

Evaluation of Proteinase-Activated Receptor-1 (PAR₁) Agonists and Antagonists Using a Cultured Cell Receptor Desensitization Assay: Activation of PAR₂ by PAR₁-Targeted Ligands

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ABSTRACT

We developed a calcium signaling-based assay, using cultured human embryonic kidney cells (HEK), that evaluates simultaneously, the activation/desensitization or blockade of the proteinase-activated receptors, PAR₁ and PAR₂. Using this assay, we analyzed the actions of a number of previously described putative PAR₁-targeted peptide agonists and antagonists. We found that most of the previously described PAR₁-targeted agents can also activate/desensitize PAR₂, and most of these peptides can also activate a calcium signaling pathway in a target cell that possesses PAR₂ along with PAR₁. Furthermore, we used this assay to develop a PAR₁ receptor-activating

probe [Ala-parafluoroPhe-Arg-Cha-Cit-Tyr-NH₂ (Cit-NH₂)], which displays a high degree of specificity for PAR₁ over PAR₂, and we used the assay to quantitate the ability of trypsin to disarm the activation of PAR₁ by thrombin. The abilities of the PAR₁-targeted agents to desensitize or block PAR₁ in the HEK cell assay were compared with their activities in a human platelet aggregation assay. Our data illustrate the usefulness of the HEK cell assay for evaluating the PAR₁/PAR₂ selectivity of PAR-activating agonists. The PAR₁-selective agonist that we developed using the assay should prove useful for studying the effects of selectively activating PAR₁ in vivo.

The G-protein-coupled receptors stimulated by thrombin (proteinase-activated receptor-1: PAR₁) or by trypsin (PAR₂) are activated by the proteolytic unmasking of anchored N-terminal cryptic receptor-activating sequences (SLIGKV and SLIGRL for human and rodent PAR₂; SFLLR and SFFLR for human and rodent PAR₁) (Vu et al., 1991; Coughlin et al., 1992; Nystedt et al., 1994, 1995; Al-Ani et al., 1995; Böhm et al., 1996; Saifeddine et al., 1996). The G-protein-coupled

receptors for thrombin are distinct from the GPIIb/IX-V platelet binding site for thrombin, which may also play a role in thrombin action (Harmon and Jamieson, 1986; Jamieson, 1997). Strikingly, short synthetic peptides based on these revealed N-terminal activating sequences of the G-protein-coupled PARs (so-called PAR₁- or PAR₂-activating peptides, or PAR-APs) can, in isolation, activate either PAR₁ or PAR₂ (Vu et al., 1991; Nystedt et al., 1994; Hollenberg et al., 1996, 1997). The PAR₂-activating peptides, derived from either the human (SLIGKV-NH₂) or rodent (SLIGRL-NH₂) receptor sequences, can mimic the action of trypsin in activating PAR₂, but they are unable to activate the PAR₁ thrombin receptor because of a lack of an essential aromatic amino acid substituent at position 2 of the activating peptide (Hollenberg et al., 1993; Natarajan et al., 1995). In contrast, thrombin

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ABBREVIATIONS: PAR, proteinase-activated receptor; PAR-AP, proteinase-activated receptor-activating peptide; PAR₁, proteinase-activated receptor-1/thrombin receptor; PAR₂, proteinase-activated receptor-2/trypsin-activated receptor; PAR₃, proteinase-activated receptor-3/thrombin receptor; PCR, polymerase-chain reaction; SF-NH₂, SFLLR-NH₂; SF₁₄-NH₂, SFLLRNPNDKYEPF-NH₂; SL-NH₂, SLIGRL-NH₂; tc-ff-NH₂, *trans*-cinnamoyl-parafluoroPhe-Arg-Leu-Arg-Orn-NH₂; TF-NH₂, TFLLR-NH₂; TG, thapsigargin; TRAP, thrombin receptor-activating peptide. Amino acids are abbreviated by their one-letter or three-letter codes; Cha, cyclohexylalanine; Cit-NH₂, Ala-parafluoroPhe-Arg-Cha-Cit-Tyr-NH₂; hArg, homoarginine; hArg-NH₂, Ala-parafluoroPhe-Arg-Cha-hArg-Tyr-NH₂; HEK, human embryonic kidney cells, 293; LPA, lysophosphatidic acid; Met-OH, Met-Ser-Arg-Pro-Asn-Asp-Lys-Tyr-Glu-OH; Mpr-NH₂, mercaptopropionyl-Phe-Cha-Cha-Arg-Lys-Pro-Asn-Asp-Lys-NH₂; Mpr, mercaptopropionyl.

receptor-activating peptides derived from the human PAR₁ receptor sequence (e.g., SFLLR-NH₂) have been observed by us and by others to activate both PAR₁ and PAR₂ (Blackhart et al., 1996; Hollenberg et al., 1997). Nonetheless, we and others have shown that peptides such as TFLLR-NH₂ (TF-NH₂) (Hollenberg et al., 1997) or TFLLRNPNNDK-NH₂ (Blackhart et al., 1996) can selectively activate PAR₁ but not PAR₂. One approach to evaluating the selectivity (or lack thereof) of agents targeted to either PAR₁ or PAR₂ has employed a xenopus oocyte receptor expression system, in which only one of the two receptors are expressed (Blackhart et al., 1996). As an alternative approach, we have used a receptor desensitization paradigm, employing an intact contractile tissue bioassay to illustrate, for instance, the selective activation of PAR₂ but not PAR₁ by the PAR₂-AP, SLIGRL-NH₂ (Saifeddine et al., 1996). We sought to extend the receptor-desensitization paradigm for use with a cultured cell system, in which an intracellular calcium signal rather than a contractile response might be used as an index of receptor activation. Furthermore, we reasoned that, provided receptor cross-desensitization did not occur, it would be advantageous to assess PAR-targeted ligands in a cell that expressed both receptors. With both receptors present in the same cell, the selectivity or nonselectivity of a variety of compounds that would affect PAR₁ and/or PAR₂ could be efficiently evaluated in a single experiment. To this end, we developed a calcium-signaling assay, employing cultured human embryonic kidney cells (HEK293) in which the action of PAR₁ and PAR₂ agonists and antagonists could be evaluated simultaneously. Using this assay, we expected to evaluate the PAR₁/PAR₂ selectivity of the compounds listed in Table 1. Many of these agents have been previously described either as potent thrombin-receptor ligands (Feng et al., 1995) or as thrombin (PAR₁) receptor antagonists (Doorbar and Winter, 1994; Seiler et al., 1995; Bernatowicz et al., 1996). We also wished to evaluate the PAR₁/PAR₂ selectivity of the originally-described thrombin receptor-activating peptides SFLLRNPNNDKYEPF-NH₂ (SF₁₄-NH₂) and SFLLR-NH₂(SF-NH₂) (so-called TRAPs: Vu et al., 1991; Hollenberg et al., 1992; Hui et al., 1992; Sabo et al., 1992; Scarborough et al., 1992; Vassallo et al., 1992; Chao et al., 1993).

Materials and Methods

Cell Culture and Fluorescence Measurements. Human embryonic kidney cells (HEK293) that express the SV40 T-antigen were

TABLE 1
Compounds evaluated for PAR₁/PAR₂ selectivity

Compound	Comment
Thrombin	Selectively activates PAR ₁ and PAR ₃
Trypsin	Activates all PARs; selective for PAR ₂
Mpr-NH ₂	Reported as a thrombin antagonist ^a
tc-fF-NH ₂	Reported as a thrombin antagonist ^b
h Arg-NH ₂	Reported as a thrombin receptor ligand ^c
TF-NH ₂	Reported as a PAR ₁ -selective agonist ^d
Cit-NH ₂	Putative PAR ₁ -selective agonist
SF ₁₄ -NH ₂	Originally-described PAR ₁ agonist (TRAP) ^e
SF-NH ₂	Short PAR ₁ agonist (TRAP), based on SF ₁₄ -NH ₂ ^f

^a Seiler et al., 1995.

^b Bernatowicz et al., 1996.

^c Feng et al., 1995; Ahn et al., 1997.

^d Hollenberg et al., 1997.

^e Vu et al., 1991.

^f Hollenberg et al., 1992.

kindly provided by Dr. Jonathan Lytton, University of Calgary, Faculty of Medicine, Calgary, AB Canada. Cells were propagated without the use of trypsin at 37°C under an atmosphere of 5% CO₂ in room air in 80 cm² T-flasks using Dulbecco's minimal medium supplemented with 10% v/v fetal calf serum. Confluent cell monolayers to be used for calcium-signaling measurements were rinsed free of growth medium and disaggregated with calcium-free isotonic phosphate-buffered saline. Disaggregated cells were pelleted by centrifugation and were resuspended in 1 ml Dulbecco's minimal medium/10% fetal calf serum for loading with the calcium indicator, fluo-3 (Molecular Probes Inc., Eugene, OR) at a final concentration of 22 μM (25 μg/ml) of fluo-3 acetoxymethyl (AM) ester. Indicator uptake was allowed to proceed for 20–25 min at room temperature in the presence of 0.25 mM sulfapyrazone, after which cells were washed two times by centrifugation and resuspension with the buffer described below, so as to remove excess dye. Cells loaded with fluo-3 were then resuspended to yield a stock suspension of about 6 × 10⁶ cells/ml in a buffer, pH 7.4, of the following composition: NaCl (150 mM), KCl (3 mM), CaCl₂ (1.5 mM), HEPES (20 mM), glucose (10 mM), and sulfapyrazone (0.25 mM). Fluorescence measurements, reflecting elevations of intracellular calcium, were conducted at 24°C, using a Perkin-Elmer fluorescence spectrophotometer, with an excitation wavelength of 480 nm and an emission recorded at 530 nm. Cell suspensions (about 2 ml at a concentration of approximately 3 × 10⁵ cells/ml) were maintained in suspension in a stirred (magnetic flea bar) thermostatted plastic cuvette (total volume 4 ml), and peptide stock solutions were added directly to the cuvette to monitor peptide-induced changes in fluorescence. To construct concentration-effect curves for fluorescence yield, the signals caused by a test peptide were expressed as a percentage (% A23187) of the fluorescence peak height yielded by replicate cell suspensions, when treated with 2 μM of ionophore, A23187 (Sigma, St. Louis, MO). This concentration of A23187 was at the plateau of its concentration-effect curve for a fluorescence response. Under the assay conditions, we established by high performance liquid chromatography analysis (as in the past, Tay-Uyboco et al., 1995) that peptide degradation did not occur, and we determined that the presence of proteinase inhibitors (e.g., amastatin) did not potentiate the action of peptides in the assay.

