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Inhibition of Cellular Action of Thrombin by *N*3-Cyclopropyl-7-{[4-(1-methylethyl)phenyl]methyl}-7*H*-pyrrolo[3,2-*f*]quinazoline-1,3-diamine (SCH 79797), a Nonpeptide Thrombin Receptor Antagonist

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ABSTRACT. A growing body of evidence suggests an important contribution of the cellular actions of thrombin to thrombosis and restenosis following angioplasty. Recently we reported on SCH 79797 (*N*3-cyclopropyl-7-{[4-(1-methylethyl)phenyl]methyl}-7*H*-pyrrolo[3,2-*f*]quinazoline-1,3-diamine) and its analogs as new potent, nonpeptide thrombin receptor antagonists. This study further characterizes the biochemical and pharmacological actions of pyrroloquinazoline inhibitors of protease activated receptor-1 (PAR-1) in human platelets and coronary artery smooth muscle cells (hCASMC). SCH 79797 and its *N*-methyl analog (SCH 203099) inhibited binding of a high-affinity thrombin receptor-activating peptide ([^{3}H]haTRAP, Ala-Phe(p-F)-Arg-ChA-HArg-[^{3}H]Tyr-NH_2) to PAR-1 with IC_{50} values of 70 and 45 nM, respectively. SCH 79797 inhibited [^{3}H]haTRAP binding in a competitive manner. SCH 79797 and SCH 203099 inhibited α-thrombin- and haTRAP-induced aggregation of human platelets, but did not inhibit human platelet aggregation induced by the tethered ligand agonist for protease-activated receptor-4 (PAR-4), γ-thrombin, ADP, or collagen. SCH 203099 inhibited surface expression of P-selectin induced by haTRAP and thrombin, and it did not increase P-selectin expression or prevent thrombin cleavage of the receptor. Thrombin and TFLLRNPNDK-NH_2 (TK), a PAR-1-selective agonist, produced transient increases in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) in hCASMC. This increase in $[Ca^{2+}]_i$ was inhibited effectively by SCH 79797. However, the Ca^{2+} transients induced by SLIGKV-NH_2, a PAR-2-selective agonist, were not inhibited by SCH 79797. Thrombin- and TK-stimulated [^{3}H]thymidine incorporation also was inhibited completely by SCH 79797. The results of this study demonstrate that SCH 79797 and SCH 203099 are potent, selective antagonists of PAR-1 in human platelets and hCASMC. These data also suggest that the thrombin stimulation of Ca^{2+} transients and mitogenesis in hCASMC is mediated primarily through activation of PAR-1. BIOCHEM PHARMACOL **60**;10: 1425–1434, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. thrombin receptor antagonist; protease-activated receptor; vascular smooth muscle cells; calcium transients; mitogenesis; SCH 79797

Thrombin is a plasma serine protease that plays a critical role in hemostasis, as well as in thrombotic disorders and atherosclerosis [1–4]. Thrombin stimulates fibrin generation, platelet aggregation, smooth muscle cell contraction and proliferation, and monocyte chemotaxis [1, 5]. Whereas fibrin generation is the result of direct cleavage of fibrinogen by thrombin, many of the cellular actions of thrombin are mediated by a G-protein-coupled receptor, PAR-1† [6]. When PAR-1 is cleaved by thrombin, a new N-terminus is generated that acts as the tethered ligand to activate PAR-1. TRAPs containing 5–14 amino acid residues corresponding to the newly exposed amino-terminus of thrombin receptor mimic many of the actions of thrombin, including aggregation of platelets [6–8], vascular smooth muscle contractility [9, 10], and proliferation [11, 12]. Three additional members (PAR-2, -3, and -4) of the PAR receptor family have been reported. PAR-2 [13, 14] is stimulated by trypsin and tryptase, whereas PAR-3 [15] and PAR-4 [16, 17] are stimulated by thrombin. PAR-2 and PAR-4 are also stimulated by their respective tethered ligand analogs [13, 16]. Current research focuses on the relative importance of the three protease-activated thrombin receptors in various cell types.

Several lines of evidence suggest that thrombin, acting through PAR-1, contributes to thrombosis and restenosis in

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† *Abbreviations*: PAR-1, -2, -3, and -4, protease-activated receptor-1, -2, -3, and -4; TRAP, thrombin receptor-activating peptides; [^{3}H]haTRAP, Ala-Phe(p-F)-Arg-ChA-HArg-[^{3}H]Tyr-NH_2, $[Ca^{2+}]_i$, cytosolic free calcium concentration; hCASMC, human coronary artery smooth muscle cells; and PE, phycoerythrin.

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patients after angioplasty procedures [4, 18–21]. Increased levels of thrombin generation in addition to a high level of thrombin receptor have been detected at the sites of vascular lesions [4, 18, 19]. TRAP antagonists and antibodies to the thrombin receptor (PAR-1) inhibit thrombin- or TRAP-stimulated platelet aggregation *in vitro* [21–25], as well as experimental arterial thrombosis in primates [4, 21]. Studies such as these suggest that PAR-1 antagonists could be useful drugs for the prevention of thrombosis and restenosis.

Peptides [26–29] and peptidomimetic compounds [22–25, 30, 31] derived from the PAR-1 tethered ligand have been shown to be PAR-1 antagonists. Although useful as tools, they display one or more of the following limitations: lack of reproducible activity and lack of specificity, partial agonist activity, or low affinity [23, 24, 26, 29, 31–33]. Although nonpeptide PAR-1 antagonists have been reported, they are of low affinity (IC_{50} values of 0.6 to 1.0 mM) and unknown selectivity [34]. Using a tritiated high-affinity TRAP analog, [^{3}H]haTRAP, we developed a PAR-1 ligand-binding assay [35] and used this assay to identify small molecule antagonists of PAR-1 [36]. Shortly after publication of our study on the nonpeptide PAR-1 antagonist SCH 79797 (*N*3-cyclopropyl-7-{[4-(1-methylethyl)phenyl]methyl}-7*H*-pyrrolo[3,2-*f*]quinazoline-1,3-diamine [36]) another nonpeptide thrombin receptor antagonist, 3-(4-chlorophenyl)-2-(2,4-dichlorobenzoylimino)-5-(methoxycarbonylmethylene)-1,3-thiazolidin-4-one (FR171113) [37], was reported. This compound exhibits selective thrombin receptor antagonism, as evidenced by its inhibitory action on aggregation of human platelets induced by thrombin and SFLLRN (a thrombin receptor agonist) but not by ADP or collagen [37]. However, it is not known whether it exhibits selectivity towards PAR-1 over other PARs, such as PAR-2 and PAR-4. This study was initiated to characterize the biochemical and pharmacological actions of the pyrroloquinazoline class of molecules, typified by SCH 79797, a potent, nonpeptide PAR-1 antagonist, in human platelets and hCASMC. The results reported here indicate that SCH 79797 and its *N*-methyl analog, SCH 203099, can effectively antagonize the PAR-1 receptor-mediated actions of thrombin on human platelets and hCASMC.

MATERIALS AND METHODS

Reagents

HaTRAP (Ala-Phe(p-F)-Arg-ChA-HArg-Tyr-NH_2), SLIGKV-NH_2, Gly-Tyr-Pro-Gly-Gln-Val-NH_2 (a PAR-4 agonist), and TFLLRNPNDK-NH_2 were custom-synthesized by AnaSpec. Human α-thrombin and γ-thrombin were purchased from Enzyme Research Laboratories. Unless otherwise indicated, other chemicals were obtained from the Sigma Chemical Co.

Thrombin Receptor [^{3}H]haTRAP Binding Assay

The filtration binding assay was performed as previously described [35]. Briefly, human platelet membranes (40 μg/0.2 mL reaction mixture) were incubated with 10 nM [^{3}H]haTRAP and various concentrations of SCH 79797 and SCH 203099 at room temperature for 1 hr. Human platelet membranes (700 mg) were prepared by ABS (Analytical Biological Services Inc.) from 40 units of fresh human platelets according to a published method [35].

