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Functional expression of a human thrombin receptor in Sf9 insect cells: evidence for an active tethered ligand

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Desensitization of recombinant human thrombin receptors expressed in Sf9 insect cells was compared with native thrombin receptors in megakaryoblast erythroleukaemia (HEL) cells. Addition of thrombin (2 units/ml) or agonist peptide SFLLRN (10 μM) to HEL cells, or to Sf9 cells infected with recombinant baculovirus containing the thrombin receptor cDNA, produced an increase in the free cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) as measured by fura-2. The response in HEL cells was transient, reflecting a rapid homologous desensitization. In contrast, $[Ca^{2+}]_i$ in Sf9 cells expressing the thrombin receptor increased rapidly to a peak value that slowly declined, but remained elevated for at least 12 min following stimulation by thrombin. The sustained $[Ca^{2+}]_i$ response to thrombin was not reversed by washout of thrombin or by subsequent addition of hirudin. Pretreatment of Sf9 cells with either thrombin (2 units/ml) or SFLLRN (10 or 50 μM) for 5 min produced a shift in the ED_{50} for SFLLRN (added 10 min after washout) from 0.4 μM to 20 and 7 μM, respectively. Thus, desensitization of thrombin receptors expressed in Sf9 cells occurs slowly and reflects a decrease in receptor affinity. The sustained $[Ca^{2+}]_i$ response in Sf9 cells stimulated by thrombin may reflect continuous activation by the tethered ligand. To test this hypothesis, the effect of protease treatment during the sustained phase of the response was examined. Addition of either aminopeptidase M or thermolysin reversed the sustained response to SFLLRN, but only thermolysin reversed the sustained response to thrombin. Thermolysin had no effect on the change in $[Ca^{2+}]_i$ observed following carbachol stimulation of Sf9 cells expressing the M_5 muscarinic receptor. Furthermore, following thermolysin treatment, the cells remained responsive to a subsequent application of SFLLRN. These results demonstrate that the tethered ligand remains active for extended periods of time after thrombin stimulation and suggests that further hydrolysis by extracellular proteases may represent an important mechanism of rapid receptor deactivation.

INTRODUCTION

Thrombin, a multifunctional serine protease, elicits biological responses from a variety of cells, including platelets, vascular endothelial and smooth-muscle cells [1]. The thrombin receptor has been cloned and the predicted amino acid sequence revealed a new member of the G-protein-coupled receptor superfamily that displays an α-thrombin cleavage site in the N-terminal extracellular domain [2]. According to the current model, cleavage of the receptor by thrombin unmasks a new N-terminus which functions as a tethered ligand and leads to activation of the receptor [2,3]. The thrombin receptor can also be activated in the absence of proteolytic cleavage by exogenous addition of the agonist peptide, SFLLRN, which corresponds to the amino acid sequence of the endogenous tethered ligand normally revealed by thrombin [4,5].

Unlike normal hormone–receptor interaction, the activating ligand of the thrombin receptor remains tethered and cannot diffuse away. Thus, understanding the mechanism(s) responsible for 'shut-off' and/or receptor deactivation is crucial for understanding receptor function. One mechanism involves homologous desensitization in which the immediate re-addition of either thrombin or agonist peptide fails to evoke a second response. However, by examining the recovery from homologous desensitization in HEL cells, Brass [6] found that resensitization to agonist peptide, but not to thrombin, occurred within 30 min of a brief exposure to thrombin and did not require protein synthesis. This resensitization to agonist peptide was blocked by phosphatase inhibitors, suggesting that the thrombin-cleaved receptor exists in a dephosphorylated state that is desensitized to thrombin, but not to exogenous agonist peptide. This result suggests that the tethered ligand is not active, but that the receptor is otherwise intact. Brass hypothesized that a 'secondary' proteolysis of the N-terminus could explain the failure of the thrombin-cleaved receptor to reactivate itself once dephosphorylated, or that the presence of thrombin is necessary for 'guiding' the tethered ligand to its binding domain [7]. These potential extracellular mechanisms of receptor deactivation could be tested on recombinant thrombin receptors expressed in cells lacking the normal intracellular mechanisms responsible for rapid receptor deactivation.

Several G-protein-coupled membrane receptors have been expressed in the Sf9 insect cell following infection with recombinant baculovirus, including rat odorant receptors [8], α_2-adrenergic receptors [9], β-adrenergic receptors [10,11], *N*-formyl-peptide receptor [12] and muscarinic receptors [10,13–15]. Recently we demonstrated that stimulation of M_5 muscarinic receptors expressed in Sf9 cells by carbachol results in a biphasic increase in free cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) [16]; the initial rise in $[Ca^{2+}]_i$ reflects the release of Ca^{2+} from internal stores, whereas a sustained phase reflects the influx of Ca^{2+} from the extracellular space. As in mammalian non-excitable cells, the carbachol-induced influx of Ca^{2+} is blocked by low concentrations of La^{3+} or by the subsequent addition of receptor antagonist, atropine. These results demonstrate that (1) Ca^{2+} signalling mechanisms in Sf9 insect cells are essentially identical to those in

Abbreviations used: $[Ca^{2+}]_i$, free cytosolic Ca^{2+} concentration; FBS, fetal bovine serum; fura-2/AM, fura-2 acetoxymethyl ester; HBS, Hepes-buffered saline; MBS, Mes-buffered saline; RT-PCR, reverse-transcription PCR.

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mammalian non-excitable cells, and (2) the baculovirus expression system can be used to study the function of heterologous phospholipase C-coupled membrane receptors.

The purpose of the present study was to investigate the function of the human thrombin receptor expressed in Sf9 insect cells and to examine thrombin receptor desensitization. The results suggest that human thrombin receptors expressed in Sf9 cells are functionally indistinguishable from native thrombin receptors, but that the tethered ligand exposed by thrombin remains intact and active in Sf9 cells after washout of thrombin. Furthermore, the thrombin-activated receptor can be deactivated by subsequent proteolysis with thermolysin. Thus, rapid receptor deactivation may be accomplished *in vivo* by extracellular proteases.

MATERIALS AND METHODS

Solution and reagents

Mes-buffered saline (MBS) contained the following: 10 mM NaCl, 60 mM KCl, 17 mM $MgCl_2$, 10 mM $CaCl_2$, 4 mM D-glucose, 110 mM sucrose, 0.1% BSA and 10 mM Mes, pH adjusted to 6.2 at room temperature with Trizma-base. The total osmolarity of MBS was approx. 340 mosM. Hepes-buffered saline (HBS) contained 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM D-glucose, 1.8 mM $CaCl_2$, 0.1% BSA and 15 mM Hepes, pH adjusted to 7.40 at 37 °C. Human plasma thrombin and hirudin were obtained from Sigma (St. Louis, MO, U.S.A.). Thrombin receptor agonist peptide, SFLLRN, was purchased from Bachem Bioscience Inc. (Philadelphia, PA, U.S.A.). *Taq* DNA polymerase was purchased from Perkin-Elmer. Fura-2 acetoxymethyl ester (fura-2/AM) was obtained from Molecular Probes (Eugene, OR, U.S.A.).

