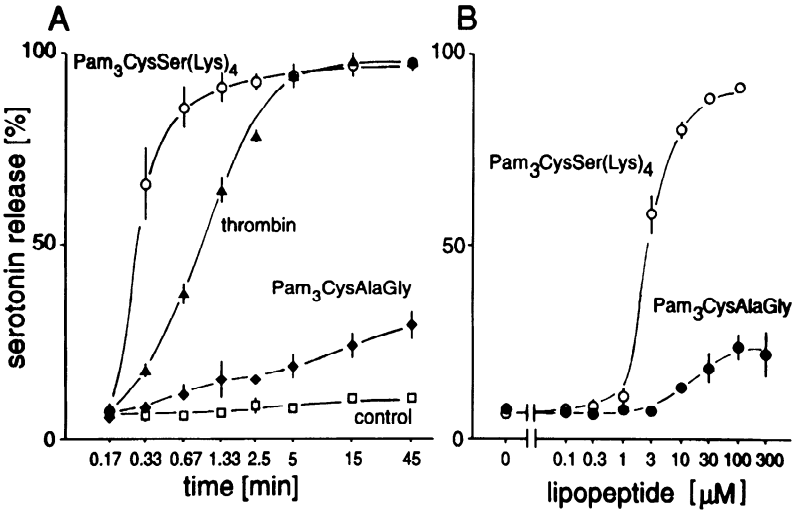

Fig. 2. Effects of lipopeptides on serotonin release in human platelets. About 1×10^7 platelets were incubated under indicated conditions. Incubation of platelets with [^{3}H]serotonin and determination of released [^{3}H]serotonin were performed as described in MATERIALS AND METHODS. *A*: [^{3}H]serotonin-loaded platelets were incubated with 30 μM lipopeptides $Pam_3CysSer(Lys)_4$ and $Pam_3CysAlaGly$, with 1.5 U/ml thrombin, or in absence of stimuli (control) as indicated for increasing time periods. *B*: platelets were incubated with increasing concentrations of $Pam_3CysSer(Lys)_4$ and $Pam_3CysAlaGly$ for 15 min. Values are means ± SE of triplicates.

effective with regard to its ability to release serotonin, and the maximal effect was not reached until 45 min after addition of the lipopeptide. The effect of both lipopeptides occurred in a concentration-dependent manner (Fig. 2*B*). $Pam_3CysSer(Lys)_4$ was more potent and more effective than $Pam_3CysAlaGly$ with regard to stimulation of serotonin release. Although $Pam_3CysSer(Lys)_4$ induced serotonin release at a half-maximal concentration of 2–3 μM, half-maximal effective concentrations of $Pam_3CysAlaGly$ amounted to ~20 μM.

Figure 3*A* shows the effect of $Pam_3CysSer(Lys)_4$ at increasing concentrations on $[Ca^{2+}]_i$. $Pam_3CysSer(Lys)_4$ elevated $[Ca^{2+}]_i$ in a concentration-dependent manner from a basal level of ~100 nM to ~1 μM at a lipopeptide concentration of 10 μM. The increase in $[Ca^{2+}]_i$ caused by 10 μM of the lipopeptide was rapid in onset and was sustained. Similar to the effect of thrombin on $[Ca^{2+}]_i$ (Ref. 23 and Fig. 3*B*), the magnitude of the rise in $[Ca^{2+}]_i$ induced by $Pam_3CysSer(Lys)_4$ was greatly reduced in the absence of extracellular Ca^{2+} (see Fig. 3*B*), suggesting that a part of the increase in $[Ca^{2+}]_i$ occurred via an influx of calcium ion from the extracellular space.