Platelet Aggregation Assay

Platelets were obtained from blood collected by venipuncture from human volunteers who had been aspirin-free for at least 7 days. Platelets were prepared, and platelet aggregation was measured according to the procedure of Bednar *et al.* [38]. Measurements were made at room temperature using a Spectromax Plate Reader (405 nm, Molecular Devices). Plates were vortex-mixed at speed 6 on a titer plate shaker (Labline Instruments). Aggregation was calculated as percent change of transmittance, $[(T - T_0)/(T_{100} - T_0)] \times 100$.

Flow Cytometry

Surface expression of P-selectin on platelets was measured using a FACScan flow cytometer (Becton Dickinson). Washed platelets were resuspended in pH 7.4 aggregation buffer (134 mM NaCl, 3 mM KCl, 0.3 mM NaH_2PO_4, 2 mM $MgCl_2$, 5 mM HEPES, pH 7.4, 5 mM glucose, 12 mM $NaHCO_3$, and 3.5 mg/mL of BSA). Platelets ($2–3 \times 10^8$/mL) were preincubated with drugs for 30 min at room temperature. Agonists were added for 5 min followed by the addition of 10 μL of PE-conjugated anti-CD62 (Becton Dickinson) for 30 min. This concentration of antibody is saturating. To measure thrombin cleavage of PAR-1, human PAR-1 was cloned with a FLAG-tag epitope (N-Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys-C, Kodak) added at the N-terminus using standard procedures. The clone was inserted into the baculovirus vector pVL 1392 (PharMingen, where recombinant baculovirus was produced). Sf9 insect cells ($3–4 \times 10^6$/mL) were infected for 48 hr with baculovirus (multiplicity of infection = 1). Infected cells (10^6) were preincubated with 10 μM SCH 203099 for 30 min, and 10 nM thrombin was added at room temperature for 1 min. The assay was terminated by the addition of 15 nM D-Phe-Pro-Arg-chloromethylketone (Sigma), an inhibitor of thrombin activity. The presence or absence of the FLAG-tag was detected by incubating with 10 μg/mL of M1 anti-FLAG mouse monoclonal antibody (Sigma) followed by a 1:40 dilution of PE-conjugated goat anti-mouse IgG (Sigma). Both platelets and Sf9 cells were fixed with 1 mL of 1% paraformaldehyde, and mean fluorescence was measured within 24 hr. Platelets and insect cells were identified by their characteristic forward and side-scattering light patterns, and 10,000 platelets or cells were counted for each assay.

Cell Cultures

Primary hCASMC were purchased from Clonetics. hCASMC were grown in SmGM2 growth medium (Clonetics) containing 5% fetal bovine serum (Gibco-BRL), 50 mg/L of gentamicin (Clonetics), and 50 μg/L of amphotericin-B (Clonetics). Sf9 cells were cultured in suspension at 27° in TNM-FH insect medium (PharMingen No. 21227), which contained fully supplemented Grace's medium, 10% fetal bovine serum, and gentamicin.

Cytosolic Ca^{2+} Measurement

Intracellular calcium mobilization was measured by using a fluorescence technique. Twenty-four hours prior to the assay, hCASMC at passages 2 to 5 were plated on poly-d-lysine-coated Biocoat 96-well plates (Becton Dickinson) at a density of 10,000 cells/well. Cells were incubated for 1 hr with 4 μM Fluo 3-AM dye (Molecular Probes) in Hanks' buffered salt solution (HBSS) containing 1% BSA, 20 mM HEPES, pH 7.4, and 2.5 mM probenecid. Cells were washed three times in this buffer without dye using a Denley cell plate washer. After washing, cells were preincubated with vehicle or an antagonist in HBSS for 30 min. Thrombin or agonist peptide was added, and the fluorescence signal was recorded for 2 min. Net peak calcium, expressed in arbitrary fluorescence units, was measured by using a Fluorescence Imaging Plate Reader (FLIPR; Molecular Devices Corp.).

DNA Synthesis ([^{3}H]Thymidine Incorporation)

Cell proliferation was measured by [^{3}H]thymidine incorporation. hCASMC were seeded on 48-well plates at 6000 cells/well in growth medium (see the section on Cell Cultures). After 3 days of growth, cells were washed two times with Dulbecco's PBS (Gibco) and transferred into serum-free SmBM medium supplemented with ITS (10 mg/L of insulin, 5.5 mg/L of transferrin, 5 μg/L of selenium; Sigma) and GA-1000 (50 mg/L of gentamicin and 50 μg/L of amphotericin-B; Clonetics). After the 3-day serum depletion, fresh serum-free medium was added to these cells. A PAR-1 antagonist was added 30 min before the addition of thrombin or PAR-1 agonist. After 18–20 hr of incubation, [^{3}H]thymidine (0.1 μCi, New England Nuclear) was added, and incubation was continued for 20 hr. Fifty micromolar amastatin was included in the incubation mixture to prevent degradation of the peptide. The cells were washed successively with Dulbecco's PBS, 5% trichloroacetic acid, and water, and solubilized with 0.5 N NaOH. The cell lysates were transferred to glass counting vials containing 5.2 mL of Packard's Ultima-Gold liquid scintillation fluid for radioactive counting in a beta counter.

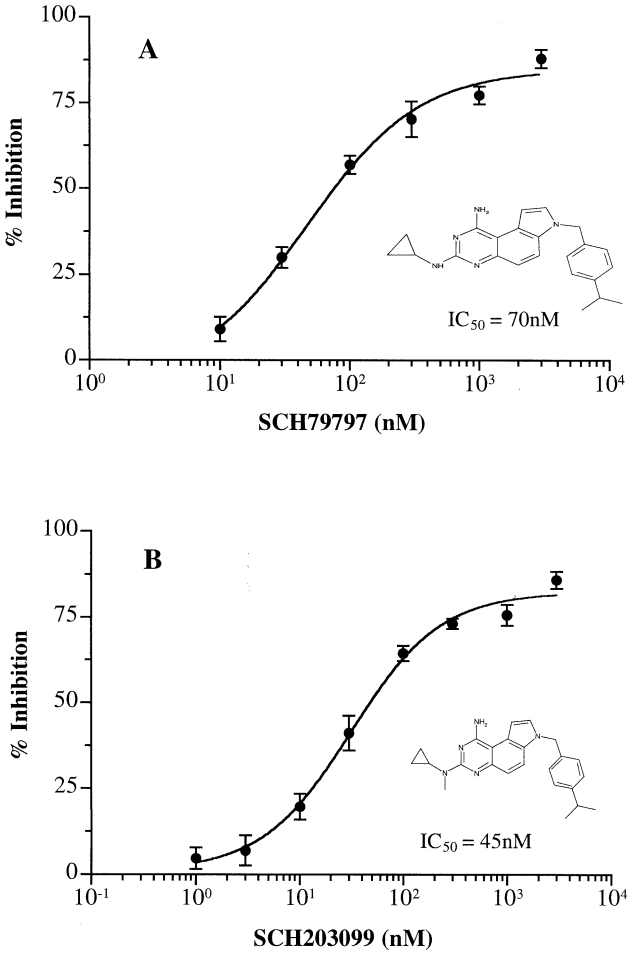

FIG. 1. Inhibition of [^{3}H]haTRAP binding to PAR-1 on human platelet membranes by SCH 79797 and SCH 203099. Human platelet membranes (40 μg/0.2 mL) were incubated for 60 min at 20° with 10 nM [^{3}H]haTRAP and various concentrations of SCH 79797 (A) and SCH 203099 (B). The final concentration of vehicle (DMSO) was 5%. The K_i was calculated using the following Cheng–Prusoff equation [39]: $K_i = IC_{50}/(1+[L]/K_d)$, where IC_{50} is the concentration of drug required for 50% inhibition of the binding, [L] is the radioligand concentration (10 nM), and K_d is 10 nM. Each value represents the mean of three determinations, with SEM of less than 5%.

Statistical Analysis

Data are presented as means ± SEM of the indicated number (N) of determinations. The significance of drug effects was assessed by a paired *t*-test. A *P* value of less than 0.05 was considered to be statistically significant.