Cell culture

Sf9 cells were obtained from Invitrogen (San Diego, CA, U.S.A.) and were cultured as described previously [16,17] using Grace's Insect Medium (Gibco) supplemented with lactalbumin hydrolysate, yeastolate, L-glutamine, 10% (v/v) heat-inactivated fetal bovine serum (FBS) and 1% penicillin-streptomycin solution (Gibco). Cells were grown either in spinner flasks (Bellco Glass, Vineland, NJ, U.S.A.) or in 100-mm-diam. plastic tissue-culture dishes (Falcon). The cell cultures were incubated at 27 °C in a humidified atmosphere. HEL cells, obtained from the American Type Culture Collection (Rockville, MD, U.S.A.), were grown in RPMI 1640 medium (Gibco) containing 10% (v/v) FBS and 1% penicillin-streptomycin solution in 100-mm-diam. culture dishes. The cell cultures were incubated at 37 °C in a humidified atmosphere containing 5% CO_2.

Cloning of the human thrombin receptor cDNA

To isolate the cDNA encoding the human thrombin receptor, four oligonucleotides were synthesized and used as primers for reverse-transcription PCR (RT-PCR) using human blood cell total RNA as template. The thrombin receptor cDNA was isolated in two fragments with an overlapping, centrally located, *Pst*I restriction enzyme site. The 5′ half of the thrombin receptor cDNA, consisting of 552 bp, was obtained using oligos TR1 (sense oligo, 5′-ACAATGGGGCCGCGGCGGCTGCTGCTGGTG-3′; position 222 to 251 in the original clone of [2]), and TR4 (antisense oligo, 5′-AGTAAAATGCTGCAGTGACGAA-3′; position 753 to 774 in the original clone of [2]). The 3′ half of the receptor cDNA, consisting of 747 bp, was obtained using oligos TR3 (sense oligo, 5′-CGTCACTGCAGCATTTTACTGT-3′; position 755 to 776 in the original clone of [2]) and TR2 (antisense oligo, 5′-CTAAGTTAACAGCTTTTTGTATATGCTGTT-3′; position 1473 to 1502 in the original clone of [2]). Each PCR product was subcloned into plasmid pCRII (Invitrogen) to produce pCRII-TR5′ and pCRII-TR3′ respectively. To obtain the full-length cDNA of the thrombin receptor, the cDNA fragments were excised from pCRII-TR5′ by digestion with restriction enzymes *Eco*RI and *Pst*I and from pCRII-TR3′ by digestion with *Pst*I and *Hin*dIII. Plasmid pBSK (Stratagene) was linearized by digestion with *Eco*RI and *Hin*dIII. The full-length thrombin receptor cDNA was obtained following a three-piece ligation reaction with TR5′, TR3′ and linearized pBSK, yielding pBSK-TR(+). The nucleotide sequence was obtained by the dideoxynucleotide method using Sequenase Version II (U.S. Biochemical Corp.). One nucleotide substitution, A to G, at position 780 was found (the nucleotide number was assigned with the initiation methionine as number one). This substitution was probably introduced by *Taq* polymerase during PCR. Since this substitution did not result in amino acid change, no attempt was made to correct this substitution.

Generation of recombinant baculovirus

The thrombin receptor cDNA was subcloned into baculovirus transfer vector pVL1392 [designated pVL-TR(+)] using standard techniques [18]. The orientation was confirmed by restriction enzyme digestion. Recombinant viruses were produced using the BaculoGold Transfection Kit (PharMingen, San Diego, CA, U.S.A.) by co-transfecting Sf9 cells with pVL-TR(+) and linearized BaculoGold viral DNA as described in the instructions provided with the kit. Single viral plaques were isolated and amplified 2–4 times to obtain a high-titre viral stock solution which was stored at 4 °C until use.

Expression of thrombin receptors in Sf9 cells

For routine infection, Sf9 cells in Grace's medium were allowed to attach to the bottom of a 100-mm-diam. plastic culture dish (10^7 cells/dish). Following incubation for 15 min, an aliquot of viral stock was added (multiplicity of infection was 10) and the cells were maintained at 27 °C in a humidified air atmosphere. Unless otherwise indicated, cells were used 36–40 h post-infection.

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

$[Ca^{2+}]_i$ was measured in Sf9 cells using the fluorescent indicator, fura-2, as described previously [16,17]. Briefly, cells were dispersed, washed and resuspended at a concentration of $(1.5–2)\times10^6$ cells/ml in MBS containing 2 μM fura-2/AM. After 30 min incubation at room temperature (22 °C), the cell suspension was subjected to centrifugation, resuspended in an equal volume of MBS and incubated for an additional 30 min. The cells were washed and fluorescence was measured using an SLM 8000 spectrophotofluorimeter. Excitation wavelength alternated between 340 and 380 nm and fluorescence intensity was monitored at an emission wavelength of 510 nm. All measurements on Sf9 cells were performed at room temperature (22 °C). Calibration of the fura-2 associated with the cells was accomplished using Triton lysis in the presence of a saturating Ca^{2+} concentration followed by addition of EGTA (pH 8.5). An identical procedure to that described above for Sf9 cells was used to estimate $[Ca^{2+}]_i$ in HEL cells with the exception that the extracellular buffer was HBS at 37 °C. $[Ca^{2+}]_i$ was calculated by the equation of [19] using K_d values for Ca^{2+} binding to fura-2 of 278 and 224 nM at 22 and 37 °C respectively [20]. The Figures

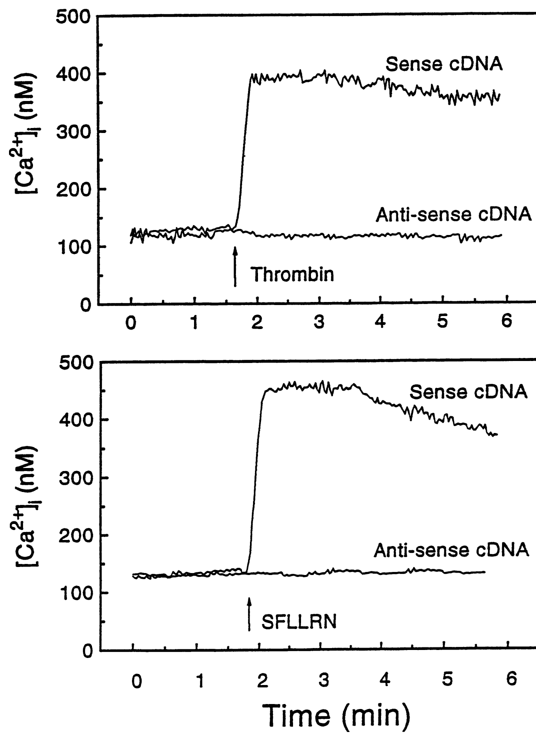

Figure 1 Effects of thrombin receptor stimulation on $[Ca^{2+}]_i$ in Sf9 insect cells

Two traces are superimposed in each panel. Thrombin (2 units/ml, upper panel) or SFLLRN (20 μM, lower panel) was added at the time indicated by the arrow to fura-2-loaded Sf9 insect cells infected with recombinant baculovirus containing the human thrombin receptor cDNA in either the sense or antisense orientation and the fluorescence was monitored as described in the Materials and methods section. In this, and all subsequent Figures, traces are representative of at least three experiments on Sf9 cells at 36–40 h post-infection.