Peptides and Other Reagents. All peptides were synthesized by solid phase methods at the Peptide Synthesis Facility, The University of Calgary, Faculty of Medicine, Calgary, AB Canada, (Director, Dr. D. McMaster), or were provided through the courtesy of Dr. L. Leblond, via the peptide synthesis facility at BioChem Therapeutic, Laval, PQ Canada. The composition and purity of all peptides were confirmed by high performance liquid chromatography analysis, mass spectral analysis, and amino acid analysis. Stock solutions, prepared in 25 mM HEPES buffer pH 7.4, were analyzed by quantitative amino acid analysis to verify peptide concentrations and purity. Thapsigargin (TG) was from Sigma (St. Louis, MO).

Evaluation of Receptor Desensitization and Cross-Desensitization of PAR₁ and PAR₂. The receptor desensitization assay made use of the principle that repeated exposure of a tissue to an agonist that is receptor-selective leads to a diminution/desensitization of the receptor's response to that agonist, but not to the tissue's response to a second agonist, which activates a distinct receptor system. The desensitization approach that we employed previously to demonstrate the independent activation of PAR₁ and PAR₂ in a gastric longitudinal muscle strip bioassay (Saifeddine et al., 1996) was expanded upon, using the calcium signal yielded by receptor activation in HEK cells as an index of agonist activity. The key to the analysis of the PAR₁-targeted ligands that we evaluated in this study lies in the use of the PAR₁-selective agonist, TF-NH₂ and the PAR₂-selective agonist, SLIGRL-NH₂ (SL-NH₂). In the desensitization protocol described in detail in the following paragraph, both of these receptor-selective agonists were used at concentrations in the mid-range of their concentration-effect curves (10–30 μM). These concentrations selectively activate either PAR₁ (TF-NH₂) or PAR₂ (SL-

NH₂). At these concentrations of receptor-selective agonists, the pretreatment of cells with any test compound that activated/desensitized or blocked one of the receptors would result in a diminution of the calcium signal subsequently generated by adding the receptor-selective agonist (TF-NH₂ or SL-NH₂) cumulatively, without removing the test compound from the cuvette.

Before evaluating the effects of the putative PAR₁-targeted reagents, each fluo-3-loaded HEK cell suspension was first assessed for the fluorescence response to the PAR₁- and PAR₂-selective agonists, TF-NH₂ (10 μM) and SL-NH₂ (30 μM). Only preparations yielding a fluorescence response to TF-NH₂ and SL-NH₂ that was at least 60% of the response caused by the addition of 2 μM A23187 were used for an extensive assay. This concentration of the ionophore (2 μM) was at the plateau of its concentration-effect curve. After quantitating a "standardized" fluorescence response to each of the receptor-selective agonists, the following protocol was used to evaluate the actions of the test compounds we wished to study for their effect on either PAR₁ or PAR₂. 1) The test compound of interest was first added to a cell suspension, and the calcium signal generated (peak height) was recorded. 2) The fluorescence signal was allowed to return to baseline (e.g., see Fig. 1), and exactly 10 min thereafter (a time required for the refilling of intracellular calcium stores), a standard concentration of either TF-NH₂ (10 μM) or SL-NH₂ (30 μM) was added to the cuvette in the continued presence of the test compound. 3) Immediately thereafter, the response of a fresh cell suspension to the same standard concentration of either TF-NH₂ or SL-NH₂ was monitored. The diminution of the fluorescence response to the standard concentration of either TF-NH₂ (10 μM) or SL-NH₂ (30 μM), caused by the earlier addition of the test compound, served as an indicator of the test compound's ability to block/desensitize either PAR₁ (TF-NH₂ signal) or PAR₂ (SL-NH₂ signal) respectively. The ability of any test compound to desensitize or block either PAR₁ or PAR₂ was expressed as a residual percentage (% control) of the fluorescence signal caused by either the PAR₁-selective (TF-NH₂) or PAR₂-selective (SL-NH₂) agonist in the presence of a test compound, relative to the signal measured for either PAR₁ (TF-NH₂) or PAR₂ (SL-NH₂) activation in the absence of test compound. In this manner, with a single assay series, concentration-effect curves were constructed for each individual test compound for their ability to: 1) cause a Ca⁺⁺ signal in HEK cells, 2) desensitize or block PAR₁, and 3) desensitize or block PAR₂. In a similar manner, by varying the time of addition of TG, after the earlier addition of an agonist that yielded a strong fluorescence signal (e.g., lysophosphatidic acid, TF-NH₂, or SL-NH₂), it was possible to assess, albeit indirectly, the time course of the refilling of intracellular calcium stores that could be mobilized by agonist action.

Preparation of HEK Cell RNA and Detection of PAR₁, PAR₂, and PAR₃ mRNA by the Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR). HEK cell monolayers were grown to confluence, and total RNA was prepared using the TRI-reagent (Molecular Research Center, Cincinnati, OH). The RNA was reverse transcribed (RT) with a first strand cDNA synthesis kit using pd(N)6 primer (Pharmacia LKB Biotechnology, Uppsala, Sweden) according to manufacturers recommendations at 37°C for 60 min; 3 μl of this solution was used with primer pairs targeted to human (h)PAR₁, PAR₂, and PAR₃. For (h)PAR₁, the primer pairs were: forward primer, PAR₁ F: 5' AAAAGCTTCCCGCTCATTTTCTCAGGAA 3', and reverse primer PAR₁ R: 5' GGGAATCAATCGGTGCCG-GAGAAGT3'. For (h)PAR₂, the primer pairs were: forward primer, PAR₂ F: 5' CACCACCTGTCACGATGTGCT3' and reverse primer, (h)PAR₂ R: 5'CCCGGGCTCAGTAGGAGGTTTAAACAC3'. For (h)PAR₃, the primer pairs were: forward primer, PAR₃ F: 5'TTT(T/G)TCAT(A/C)CTGAAGCAGGA3'; and for the reverse primer, PAR₃ R: 5'CTATTTTGTAAAGGTAAGCAG3'. The signal yielded by the three sets of PAR primer pairs were normalized to the polymerase chain reaction (PCR) signal generated concurrently by an actin primer pair (Watson et al., 1992) that spanned an actin intron: forward primer: 5'CGT GGG CCG CCC TAG GCA CCA3'; reverse

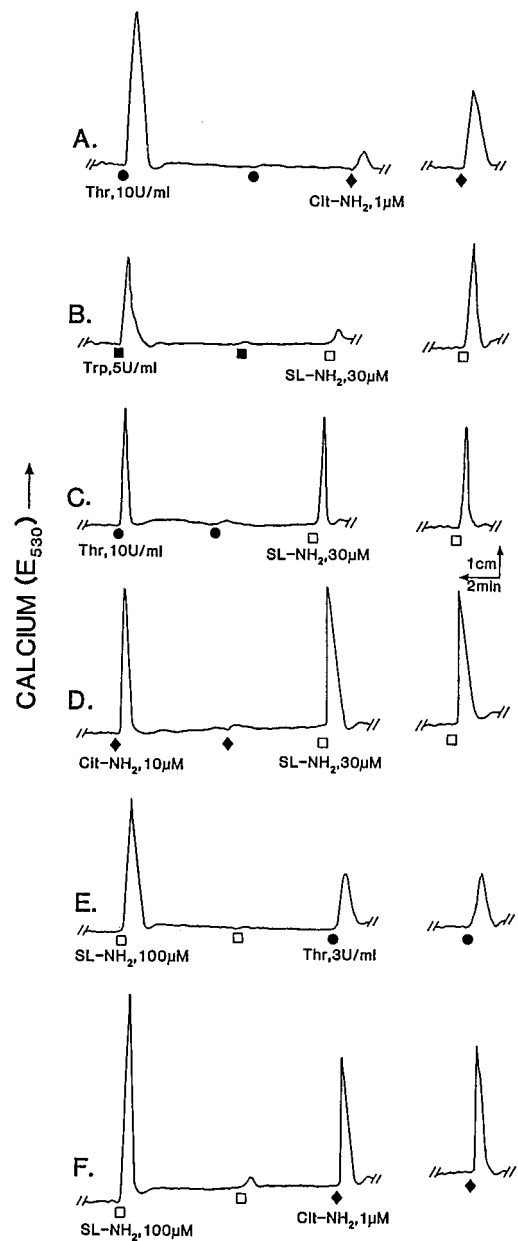


Fig. 1. Homologous desensitization of PAR₁ by thrombin and PAR₂ by trypsin, as well as lack of heterologous desensitization either of PAR₂ by PAR₁ activation due to thrombin or Cit-NH₂, or of PAR₁ by SL-NH₂. HEK cell suspensions were first exposed twice to receptor-desensitizing concentrations of thrombin (Thr, ●, 10 U/ml or 100 nM, to desensitize PAR₁: tracings A and C), trypsin (■, 5 U/ml or 10 nM, to desensitize PAR₂: tracing B), Cit-NH₂ (◆, 10 μM to desensitize PAR₁: tracing D), or SL-NH₂ (□, 100 μM, to desensitize PAR₂: tracings E and F), with continuous recording of the fluorescence tracing (E₅₃₀). At 10 min after the addition of the first agonist to the cuvette, a test concentration of the second agonist was added (e.g., thrombin, followed by a test concentration of the PAR₁-AP, Cit-NH₂: tracing A). The fluorescence signal caused by the sequential addition of the test compound after receptor-specific desensitization was compared with the signal caused by the same concentration of test compound without earlier desensitization of the cells (right-hand portion of tracings A to F.)

primer: 5'TTG GCC TTA GGG TTC AGG GGG3'. The detection of an intron-free 243 base-pair product using this primer pair can confirm the absence of DNA-derived intron sequences in the RT product obtained from tissue RNA. Routinely, amplification was obtained using 2.5 units of *Taq* DNA polymerase (Promega, Madison, WI) in a 10 mM Tris-HCl buffer, pH 9.0 (50 μl, final volume) containing

MgCl₂ (1.5 mM), KCl (50 mM), 0.1% v/v Triton X-100, and 0.2 mM each of deoxynucleotide triphosphates. The amplification reaction was allowed to proceed for 40 cycles, beginning with a 1-min denaturing period at 94°C, followed by a 1-min reannealing time at 55°C, then a 1-min primer extension period at 72°C. The PCR products were separated by 1.5% agarose gel electrophoresis and visualized by ethidium bromide. PCR products were subcloned into the pGEM-T Vector (Promega, Madison WI) for sequencing by the dideoxynucleotide chain termination method (Sanger et al., 1977), employing a T₇DNA sequencing kit (Pharmacia). Sequencing was done in both the 5' and 3' directions.