RESULTS

Inhibition of [^{3}H]haTRAP Binding by SCH 79797 and SCH 203099

SCH 79797 and its *N*-methyl analog, SCH 203099, competed with [^{3}H]haTRAP for binding to thrombin receptor (PAR-1) on human platelet membranes with IC_{50} values of 70 nM (K_i = 35 nM) and 45 nM (K_i = 22 nM), respectively (Fig. 1). Analysis of saturation binding of

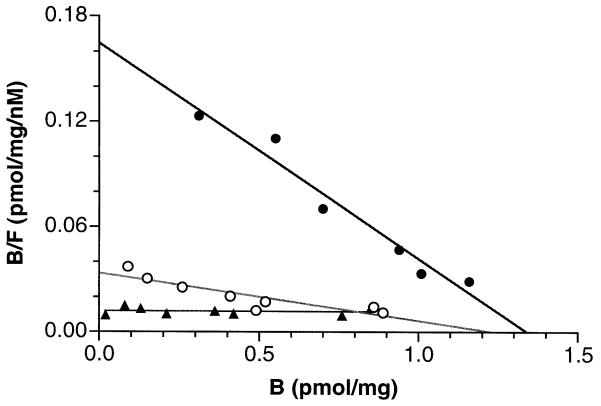

FIG. 2. Scatchard plots of saturation binding of [^{3}H]haTRAP to PAR-1 on human platelets in the presence of SCH 79797. Key: (●) control; (○) 0.1 μM SCH 79797; and (▲) 0.5 μM SCH 79797. Binding studies were performed as described in the legend of Fig. 1 using 2.5, 5, 10, 20, 30, 40, 60, and 80 nM [^{3}H]haTRAP.

[^{3}H]haTRAP in the presence and absence of SCH 79797 indicated that this compound is a competitive inhibitor of PAR-1 (Fig. 2).

Inhibition of Platelet Aggregation and P-Selectin Expression

SCH 79797 and SCH 203099 blocked platelet aggregation induced by the PAR-1-selective agonist haTRAP in a concentration-dependent fashion (Fig. 3, A and B), with IC_{50} values of 300 and 150 nM, respectively [36]. SCH 79797 and SCH 203099 selectively inhibited platelet aggregation mediated by PAR-1, since at 10 μM they had no effect on aggregation induced by 100 μM ADP or 5 μM collagen (data not shown). Both compounds also inhibit aggregation induced by α-thrombin, with ic_{50} values of 3000 and 700 nM, respectively [36]. In contrast to the sustained inhibition of haTRAP-induced aggregation, the inhibition of thrombin-induced aggregation was transient (Fig. 3, C and D), and the observed delay in aggregation was dependent on the concentration of thrombin used. At 0.5 nM thrombin (Fig. 4A), full aggregation was delayed by several minutes, whereas at 10 nM thrombin, no significant delay was seen (Fig. 4B). To explore possible explanations for this phenomenon, we looked at the ability of SCH 79797 to block PAR-4. SCH 79797 did not inhibit aggregation induced by PAR-4 tethered ligand peptide (Fig. 4C). In addition, SCH 79797 had no effect on platelet aggregation induced by γ-thrombin (Fig. 4D), a proteolytically modified thrombin lacking the high-affinity binding site for PAR-1 [40]. Binding of these drugs to platelets was reversible. Using 1 μM SCH 79797, a concentration that produces complete inhibition of aggregation, it was necessary to preincubate platelets with the drug for 60 min to obtain full inhibition (Fig. 5A), and full reversal of inhibition required platelets to be washed free of drug for 20 min (Fig. 5B).

Some reported peptide antagonists of PAR-1 are actually partial agonists [23, 24, 32]. Flow cytometric determination of P-selectin expression can be used as a measure of platelet activation [41]. Using this assay, we showed that SCH 203099 has no agonist properties at concentrations as high as 3 μM, although a slight increase in P-selectin expression was observed at a 10 μM concentration of drug (Fig. 6A). The drug was effective at blocking haTRAP- or thrombin-induced P-selectin expression. In addition, using a FLAG-tag-labeled PAR-1 receptor expressed in Sf9 cells, it was possible to demonstrate that SCH 203099 did not inhibit the catalytic activity of thrombin, since thrombin cleavage of the N-terminus of the receptor occurred in the presence of a 10 μM concentration of drug (Fig. 6B). Similar results were obtained with SCH 79797.

Inhibition of Thrombin- and TFLLRNPNDK-NH_2-Induced Calcium Transients in Vascular Smooth Muscle Cells

Thrombin and a PAR-1-selective tethered ligand analog, TFLLRNPNDK-NH_2 [42], elevated the intracellular calcium concentration in a concentration-dependent manner in hCASMC (data not shown). The EC_{50} values of thrombin and TFLLRNPNDK-NH_2 were 0.9 ± 0.2 nM (N = 6) and 12 ± 2.6 μM (N = 5), respectively. The effects of SCH 79797 on calcium transients in hCASMC were assessed. Because of the slow equilibration of drug with PAR-1 receptor on platelets, cells were preincubated with drug for 30 min before the addition of agonist. SCH 79797 inhibited the calcium transients induced by thrombin (3 nM) and TFLLRNPNDK-NH_2 (30 μM) with respective K_i values of 82 and 55 nM (Fig. 7). In contrast to platelets, where inhibition of thrombin-induced effects was transient, inhibition of the calcium transients in hCASMC was sustained over the time course of the assay. Platelets are known to possess multiple thrombin receptors [16, 17], but the thrombin receptors in hCASMC have not been characterized yet. To assess whether another tethered ligand thrombin receptor exists in these cells, the effects of a human PAR-4 agonist, GYPGQV-NH_2 [16, 17], on the intracellular calcium concentration were examined. GYPGQV-NH_2 at concentrations up to 1 mM did not stimulate calcium transients (data not shown), consistent with a lack of PAR-4 thrombin receptors in these cells. SLIGKV-NH_2, a selective PAR-2 agonist, induced calcium transients, an effect that was not inhibited by SCH 79797 at concentrations of up to 10 μM (data not shown). These data indicate the presence of PAR-2 and the selective inhibition of PAR-1 over PAR-2 by SCH 79797 in these cells.

Inhibition of Thrombin-Stimulated [^{3}H]Thymidine Incorporation in hCASMC

We next examined the effect of thrombin and TFLLRN-PNDK-NH_2 on [^{3}H]thymidine incorporation into hCASMC as a measure of smooth muscle cell proliferation.