Because platelet activation by different agents is known to involve the reversible phosphorylation of several platelet proteins on tyrosine residues (7, 9, 19), we tested the effect of lipopeptides on tyrosine phosphorylation in human platelets. $Pam_3CysSer(Lys)_4$ and $Pam_3CysAlaGly$ at 30 μM induced tyrosine phosphorylation of several proteins with molecular masses between 29 and 170 kDa (Figs. 4 and 5). Proteins found to be tyrosine phosphorylated in response to both lipopeptides exhibited molecular masses very similar to proteins tyrosine phosphorylated after addition of thrombin. Pam_3Cys coupled to polyethylene glycol and the hexapeptide $CysSer(Lys)_4$ were without effect on tyrosine phosphorylation (Fig. 4*A*). The effect of $Pam_3CysSer(Lys)_4$ and $Pam_3CysAlaGly$ occurred in a concentration-dependent manner (Fig. 4*B* and data not shown, respectively). $Pam_3CysSer(Lys)_4$ was maximally effective with regard to tyrosine phosphorylation at a concentration of ~10 μM, and, like the effect of $Pam_3CysSer(Lys)_4$ on platelet aggregation and serotonin release, $Pam_3CysSer(Lys)_4$-induced tyrosine phosphorylation was observed very shortly after addition of the lipopeptide (Fig. 5). Tyrosine phosphorylation in response to $Pam_3CysSer(Lys)_4$ was detectable after 10 s, and tyrosine phosphorylation of most proteins reached a plateau after ~30–90 s, whereas tyrosine phosphorylation in response to $Pam_3CysAlaGly$ was detectable not until 3–5 min after addition of the lipopeptide (data not shown). There are no obvious differences between the proteins tyrosine phosphorylated after addition of $Pam_3CysSer(Lys)_4$ and thrombin, and increased tyrosine phosphorylation in response to $Pam_3CysSer(Lys)_4$ and thrombin occurred with similar time courses.

In a series of experiments we tested whether the lipid moiety or the peptide moiety of $Pam_3CysSer(Lys)_4$ alone

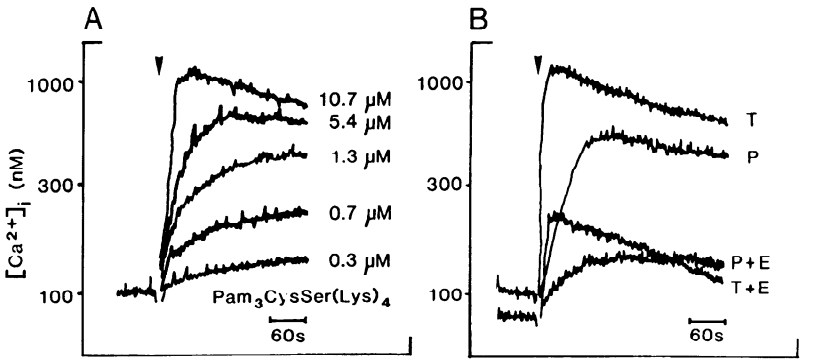

Fig. 3. Effect of $Pam_3CysSer(Lys)_4$ on cytosolic calcium concentration ($[Ca^{2+}]_i$) in human platelets. Platelets were loaded with acetoxymethyl ester of fura 2, and effect of stimuli on $[Ca^{2+}]_i$ was measured as described in MATERIALS AND METHODS. *A*: effect of indicated increasing concentrations of $Pam_3CysSer(Lys)_4$ on $[Ca^{2+}]_i$. *B*: platelets were incubated in presence of extracellular $CaCl_2$ with 5 μM $Pam_3CysSer(Lys)_4$ (P) and 1.5 U/ml thrombin (T), or incubation was performed in absence of $CaCl_2$ in buffer containing 1 mM EGTA with same concentrations of $Pam_3CysSer(Lys)_4$ (P + E) and thrombin (T + E). Arrowheads: addition of stimuli.