Measurement of Platelet Aggregation. Washed platelets were isolated from citrate-anticoagulated plasma obtained from healthy volunteers who denied the use of nonsteroidal anti-inflammatory agents or other platelet-targeted drugs over at least the preceding 2 weeks. Washed platelet suspensions were prepared as outlined previously (Mustard et al., 1972), and aggregation was monitored by light scattering measurements done at 37°C using stirred platelet suspensions (400 μl) in a Bio Data aggregometer. Peptides in a volume of 50 μl were added directly to the stirred platelet suspension, and the degree of aggregation observed after a 3-min time period was expressed as a percentage of the maximum aggregation caused by each agonist. The use of the washed platelet suspension eliminated the need to add an aminopeptidase inhibitor (e.g., amastatin), as required for the use of platelet-rich plasma samples. Concentration-effect curves for the aggregating activity of peptides were thereby constructed, and the potency of each peptide was expressed as a concentration for which aggregation was half-maximal (EC₅₀). To assess the activity of putative PAR₁ inhibitors, each compound (in 50 μl) was added to the platelet suspension 1 min before the addition of either thrombin (in 50 μl of buffer: 0.015–0.1 U/ml, final concentration) or SFLLR-NH₂, (final concentration 3 μM). The concentration of thrombin used for platelets derived from different

donors was based on the minimum thrombin concentration required to cause maximal platelet aggregation. Once determined, this minimum effective thrombin concentration was used to estimate the IC₅₀ for the putative PAR₁ inhibitors.

Results

RT-PCR Detection of PARs in HEK Cells. We wished to obtain a semiquantitative estimate of the abundance of PAR₁ and PAR₂ receptors in the HEK cells by a biochemical method. To this end, we used an RT-PCR approach, with amplimers targeted to these receptors; we also used primer pairs targeted to the more recently-described thrombin receptor, PAR₃ (Ishihara et al., 1997) to assess its abundance in the HEK cells, relative to PAR₁ and PAR₂. Relative to the PCR signal for actin, the relative intensities of the PCR signals for the three PARs were: PAR₃ > PAR₂ > PAR₁ (data not shown). It was not possible, with anti-receptor antibodies, to detect receptor protein for any of the PARs in the HEK cell line. Because PAR₃ can be activated only by thrombin and not by a variety of receptor-derived activating peptides related to SF-NH₂ or Cit-NH₂ (Ishihara et al., 1997), we anticipated that the presence of PAR₃ in the HEK cells would not be an interfering factor in the desensitization assay, which is described in the sections to follow.

Validation of the HEK Assay: Lack of Heterologous Receptor Cross-Desensitization and Adequacy of Calcium Stores for Repetitive Cell Activation. Before proceeding with the HEK calcium signaling assay, which depends upon the principle of homologous receptor activation/

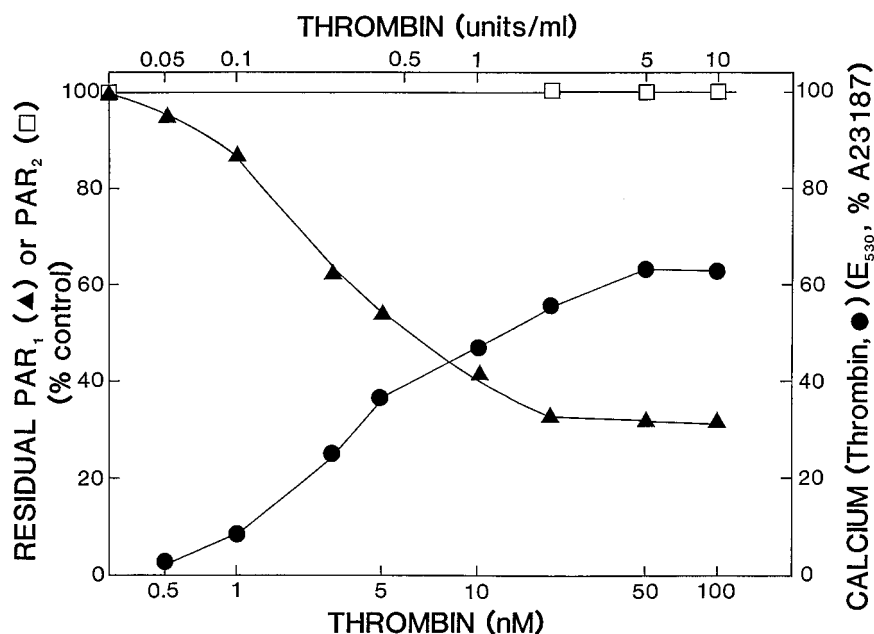


Fig. 2. Concentration-Desensitization (▲, □) and concentration-stimulation (●) curves for thrombin-mediated activation of HEK cells. As illustrated in Fig. 2, cells were first stimulated by the addition of thrombin (concentrations ranging from 0.5–100 nM; 0.05–10 U/ml), and the magnitude of the calcium signal (% A23187, ●) relative to that caused by 2 μM A23187 in an aliquot of the indicator HEK cells was monitored. At 10 min after the addition of thrombin, a test concentration of either the PAR₁-AP, TF-NH₂ (▲, 10 μM), or the PAR₂-AP, SL-NH₂ (□, 30 μM) was added to the same cells that had been pretreated with thrombin, and the fluorescence signal caused by the second addition of agonist was monitored. The residual fluorescence signal measured after thrombin pretreatment was expressed as a percentage [% residual PAR₁ (▲) or PAR₂ (□)] of the control fluorescence signal observed by activating either PAR₁ or PAR₂ in HEK cell aliquots with the addition of either 10 μM TF-NH₂ or 30 μM SL-NH₂ to cells without thrombin pretreatment. Data points represent average values for measurements done with three or more separate cell suspensions, for which the standard errors of the mean fell within the size of the symbols shown. The figure is representative of experiments done on three or more independently grown crops of HEK cells. Thrombin pretreatment did not affect the subsequent activation of PAR₂ by SL-NH₂ (□), but desensitized 70% of the subsequent activation of PAR₁ by TF-NH₂ (▲).

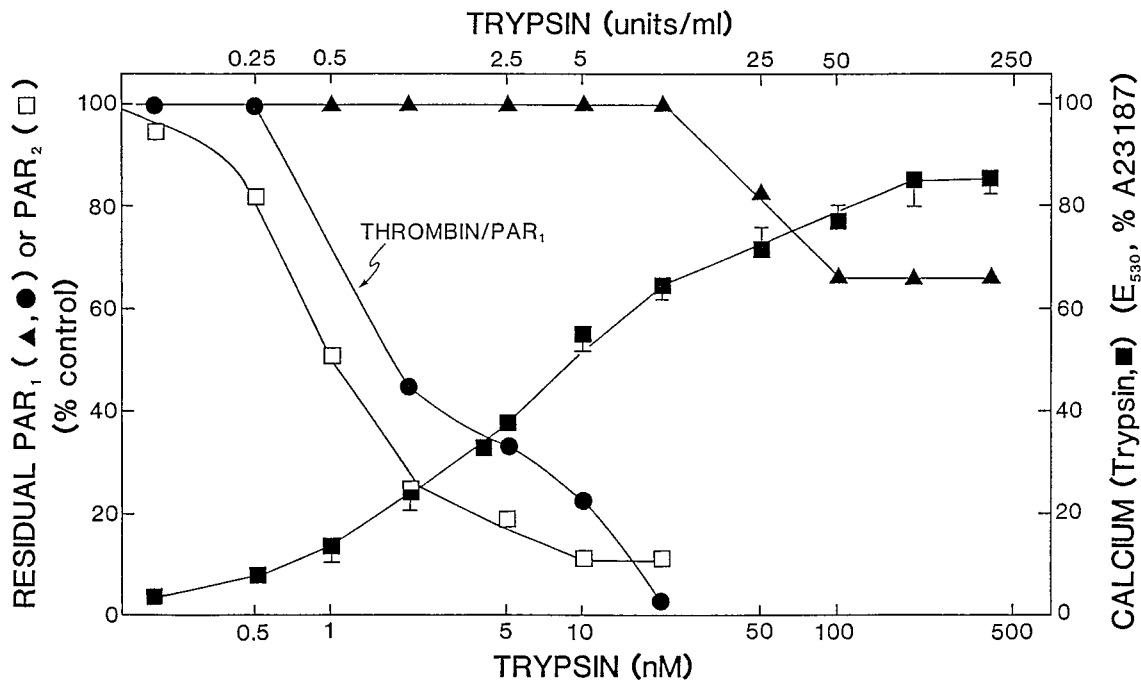


Fig. 3. Concentration-desensitization (▲, □, ●) and concentration-stimulation (■) curves for trypsin-mediated activation of HEK cells. As for thrombin (Fig. 2), HEK cell suspensions were first stimulated by the addition of trypsin (0.2–500 nM; 0.1–250 U/ml), and the calcium signal (% A23187, ■) relative to that caused by 2 μM A23187 was measured. Following trypsin treatment, cells were exposed to either (10 nM; 1 U/ml) thrombin (●) or 10 μM TF-NH₂ (▲) to monitor PAR₁ activation, and to 30 μM SL-NH₂ for activating PAR₂ (□). The residual fluorescence signal measured after trypsin pretreatment was expressed as a percentage [% residual PAR₁ monitored by TF-NH₂ (▲) or thrombin (●) stimulation; or % residual PAR₂ monitored by SL-NH₂ (□) stimulation] of the control fluorescence signal measured without earlier trypsin treatment, by activating PAR₁ with either thrombin (●) or TF-NH₂ (▲) and by activating PAR₂ with SL-NH₂ (□). Values represent the means (±S. E. M., bars, except where error bars were smaller than the size of the symbols) for measurements done with three or more cell aliquots. The data are representative of experiments done with two or more separately grown crops of HEK cells.