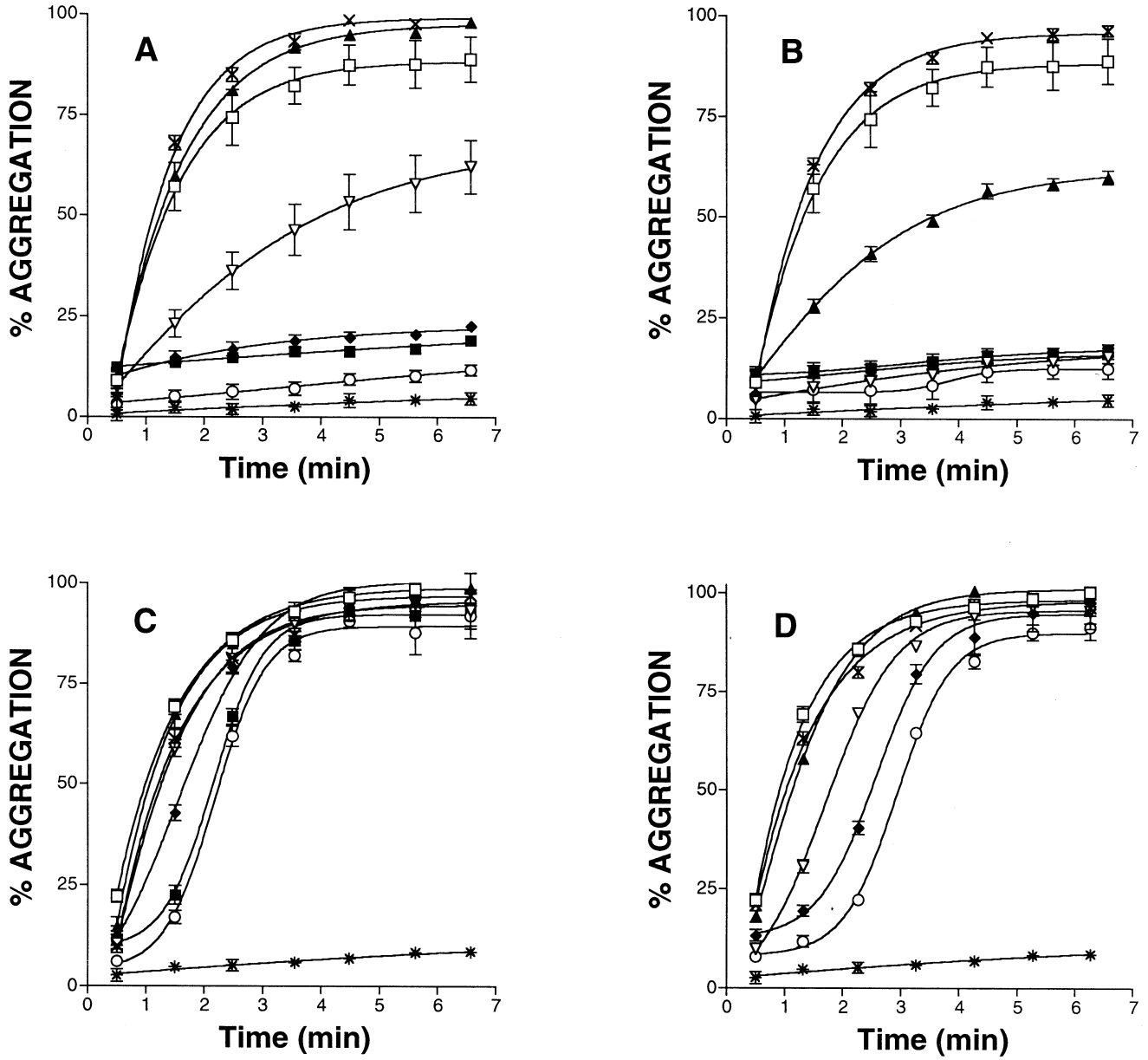

FIG. 3. Inhibition of platelet aggregation induced by haTRAP and α-thrombin. (A) Inhibition by SCH 79797 of aggregation induced by 0.3 μM haTRAP. Platelets were preincubated for 60 min with: (* and □) vehicle; (X) 0.03 μM SCH 79797; (▲) 0.1 μM SCH 79797; (▽) 0.3 μM SCH 79797; (♦) 1 μM SCH 79797; (○) 3 μM SCH 79797; and (■) 10 μM SCH 79797. Except for *, which was a vehicle control, all samples received 0.3 μM haTRAP. (B) Inhibition by SCH 203099 of aggregation induced by 0.3 μM haTRAP. Platelets were preincubated for 60 min with: (* and □) vehicle; (X) 0.03 μM SCH 203099; (▲) 0.1 μM SCH 203099; (▽) 0.3 μM SCH 203099; (♦]) 1 μM SCH 203099; (○) 3 μM SCH 203099; and (■) 10 μM SCH 203099. Except for *, which was a vehicle control, all samples received 0.3 μM haTRAP. (C) Inhibition by SCH 79797 of aggregation induced by 3 nM α-thrombin. Conditions were identical to those of panel A, except that 3 nM α-thrombin was substituted for haTRAP. (D) Inhibition by SCH 203099 of aggregation induced by 3 nM α-thrombin. Conditions were identical to those of panel B, except that 3 nM thrombin was substituted for haTRAP. Data are means ± SD, N = 4. Similar results were obtained in repeat experiments using platelets from different individuals.

Thrombin and TFLLRNPNDK-NH_2 stimulated [^{3}H]thymidine incorporation into these cells in a concentration-dependent manner and to a similar extent, with EC_{50} values of 0.0021 and 22 μM, respectively (data not shown). SCH 79797 completely inhibited thrombin- and TFLLRN-PNDK-NH_2-stimulated [^{3}H]thymidine incorporation, with respective apparent K_i values of 88 and 32 nM (Fig. 8). Similar inhibition of [^{3}H]thymidine incorporation was observed for SCH 203099 (data not shown). As observed with the calcium flux experiments, inhibition of thrombin-induced thymidine incorporation was sustained throughout the incubation period of the assay.