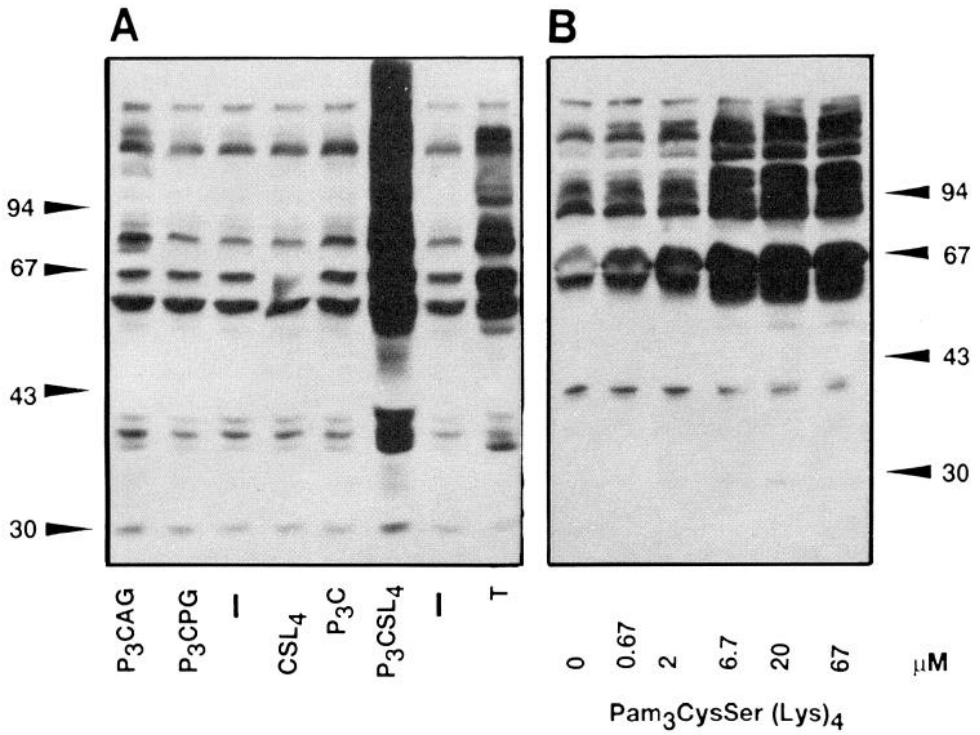

Fig. 4. Tyrosine phosphorylation stimulated by lipopeptides in human platelets. Platelets ($6-7 \times 10^7$/tube) were incubated in absence or presence of stimuli, platelets were lysed, and proteins were analyzed as described in MATERIALS AND METHODS. *A*: platelets were incubated for 20 min in presence of 1.5 U/ml thrombin (T), 30 μM $Pam_3CysSer(Lys)_4$ (P_3CSL_4), 30 μM Pam_3Cys (P_3C), 30 μM $CysSer(Lys)_4$ (CSL_4), 30 μM $Pam_3CysPEG$ (P_3CPG), and 30 μM $Pam_3CysAlaGly$ (P_3CAG). *B*: effect of increasing concentrations (μM, abscissa) of $Pam_3CysSer(Lys)_4$ on tyrosine phosphorylation of platelet proteins. Shown are autoluminograms of blots. Numbers on *left* and *right* indicate molecular masses of marker proteins (kDa). See text for definitions.

was able to induce platelet activation. Neither the lipid moieties Pam_3Cys and $Pam_3CysPEG$ nor the hexapeptide $CysSer(Lys)_4$ led to platelet aggregation or serotonin release in human platelets (Table 1). Each substance was tested at concentrations to 100 μM (data not shown).

To study the relevance of lipopeptide-induced tyrosine phosphorylation, we assessed the effect of the tyrosine kinase inhibitor genistein (1) on lipopeptide-induced platelet activation. Genistein has been shown to inhibit activation of platelets induced by different stimuli (6, 2). Figure 6*A* shows the effect of genistein (30 and 100 μM) on $Pam_3CysSer(Lys)_4$- and thrombin-induced tyrosine phosphorylation. Preincubation of platelets with 100 μM genistein blocked the effect of $Pam_3CysSer(Lys)_4$ and considerably inhibited the effect of thrombin on tyrosine phosphorylation. Genistein at the same concentration led to the marked inhibition of lipopeptide- and thrombin-induced platelet aggregation (Fig. 6, *B* and *C*, respectively) and serotonin release (Fig. 6*D*). In all cases the inhibitory effect of genistein toward $Pam_3CysSer(Lys)_4$-induced responses was more pronounced than toward thrombin effects. Although inhibition of aggregation induced by both stimuli was almost complete after preincubation of platelets with 300 μM genistein, 300 μM genistein by itself released some