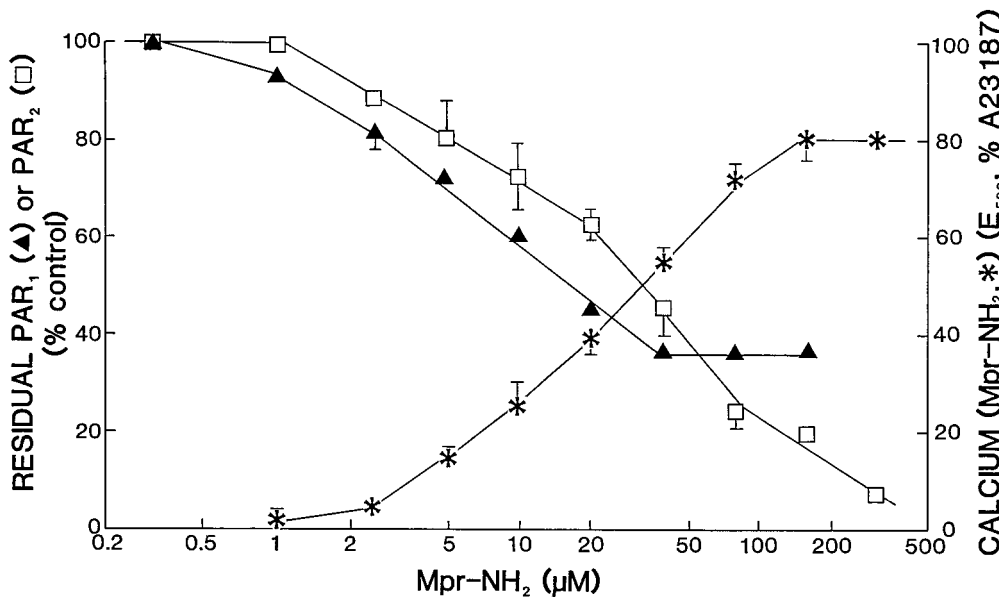


Fig. 4. Diminished calcium signal in response to the addition of either PAR₁- (▲) or PAR₂- (□) activating peptides by pretreatment of HEK cells with the putative PAR₁ antagonist, Mpr-NH₂. As illustrated for thrombin in Fig. 2, HEK cell suspensions were exposed first to Mpr-NH₂ (*, 1–300 μM) and the calcium signal (E₅₃₀, % A23187, *) relative to 2 μM A23187, was measured. At 10 minutes after the earlier addition of Mpr-NH₂, the residual PAR₁ (▲) or PAR₂- (□) mediated calcium fluorescence response (% control) relative to that observed in identical cell suspensions that had not been pretreated with Mpr-NH₂, was monitored by the addition of either the PAR₁-AP, TF-NH₂ (10 μM, ▲) or the PAR₂-AP, SL-NH₂ (30 μM, □). Values represent the means (±S. E. M., bars) for measurements done with triplicate cell suspensions derived from two or more separately grown crops of HEK cells.

desensitization and upon a receptor-mediated elevation of intracellular Ca⁺⁺, it was necessary to answer two main questions with regard to the system: 1) Did the protocol of sequential cell stimulation by one agonist/antagonist followed by another allow sufficient time for the refilling of intracellular calcium stores, such that the calcium signal yielded by the addition of the first agonist would not, simply because of inadequate calcium stores, diminish the calcium signal yielded by the sequential addition of the second ago-

nist? 2) Even if calcium stores proved adequate, was there heterologous receptor cross-desensitization between PAR₂ and PAR₁ in the HEK cell system, as has been documented in the endothelial cell, wherein PAR₂ activation can cross-desensitize PAR₁ (Mirza et al., 1996)?

To answer the first question concerning the adequate refilling of intracellular calcium stores, after agonist stimulation, we employed two approaches. In the first approach, cells were first stimulated by a strong calcium-releasing agonist

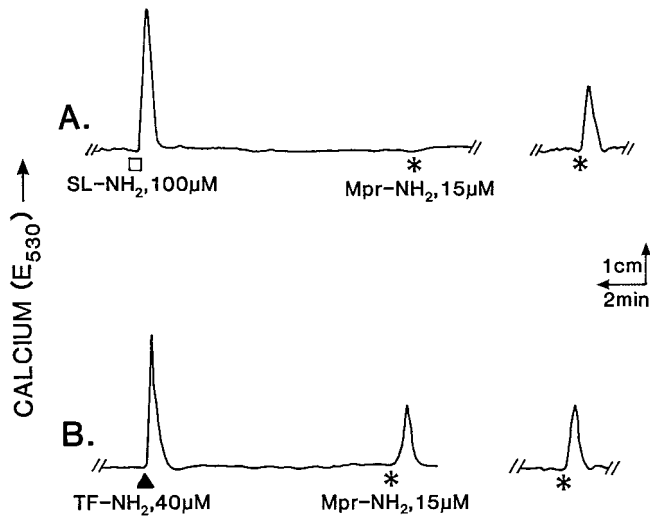


Fig. 5. Desensitization of the Mpr-NH₂-mediated calcium signal by desensitization of PAR₂ but not by desensitization of PAR₁. HEK cell suspensions were first exposed to receptor-desensitizing concentrations of either the PAR₂-AP, SL-NH₂ (□, 100 μM, tracing A) or the PAR₁-AP, TF-NH₂ (▲, 40 μM, tracing B). After 10 min, a test concentration of Mpr-NH₂ (*, 15 μM) was added to the cell suspensions, with a continuous recording of calcium-mediated fluorescence (E₅₃₀, scale for time and fluorescence shown between tracings A and B). The calcium signal caused by 15 μM Mpr-NH₂ (*) in a cell suspension that had *not* been previously exposed to either SL-NH₂ (tracing A) or TF-NH₂ (tracing B) is shown to the right of each tracing. The tracings are representative of three or more independently conducted experiments with replicates derived from different HEK cell crops.

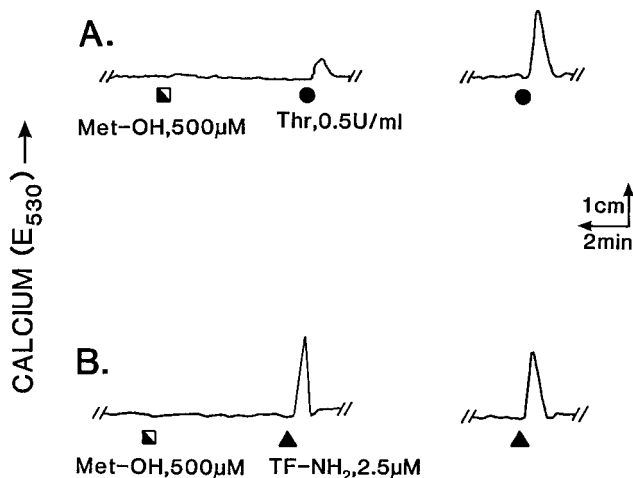


Fig. 6. Inhibition by Met-OH of PAR₁ activation by thrombin but not by TF-NH₂. HEK cell suspensions were first exposed to a concentration of Met-OH (□, 500 μM) above that previously reported to inhibit thrombin-mediated platelet activation (Doorbar and Winter, 1994). After 10 min, the residual PAR₁-mediated calcium fluorescence signal (E₅₃₀) generated by the addition of either thrombin (●, 5 nM; 0.5U/ml, tracing A) or the PAR₁-AP, TF-NH₂ (▲, 2.5 μM, tracing B) was monitored. The calcium signal caused in an identical cell aliquot by the addition of either thrombin (●, tracing A) or TF-NH₂ (▲, tracing B) to cells that had *not* been pretreated with Met-OH is shown in the right-hand portion of each tracing. The tracings are representative of two independently conducted experiments. The scale for time and calcium signal is shown between the tracings.

(e.g., lysophosphatidic acid, LPA) as well as by selected PAR-APs; thereafter, at timed intervals, cells were treated with TG, and the calcium signal was monitored. In HEK cells, TG caused an elevation of intracellular calcium, presumably by

blocking calcium store reuptake and allowing the depletion of stored intracellular calcium. In the second approach, we used LPA to cause a strong receptor-mediated signal derived from intracellular calcium stores, followed at timed intervals by the selective activation of either PAR₁ or PAR₂. The question concerning PAR₁/PAR₂ cross-desensitization was answered by observing the calcium response caused by the sequential stimulation of PAR₁ and PAR₂, at increasing time intervals, with the receptor-selective agonists, TF-NH₂ and SL-NH₂.

Our validation study revealed the following information, which provided the basis for the desensitization assay illustrated in the following sections: 1) a time interval of 10 min was required after the addition of a single agonist to the assay cuvette to permit the refilling of calcium stores, as assessed by a TG-induced calcium signal; and 2) a time interval of at least 10 min between the sequential addition of different agonists to the assay cuvette was also required for each agonist to cause a full calcium signal. This time interval was presumably due to the time frame required for refilling the calcium stores. This presumption was supported by the observation that pretreatment of cells with TG (0.2 μM) abolished the calcium signal caused by the subsequent addition of LPA, TF-NH₂, or SL-NH₂ (data not shown); the omission of extracellular calcium from the suspension buffer did not diminish the fluorescence signal caused by the first addition of agonist (LPA, TF-NH₂, or SL-NH₂), relative to the signal observed in the routine calcium-containing (1.5 mM) medium (not shown). We therefore concluded that, for the purpose of our assay, a time period of 10 min between the cumulative addition of the first and second agonist to the assay cuvette was sufficient for the cells to replenish the agonist-sensitive calcium stores responsible for the fluorescence signal. Furthermore, our indirect approach suggested that a TG-sensitive intracellular calcium store was responsible for the fluorescence signal generated by activation of the PAR₁, PAR₂, and LPA receptors.

Homologous Versus Heterologous PAR Desensitization in HEK Cells. Because a cross-desensitization between PAR₂ and PAR₁ had been observed in human endothelial cell cultures (Mizra et al., 1996), it was important to establish in the HEK cell system that the activation of PAR₁ did not simultaneously cross-desensitize PAR₂ and vice versa. To deal with this issue, we first used thrombin and SL-NH₂ as selective activators of PAR₁ and PAR₂, respectively. We then used a new PAR₁-selective receptor-activating peptide (Ala-parafluoroPhe-Arg-Cha-Cit-Tyr-NH₂) (Cit-NH₂) as well as the previously described PAR₁-selective agonist, TF-NH₂ (Hollenberg et al., 1997), and the PAR₂-selective agonist, SL-NH₂, to assess the cross-desensitization of PAR₁ and PAR₂ in the HEK cell system. The principle of the desensitization assay is illustrated by the data in Fig. 1. As expected, activation of PAR₁ by thrombin desensitized the subsequent HEK cell response to the selective PAR₁ agonist, Cit-NH₂ (tracing A, Fig. 1). Activation of PAR₂ by trypsin similarly desensitized the subsequent HEK cell response to the selective PAR₂ agonist, SL-NH₂ (tracing B, Fig. 1). However, as shown in tracing C of Fig. 1, activation of PAR₁ by thrombin did not affect the PAR₂ signal caused by the PAR₂-selective agonist, SL-NH₂, nor did activation of the thrombin receptor by the PAR₁ receptor-selective agonist, Cit-NH₂, diminish the response to SL-NH₂ (tracing D, Fig. 1). Furthermore, activation of PAR₂ by SL-NH₂ did not cause cross-desensiti-

zation of the response to thrombin; nor did PAR₂ activation cross-desensitize the HEK cell response to Cit-NH₂ (tracings E and F, Fig. 1). In view of the results described in the above paragraphs, we were able to arrive at the standard cross-desensitization protocol described in detail in the *Materials and Methods* section. The compounds that we wished to evaluate for their ability to affect either PAR₁ or PAR₂ are summarized in Table 1.