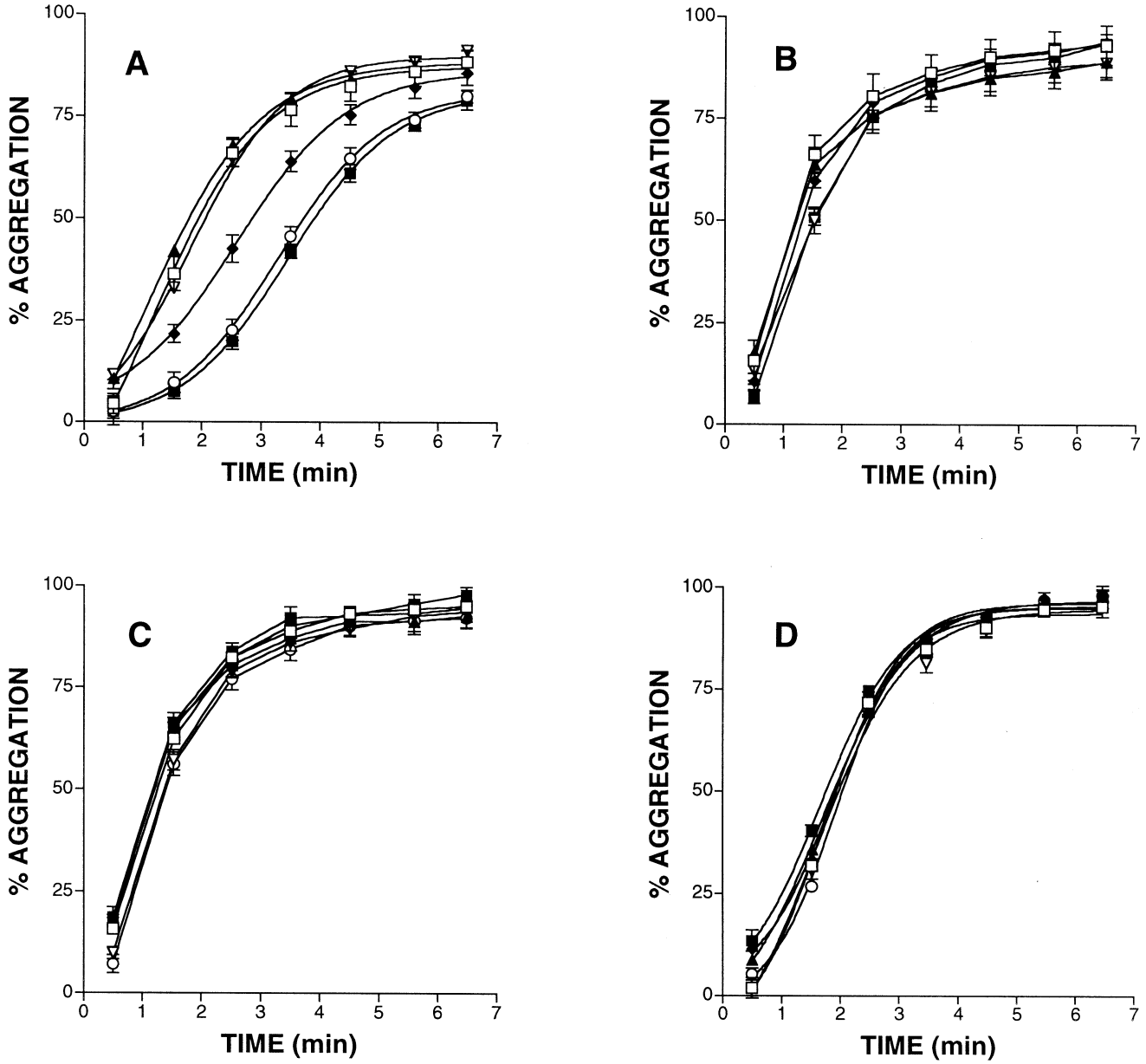

FIG. 4. Inhibition of platelet aggregation induced by α-thrombin, PAR-4 TRAP, and γ-thrombin. (A) Inhibition by SCH 79797 of aggregation induced by 0.5 nM α-thrombin. Platelets were preincubated for 60 min with: (□) vehicle; (▲) 0.1 μM SCH 79797; (▽) 0.3 μM SCH 79797; (♦) 1 μM SCH 79797; (○) 3 μM SCH 79797; and (■) 10 μM SCH 79797. All samples received 0.5 nM α-thrombin. (B) Inhibition by SCH 79797 of aggregation induced by 10 nM α-thrombin. Conditions were identical to those of panel A, except that all samples received 10 nM α-thrombin. (C) Inhibition by SCH 79797 of aggregation induced by 1 mM PAR-4 TRAP. Conditions were identical to those of panel A, except that all samples received 1 mM PAR-4 TRAP instead of thrombin. (D) Inhibition by SCH 79797 of aggregation induced by 30 nM γ-thrombin. Conditions were identical to those of panel A, except that 30 nM γ-thrombin was substituted for α-thrombin. Data are means ± SD, N = 4.

Lack of Effect of a PAR-2 Agonist and PAR-4 Tethered Ligand Analogs on [^{3}H]Thymidine Incorporation

To confirm our observations of the absence of PAR-4 in hCASMC, we examined the effect of the PAR-4 agonist GYPGQV-NH_2 [16, 17] on [^{3}H]thymidine incorporation. There have also been reports of a PAR-2 agonist stimulating [^{3}H]thymidine incorporation in vascular smooth muscle cells from other sources [43, 44]. Therefore, we tested a human PAR-2 agonist, SLIGKV-NH_2 [13], head to head with the PAR-1 and PAR-4 agonists. Whereas the PAR-1 agonist TFLLRNPNDK-NH_2 stimulated [^{3}H]thymidine incorporation, the PAR-2 and PAR-4 agonists did not (Fig. 9).

DISCUSSION

The thrombin receptor PAR-1 is distributed widely in various cell types [45], and activation of this receptor following vascular injury contributes to thrombosis, restenosis, and atherosclerosis [1–4]. Thus, the development of potent, small-molecule PAR-1 antagonists would allow us

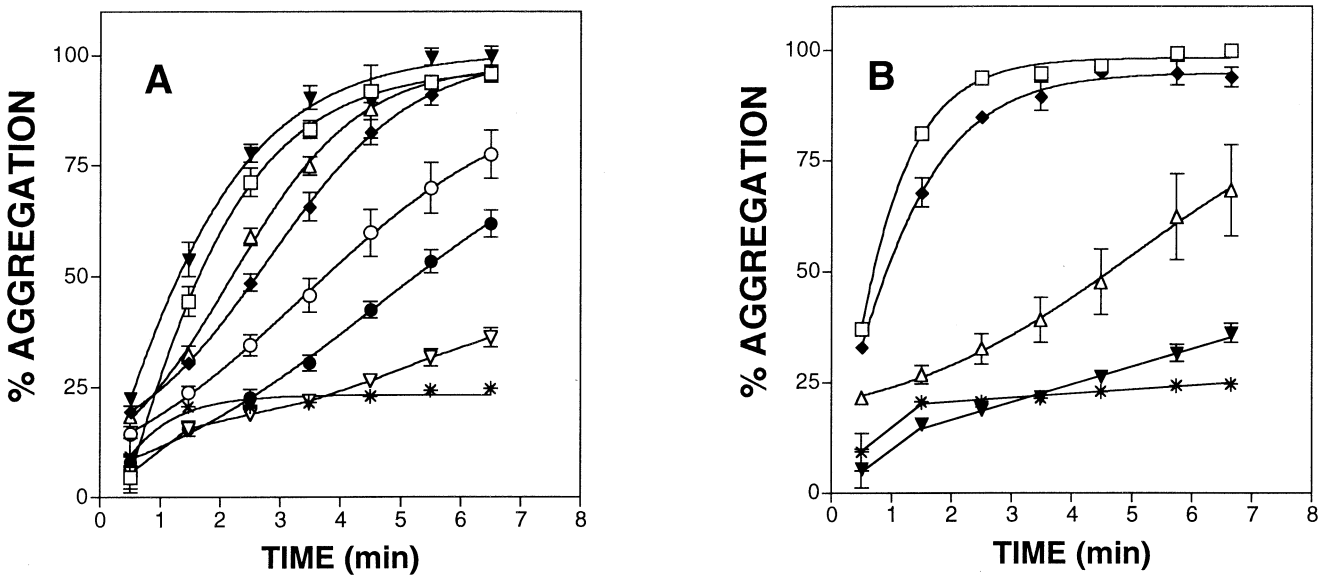

FIG. 5. Time course of inhibition of platelet aggregation by SCH 79797. (A) Platelets were preincubated with 1 μM SCH 79797 for the times indicated: (▽) 60 min; (●) 45 min; (○) 30 min; (♦) 20 min; (△) 10 min; and (▼) 1 min. Controls (□ and *) were incubated for 60 min. without drug. Platelet aggregation was initiated by the addition of 0.3 μM haTRAP except for *, which received no agonist. Aggregation was monitored as described in Materials and Methods. (B) Platelets were preincubated with 1 μM SCH 79797 (♦, △, ▼, *) or vehicle (□, 0.1% DMSO) for 60 min at room temperature in pH 6.5 wash buffer (see Materials and Methods). Platelets were pelleted, and the supernatant solutions were removed carefully. Platelets were resuspended in pH 7.4 aggregation buffer without drug and incubated at room temperature for the times shown [(▼) 0 min; (△ and □) 10 min; (* and ♦) 20 min]. Aggregation was then initiated by the addition of 0.3 μM haTRAP (□, ♦, △, ▼) or vehicle (*) and monitored as described in Materials and Methods. Each value represents the mean of three determinations, with SEM of less than 5%.

to test the potential therapeutic effect of blocking cellular actions of thrombin. SCH 79797 and SCH 203099 represent the first potent, nonpeptide class of competitive PAR-1 receptor antagonists. SCH 79797 and SCH 203099 do not inhibit thrombin enzyme activity or cause platelet activation. They are functionally selective for PAR-1 inhibition, showing no activity against PAR-4, PAR-2, and other receptors (ADP, collagen) involved in platelet activation.

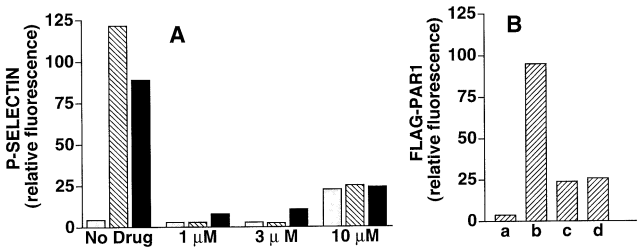

FIG. 6. (A) Effect of SCH 203099 on P-selectin expression in platelets. P-selectin expression was measured using flow cytometry as described in Materials and Methods. Platelets were preincubated for 30 min with drug. Vehicle (gray bars), 0.3 μM haTRAP (downward cross-hatched bars), or 1 nM thrombin (solid bars) was added, and P-selectin expression was determined by binding of fluorescent labeled antibody against P-selectin. (B) Effect of SCH 203099 on thrombin cleavage of PAR-1 thrombin receptors expressed in Sf9 cells. Cells expressing FLAG-tag-labeled PAR-1 were preincubated for 30 min with drug. Thrombin (10 nM) was added for 1 min, and the reaction was stopped with d-Phe-Pro-Arg-chloromethylketone as described in Materials and Methods. Cleavage of the receptor was measured as loss of the FLAG-tag, determined by selective antibody binding. This experiment was repeated once with essentially the same results. Conditions: (a) no primary antibodies; (b) vehicle; (c) 10 nM α-thrombin; and (d) 10 nM α-thrombin plus 10 μM SCH 203099.