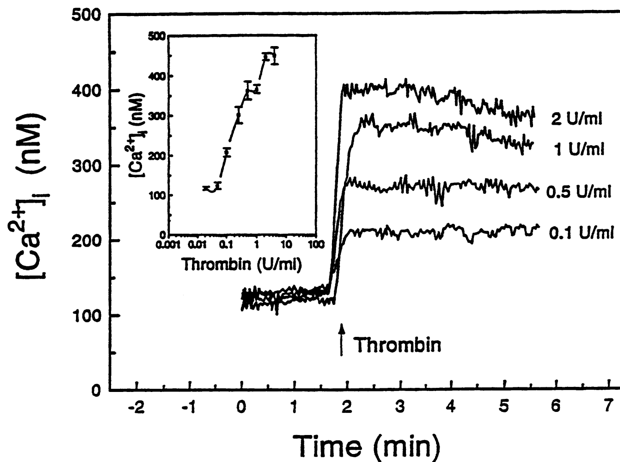

Figure 2 Concentration–response relationship for thrombin-induced change in $[Ca^{2+}]_i$

Four traces are superimposed. Thrombin was added at the indicated concentrations to fura-2-loaded Sf9 cells expressing the thrombin receptor. Insert: The values represent mean ± S.E.M. peak $[Ca^{2+}]_i$ response to various concentrations of thrombin ($n = 4$).

show representative traces from experiments performed at least three times. Statistical differences were determined using Student's *t*-test.

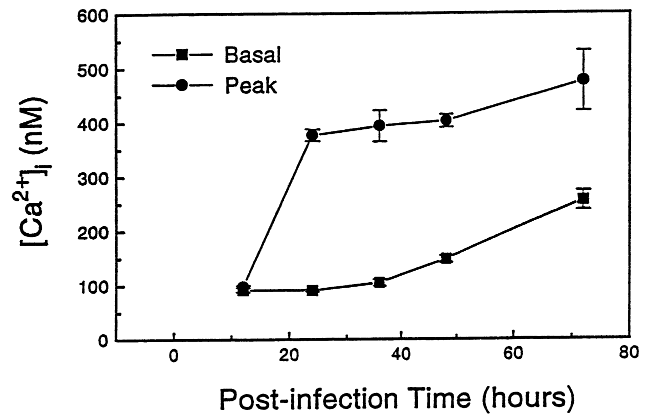

Figure 3 Effect of post-infection time on the functional expression of thrombin receptor

The effect of SFLLRN (20 μM) on $[Ca^{2+}]_i$ in Sf9 insect cells expressing the thrombin receptor was determined at the indicated times post-infection. Values represent the mean ± S.E.M. basal $[Ca^{2+}]_i$ (■) and peak change in $[Ca^{2+}]_i$ (●); $n = 4$.

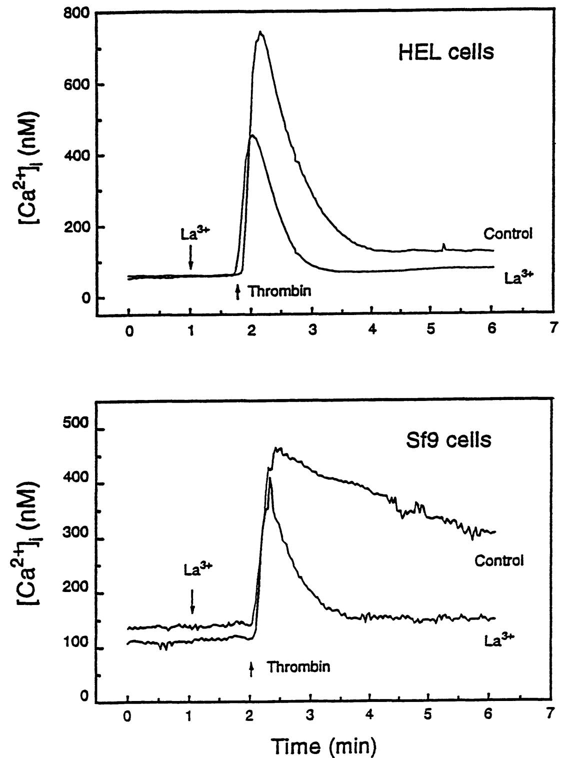

Figure 4 Comparison of the thrombin response in HEL and Sf9 cells

Two traces are superimposed in each panel. Thrombin (2 units/ml) was added to fura-2-loaded HEL cells (upper panel) or Sf9 cells expressing the thrombin receptor (lower panel) in the absence (control) or presence of La^{3+} (10 μM) added at the time indicated by the arrow.

Receptor desensitization assay

To test for homologous and heterologous receptor desensitization, an aliquot of HEL or Sf9 cells expressing the thrombin receptor was incubated with the indicated concentration of either thrombin or the agonist peptide, SFLLRN. At time 5 min, the

cells were subjected to centrifugation and resuspended in the same volume of fresh buffer without agonist agents. This wash procedure was repeated twice to ensure complete removal of the agonist. After 10 min, the cells were placed in a cuvette for fluorescence measurement and challenged again with either thrombin, SFLLRN, or the purinergic agonist, ATP, at the concentrations indicated in the text.

RESULTS

Thrombin- or SFLLRN-induced $[Ca^{2+}]_i$ response in Sf9 cells

Thrombin or agonist peptide, SFLLRN, produced a sustained increase in $[Ca^{2+}]_i$ in Sf9 cells infected with recombinant baculovirus containing the thrombin receptor cDNA under control of the polyhedrin promoter (Figure 1). Thrombin or SFLLRN had no effects on Sf9 cells infected with baculovirus containing an antisense thrombin receptor cDNA insert. The response to thrombin was concentration-dependent and saturable, with a maximal response obtained at 2 units/ml, and an EC_{50} of 0.5 unit/ml (Figure 2). The change in $[Ca^{2+}]_i$ in response to SFLLRN was also concentration-dependent; the maximal response was obtained at a SFLLRN concentration of 20 μM, with an EC_{50} of 0.4 μM (see Figure 7). The $[Ca^{2+}]_i$ response to SFLLRN was dependent on post-infection time (Figure 3). No response to SFLLRN was observed before 12 h post-infection. However, maximal response was seen 24 h after infection, and remained unchanged until 72 h post-infection. This profile is consistent with expression of a protein under control of the polyhedrin promoter and is similar to that previously reported for expression of the M_5 muscarinic receptor in Sf9 insect cells [16]. Basal $[Ca^{2+}]_i$ was unchanged for 36 h, but was elevated at 48