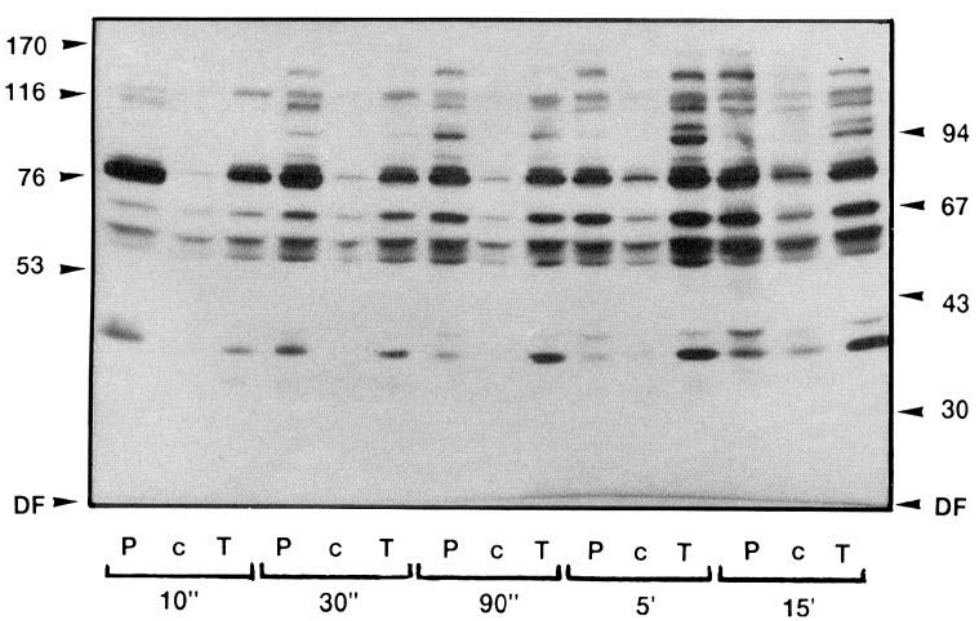

Fig. 5. Time course of $Pam_3CysSer(Lys)_4$- and thrombin-induced tyrosine phosphorylation in human platelets. Platelets ($6-7 \times 10^7$/tube) were incubated with 30 μM Pam_3Cys-$Ser(Lys)_4$ (P) or 1.5 U/ml thrombin (T) for indicated time periods. Samples were processed as described in MATERIALS AND METHODS. Shown is autoluminogram of blot with molecular masses of standard proteins (kDa) on *left* and *right*. DF, dye front; c, control (without stimulus).

Table 1. *Effect of different substances on platelet aggregation and serotonin release from platelets*

Condition	Platelet Aggregation, %	Serotonin Release, %
Thrombin	96.0 ± 3.8	99.8 ± 0.7
$Pam_3CysSer(Lys)_4$	91.7 ± 5.8	98.3 ± 1.0
$CysSer(Lys)_4$	4.3 ± 3.7	8.7 ± 0.3
Control	4.0 ± 5.4	8.8 ± 1.4
Pam_3Cys	6.5 ± 4.3	8.1 ± 0.5
$Pam_3CysPEG$	4.3 ± 3.3	8.6 ± 0.7
$Pam_3CysAlaGly$	50.2 ± 5.2	12.5 ± 1.0

Values are means ± SD of triplicates. Platelet aggregation and serotonin release were measured 10 min after addition of stimuli as described in Figs. 1 and 2 and MATERIALS AND METHODS. Thrombin was used at a concentration of 1 U/ml, and all other substances were applied at a concentration of 30 μM. Control, without stimulus. See text for other definitions.

[^{3}H]serotonin from platelets, indicating that genistein at this concentration may lead to unspecific effects. The effect of genistein on a lipopeptide-induced rise in $[Ca^{2+}]_i$ could not be studied since genistein interfered with fluorescence measurements.