Two additional thrombin receptors with close homology to PAR-1, namely PAR-3 [15] and PAR-4 [16, 17], have been cloned and identified in various cell types and tissues (see Ref. 45, a review paper on protease-activated receptors). Whereas human platelets aggregate in response to both PAR-1 and PAR-4 agonists, we showed in the current study that hCASMC mobilize calcium only in response to PAR-1 agonists. In contrast to its effect on human platelets, SCH 79797 blocked thrombin-induced calcium mobilization completely in hCASMC. These results clearly demonstrate that PAR-1 is the only thrombin-sensitive receptor coupled to calcium mobilization in hCASMC. An essentially similar finding has been reported for rat aortic smooth muscle cells [25].

The proliferation of arterial smooth muscle cells is a key event in the development of arterial lesions after vascular injury and angioplasty [4, 46]. Coronary arterial smooth muscle cells are a major target for thrombin, and proliferation of these smooth muscle cells is a hallmark of intimal thickening and restenosis after angioplasty. Thrombin is known to promote vascular smooth muscle cell proliferation *in vitro* [11, 12]. Thrombin also has been reported to stimulate thymidine incorporation in cultured human and porcine coronary artery smooth muscle cells [47, 48]. To date, no information is available regarding the type of thrombin receptor that mediates the mitogenic action of

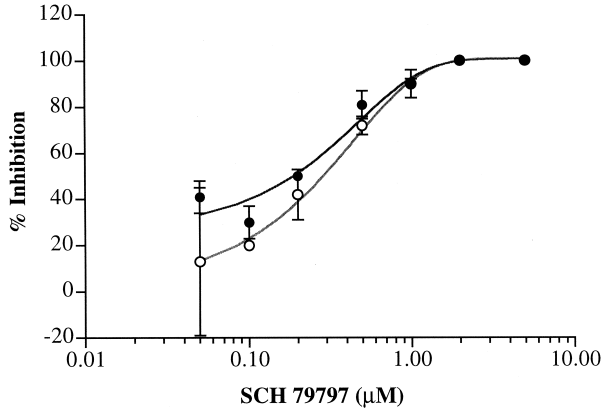

FIG. 7. Inhibition of thrombin- and TFLLRNPNDK-NH_2-stimulated calcium transients in hCASMC by SCH 79797. SCH 79797 was added 30 min before the addition of thrombin (3 nM) and TFLLRNPNDK-NH_2 (30 μM). The final concentration of vehicle (DMSO) was 0.2%. Key: (●) 3 nM thrombin; and (○) 30 μM TFLLRNPNDK-NH_2. The K_i was calculated using the following Cheng–Prusoff equation [39]: $K_i = IC_{50}/(1+[A]/[EC_{50}])$, where IC_{50} is the antagonist concentration for inhibition of an agonist effect by 50%, [A] is the concentration of the agonist used, and EC_{50} is the agonist concentration for half-maximal stimulation. Each value is the mean ± SEM of 4 determinations. All points (% inhibition) with the exception of one point (0.03 μM SCH 79797 against TFLLRNPNDK-NH_2) were significantly different from the control (no SCH 79797) ($P < 0.05$). These data are representative of three separate experiments with essentially similar results.

thrombin in hCASMC. Thrombin and a PAR-1 agonist, but not PAR-2 or PAR-4 agonists, induced stimulation of mitogenesis in hCASMC, and this response was inhibited completely by the selective PAR-1 antagonists SCH 79797 and SCH 203099. The predominant role of PAR-1 in mediating thrombin-induced mitogenesis of hCASMC is in agreement with a recent report showing complete inhibition of thrombin-stimulated rat aortic smooth muscle proliferation by a selective peptidomimetic PAR-1 antagonist, RWJ-56110 [25]. Earlier studies [47, 49] failed to show a stimulatory effect of a PAR-1 agonist peptide on the mitogenesis of bovine coronary artery smooth muscle cells, despite the fact that thrombin stimulated mitogenesis. However, this may have resulted in part from degradation of the peptide by aminopeptidases during a long incubation period. Even in our study, TFLLRNPNDK-NH_2 or SFLLRN-NH_2 failed to stimulate thymidine incorporation consistently unless amastatin, an aminopeptidase inhibitor, was present at a concentration of 50 μM or higher (unpublished observation). Species differences in the mitogenic response of vascular smooth muscle cells to PAR-1 agonist peptides have also been reported [12].

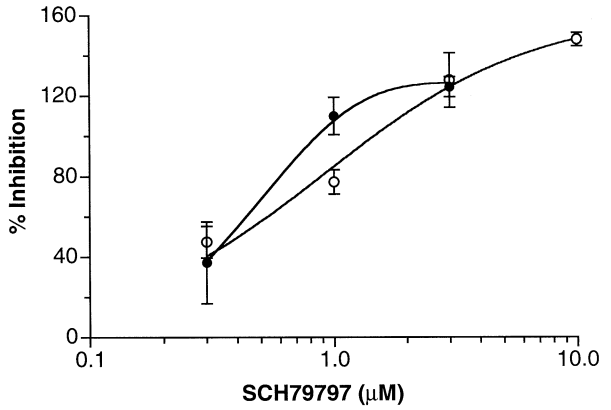

FIG. 8. Inhibition of thrombin- and TFLLRNPNDK-NH_2-stimulated [^{3}H]thymidine incorporation in hCASMC by SCH 79797. SCH 79797 was added 30 min before the addition of PAR-1 agonists. The final concentration of vehicle (DMSO) was 0.2%. Key: (●) 3 nM thrombin; and (○) 10 μM TFLLRNPNDK-NH_2. Amastatin (50 μM) was present in the incubation medium when TFLLRNPNDK-NH_2 was tested. Each value is the mean ± SEM of 4 determinations. These data are representative of three experiments with similar results. The K_i values were determined as described in the legend to Fig. 7.

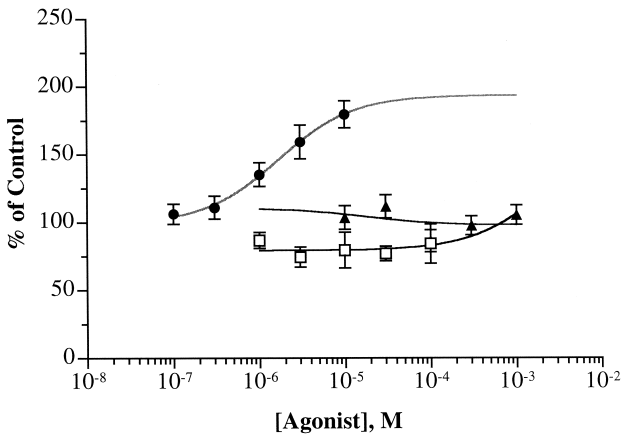

FIG. 9. Lack of effect of PAR-2 and PAR-4 agonist peptides on thymidine incorporation in hCASMC. Key: (●) TFLLRNPNDK-NH_2; (□) SLIGKV-NH_2; and (▲) GYPGQV-NH_2. Amastatin (50 μM) was present in the incubation medium. Each value represents the mean of four determinations, with SEM of less than 12%. The mean ± SEM of the control value ([^{3}H]thymidine incorporated into the cells, dpm) was 1639 ± 124 (N = 12). This experiment was repeated once with similar results.