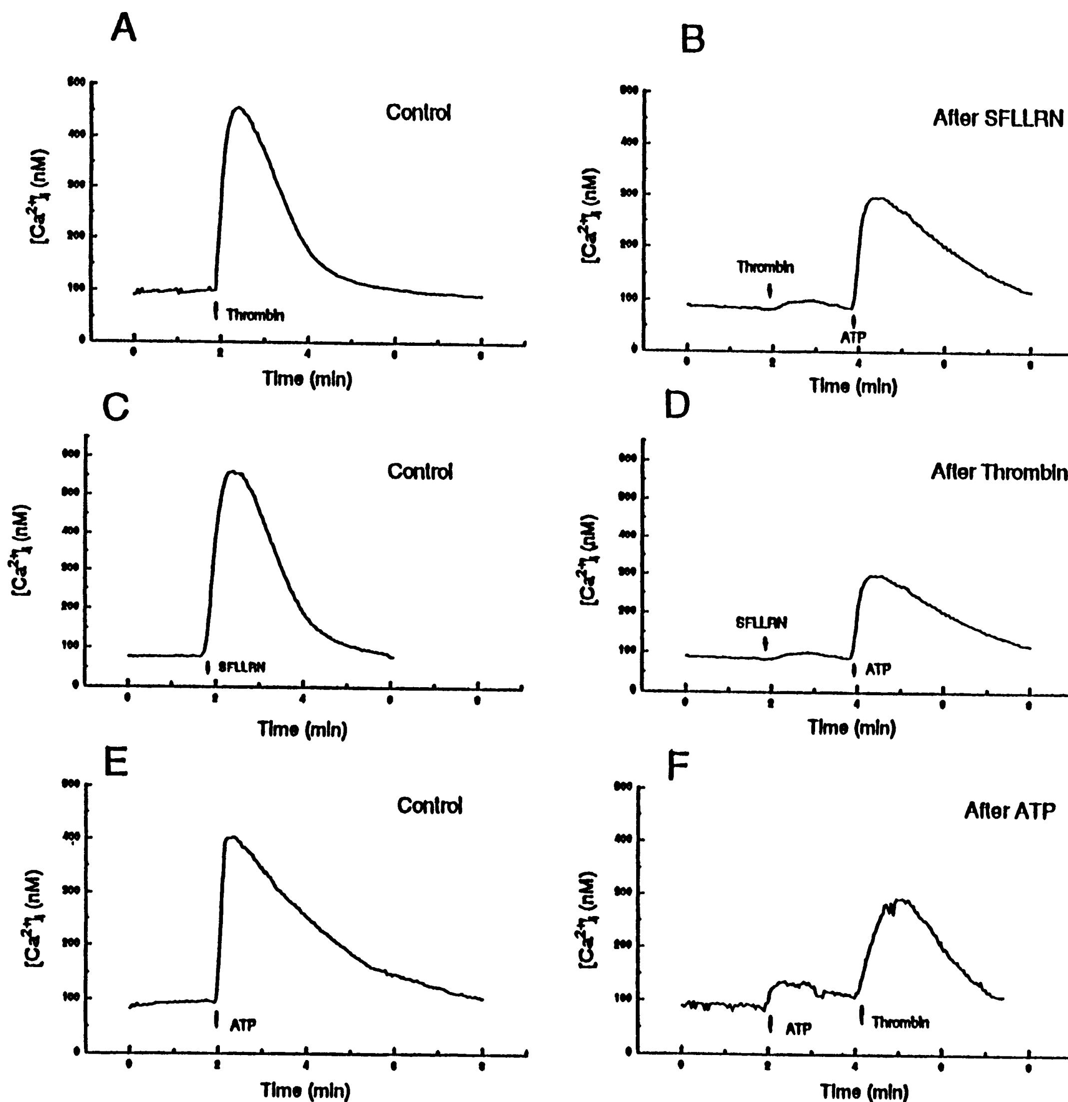

Figure 5 Desensitization of human thrombin and purinergic receptors in HEL cells

(**A**), (**C**) and (**E**): At the times indicated by the arrow, thrombin (2 units/ml), SFLLRN (10 μM), or ATP (1 mM) was added to HEL cells. (**B**), (**D**) and (**F**): HEL cells were first incubated with each agonist for 5 min, washed twice and resuspended in fresh HBS. After 10 min, the cell suspensions were placed in a cuvette for fluorescence measurement and thrombin (2 units/ml), SFLLRN (10 μM) or ATP (1 mM) were added at the time indicated in each panel by the arrows.

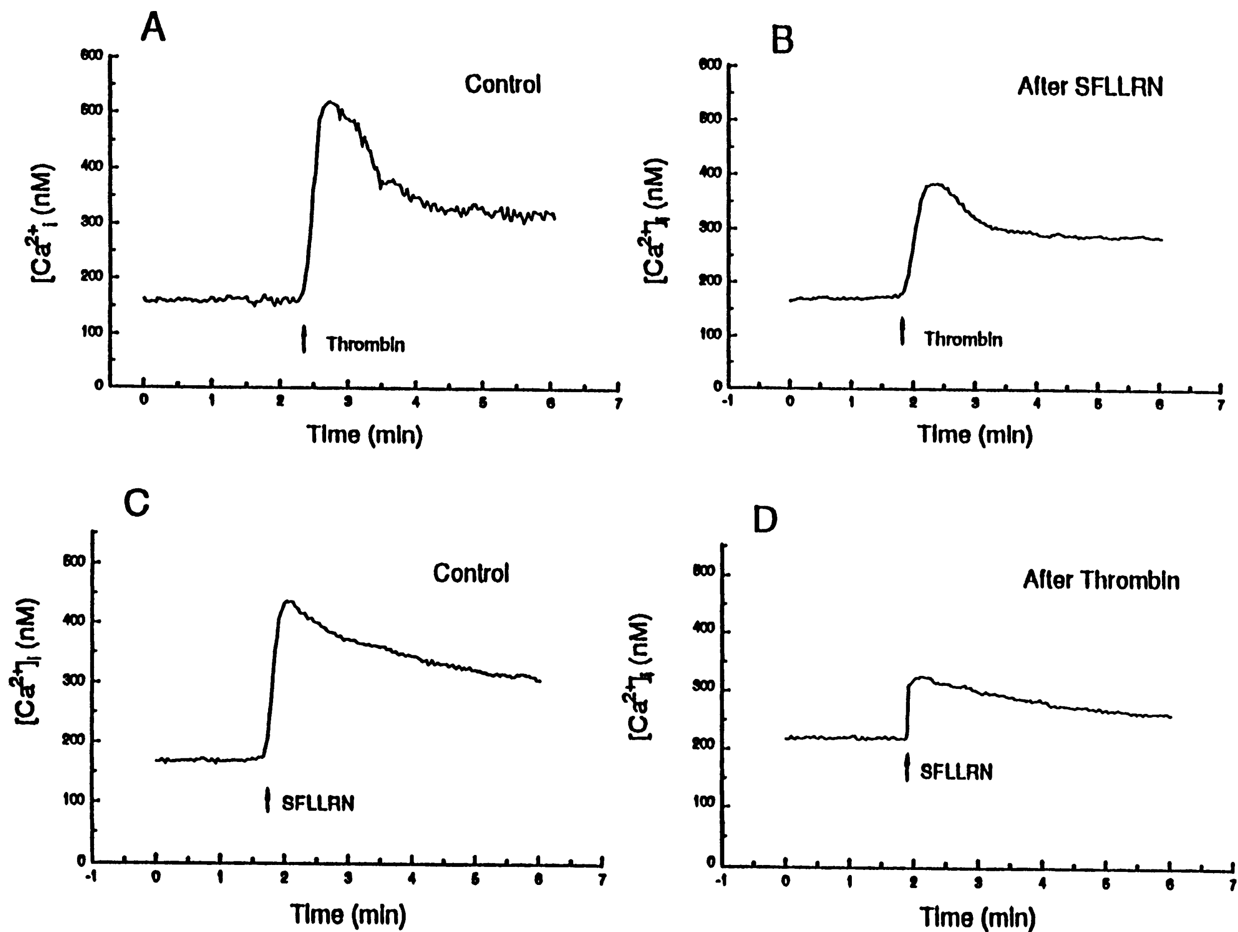

Figure 6 Desensitization of human thrombin receptors in Sf9 cells

(**A**) and (**C**): At the times indicated by the arrow, thrombin (2 units/ml) or SFLLRN (10 μM) was added to Sf9 cells expressing the thrombin receptor. (**B**) and (**D**): The Sf9 cells were first incubated with each agonist for 5 min, washed twice, and resuspended in fresh MBS. After 10 min, the cell suspensions were placed in a cuvette for fluorescence measurement and thrombin (2 units/ml), or SFLLRN (10 μM) was added at the times indicated.

and 72 h post-infection. The increase in $[Ca^{2+}]_i$ seen at the later times post-infection has been previously noted and appears to be associated with decreased cell viability [16,21].