Selective Desensitization of PAR₁ by Thrombin. Having established the working protocol for the assay of the PAR₁/PAR₂ selectivity of peptide agonists, we sought to deal with the following questions: 1) What type of desensitization of PAR₁ and PAR₂ was caused by the enzymes, thrombin (Fig. 2) and trypsin (Fig. 3)? 2) Could we obtain evidence for the activation of PAR₃ in the HEK cells? 3) What was the selectivity/activity of previously described thrombin receptor antagonists (Figs. 4–8)? 4) What was the relative PAR₁/PAR₂ selectivity of previously described PAR₁-targeted agonists (Fig. 9)? 5) What was the relative PAR₁/PAR₂ selectivity of the originally described thrombin receptor-activating peptides (referred to in the literature as TRAPs), SF-NH₂, and SF₁₄-NH₂ (Fig. 10)? Finally, we wished to determine if the desensitization protocol that we had so far developed (see above sections) might depend on the nature of the receptor-selective agonist used to generate the standard PAR₁ or PAR₂ signal (steps 1 and 2 described in the preceding paragraph.) (See Fig. 11).

Our attention was focused first on questions 1 and 2, using thrombin, known as a selective activator of either PAR₁ or PAR₃, and trypsin, an enzyme known to activate both PAR₁ and PAR₂. In previous work we had established that 40 μM concentrations of either TF-NH₂ or SF-NH₂ were able to desensitize the HEK cell response to other PAR₁-activating peptides or to thrombin (Hollenberg et al., 1997). But we had not evaluated fully the ability of increasing concentrations of

thrombin to desensitize the HEK cell response to PAR-activating peptides. As shown in Fig. 2, increasing concentrations of thrombin, up to 100 nM (10 U/ml) were able selectively to desensitize the HEK cell response to the selective PAR₁-activating peptide, TF-NH₂ (▲, Fig. 2) without affecting at all, the response to the PAR₂-selective peptide, SL-NH₂ (□, Fig. 2). The EC₅₀ for the ability of thrombin to generate a calcium signal in the HEK cells (about 3.3 nM: right-hand ordinate, Fig. 2) reflected the IC₅₀ for the ability of thrombin to desensitize the cellular response to TF-NH₂ (about 2.5 nM: left-hand ordinate, Fig. 2). Nonetheless, even at its plateau concentration, thrombin was not able to desensitize the response to TF-NH₂ by more than 70% (▲, Fig. 2).

Cross-Desensitization of PAR₁ by Trypsin: Quantitative Estimate of Receptor Selectivity. In contrast with thrombin, which cannot activate PAR₂, elevated concentrations of trypsin (greater than about 20 U/ml or 40 nM) can also activate PAR₁ (Vu et al., 1991). Therefore, with the cross-desensitization assay, we used trypsin to illustrate quantitatively the dual specificity of its ability to activate/desensitize PAR₂ at concentrations lower than about 5 U/ml (10 nM) and to activate/desensitize PAR₁ at concentrations higher than about 20 U/ml (>40 nM) (Fig. 3). Although trypsin was able to desensitize almost completely the response of PAR₂ to SL-NH₂, (Fig. 3), this protease was not able to desensitize more than about 35% of the PAR₁ response to TF-NH₂ (□, Fig. 3). Nonetheless, prior treatment of the cells with concentrations of trypsin that were lower than those required to desensitize the response to TF-NH₂, “disarmed” completely the ability of thrombin (●, Fig. 3) to activate PAR₁. In Fig. 3, the response of the HEK cells to trypsin, in terms of the fluo-3 calcium signal, could be the result of the combined activation of PAR₁ and PAR₂.

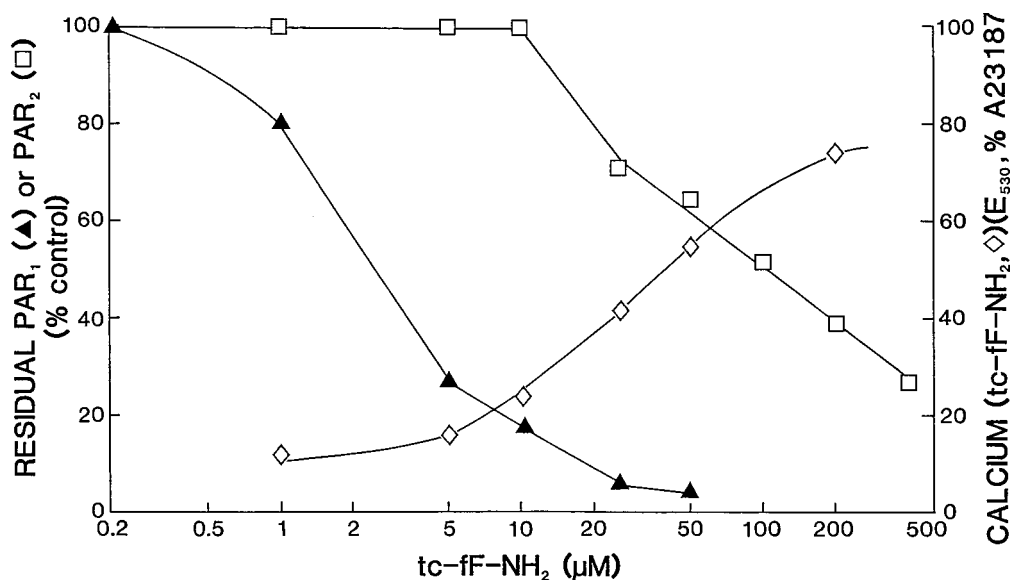


Fig. 7. Desensitization of PAR₁ (▲) and PAR₂ (□) by pretreatment of HEK cells with the putative PAR₁ antagonist, *trans*-Cinnamoyl-(f)F-R-L-R-O-NH₂. As illustrated for Mpr-NH₂ in Fig. 4, HEK cell suspensions were first exposed to tc-fF-NH₂ (◇, 1–400 μM) and the calcium signal thereby generated (E₅₃₀) was measured as a percentage (% A23187,) relative to 2 μM A23187. At 10 min after the earlier addition of tc-fF-NH₂, the residual PAR₁- (▲) or PAR₂- (□) mediated calcium fluorescence response was measured as a percentage (% control ▲, □) of the control fluorescence response measured upon adding the test concentrations of either the PAR₁-AP, TF-NH₂ (▲, 10 μM), or the PAR₂-AP, SL-NH₂ (□, 30 μM) to identical cell suspensions that had not been pretreated with tc-fF-NH₂. Values represent the means for measurements done with triplicate cell suspensions (±S. E. M. smaller than size of symbols) obtained from two or more HEK cell crops.

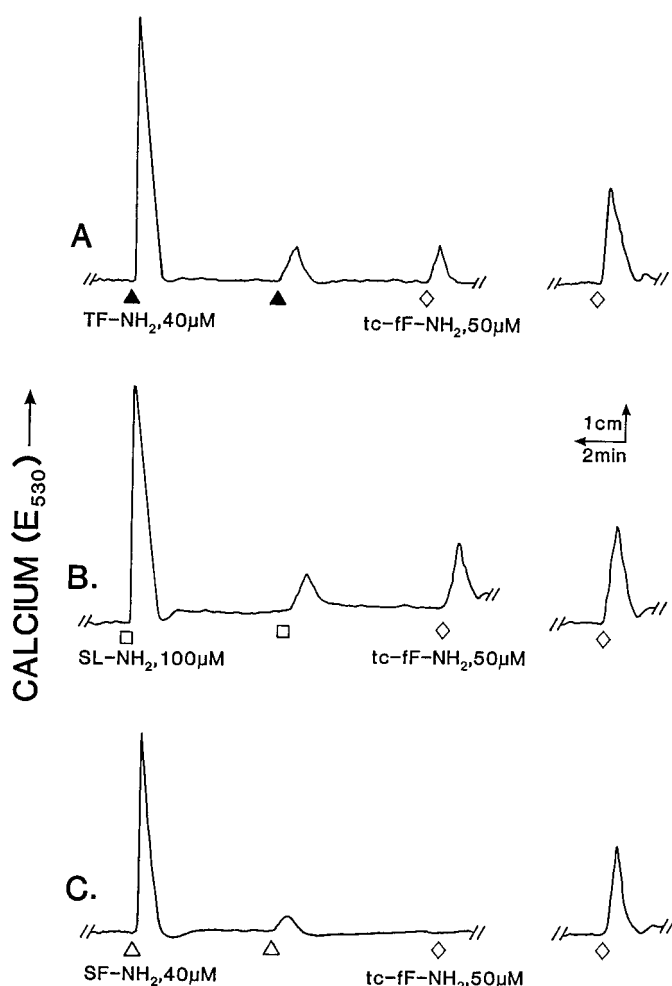


Fig. 8. Partial desensitization of the HEK cell calcium signal caused by *tc-fF-NH₂* caused by pretreatment with the PAR₁- and PAR₂-selective agonists, TF-NH₂ and SL-NH₂, as well as full desensitization by pretreatment with the nonselective PAR₁/PAR₂ agonist, SF-NH₂. HEK cell suspensions were first pretreated twice with desensitizing concentrations of either the PAR-selective agonists, TF-NH₂ (▲, 40 μM for PAR₁, tracing A) and SL-NH₂ (□, 100 μM for PAR₂, tracing B) or the nonselective PAR₁/PAR₂ agonist, SF-NH₂ (Δ, 40 μM, tracing C). After 10 min, a test concentration of *tc-fF-NH₂* close to its EC₅₀ for causing a calcium signal (star), 50 μM) was added to the cell suspension, with continuous monitoring of all the calcium fluorescence (E₅₃₀). The calcium signal caused by 50 μM *tc-fF-NH₂* (◇) in the agonist-pretreated cells (left-hand set of tracings A to C) was compared with the signal caused in identical cell suspensions that had not been desensitized by agonist pretreatment (right-hand set of tracings A to C). The experiment shown is representative of measurements done with two sets of independently prepared HEK cell suspensions. The scale for time and fluorescence is shown between tracings A and B.