We further studied the effect of the prostacyclin receptor agonist cicaprost on $Pam_3CysSer(Lys)_4$-induced platelet activation. Prostacyclin receptor-mediated elevation of the intracellular adenosine 3′,5′-cyclic monophosphate (cAMP) concentration is one of the physiological pathways leading to inhibition of platelet activation (31). Preincubation of platelets with cicaprost resulted in inhibition of thrombin- and $Pam_3CysSer(Lys)_4$-induced platelet aggregation and serotonin release (Fig. 7). The inhibitory effects of cicaprost toward the action of thrombin were more pronounced than the inhibitory effects toward $Pam_3CysSer(Lys)_4$-induced platelet activation. Because stimulation of cAMP production by different mechanisms is known to inhibit tyrosine phosphorylation and $[Ca^{2+}]_i$ elevation induced by different stimuli in platelets (10, 22, 31), we additionally tested the effect of cicaprost on tyrosine phosphorylation and $[Ca^{2+}]_i$ elevation induced by $Pam_3CysSer(Lys)_4$ and thrombin (Fig. 7, *C* and *D*). Although thrombin-induced tyrosine phosphorylation was totally abolished by cicaprost, the rise in $[Ca^{2+}]_i$ produced by thrombin and the effects of $Pam_3CysSer(Lys)_4$ on $[Ca^{2+}]_i$ and tyrosine phosphorylation were markedly reduced by the prostacyclin analogue cicaprost.

DISCUSSION

Synthetic lipopeptide analogues of bacterial lipoprotein, which constitutes a major component of the outer membrane of gram-negative bacteria, are well known activators of B-lymphocytes, macrophages, and neutrophils (3, 12, 28). The data presented here indicate that lipopeptides also activate human platelets. The activation of platelets was examined by studying the effect of lipopeptides on the main platelet responses to stimuli, i.e., the aggregation of platelets and the release of granule content measured as serotonin secretion from [^{3}H]serotonin-loaded platelets. The lipopeptide $Pam_3CysSer(Lys)_4$ rapidly induced aggregation of platelets and secretion of serotonin from platelets (Figs. 1 and 2). Effects could be observed at $Pam_3CysSer(Lys)_4$ concentrations of <1 μM. Half-maximal and maximal effects occurred at concentrations of 2–3 and 10–30 μM, respectively (Figs. 1 and 2). The maximum response of $Pam_3CysSer(Lys)_4$ was of similar magnitude to that obtained using 1.5 U/ml thrombin, a potent platelet stimulus. Platelet activation in response to $Pam_3CysSer(Lys)_4$ differed from activation induced by thrombin, in that it occurred significantly earlier.

To exclude the possibility that platelet activation was dependent on the positively charged lysine residues of $Pam_3CysSer(Lys)_4$ and to elucidate which part of the lipopeptide molecule is necessary for its biological activity, $Pam_3CysPEG$, the lipopeptide $Pam_3CysAlaGly$, and