Comparison of $[Ca^{2+}]_i$ response in HEL cells and Sf9 cells

Addition of thrombin to HEL cells produced an increase in $[Ca^{2+}]_i$ that peaked within 10 s and rapidly returned toward basal values within 2 min (Figure 4). Pretreatment of HEL cells with Ca^{2+}-influx blocker, La^{3+}, decreased the maximal response to thrombin as well as the duration of the $[Ca^{2+}]_i$ transient (Figure 4, upper panel). Similar results were obtained for the agonist peptide SFLLRN. In contrast to HEL cells, $[Ca^{2+}]_i$ in Sf9 cells expressing the thrombin receptor increased rapidly to a peak value and subsequently declined slowly with time, but remained elevated over the basal level for at least 12 min following thrombin receptor activation (Figure 4, lower panel). Pretreatment with La^{3+}, had little effect on the peak response, but essentially eliminated the sustained component, suggesting that the initial increase in $[Ca^{2+}]_i$ reflects release of Ca^{2+} from intracellular stores, whereas the sustained increase in $[Ca^{2+}]_i$ reflects influx of Ca^{2+} from extracellular spaces.

Desensitization of the thrombin receptor

Pretreatment of HEL cells with either thrombin (2 units/ml) or SFLLRN (50 μM) for 5 min essentially abolished the subsequent (after 10 min washout) response to both activators, but the response to ATP (1 mM) was only partially attenuated relative to the control (Figures 5A–5D). Similarly, pretreatment of HEL cells with ATP for 5 min dramatically attenuated the subsequent response to ATP (1 mM), but only moderately decreased the response to thrombin (Figures 5E and 5F). These results demonstrate rapid homologous desensitization of the thrombin and purinergic receptors in HEL cells. Since the response to ATP shown in Figures 5(B) and 5(D) and the response to thrombin in Figure 5(F) are decreased relative to the control there also appears to be some heterologous receptor desensitization in the HEL cell over the time frame examined.

In contrast to HEL cells, pretreatment of Sf9 cells with SFLLRN (50 μM) for 5 min produced only a slight attenuation of the subsequent (after 10 min washout) response to thrombin (2 units/ml; Figures 6A and 6B). Likewise, pretreatment with thrombin (2 units/ml) for 5 min attenuated the subsequent response to SFLLRN (50 μM, Figures 6C and 6D). These results

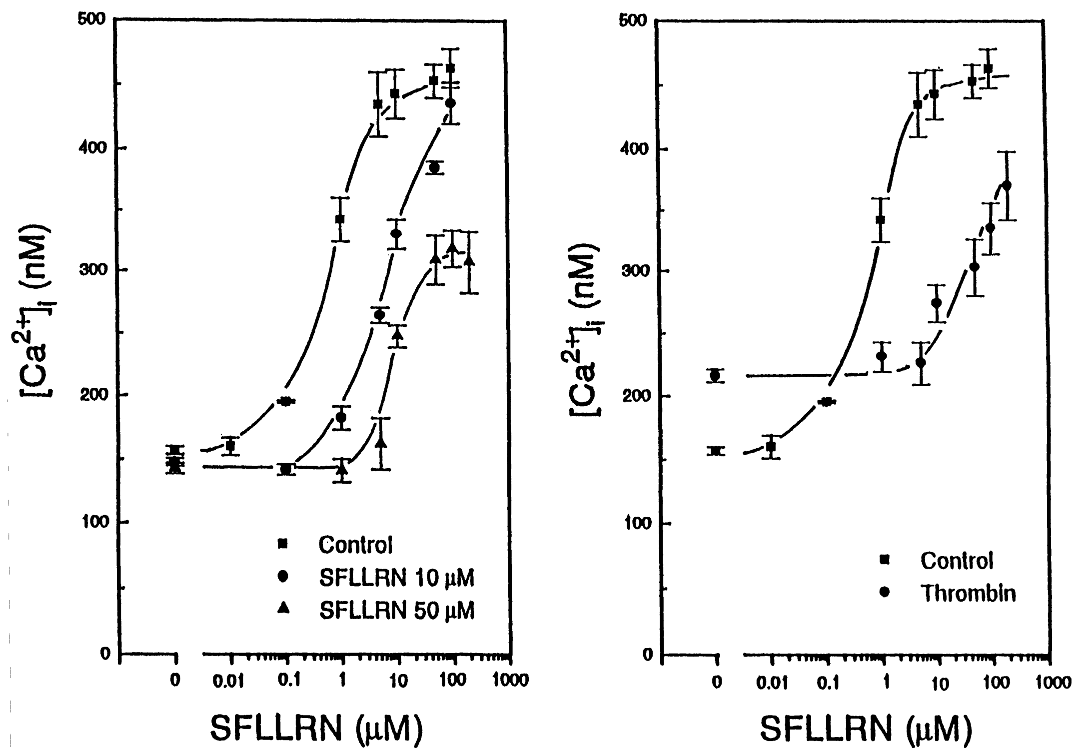

Figure 7 Concentration–response curves before and after thrombin receptor desensitization in Sf9 cells

Right-hand panel: Sf9 cells expressing the thrombin receptor were pretreated as described in the legend to Figure 6 with SFLLRN (10 and 50 μM) for 5 min followed by washout for 10 min. The cells were subsequently challenged with the indicated concentrations of SFLLRN and the peak change in $[Ca^{2+}]_i$ was recorded. Left-hand panel: Sf9 cells expressing the thrombin receptor were pretreated with thrombin (2 units/ml) for 5 min followed by washout for 10 min. The cells were subsequently challenged with the indicated concentrations of SFLLRN and the peak change in $[Ca^{2+}]_i$ was recorded. The control cells (which are the same in each panel) were not pretreated with agonist. Values represent the means ± S.E.M. ($n = 4$).

suggest that agonist-induced desensitization of the thrombin receptors occurs in the Sf9 cells, but the degree of desensitization is much less than that in HEL cells, and/or desensitization occurs at a much lower rate.

In order to understand the mechanism of receptor desensitization, a concentration–response profile for SFLLRN was determined after pretreatment with either thrombin or SFLLRN (Figure 7). Pretreatment of Sf9 cells with SFLLRN (10 μM) for 5 min increased the EC_{50} value for SFLLRN (added 10 min after washout) from 0.4 to 7 μM without changing the maximal response. Similar treatment with SFLLRN (50 μM) further increased the EC_{50} for SFLLRN to 11 μM and decreased the maximal response to SFLLRN (Figure 7, left-hand panel). Pretreatment with thrombin (2 units/ml, 5 min) increased EC_{50} for SFLLRN (added 10 min after washout) from 0.4 μM to more than 20 μM, (Figure 7, right-hand panel). These results suggest that desensitization of the thrombin receptor expressed in Sf9 cells reflects, at least in part, a decrease in receptor affinity for the agonist peptide.