Test for Thrombin-Activated PAR₃ Signaling in HEK Cells. Because PAR₃ is evidently insensitive to activation by PAR-activating peptides (Ishihara et al., 1997), we reasoned that the peptide, SFLLR-NH₂ (SF-NH₂), which can activate/desensitize both PAR₁ and PAR₂ (Hollenberg et al., 1997), would be able to desensitize both PAR₁ and PAR₂ without activating PAR₃. If at these concentrations of SF-NH₂ (about 40 μM), PAR₃ were not activated, we reasoned that, although a selective PAR₁-activating peptide would no longer yield a calcium signal, thrombin should still be able to activate PAR₃ to yield an increase in fluo-3 fluorescence. We found that, at concentrations of SF-NH₂ that desensitized the HEK cell response to both TF-NH₂ and SL-NH₂ (i.e., both PAR₁ and

PAR₂ were desensitized: not shown), no further fluorescence response to thrombin (10 U/ml; 100 nM) was observed. Similarly, after fully desensitizing PAR₁ with Cit-NH₂, thrombin (10 U/ml; 100 nM) failed to cause a fluorescence response (not shown). This concentration of thrombin (10 U/ml) would have been more than sufficient to activate PAR₃ (Ishihara et al., 1997).

Relative Selectivity of Putative PAR₁-Targeted Antagonists for PAR₁ Compared with PAR₂. Previous work had described a PAR₁ receptor antagonist, Mpr-Phe-Cha-Cha-Arg-Lys-Pro-Asn-Asp-Lys-NH₂ (Mpr-NH₂) (Seiler et al., 1995). We assessed the PAR₁/PAR₂ selectivity of Mpr-NH₂ using the HEK cell assay, as shown in Fig. 4. Prior treatment of HEK cells with Mpr-NH₂ led to a reduction in the subsequent ability of both TF-NH₂ and SL-NH₂ to activate PAR₁ and PAR₂ respectively, with comparable IC₅₀s for blocking or desensitizing both receptors. Mpr-NH₂ was able to block PAR₂ completely (□, Fig. 4), but was only able to block about 60% of the HEK cell PAR₁ response to TF-NH₂ (▲, Fig. 4).

On its own, Mpr-NH₂ activated the HEK cell calcium signal, with an EC₅₀ of about 20 μM. We wondered if the calcium-induced fluo-3 signal in response to Mpr-NH₂ was due to the activation of PAR₁, PAR₂ or perhaps both receptors. Therefore, we assessed the ability of Mpr-NH₂ to cause a fluorescence signal in HEK cells in which either PAR₁ or PAR₂ had previously been activated/desensitized by treatment of the cells with high concentrations of either the selective PAR₁-selective agonist, TF-NH₂ or the PAR₂-selective agonist, SLIGRL-NH₂ (SL-NH₂) (Fig. 5). We observed that exposure of the HEK cells to high concentrations SL-NH₂ (100 μM) abolished the calcium signal caused by Mpr-NH₂, (Fig. 5A), whereas pretreatment with comparatively high concentrations of TF-NH₂ (40 μM) did not affect the Mpr-NH₂-induced calcium signal (Fig. 5B). These data indicated that Mpr-NH₂ was an agonist only for PAR₂ and not for PAR₁; and that Mpr-NH₂ was a PAR₁ antagonist.

After evaluating the activity of the PAR₁ antagonist, Mpr-NH₂, we turned our attention to two other PAR₁ receptor antagonists that had also been previously described: the peptide, Met-Ser-Arg-Pro-Ala-Cys-Pro-Asn-Asp-Lys-Tyr-Glu, (Met-OH), discovered by a phage display approach (Doorbar and Winter, 1994) and the peptide, *trans*-cinnamoyl-parafluoroPhe-Arg-Leu-Arg-NH₂, which had been prepared by rational drug design (compound 76, Bernatowicz et al., 1996). In keeping with the data of Bernatowicz et al. (1996), we therefore tested the peptide, *trans*-cinnamoyl-parafluoroPhe-Arg-Leu-Arg-Orn-NH₂ (*tc-fF-NH₂*) as a potential PAR₁ ligand along with Met-OH. Met-OH, at concentrations as high as 800 μM, showed no ability either to cause a calcium signal in the HEK cells or to affect the action of SL-NH₂ in the HEK cell assay (data not shown). However, Met-OH between 400 and 800 μM was able to attenuate the calcium signal caused by 0.5 U/ml (5 nM) thrombin (70 ± 6% inhibition: average ± 1/2 range for duplicate measurements: Fig. 6, tracing A). Nonetheless, over the same concentration range, Met-OH potentiated (29 ± 3%: average ± 1/2 range for duplicate measurements) rather than inhibited the action of the PAR₁-activating peptide, TF-NH₂, even at comparatively low concentrations of TF-NH₂ (Fig. 6, tracing B).

In contrast with Met-OH, *tc-fF-NH₂* on its own activated a calcium signal in the HEK cells and was able to diminish the HEK cell calcium response to both the PAR₁-activating peptide, TF-NH₂, and the PAR₂-activating peptide, SL-NH₂ (Fig.

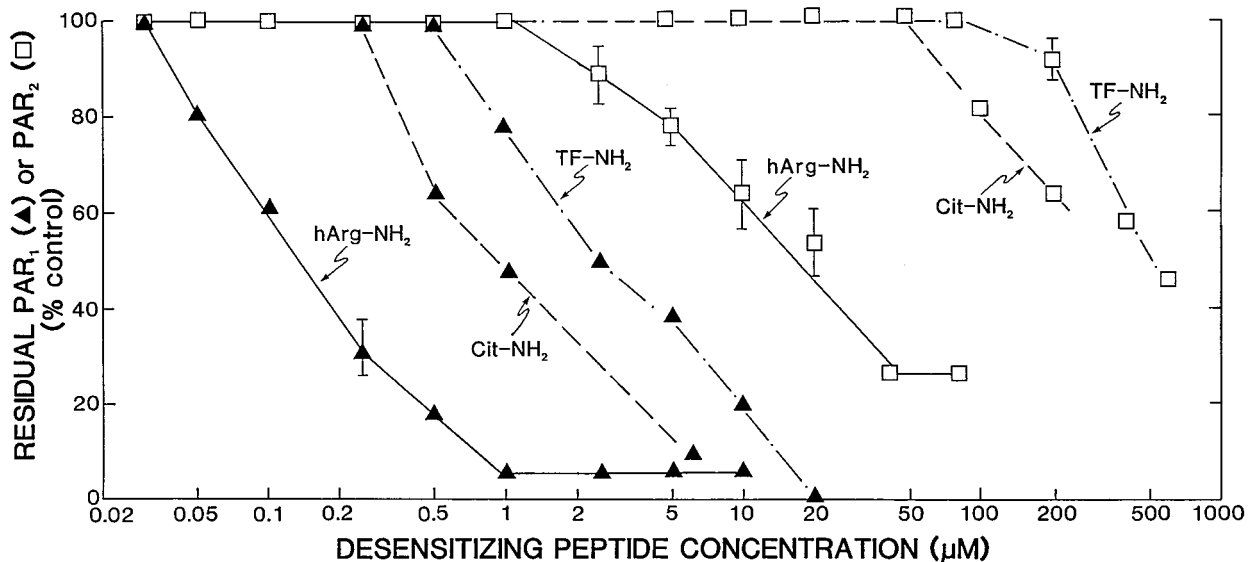


Fig. 9. Desensitization of PAR₁ (▲) and PAR₂ (□) by PAR₁-selective agonists: concentration-desensitization curves. As illustrated for thrombin in Fig. 2, HEK cell suspensions were first exposed to different concentrations of three PAR₁-selective agonists: hArg-NH₂ (solid lines), Cit-NH₂ (dash-dot lines), and TF-NH₂ (dashed lines). After 10 min, the cell suspension was challenged by the sequential addition of test concentrations of either the PAR₁-selective agonist, TF-NH₂ (▲, 10 µM) or the PAR₂-selective agonist, SL-NH₂ (□, 30 µM). The residual PAR₁ (▲) or PAR₂ (□) calcium signal (E_{530}) observed in the agonist-pretreated cells was measured as a percentage (% control) of the calcium signal caused by either 10 µM, TF-NH₂ (▲) or 30 µM, SL-NH₂ (□) in identical cell suspensions that had *not* been pretreated with the PAR₁-selective agonists. Values represent the means (\pm S.E.M., bars) for measurements done with three or more replicate cell suspensions coming from two or more independently grown HEK cell crops. Error bars smaller than the symbol size are not shown.

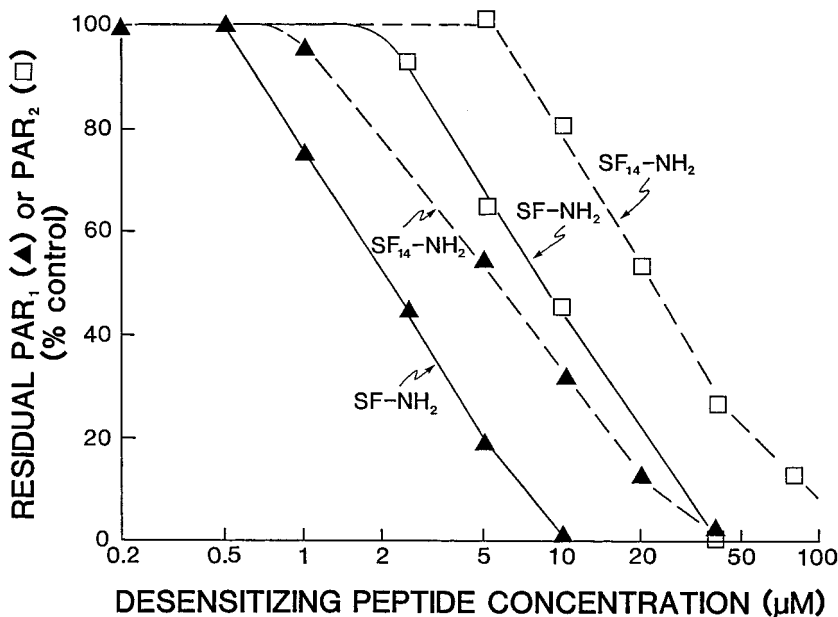


Fig. 10. Desensitization of PAR₁ (▲) and PAR₂ (□) by the originally described thrombin receptor-activating peptides SFLLR-NH₂ and SFLLRNPNDKYEPF-NH₂: concentration-desensitization curves. As illustrated for the PAR₁-selective agonists in Fig. 9, concentration-desensitization curves were obtained for the ability of the originally described thrombin receptor-activating peptides, SF₁₄-NH₂ (dashed lines) and SF-NH₂ (solid lines) to desensitize either PAR₁ (▲) or PAR₂ (□). Values represent the means of measurements done with triplicate cell suspensions obtained from two or more separately grown HEK cell crops; standard error bars were smaller than the symbols shown.