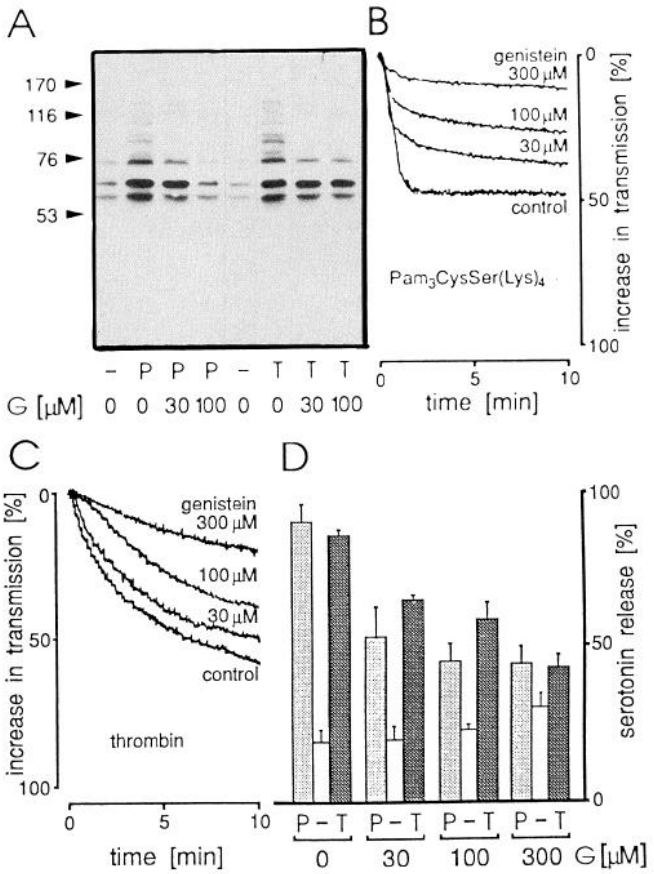

Fig. 6. Influence of genistein on $Pam_3CysSer(Lys)_4$- and thrombin-induced activation of human platelets. *A*: after preincubation of platelets in absence or presence of indicated concentrations of genistein (G), platelets were incubated without (−) or with 20 μM $Pam_3CysSer(Lys)_4$ (P) and 1 U/ml thrombin (T) for further 2 min. Thereafter, tyrosine-phosphorylated proteins were detected as described. Shown is autoluminogram of blot with molecular masses (kDa) of marker proteins on *left*. *B* and *C*: platelets were preincubated with indicated concentrations of genistein (control: without genistein) and then stimulated with 5 μM $Pam_3CysSer(Lys)_4$ (*B*) or with 0.5 U/ml thrombin (*C*). Platelet aggregation was measured as described in MATERIALS AND METHODS. *D*: serotonin release after addition of vehicle (−) or 20 μM $Pam_3CysSer(Lys)_4$ (P) and 1 U/ml thrombin (T) was measured in platelets preincubated in absence or presence of indicated concentrations of genistein (G) as described in Fig. 2 and in MATERIALS AND METHODS. Serotonin release was measured 2 min after addition of stimuli. In all experiments shown, cells were preincubated for 15 min with indicated genistein concentrations.