One interesting finding, evident in Figures 6 and 7, was that basal $[Ca^{2+}]_i$ increased from 157 ± 9 nM ($n = 19$) to 218 ± 5 nM ($n = 22$; $P < 0.05$) after pretreatment of Sf9 cells with thrombin (2 units/ml) followed by wash and incubation in the absence of thrombin for 10 min, whereas pretreatment of Sf9 cells with SFLLRN (50 μM) had no effect on the basal $[Ca^{2+}]_i$ (148 ± 4 nM; $n = 24$). In contrast, pretreatment of HEL cells with either thrombin or SFLLRN had no effect on the basal $[Ca^{2+}]_i$ (Figure 5). To determine whether the sustained elevation of $[Ca^{2+}]_i$ reflects the presence of thrombin that was not completely removed by the wash procedure, the effect of hirudin was examined. The sustained response to thrombin was not reversed after adding hirudin at 5 units/ml, a concentration shown to block the response if added before thrombin (results not shown). In mammalian non-excitable cells and in Sf9 cells expressing the M_5 muscarinic receptor, addition of receptor antagonist rapidly decreases the sustained component of the response, demonstrating that the change in $[Ca^{2+}]_i$ is dependent on continuous receptor occupation. We hypothesized that the sustained increase in $[Ca^{2+}]_i$ observed after thrombin may also reflect continuous activation of the thrombin receptors in Sf9 cells.

Effects of protease, aminopeptidase M (AmM), or thermolysin on the $[Ca^{2+}]_i$ response

Since thrombin receptor antagonists are not currently available, we examined the effect of exogenous protease treatment on the sustained response to thrombin. AmM has been reported to inactivate the agonist peptide by removing the N-terminal serine residue [22,23]. Addition of AmM (2 units/ml) abolished the sustained increase in $[Ca^{2+}]_i$ following SFLLRN (Figure 8, upper panel), suggesting that intact ligand peptide and continuous receptor occupancy is required for the sustained increase in $[Ca^{2+}]_i$. In contrast to SFLLRN, the sustained increase in $[Ca^{2+}]_i$ after thrombin in Sf9 cells was not reversed by adding AmM (up to 10 units/ml; Figure 8, lower panel). These results suggest that

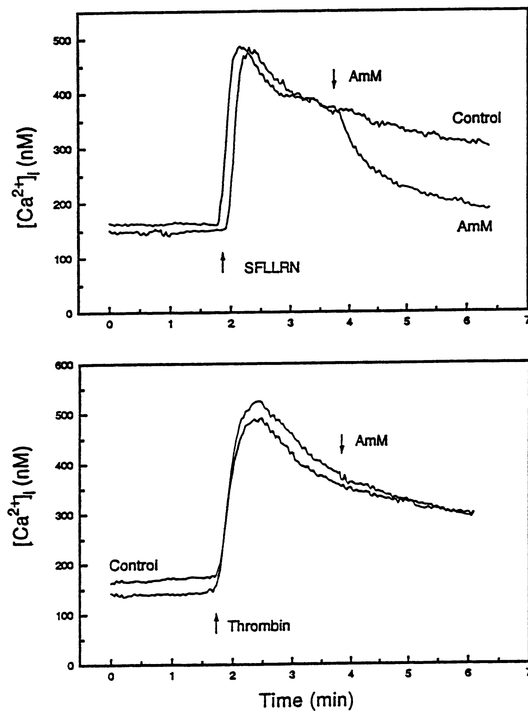

Figure 8 Effect of protease, AmM, on the sustained increase in $[Ca^{2+}]_i$

Two traces are superimposed in each panel. SFLLRN (10 μM; upper panel) or thrombin (2 units/ml; lower panel) were added to Sf9 cells expressing the thrombin receptor. At the time indicated by the arrow, AmM (2 units/ml) was added to one trace.

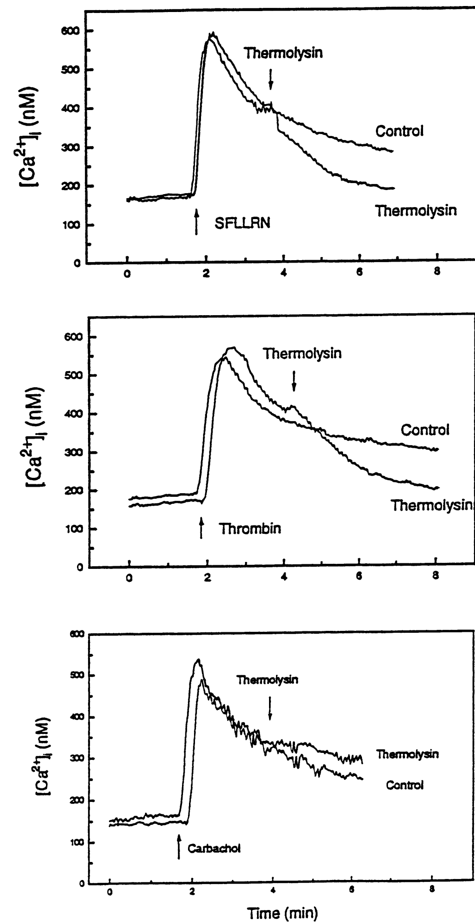

Figure 9 Effect of protease, thermolysin, on the sustained increase in $[Ca^{2+}]_i$

Two traces are superimposed in each panel. SFLLRN (10 μM; upper panel) or thrombin (2 units/ml; middle panel) were added to Sf9 cells expressing the thrombin receptor. At the time indicated by the arrow, thermolysin (5 units/ml) was added to one trace. In the lower panel, carbachol (1 mM) was added to cells expressing the M_5 muscarinic receptor. At the time indicated by the arrow, thermolysin (5 units/ml) was added to one trace.

AmM has no effect on the tethered ligand of the thrombin receptor.

The inability of AmM to modify the response after thrombin treatment prompted us to look for other proteases which may act on the tethered ligand. Primary sequence analysis (Proteolytic Digestion Program, IntelliGenetics Suite), identified three additional proteases which may cleave the thrombin receptor: thermolysin, chymotrypsin and pepsin. For each of these enzymes, there is a potential cleavage site in the N-terminus at position Phe-43/Leu-44. Thermolysin has an additional site at Leu-44/Leu-45. Since the minimum effective agonist peptide is the 5-amino-acid peptide SFLLR, removal of any amino acid from the tethered ligand will cause the loss of agonist activity [5,24]. We reasoned that these proteases may digest the agonist peptide and/or tethered ligand and terminate the sustained Ca^{2+} response.

Similar to AmM, thermolysin (5 units/ml) reversed the sustained increase in $[Ca^{2+}]_i$ induced by SFLLRN (Figure 9). Furthermore, thermolysin also reversed the thrombin-induced sustained increase in $[Ca^{2+}]_i$. As a control, thermolysin (5 units/ml) had no effect on the sustained response after carbachol stimulation of the Sf9 cells expressing the M_5 muscarinic receptor. These results suggest that thermolysin can inactivate both the agonist peptide SFLLRN and tethered ligand generated by thrombin proteolysis. The effect of thermolysin to reverse the sustained response to SFLLRN and thrombin, may reflect cleavage of the tethered ligand or a change in the overall receptor structure. As shown in Figure 10, treatment of Sf9 cells with thermolysin (5 units/ml) abolished the response to thrombin (2 units/ml), as well as the response to SFLLRN (20 μM). The response to SFLLRN, but not to thrombin, was recovered by treatment with the thermolysin inhibitor, thiorphan (100 μM) [25], added after thermolysin, suggesting that thermolysin has no effect on the overall structure of the thrombin receptor. Thiorphan alone had no effects on the $[Ca^{2+}]_i$ response to thrombin or SFLLRN (results not shown).