7). Unlike Mpr-NH₂ (Fig. 5), the calcium signal caused by tc-fF-NH₂ was diminished (but not eliminated) by pretreating the cells twice in succession with either TF-NH₂ or SL-NH₂ (tracings A and B, Fig. 8). Pretreating the cells with 40 µM SFLLR-NH₂ (this peptide simultaneously desensitizes both PAR₁ and PAR₂) completely eliminated the signal generated by tc-fF-NH₂ (tracing C, Fig. 8). Thus, tc-fF-NH₂ was a partial agonist at both PAR₁ and PAR₂.

Relative Selectivity of PAR₁-Targeted Agonists for PAR₁ Compared with PAR₂. In previous work, the peptide, Ala-parafluoroPhe-Arg-Cha-hArg-Tyr-NH₂ (hArg-NH₂) had been synthesized as a high potency PAR₁ agonist for use as a PAR₁ receptor binding probe (Feng et al., 1995; Ahn et

al., 1997). In accord with these studies, we synthesized the peptide Ala-parafluoroPhe-Arg-Cha-Cit-Tyr-NH₂ (Cit-NH₂) as a potential alternative to hArg-NH₂ for use as a PAR₁ receptor binding probe; and we had already synthesized the peptide, TF-NH₂, which in preliminary work appeared to be a PAR₁-selective agonist (Hollenberg et al., 1997). We first assessed the relative PAR₁/PAR₂ specificity of hArg-NH₂ and, in parallel, evaluated the receptor selectivity of Cit-NH₂ and TF-NH₂ (Fig. 9), although as expected, both hArg-NH₂ and Cit-NH₂ were able to activate/desensitize PAR₁, hArg-NH₂ at concentrations between 2 and 40 µM was also able to activate/desensitize PAR₂ by more than 60%. In contrast, Cit-NH₂, at concentrations as high as 50 µM did not affect

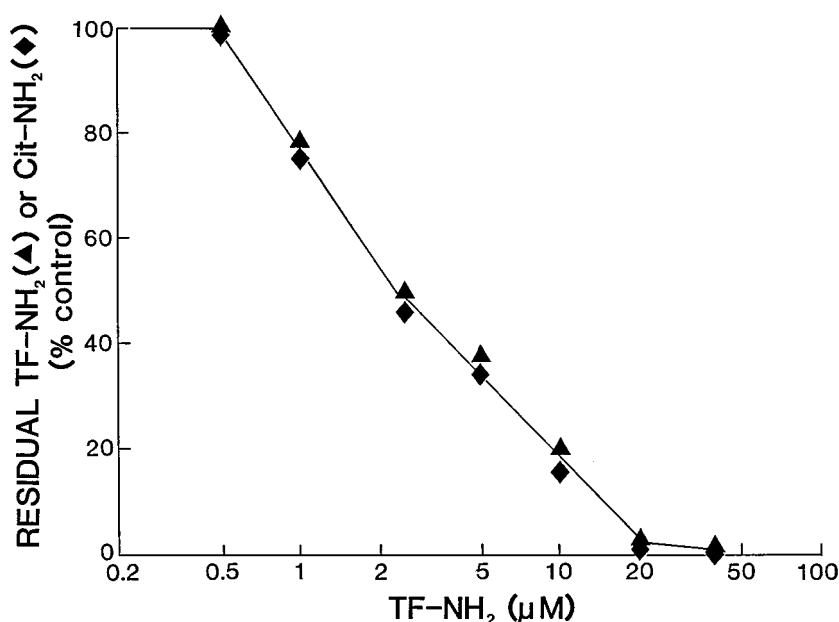


Fig. 11. Concentration-desensitization curves for TF-NH₂ using test concentrations of either Cit-NH₂ (◆, 1 μM) or TF-NH₂ (▲) to monitor PAR₁ desensitization. As outlined for the agonists shown in Figs. 9 and 10, a concentration-desensitization curve was determined for pretreatment of HEK cell suspensions with different concentrations of TF-NH₂ (0.5–40 μM), followed by monitoring the residual calcium signal (E₅₃₀) yielded by the subsequent addition of a test concentration of either Cit-NH₂ (◆, 1 μM) or TF-NH₂ (▲, 10 μM). The residual PAR₁-stimulated fluorescence signal (E₅₃₀) observed for the two different PAR₁-APs was expressed as a percentage (% control) of the signal observed upon adding either Cit-NH₂ (◆) or TF-NH₂ (▲, 10 μM) to identical HEK cell suspensions that had *not* been pretreated with the different concentrations of TF-NH₂ (x-axis). Values represent the means of measurements done with triplicate cell suspensions obtained from two independently grown HEK cell crops. Error bars were smaller than the symbols shown.

PAR₂ (Fig. 9). In the HEK cell assay, the relative selectivity of Cit-NH₂ for the PAR₁ receptor, compared with PAR₂ was about 280:1, whereas the PAR₁-selectivity of hArg-NH₂ was lower by about 2-fold (about 120:1, Fig. 9 and Table 2). Both hArg-NH₂ and Cit-NH₂ were found to be full agonists for the PAR₁ receptor (not shown). In comparison with hArg-NH₂ and Cit-NH₂, the much simpler peptide TF-NH₂, composed entirely of naturally occurring amino acids, demonstrated a PAR₁/PAR₂-selectivity of about 220:1 (Fig. 9 and Table 2).

Relative Receptor Selectivity of Receptor-Activating Peptides Derived from Human PAR₁. Because many previous studies, including our own (Yang et al., 1992; Tay-Uyboco et al., 1995), have used peptides derived from the human PAR₁ activating sequence as surrogates for the action of thrombin (previously termed thrombin receptor activating peptides, or TRAPs), we wished to evaluate the PAR₁/PAR₂ receptor selectivity of TRAPs that had been used extensively in previous work without the knowledge that such agonists could affect both PAR₁ and PAR₂: SFLLR-NH₂ (SF-NH₂) and

TABLE 2
PAR₁/PAR₂ Selectivity of PAR₁-targeted ligands

Compound	Inhibitory potency ^a		PAR ₁ -selectivity ^b
	PAR ₁	PAR ₂	
	(IC ₅₀ , μM)		S-value ^b
SF-NH ₂	2.2	8.7	4
SF ₁₄ -NH ₂	5.5	22	4
TF-NH ₂	2.5	540	220
hArg-NH ₂	0.14	17	120
Cit-NH ₂	1.0	>200	280
tc-fF-NH ₂	2.4	100	42
Mpr-NH ₂	17	33	2

^a The inhibitory potencies (IC₅₀ values) were determined from the activation/desensitization curves shown in Fig. 13 and 14. IC₅₀ = the concentration of test compound required to diminish the PAR₁ (TF-NH₂) or PAR₂ (SL-NH₂) calcium signal by 50%.

^b The selectivity (S) values were obtained from the concentration-inhibition curves (Figs. 4, 7, 9, and 10) from the equation: $S = IC_{50\text{PAR}_2} / IC_{50\text{PAR}_1}$, wherein the concentration of a given agonist to inhibit the PAR₂ signal (IC_{50PAR2}) by a given percentage was compared relative to the concentration of that agonist required to cause an equivalent reduction (IC_{50PAR1}) of the PAR₁ signal. Values of S greater than unity reflect a selectivity for PAR₁ relative to PAR₂. The S-values were estimated at three or more points along the parallel portions of the concentration-inhibition curves shown in Figs 4, 7, 9 and 10. Average values are recorded.

TABLE 3
Potencies of PAR₁-targeted ligands in platelets and HEK cells

Compound	Platelet assay		HEK cell PAR ₁ Ca ²⁺ signal
	EC ₅₀ (μM)	IC ₅₀ (μM)	IC ₅₀ (μM)
SF-NH ₂	0.90		2.2
SF ₁₄ -NH ₂	9.3		5.5
TF-NH ₂	1.9		2.5
hArg-NH ₂	0.12		0.14
Cit-NH ₂	0.20		1.0
tc-fF-NH ₂	2.0 ^a	1.6 ^b	2.4
Mpr-NH ₂		0.7 ^c /6.4 ^d	17
Met-OH		48 ^e	Potentiates

^a Concentration causing a half-maximal reversible aggregation.

^b Concentration that completely blocks aggregation caused by 3 μM SF-NH₂, but blocks 50% of aggregation caused by 0.05 U/ml thrombin.

^c IC₅₀ for aggregation caused by 0.015 U/ml thrombin.

^d IC₅₀ for aggregation caused by 0.04 U/ml thrombin.

^e IC₅₀ for aggregation caused by 0.025 U/ml thrombin.

SFLLRNPNDKYEPF-NH₂ (SF₁₄-NH₂) (originally described by Vu et al., 1991). As shown in Fig. 10, both SF₁₄-NH₂ and SF-NH₂ were somewhat selective for PAR₁ over PAR₂ (about 2- to 4-fold), but neither of the originally described TRAPs were anywhere near as PAR₁-selective as hArg-NH₂ (120:1), TF-NH₂ (220:1), or Cit-NH₂ (280:1). Table 2 summarizes the activities of all peptides studied in terms of their PAR₁/PAR₂ selectivity and their IC₅₀s for attenuating the calcium signal in HEK cells caused by subsequent activation of either PAR₁ (TF-NH₂) or PAR₂ (SL-NH₂).

Evaluation of the Use of Different PAR-Selective Standard Agonists in the HEK Cell Assay. Because the desensitization assay depends primarily on the activity of the unknown compound added to the cells before the PAR-selective test compound (routinely, TF-NH₂, in the PAR₁ assay we have developed), the desensitization profile for a given unknown compound should not, in principle, depend on the PAR-selective receptor probe that is used to generate the standard signal (step 2 in the procedure outlined above). To test this principle, we measured the PAR₁ desensitization profile for TF-NH₂, (as an unknown), using either the standard concentration of TF-NH₂ itself (10 μM) or, the more

potent PAR₁-selective agonist, Cit-NH₂ (1 μM). As shown in Fig. 11, the PAR₁ desensitization profile for TF-NH₂ was virtually superimposable, irrespective of which selective PAR₁ receptor probe was used to generate the standard calcium signal.