Since the actual ligands of protease-activated receptors are new N-terminal peptides generated by enzymatic cleavage of the receptors themselves, it was originally thought that the entropic advantage of such "tethered" ligands would make them difficult or impossible to block [50]. However, this does not appear to be the case. The complete inhibition of thrombin-induced PAR-1 activation in hCASMC by SCH 79797 with a K_i value of 82 nM demonstrates that these antagonists functionally antagonize the thrombin-generated tethered ligand. Thus, it is likely that the failure of these compounds to inhibit thrombin activation of platelet aggregation for more than a few minutes is due to the action of thrombin on a second thrombin receptor such as PAR-4 [16, 17, 51]. This conclusion is supported by the observation that the delay of platelet aggregation was greatest at a low thrombin concentration (0.5 nM) and disappeared at a high thrombin concentration (10 nM). These results are consistent with

the observations of Kahn et al. [17], who showed that PAR-4 is less sensitive to cleavage by thrombin than PAR-1. They concluded that at low concentrations of thrombin, PAR-1 is the primary mediator of aggregation, whereas at high concentrations, activation of either PAR-1 or PAR-4 can produce full aggregation of platelets [51]. Therefore, in the presence of drug at 10 nM thrombin, aggregation most likely proceeds through activation of PAR-4. The delayed aggregation in the presence of SCH 79797 at 0.5 nM thrombin probably results from a slow cleavage of PAR-4 at this enzyme concentration. Further evidence for the role of PAR-4 comes from the experiment with γ-thrombin. Unlike α-thrombin, γ-thrombin is equally active in stimulating PAR-1 and PAR-4 [16]. Since SCH 79797 has no effect on γ-thrombin-induced platelet aggregation, the effect of γ-thrombin appears to be mediated primarily through PAR-4.

The only other class of thrombin receptor antagonists reported to date with potencies similar to those of SCH 79797 and SCH 203099 are peptidomimetics based on the structure of the tethered ligand peptide SFLLRN [25]. The pyrroloquinazoline compounds shown here demonstrate that inhibition can be achieved with smaller molecules without peptidomimetic properties. Thrombin receptor antagonists have potential advantages over other thrombin-inhibitory drugs in treating vascular diseases because they leave the fibrinolytic function of thrombin and other non-thrombin-mediated platelet aggregation mechanisms intact. Unlike GPIIbIIIa antagonists and thrombin inhibitors, thrombin receptor inhibitors are expected to have minimal bleeding side-effects [52]. In addition, receptor antagonists will have a direct effect on thrombin-induced mitogenesis in smooth muscle cells. Thus, this class of compounds will have a dual action on platelets and smooth muscle cells that should reduce the risk of restenosis after angioplasty.

In summary, SCH 79797 and SCH 203099 represent the first potent, non-peptidic class of selective PAR-1 antagonists that effectively inhibit PAR-1 on human platelets and hCASMC. The present study demonstrates for the first time that thrombin stimulation of calcium mobilization and mitogenesis of hCASMC is mediated by PAR-1 activation, in contrast to the involvement of multiple thrombin receptors in platelet activation. This new pyrroloquinazoline class of selective thrombin receptor antagonists will serve as useful tools to clarify the role of PAR-1 in restenosis and other pathophysiological conditions.

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References

1. Fenton JW II, Regulation of thrombin generation and functions. *Semin Thromb Hemost* 14: 234–240, 1988.
2. Hanson SR and Harker LA, Interruption of acute platelet-dependent thrombosis by the synthetic antithrombin D-phenylalanyl-L-prolyl-L-arginyl chloromethyl ketone. *Proc Natl Acad Sci USA* 85: 3184–3188, 1988.
3. Eidt JF, Allison P, Noble S, Ashton J, Golino P, McNatt J, Buja LM and Willerson JT, Thrombin is an important mediator of platelet aggregation in stenosed canine coronary arteries with endothelial injury. *J Clin Invest* 84: 18–27, 1989.
4. Harker LA, Hanson SR and Runge MS, Thrombin hypothesis of thrombus generation and vascular lesion formation. *Am J Cardiol* **75:** 12B–17B, 1995.
5. Coughlin SR, Vu TK, Hung DT and Wheaton VI, Characterization of a functional thrombin receptor. Issues and opportunities. *J Clin Invest* **89:** 351–355, 1992.
6. Vu TK, Hung DT, Wheaton VI and Coughlin SR, Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. *Cell* **64:** 1057–1068, 1991.
7. Scarborough RM, Naughton MA, Teng W, Hung DT, Rose J, Vu TK, Wheaton VI, Turck CW and Coughlin SR, Tethered ligand agonist peptides. Structural requirements for thrombin receptor activation reveal mechanism of proteolytic unmasking of agonist function. *J Biol Chem* **267:** 13146–13149, 1992.
8. Vassallo RR Jr, Kieber-Emmons T, Cichowski K and Brass LF, Structure-function relationships in the activation of platelet thrombin receptors by receptor-derived peptides. *J Biol Chem* **267:** 6081–6085, 1992.
9. Yang SG, Laniyonu A, Saifeddine M, Moore GJ and Hollenberg MD, Actions of thrombin and thrombin receptor peptide analogues in gastric and aortic smooth muscle: Development of bioassays for structure-activity studies. *Life Sci* **51:** 1325–1332, 1992.
10. Antonaccio MJ, Normandin D, Serafino R and Moreland S, Effects of thrombin and thrombin receptor-activating peptides on rat aortic vascular smooth muscle. *J Pharmacol Exp Ther* **266:** 125–132, 1993.
11. Fager G, Thrombin and proliferation of vascular smooth muscle cells. *Circ Res* **77:** 645–650, 1995.
12. McNamara CA, Sarembock IJ, Bachhuber BG, Stouffer GA, Ragosta GA, Barry W, Gimple LW, Powers ER and Owens GK, Thrombin and vascular smooth muscle cell proliferation: Implications for atherosclerosis and restenosis. *Semin Thromb Hemost* **22:** 139–144, 1996.
13. Nystedt S, Emilsson K, Wahlestedt C and Sundelin J, Molecular cloning of a potential proteinase activated receptor. *Proc Natl Acad Sci USA* **91:** 9208–9212, 1994.
14. Nystedt S, Emilsson K, Larsson AK, Strombeck B and Sundelin J, Molecular cloning and functional expression of the gene encoding the human proteinase-activated receptor 2. *Eur J Biochem* **232:** 84–89, 1995.
15. Ishihara H, Connolly AJ, Zeng D, Kahn ML, Zheng YW, Timmons D, Tram T and Coughlin SR, Protease-activated receptor 3 is a second thrombin receptor in humans. *Nature* **386:** 502–506, 1997.
16. Xu WF, Andersen H, Whitmore TE, Presnell SR, Yee DP, Ching A, Gilbert T, Davie EW and Foster DC, Cloning and characterization of human protease-activated receptor 4. *Proc Natl Acad Sci USA* **95:** 6642–6646, 1998.
17. Kahn ML, Zheng Y-W, Huang W, Bigornia V, Zeng D, Moff S, Farese RV Jr, Tam C and Coughlin SR, A dual thrombin receptor system for platelet activation. *Nature* **394:** 690–694, 1998.
18. Nelken NA, Soifer SJ, O'Keefe J, Vu TK, Charo IF and Coughlin SR, Thrombin receptor expression in normal and atherosclerotic human arteries. *J Clin Invest* **90:** 1614–1621, 1992.
19. Wilcox JN, Rodriguez J, Subramanian R, Ollerenshaw J, Zhong C, Hayzer DJ, Horaist C, Hanson SR, Lumsden A, Salam TA, Kelly AB, Harker LA and Runge M, Characterization of thrombin receptor expression during vascular lesion formation. *Circ Res* **75:** 1029–1038, 1994.
20. Baykal D, Schmedtje JF Jr and Runge MS, Role of the