The above experiments (Figures 8–10) suggest that the tethered ligand remains active for 2–3 min after thrombin treatment, but is this the mechanism responsible for the long-lasting increase in basal $[Ca^{2+}]_i$ observed in Sf9 cells following thrombin pretreatment and washout as seen in Figures 6 and 7? As shown in Figure 11, pretreatment with thrombin (2 units/ml, 5 min) followed by washout for 10 min produced an increase in basal $[Ca^{2+}]_i$ from 157 ± 9 nM to 218 ± 9 nM ($P < 0.05$). The increased basal $[Ca^{2+}]_i$ was decreased from 237 ± 9 to 155 ± 4 nM ($P < 0.05$, $n = 3$) by addition of La^{3+} (10 μM), suggesting that the long-lasting component is due to the influx of Ca^{2+} from extracellular spaces. AmM (up to 10 units/ml) had no effects on the increased basal $[Ca^{2+}]_i$ after thrombin treatment, whereas addition of thermolysin (5 units/ml) decreased $[Ca^{2+}]_i$ from 236 ± 13 to 170 ± 16 nM ($P < 0.05$, $n = 3$). These results suggest that the long-lasting increase in basal $[Ca^{2+}]_i$ is due to sustained

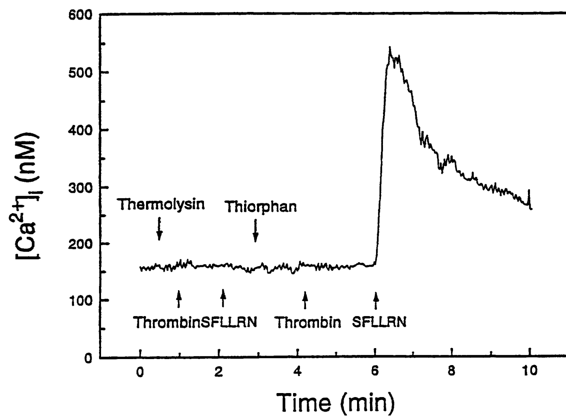

Figure 10 Effects of the thermolysin inhibitor, thiorphan

At the times indicated by the arrows, thermolysin (5 units/ml), thiorphan (100 μM), thrombin (2 units/ml) and SFLLRN (10 μM) were added to suspensions of Sf9 cells expressing the thrombin receptor.

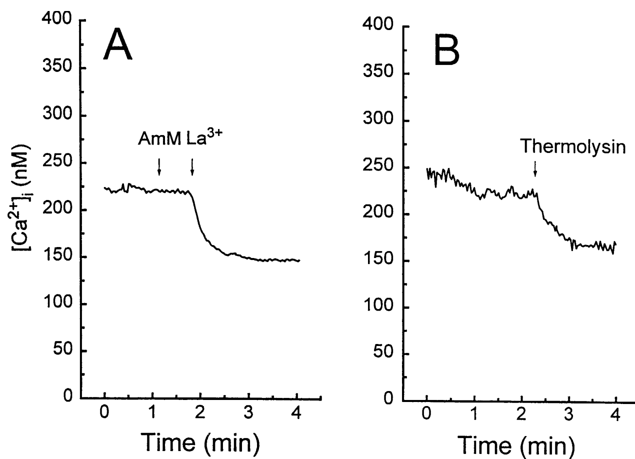

Figure 11 Effects of thrombin pretreatment on the basal $[Ca^{2+}]_i$ in Sf9 cells expressing the thrombin receptor

Sf9 cells were pretreated with thrombin (2 units/ml) for 5 min as described in the legend to Figure 6. Basal $[Ca^{2+}]_i$ was determined 10 min following washout of the agonist. AmM (2 units/ml) and La^{3+} (10 μM; **A**), or thermolysin (5 units/ml; **B**) were added at the times indicated by the arrows.

influx of Ca^{2+} from the extracellular space, reflecting activation of thrombin receptor by the tethered ligand.

DISCUSSION

The baculovirus expression system has been used successfully to express a variety of active proteins, including nuclear-localized, cytoplasmic, membrane-associated and secreted proteins. At the functional level, baculovirus-encoded thrombin receptors are very similar to thrombin receptors expressed in mammalian systems. The recombinant receptors are linked to changes in $[Ca^{2+}]_i$ and can be activated by both thrombin and by the agonist peptide SFLLRN. Furthermore, the EC_{50} for thrombin and SFLLRN in Sf9 cells is similar to the EC_{50} reported in mammalian cells [5,26]. These results demonstrate that thrombin receptors expressed in Sf9 cells using the baculovirus system are synthesized, folded and targeted to the surface membrane, and interact with endogenous Ca^{2+} signal-transduction mechanisms in a manner similar to native thrombin receptors in mammalian cells.

One striking difference between the response of native thrombin receptors in HEL cells and those expressed in Sf9 insect cells is the time course of $[Ca^{2+}]_i$ change following receptor stimulation. In HEL cells, the response to thrombin or SFLLRN was transient, whereas the response of recombinant receptors in Sf9 cells was sustained. This difference appears to be cell-related rather than receptor-related, since the response of HEL cells to ATP was also transient and the response of the Sf9 cells expressing the muscarinic receptor to carbachol was sustained. Furthermore, the sustained response observed in Sf9 cells may in fact be more representative of the typical mammalian cell response since many other cell types (e.g. endothelial and smooth-muscle cells) respond to thrombin with a sustained change in $[Ca^{2+}]_i$ [27–29]. Previous studies in HEL cells indicate that the thrombin receptor undergoes rapid (within 1 min) internalization following stimulation by either thrombin or the agonist peptide [30]. Thus, the transient nature of the HEL cell response to thrombin or SFLLRN is unusual and appears to reflect a rapid receptor sequestration.

In order to determine the mechanism associated with the slow desensitization of the thrombin receptor in Sf9 cells, the cells were first pretreated either with agonist peptide or thrombin and subsequently (following wash) subjected to a second challenge with the agonist peptide. The response to a second challenge with SFLLRN, at a concentration that would be maximal for a first response, was attenuated, but clearly greater in magnitude than that seen in HEL cells. Using this same protocol, a complete concentration–response profile was constructed. The results suggest that slow agonist-induced desensitization reflects a decrease in the affinity of the thrombin receptor for the agonist peptide. The physiological importance of this mechanism of receptor deactivation is unknown since the change in affinity of the thrombin receptor for the active tethered ligand cannot be determined. Because the $[Ca^{2+}]_i$ response is directly related to continuous receptor activity (see below), this change in affinity may in part be responsible for the slow decrease in $[Ca^{2+}]_i$ observed during the sustained phase of the response to both thrombin and SFLLRN.

During the course of the desensitization experiments it became evident that upon washout of SFLLRN $[Ca^{2+}]_i$ returned to basal prestimulated levels, whereas following pretreatment and washout of thrombin, the $[Ca^{2+}]_i$ never returned to the basal level. It is well established in many non-excitable cell types, including Sf9 insect cells, that the sustained component of the $[Ca^{2+}]_i$ response to specific receptor stimulation is dependent upon continuous receptor occupation and reflects the influx of Ca^{2+} from the extracellular space [16,31]. Addition of either specific receptor antagonists or of Ca^{2+}-influx blockers such as La^{3+} or Gd^{3+} rapidly returns $[Ca^{2+}]_i$ to resting levels. The effect of La^{3+} addition during the sustained phase of the $[Ca^{2+}]_i$ response to thrombin and SFLLRN in the Sf9 cell demonstrates a similar Ca^{2+}-influx component and suggests that continuous thrombin receptor stimulation may be responsible for the sustained component. Brass and colleagues [6,7] found that, in HEL cells, the thrombin-cleaved receptor failed to reactivate during recovery from homologous desensitization, but the cells remained responsive to agonist peptides. They hypothesized that a critical portion of the N-terminus is no longer available and that perhaps further cleavage of the N-terminus by proteolysis may be responsible for inactivation of the tethered ligand. In this regard, Coller et al. [22,23] reported that AmM, in plasma and on endothelial cells, can inactivate the agonist peptide by cleavage of the N-terminal serine. Likewise, it is possible that proteolysis occurs during

internalization and recycling of the receptors to the membrane surface in the HEL cell. Alternatively, it has been suggested that continued receptor stimulation by the tethered ligand requires thrombin to 'guide' the ligand to its binding domain [7].