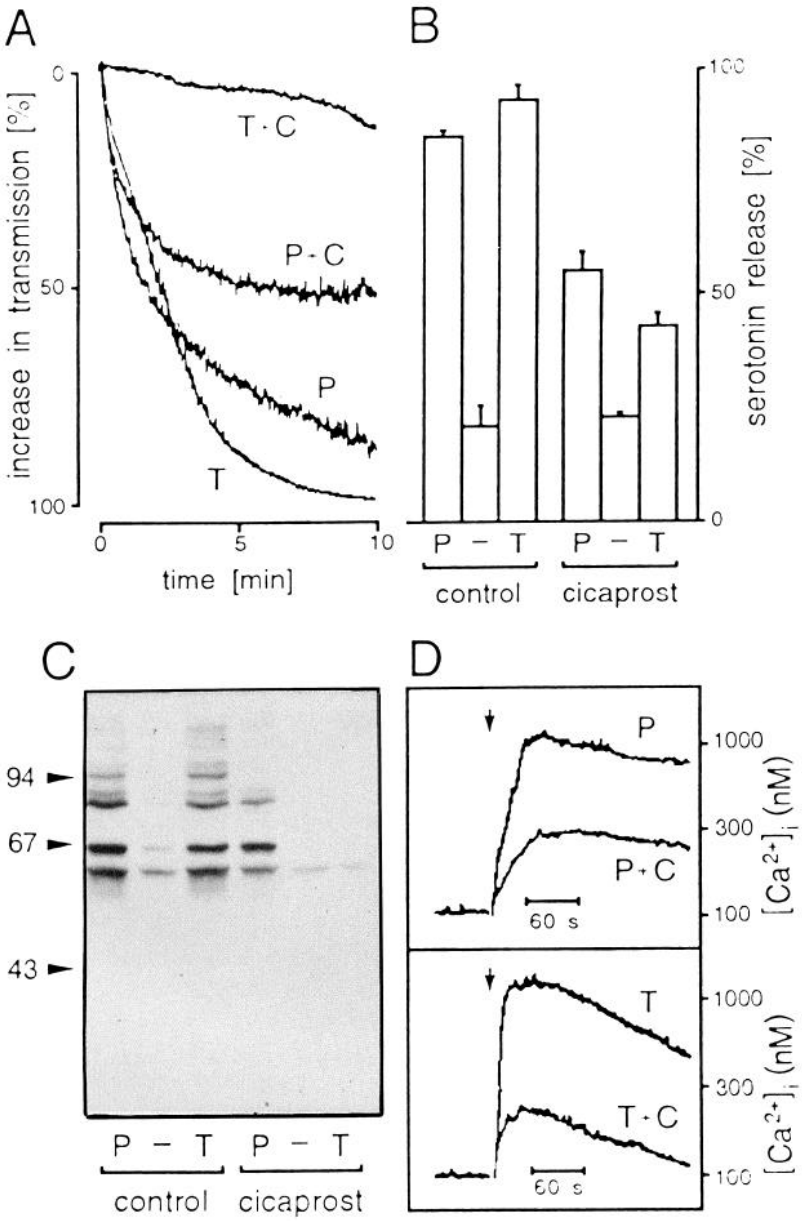

Fig. 7. Effect of cicaprost on platelet activation in response to $Pam_3CysSer(Lys)_4$ and thrombin. *A* and *B*: effect of 1 μM cicaprost (C) on platelet aggregation (*A*) and serotonin release (*B*) induced by 30 μM $Pam_3CysSer(Lys)_4$ (P) or 1.5 U/ml thrombin (T). Experiments were performed as described in MATERIALS AND METHODS. Platelets were incubated with cicaprost for 5 min before addition of stimuli. Serotonin release was measured 5 min after addition of stimuli. *C*: effect of 1 μM cicaprost on tyrosine phosphorylation of platelet proteins induced by incubation with 30 μM $Pam_3CysSer(Lys)_4$ (P) or 1.5 U/ml thrombin (T) for 3 min. Shown is autoluminogram of blot with molecular masses of marker proteins (kDa) on *left*. Tyrosine phosphorylation was determined as described. *D*: effect of 1 μM cicaprost on rise in $[Ca^{2+}]_i$ induced by 5 μM $Pam_3CysSer(Lys)_4$ (P; *top*) or 1.5 U/ml thrombin (T; *bottom*). $[Ca^{2+}]_i$ was determined as described in Fig. 3 and in MATERIALS AND METHODS. In all experiments shown, cells were preincubated for 5 min with cicaprost.

the hexapeptide $CysSer(Lys)_4$ were employed. $Pam_3CysAlaGly$ also led to the activation of platelets. However, $Pam_3CysAlaGly$-induced platelet activation occurred more slowly than that induced by $Pam_3CysSer(Lys)_4$, and $Pam_3CysAlaGly$ was less potent and less effective than $Pam_3CysSer(Lys)_4$ (Figs. 1 and 2). Neither Pam_3Cys, $Pam_3CysPEG$, nor the peptide $CysSer(Lys)_4$ induced platelet activation (Fig. 4 and Table 1). In addition, high concentrations of dilysine (applied at concentrations to 1 mM) were without any effect on platelet aggregation and serotonin secretion (data not shown). Taken together, this indicates that the characteristic lipid moiety of lipopeptides, consisting of one amide-linked and two ester-linked fatty acids attached to *S*-(2,3-dihydroxypropyl)-cysteine, is necessary but not sufficient for platelet activation by lipopeptides and that the amino acids of the peptide moiety may contribute to the potency and effectiveness of the lipopeptide-induced platelet activation.

Because elevation of $[Ca^{2+}]_i$ and tyrosine phosphorylation of cellular proteins are important molecular events involved in platelet activation by different stimuli (16, 29, 30), we studied the effect of lipopeptides on both parameters. $Pam_3CysSer(Lys)_4$ within seconds led to an increase in $[Ca^{2+}]_i$ (Fig. 3) and to tyrosine phosphorylation of cellular proteins (Figs. 4 and 5) in a concentration-dependent manner. Elevation of $[Ca^{2+}]_i$ and stimulation of tyrosine phosphorylation were achieved with half-maximal and maximal concentrations of ~2–3 and 10 μM $Pam_3CysSer(Lys)_4$, respectively (Figs. 3 and 4). The concentrations are similar to those needed for platelet aggregation and secretion (Figs. 1 and 2). The lipopeptide $Pam_3CysAlaGly$ also induced tyrosine phosphorylation of proteins with molecular masses identical to those found after incubation with $Pam_3CysSer(Lys)_4$ (Fig. 4), but significant effects of $Pam_3CysAlaGly$ on $[Ca^{2+}]_i$ could not be observed (data not shown). $Pam_3CysSer(Lys)_4$-induced elevation of $[Ca^{2+}]_i$ and tyrosine