Activity of Peptides in a Human Platelet Aggregation Assay. We wished to compare the PAR₁ selective activities of Mpr-NH₂, Met-OH, tc-ff-NH₂, hArg-NH₂, Cit-NH₂, TF-NH₂, SF-NH₂, and SF₁₄-NH₂ determined in the HEK cell assay, in terms of attenuating the PAR₁ calcium signal (Table 2), with their activities in a platelet aggregation assay (results summarized in Table 3). Platelets do not possess PAR₂, and thus the potential cross-reactivity of the peptides at PAR₂ was not an issue in the platelet assay. All peptides except Mpr-NH₂ (up to 20 μM) and Met-OH were platelet agonists, with EC₅₀ s in the range of 0.1 to 10 μM (Table 3). Mpr-NH₂, as previously described, proved to be an inhibitor of both thrombin-mediated and SF-NH₂-mediated platelet aggregation. However, the IC₅₀ for Mpr-NH₂ depended heavily on the concentration of either thrombin or SF-NH₂, which was used as a platelet agonist. For instance, at a concentration of 0.015 U/ml thrombin, the IC₅₀ for Mpr-NH₂ was 0.7 μM (Table 3), whereas at a concentration of 0.04 U/ml thrombin, the IC₅₀ was about 6 μM (Table 2). Both Mpr-NH₂ and Met-OH proved to be poor antagonists of SF-NH₂ in the platelet aggregation assay (IC₅₀ > 200 μM), and Met-OH was a weak antagonist of thrombin-mediated platelet aggregation (Table 3, and data not shown). Surprisingly, tc-ff-NH₂ on its own was a weak platelet agonist, causing a reversible aggregation at concentrations up to 20 μM. At concentrations lower than 20 μM, tc-ff-NH₂ was able to inhibit SF-NH₂-induced platelet aggregation, with an IC₅₀ of about 2 μM (Table 2). This inhibition, however, was observed only by adding tc-ff-NH₂ to the cuvette first and by waiting until the light scattering caused by this peptide had returned to baseline, before adding the full agonist (3 μM SF-NH₂ or 0.05 U/ml thrombin) that caused aggregation.

Discussion

Our data, obtained with the newly described desensitization assay, considerably extended our preliminary observations describing TF-NH₂ as a PAR₁-selective agonist (Hollenberg et al., 1997). The principal finding of our study was that ligands originally developed as PAR₁-targeted ligands can also be seen to activate PAR₂, with a greater or lesser selectivity for PAR₁. The results with the PAR₁ antagonist, Mpr-NH₂ were particularly instructive as, with comparable potencies, this peptide proved to be an antagonist for PAR₁ and full agonist for PAR₂ (Figs. 4 and 5). Confirming earlier reports (Seiler et al., 1995), Mpr-NH₂ was a PAR₁ antagonist in the platelet, where PAR₂ was absent; this compound was also an antagonist for the HEK cell PAR₁ receptor. Thus, previous work in which this PAR₁ antagonist or in which nonselective PAR₁-APs were used to evaluate tissues such as the vascular endothelium (Lum et al., 1993; Zimmerman et al., 1994), wherein PAR₁ and PAR₂ coexist (Al-Ani et al., 1995), may need to be reevaluated. The data obtained with the Mpr-NH₂ peptide also point to differences whereby this peptide docks with PAR₁ and PAR₂, on the one hand leading to receptor activation (PAR₂), on the other to inhibition (PAR₁).

The principle of the PAR₁/PAR₂ desensitization assay depends on a density of PAR₁ and PAR₂ receptors that is low enough so that the activation/desensitization of receptors by a test compound will diminish the calcium signal generated by the subsequent addition of the PAR-selective test compound. Thus, the assay we describe will only work efficiently for agonists if the proportion of spare receptors for the calcium signal response is low, relative to the total numbers of receptors present in the system. That our assay works suggests that the proportion of spare PAR₁ and PAR₂ receptors in the HEK cells is indeed appropriately low for the purposes of our new assay. However, the absolute values of the IC₅₀s for the various test compounds obtained in the desensitization assay for interacting with either PAR₁ or PAR₂ must be interpreted with caution, relative to the potencies that the peptides we have studied may exhibit in other responsive systems. For instance, differences in receptor numbers between cell types could shift the concentration-effect curves to the left (higher receptor numbers) or to the right (lower receptor numbers). Nonetheless, the relative potencies within a series of compounds that interact with either PAR₁ or PAR₂ (or both) would be expected to be the same, irrespective of the tissue in which the compounds were assayed. It is, therefore, important to note that the relative potencies of the PAR₁-targeted agonists in the PAR₁ desensitization assay (hArg-NH₂ > Cit-NH₂ > tc-ff-NH₂ ≈ TF-NH₂ ≈ SF-NH₂ > SF₁₄-NH₂) (Table 2) were in good accord with the relative EC₅₀s of the same compounds in the platelet aggregation assay (Table 3). Because of the complex dependence of the IC₅₀s for antagonists on the thrombin concentration in the platelet aggregation assay, a correlation with the HEK cell assay was problematic.

Verification of the ability of the HEK cell signaling assay to identify receptor-selective agonists can be seen in the data obtained with thrombin, the PAR₁-selective peptide, Cit-NH₂, and the PAR₂-selective agonist, SL-NH₂. As has been observed in previous work (Nystedt et al., 1994), thrombin is unable to activate PAR₂, and SL-NH₂ is unable to activate PAR₁. The PAR₁ selectivity of Cit-NH₂ (almost 300:1) documented by our assay singles this compound out as an attractive, selective PAR₁ agonist for use in studies done *in vivo*. Based on our previous data and on the results with TF-NH₂, one can predict that the peptide Thr-parafluoroPhe-Arg-Cha-Cit-Tyr-NH₂ would be an even more selective PAR₁ agonist than Cit-NH₂. A key to our assay was that PAR₁ activation did not lead to heterologous desensitization of PAR₂, nor did PAR₂ activation desensitize PAR₁. These data appear to conflict with the observations of Mirza and colleagues (1996), who observed heterologous desensitization between PAR₁ and PAR₂ receptors in cultured human endothelial cells. That this cross-desensitization did not occur between PAR₁ and PAR₂ in the HEK cell assay may be due to two factors. First, our assay allowed for a time period of 10 min or more between the sequential addition of agonists to permit a complete refilling of intracellular calcium stores; this time period, which was longer than that used by Mirza et al. (1996), may have also allowed for a resensitization of receptors that had been cross-desensitized via a heterologous receptor mechanism. Alternatively, the enzymes responsible for heterologous receptor desensitization in cultured human endothelial cells and the relative abundance of PAR₁ and PAR₂ in the endothelial cells (Mirza et al., 1996) may differ consider-

ably in comparison with the HEK cells, so as to account for the differences in heterologous receptor desensitization. This lack of heterologous receptor cross-desensitization, not only for the PAR₁/PAR₂ system, but also between the LPA receptor and the PAR receptors could allow for the simultaneous evaluation of multiple members of other receptor families that might be either present in HEK cells or coexpressed in these cells via transfection.

Because the HEK cells express sufficient wild-type human PAR₁ and PAR₂ receptors for the assay we describe, the system is far more convenient and precise than the use of PAR₁ and PAR₂ transfected xenopus oocytes, as described in work that appeared during the course of our studies (Blackhart et al., 1996). Although the data obtained with the xenopus oocyte receptor expression system can complement the results we describe in this report, the variability of response of the receptor-transfected oocytes makes difficult any precise assignment of the relative magnitude of PAR₁/PAR₂ ligand selectivity, as is possible with the HEK cell assay (Table 2). Unfortunately, the spectrum of PAR₁APs used in the oocyte expression assay (Blackhart et al., 1996) differed considerably from the compounds we studied with the HEK cell assay system, and a direct comparison of receptor selectivity was not possible. The calcium signaling assay employing the HEK cells can also be seen to complement the use of human platelets (Table 3), which represent a useful screening target for the evaluation of PAR₁ agonists and antagonists, but which are devoid of PAR₂ receptors. Furthermore, as opposed to the xenopus expression system, our assay was able to demonstrate simultaneously (Fig. 3) the activation/densitization of PAR₁ by trypsin (20–100 nM), as reflected by TF-NH₂-mediated receptor activation and, at much lower concentrations (0.5–10 nM trypsin), the disarming of the ability of thrombin to activate PAR₁, presumably by trypsin cleavage of the receptor at a site downstream from the tethered receptor-activating ligand domain. Thus, the HEK assay will also prove of value for assessing the effects of a variety of proteinases on PAR₁ and PAR₂ activation.

We were puzzled to observe that the peptide tc-ff-NH₂ was an agonist at both PAR₁ and PAR₂ in the HEK cell assay (Figs. 7 and 8) and that this peptide was a partial agonist in the platelet aggregation assay, where antagonist activity could also be measured. Given the antagonist activity reported by Bernatowicz et al. (1996) for the peptide: *trans*-cinnamoyl-parafluoroPhe-Arg-Leu-Arg-NH₂ [IC₅₀ of about 1 μM for inhibiting PAR₁AP-induced platelet aggregation: Bernatowicz et al. (1996) compound 76, Table 9] and the antagonist activity of *trans*-cinnamoyl-parafluoroPhe-paraguanidinoPhe-Leu-Arg-Orn-NH₂ [IC₅₀, about 50 nM for inhibiting PAR₁AP-induced platelet aggregation: Bernatowicz et al. (1996), compound 88, Table 11], we expected tc-ff-NH₂ to be a full antagonist. The partial agonist activity in the peptide we synthesized (tc-ff-NH₂) points to the importance of the paraguanidino-phenyl group at position 3 in PAR₁ antagonist peptides. Furthermore, it is possible that tc-ff-NH₂ may nonspecifically desensitize the platelet to other agonists (e.g., ADP).

Finally, based on our PCR data, we expected to find evidence for the activation of PAR₃ by thrombin in the HEK cell assay. Because PAR₃ has been reported not to be activated by PAR-APs (Ishihara et al., 1997), we fully anticipated that the complete desensitization of PAR₁ by TF-NH₂ or by SF-NH₂

would still allow for the sequential activation of PAR₃ by thrombin. That this was not the case may be accounted for by several possibilities. First, activation of PAR₁ may cross-desensitize PAR₃. Second, PAR₁AP, such as Cit-NH₂, TF-NH₂, or SF-NH₂, which cannot activate PAR₃, may nonetheless prove to be antagonists of the PAR₃-tethered peptide. Third, PAR₃ may not, as do PAR₁ and PAR₂, couple to the calcium signaling mechanism in HEK cells. These possibilities represent interesting topics for further study. Notwithstanding, the presumed presence of PAR₃ (we were not able to assess the HEK cell content of PAR₃ protein) in the HEK cells appears not to interfere with the PAR₁/PAR₂ desensitization assay that we have developed. We anticipate that this fluorescence-based assay will prove of considerable use for screening of PAR₁- and PAR₂-targeted agents in future studies, not only for peptidomimetic compounds but also for other proteases that may affect either PAR₁ or PAR₂.

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