thrombin receptor in restenosis and atherosclerosis. *Am J Cardiol* **75:** 82B–87B, 1995.
21. Cook JJ, Sitko GR, Bednar B, Condra C, Mellott MJ, Feng D-M, Nutt RF, Shafer JA, Gould RJ and Connolly TM, An antibody against the exosite of the cloned thrombin receptor inhibits experimental arterial thrombosis in the African green monkey. *Circ Res* **91:** 2961–2971, 1995.
22. Seiler SM, Peluso M, Michel IM, Goldenberg H, Fenton JW II, Riexinger D and Natarajan S, Inhibition of thrombin and SFLLR-peptide stimulation of platelet aggregation, phospholipase A_2 and Na^+/H^+ exchange by a thrombin receptor antagonist. *Biochem Pharmacol* **49:** 519–528, 1995.
23. Seiler SM, Peluso M, Tuttle JG, Pryor K, Klimas C, Matsueda GR and Bernatowicz MS, Thrombin receptor activation by thrombin and receptor-derived peptides in platelet and CHRF-288 cell membranes: Receptor-stimulated GTPase and evaluation of agonists and partial agonists. *Mol Pharmacol* **49:** 190–197, 1996.
24. Bernatowicz MS, Klimas CE, Hartl KS, Peluso M, Allegretto NJ and Seiler SM, Development of potent thrombin receptor antagonist peptides. *J Med Chem* **39:** 4879–4887, 1996.
25. Andrade-Gordon P, Maryanoff BE, Derian CK, Zhang H-C, Addo MF, Darrow AL, Eckardt AJ, Hoekstra WJ, McComsey DF, Oksenberg D, Reynolds EE, Santulli RJ, Scarborough RM, Smith CE and White KB, Design, synthesis, and biological characterization of a peptide-mimetic antagonist for a tethered-ligand receptor. *Proc Natl Acad Sci USA* **96:** 12257–12262, 1999.
26. Rasmussen UB, Gachet C, Schlesinger Y, Hanau D, Ohlmann P, Van Obberghen-Schilling E, Pouyssegur J, Cazenave J-P and Pavirani A, A peptide ligand of the human thrombin receptor antagonizes a thrombin and partially activates platelets. *J Biol Chem* **268:** 14322–14328, 1993.
27. Scarborough RM, Teng W, Naughton MA, Rose JW, Alves V and Aristen A, C186–65, a thrombin receptor antagonist designed from tethered ligand agonist peptides. *Circulation* **86:** I151, 1992.
28. Debeir T, Vige X and Benavides J, Pharmacological characterization of protease-activated receptor (PAR-1) in rat astrocytes. *Eur J Pharmacol* **323:** 111–117, 1997.
29. Lindahl AK, Scarborough RM, Naughton MA, Harker LA and Hanson SR, Antithrombotic effect of a thrombin receptor antagonist peptide in baboons. *Thromb Hemost* **69:** 1196, 1993.
30. Hoekstra WJ, Hushizer BL, McComsey DF, Andrade-Gordon P, Kauffman JA, Addo MF, Oksenberg D, Scarborough RM and Maryanoff BE, Thrombin receptor (PAR-1) antagonists: Heterocycle-based peptidomimetics of the SFLLR agonist motif. *Bioorg Med Chem Lett* **8:** 1649–1654, 1998.
31. Fujita T, Nakajima M, Inoue Y, Nose T and Shimohigashi Y, A novel molecular design of thrombin receptor antagonist. *Bioorg Med Chem Lett* **9:** 1351–1356, 1999.
32. Ogletree ML, Natarajan S and Seiler S, Thrombin receptors as drug discovery targets. *Perspect Drug Discov Des* **1:** 527–536, 1994.
33. McComsey DF, Hawkins MJ, Adrade-Gordon P, Addo MF, Oksenberg D and Maryanoff BE, Heterocyclic-peptide hybrid compounds: Aminotriazole-containing agonists of the thrombin receptor (PAR-1). *Bioorg Med Chem Lett* **9:** 1423–1428, 1999.
34. Alexopoulos K, Matsoukas J, Tselios T, Roumelioti P, Mavromoustakos T and Holada K, A comparative SAR study of thrombin receptor derived non peptide mimetics: Importance of phenyl/guanidino proximity for activity. *Amino Acids* **15:** 211–220, 1998.
35. Ahn H-S, Foster C, Boykow G, Arik L, Smith-Torhan A, Hesk D and Chatterjee M, Binding of a thrombin receptor tethered ligand analogue to human platelet thrombin receptor. *Mol Pharmacol* **51:** 350–356, 1997.
36. Ahn H-S, Arik L, Boykow G, Burnett D, Caplen MA, Czarniecki M, Domalski MS, Foster C, Manna M, Stamford AW and Wu Y, Structure-activity relationships of pyrroloquinazolines as thrombin receptor antagonists. *Bioorg Med Chem Lett* **9:** 2073–2078, 1999.
37. Kato Y, Kita Y, Nishio M, Hirasawa Y, Ito K, Yamanaka T, Motoyama Y and Seki J, *In vitro* antiplatelet profile of FR171113, a novel non-peptide thrombin receptor antagonist. *Eur J Pharmacol* **384:** 197–202, 1999.
38. Bednar B, Condra C, Gould RJ and Connolly TM, Platelet aggregation monitored in a 96 well microplate reader is useful for evaluation of platelet agonists and antagonists. *Thromb Res* **77:** 453–463, 1995.
39. Cheng Y-C and Prusoff WH, Relationship between the inhibition constant (K_I) and the concentration of inhibitor which causes 50 per cent inhibition (I_{50}) of an enzymatic reaction. *Biochem Pharmacol* **22:** 3099–3108, 1973.
40. Rydel TJ, Yin M, Padmanabhan KP, Blankenship DT, Cardin AD, Correa PE, Fenton JW and Tulinsky A, Crystallographic structure of human γ-thrombin. *J Biol Chem* **269:** 22000–22006, 1994.
41. Shattil SJ, Cunningham M and Hoxie JA, Detection of activated platelets in whole blood using activation-dependent monoclonal antibodies and flow cytometry. *Blood* **70:** 307–315, 1987.
42. Blackhart BD, Emilsson K, Nguyen D, Teng W, Martelli AJ, Nystedt S, Sundelin J and Scarborough RM, Ligand cross-reactivity within the protease-activated receptor family. *J Biol Chem* **271:** 16466–16471, 1996.
43. Bono F, Lamarche I and Herbert JM, Induction of vascular smooth muscle cell growth by selective activation of the proteinase activated receptor-2 (PAR-2). *Biochem Biophys Res Commun* **241:** 762–764, 1997.
44. Bretschneider E, Kaufmann R, Braun M, Wittpoth M, Glusa E, Nowak G and Schror K, Evidence for proteinase-activated receptor-2 (PAR-2)-mediated mitogenesis in coronary artery smooth muscle cells. *Br J Pharmacol* **126:** 1735–1740, 1999.
45. Dery O, Corvera CU, Steinhoff M and Bunnett NW, Proteinase-activated receptors: Novel mechanisms of signaling by serine proteases. *Am J Physiol* **274:** C1429–C1452, 1998.
46. Ross R, The pathogenesis of atherosclerosis: A perspective for the 1990s. *Nature* **362:** 801–809, 1993.
47. Bretschneider E, Wittpoth M, Weber A-A, Glusa E and Schror K, Activation of NFκB is essential but not sufficient to stimulate mitogenesis of vascular smooth muscle cells. *Biochem Biophys Res Commun* **235:** 365–368, 1997.
48. Varela O, Martínez-Gonzalez J and Badimon L, The response of smooth muscle cells to α-thrombin depends on its arterial origin: Comparison among different species. *Eur J Clin Invest* **28:** 313–323, 1998.
49. Bretschneider E, Wittpoth M, Weber A-A, Glusa E and Schror K, Thrombin but not thrombin receptor activating peptide is mitogenic for coronary artery smooth muscle cells. *Thromb Res* **87:** 493–497, 1997.
50. Brass LF, Thrombin receptor antagonists: A work in progress. *Coron Artery Dis* **8:** 49–58, 1997.
51. Kahn ML, Nakanishi-Matsui M, Shapiro MJ, Ishihara H and Coughlin SR, Protease-activated receptors 1 and 4 mediate activation of human platelets by thrombin. *J Clin Invest* **103:** 879–887, 1999.
52. Harker LA, Therapeutic inhibition of thrombin activities, receptors, and production. *Hematol Oncol Clin North Am* **12:** 1211–1230, 1998.