phosphorylation occurred with a very similar time dependence and effectiveness (Figs. 3 and 5). These data indicate that lipopeptides use common early intracellular signal transduction pathways to induce platelet activation. The relevance of $Pam_3CysSer(Lys)_4$-induced tyrosine phosphorylation for platelet activation is demonstrated by the finding that the tyrosine kinase inhibitor genistein, which largely inhibited tyrosine phosphorylation in response to $Pam_3CysSer(Lys)_4$ and thrombin, inhibited platelet aggregation and serotonin secretion in a concentration-dependent manner in response to both stimuli (Fig. 6). This is in line with earlier studies showing that tyrosine phosphorylation is implicated in platelet aggregation and secretion of serotonin (17, 26). In addition, tyrosine phosphorylation of a subset of proteins phosphorylated on tyrosine residues in the course of platelet activation has been shown to be dependent on the activation of glycoprotein IIb-IIIa and subsequent fibrinogen binding (8, 10).

Platelet activation by $Pam_3CysSer(Lys)_4$ also resembled thrombin-induced activation with regard to its dependence on the presence of extracellular calcium. Although tyrosine phosphorylation induced by both stimuli was only slightly inhibited in the absence of extracellular calcium (data not shown), platelet aggregation and $[Ca^{2+}]_i$ elevation were markedly reduced in the absence of extracellular calcium (Figs. 1 and 3). Similarly, elevation of the intracellular cAMP concentration via the prostacyclin receptor by cicaprost inhibited both $Pam_3CysSer(Lys)_4$- and thrombin-induced platelet activation (Fig. 7). These data indicate that lipopeptide-induced platelet activation is susceptible to the action of a physiological platelet inhibitor.

Although the effects of both thrombin and $Pam_3CysSer(Lys)_4$ are mediated at least in part by similar or identical mechanisms, it is obvious that the activation of platelets by lipopeptide and by thrombin exhibit slightly different susceptibilities toward the action of cicaprost and genistein and toward the removal of extracellular calcium. Genistein more effectively inhibited the effects of $Pam_3CysSer(Lys)_4$ (Fig 6), and cicaprost more effectively inhibited the effects of thrombin (Fig. 7). In addition, the thrombin-induced aggregation was more susceptible to the removal of extracellular calcium than the lipopeptide-induced aggregation (Fig. 1).

So far, it is not clear whether lipopeptides activate platelets via a surface receptor or whether lipopeptides exert their effects independently of a surface receptor. The latter possibility appears to be conceivable, since studies carried out by electron energy loss spectroscopy (32) indicate that lipopeptide can quickly enter the cell. Because lipopeptides lead to the activation of early signal transduction pathways identical to thrombin, the site of a receptor-independent platelet activation by lipopeptides can be assumed to be located at an early step of the pathways leading to platelet activation. The direct activation of guanine nucleotide-binding proteins (G proteins) by lipopeptides may represent a possible mechanism for the lipopeptide effects (28). In any case, the molecular site of lipopeptide action remains to be elucidated.

Whether lipoprotein or lipopeptides, which are a main component of the outer membrane of gram-negative bacteria and which occur in murein-bound and free form, can be released from bacteria in considerable quantities and whether they may play a pathophysiological role in inflammatory processes due to gram-negative bacteria or in gram-negative sepsis is as yet not known. The direct activation of platelets by lipopeptides could contribute to the known platelet activation in the course of endotoxemia (15, 18, 33). However, this speculation needs further support by the use of in vivo models. Interestingly, lipid A, which is also derived from gram-negative bacteria and which shows some structural similarities to lipopeptides (2 amide- and 2 ester-linked hydroxy fatty acids to a glucosamine backbone in lipid A vs. 2 ester- and 1 amide-linked palmitic acids in lipopeptides), has also been shown to activate platelets (11, 24, 25).

In summary, our data clearly demonstrate that lipopeptide analogues of bacterial lipoprotein are capable of full platelet activation and support the view that lipopeptides are activators of various types of hematopoietic cells.

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Address for reprint requests: S. Offermanns, Institut für Pharmakologie, Freie Universität Berlin, Thielallee 69–73, 14195 Berlin, Germany.

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