In the present study, the sustained response after SFLLRN could be abolished by either AmM or thermolysin, suggesting that the sustained increase in $[Ca^{2+}]_i$ was due to the continuous occupancy of the receptor by the peptide ligand. The observation that thermolysin selectively abolished the response to thrombin suggests that the tethered ligand is responsible for the continuous receptor response. The ability of the thrombin receptor to fully respond to agonist peptide following treatment with thermolysin indicates that the overall structure of the receptor remains intact and the finding that the carbachol response of Sf9 cells expressing the muscarinic receptor is unaffected by thermolysin demonstrates that protease treatment has no effect on the downstream mechanisms associated with Ca^{2+} signalling. Together these results clearly demonstrate that the tethered ligand can remain active for extended periods of time and does not require the continuous presence of thrombin for activity.

Inability of the AmM to reverse the sustained increase in $[Ca^{2+}]_i$ suggests that, in Sf9 cells, AmM does not digest the tethered ligand even at a concentration of 10 units/ml. Previous studies have shown that AmM cannot inactivate the tethered ligand produced by cleavage of the thrombin receptor in platelets [22]. Since AmM is the predominant peptidase identified in the plasma [22], it seems likely that inactivation by AmM *in vivo* plays little role in thrombin receptor responses. This does not eliminate the possibility that some other protease in plasma may function specifically to inactivate the thrombin receptor. It is unclear why AmM had no effects on the thrombin-induced sustained increase in $[Ca^{2+}]_i$. Since thermolysin appears to be capable of proteolytic cleavage, sequestration of the tethered ligand within the active binding domain may not be responsible for the lack of effect of AmM on the thrombin response. One possibility is that steric and/or charge–charge interactions between AmM and the thrombin receptor hinder access of AmM to the N-terminus. Alternatively, thermolysin may cleave the receptor at some site distal to the N-terminus that is not exposed to AmM.

In conclusion, the results of the present study demonstrate the usefulness of the baculovirus–Sf9 insect cell expression system for the functional expression of human thrombin receptors. Although we do not know the level of thrombin receptor expression, the baculovirus system is commonly used as an overexpression vector. This approach may ultimately allow for the production of large quantities of functional protein necessary for further biochemical studies on the mechanisms of receptor activation and desensitization.

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REFERENCES

1 Shuman, M. A. (1990) Ann. N. Y. Acad. Sci. **485**, 228–239
2 Vu, T.-K. H., Hung, D. T., Wheaton, V. I. and Coughlin, S. R. (1991) Cell **64**, 1057–1068
3 Vu, T.-K. H., Wheaton, V. I., Hung, D. T., Charo, I. and Coughlin, S. R. (1991) Nature (London) **353**, 674–677
4 Scarborough, R. M., Naughton, M., Teng, W., Hung, D. T., Rose, J., Vu, T. H., Wheaton, V. I., Turck, C. W. and Coughlin, S. R. (1992) J. Biol. Chem. **267**, 13146–13149
5 Vassallo, R. R., Kieber-Emmons, T., Cichowski, K. and Brass, L. F. (1992) J. Biol. Chem. **267**, 6081–6085
6 Brass, L. F. (1992) J. Biol. Chem. **267**, 6044–6050
7 Brass, L. F., Pizarro, S., Ahuja, M., Belmonte, E., Blanchard, N., Stadel, J. M. and Hoxie, J. A. (1994) J. Biol. Chem. **269**, 2943–2952
8 Raming, K., Krieger, J., Strotmann, J., Boekhoff, I., Kubick, S., Baumstark, C. and Breer, H. (1993) Nature (London) **361**, 353–356
9 Oker-Blom, C., Jansson, C., Karp, M., Lindqvist, C., Savola, J.-M., Vlak, J. and Akerman, K. (1993) Biochim. Biophys. Acta **1176**, 269–275
10 Parker, E. M., Kameyama, K., Higashijima, T. and Ross, E. M. (1991) J. Biol. Chem. **266**, 519–527
11 George, S. T., Arbabian, M. A., Ruoho, A. E., Kiely, J. and Malbon, C. C. (1989) Biochem. Biophys. Res. Commun. **163**, 1265–1269
12 Quehenberger, O., Prossnitz, E. R., Cochrane, C. G. and Ye, R. D. (1992) J. Biol. Chem. **267**, 19757–19760
13 Richardson, R. M. and Hosey, M. M. (1992) J. Biol. Chem. **267**, 22249–22255
14 Vasudevan, S., Reilander, H., Maul, G. and Michel, H. (1991) FEBS Lett. **283**, 52–56
15 Vasudevan, S., Premkumar, L., Stowe, S., Gage, P. W., Reilander, H. and Chung, S.-H. (1992) FEBS Lett. **311**, 7–11
16 Hu, Y., Rajan, L. and Schilling, W. P. (1994) Am. J. Physiol. Cell Physiol. **266**, C1736–C1743
17 Hu, Y. and Schilling, W. P. (1995) Biochem. J. **305**, 605–611
18 Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor
19 Grynkiewicz, G., Poenie, M. and Tsien, R. Y. (1985) J. Biol. Chem. **260**, 3440–3450
20 Shuttleworth, T. J. and Thompson, J. L. (1991) J. Biol. Chem. **266**, 1410–1414
21 Hu, Y., Vaca, L., Zhu, X., Birnbaumer, L., Kunze, D. L. and Schilling, W. P. (1994) Biochem. Biophys. Res. Commun. **201**, 1050–1056
22 Coller, B. S., Ward, P., Ceruso, M., Scudder, L. E., Springer, K., Kutok, J. and Prestwich, G. D. (1992) Biochemistry **31**, 11713–11720
23 Coller, B. S., Springer, K., Scudder, L. E., Kutok, J. L., Ceruso, M. and Prestwich, G. D. (1993) J. Biol. Chem. **268**, 20741–20743
24 Chao, B. H., Kalkunte, S., Maraganore, J. M. and Stone, S. R. (1995) Biochemistry **31**, 6175–6178
25 Benchetrit, T., Fournie-Zaluski, M. C. and Roques, B. P. (1987) Biochem. Biophys. Res. Commun. **147**, 1034–1040
26 Babich, M., King, K. L. and Nissenson, R. A. (1990) Endocrinology **126**, 948–954
27 Lum, H., Andersen, T. T., Siflinger-Birnboim, A., Tiruppathi, C., Goligorsky, M. S., Fenton, J. W., II and Malik, A. B. (1993) J. Cell Biol. **120**, 1491–1499
28 Goligorsky, M. S., Menton, D. N., Laszlo, A. and Lum, H. (1989) J. Biol. Chem. **264**, 16771–16775
29 Neylon, C. B. and Irvine, R. F. (1991) J. Biol. Chem. **266**, 4251–4256
30 Hoxie, J. A., Ahuja, M., Belmonte, E., Pizarro, S., Parton, R. and Brass, L. F. (1993) J. Biol. Chem. **268**, 13756–13763
31 Putney, J. W., Jr. and Bird, G. S. J. (1993) Endocr. Rev. **14**, 610–631