Differential Ca2+responses induced by thrombin and thrombin-receptor agonist peptides in HSY-EA1 cells

ARTICLE *in* CELL BIOLOGY INTERNATIONAL · FEBRUARY 2003

Impact Factor: 1.93 · DOI: 10.1016/j.cellbi.2003.10.001 · Source: PubMed

5 AUTHORS, INCLUDING:

Akihiko Tanimura
Health Sciences University of Hokkaido
87 PUBLICATIONS **953** CITATIONS
SEE PROFILE

Akiko Shitara
21 PUBLICATIONS **95** CITATIONS
SEE PROFILE

Akihiro Nezu
Health Sciences University of Hokkaido
38 PUBLICATIONS **421** CITATIONS
SEE PROFILE

ELSEVIER

Cell Biology International 27 (2003) 1017–1023

Cell Biology International

www.elsevier.com/locate/cellbi

Differential Ca^{2+} responses induced by thrombin and thrombin-receptor agonist peptides in HSY-EA1 cells

Akihiko Tanimura*, Akiko Shitara, Akihiro Nezu, Takao Morita, Yosuke Tojyo

Department of Dental Pharmacology, School of Dentistry, Health Sciences University of Hokkaido, Ishikari-Tobetsu, Hokkaido 061-0293, Japan

Received 10 February 2003; revised 3 July 2003; accepted 23 September 2003

Abstract

We examined the mechanism by which protease-activated receptor (PAR)-1 is desensitized by comparing the effect of thrombin and the soluble agonist peptide SFLLRN on Ca^{2+} responses in HSY-EA1 cells. Thrombin-induced increases in cytosolic Ca^{2+} concentrations ($[Ca^{2+}]_i$) returned to basal levels within 60 s, but SFLLRN generated a sustained $[Ca^{2+}]_i$ elevation. Interestingly, thrombin-desensitized cells partially retained their ability to respond to SFLLRN. We desensitized PAR-2 by pretreating cells with SLIGKV to confirm that this response was not due to PAR-2, which can recognize SFLLRN. The highly specific PAR-1 agonist peptide TFLLR also increased $[Ca^{2+}]_i$ in PAR-2-desensitized cells pretreated with thrombin. These observations indicate that thrombin disarms PAR-1 from further proteolytic activation, but leaves the receptor responsive for non-tethered ligands.

Keywords: Protease-activated receptor; Thrombin; Calcium; Desensitization; SFLLRN; TFLLR

1. Introduction

Protease-activated receptors (PAR) belong to the G-protein-coupled receptor (GPCR) family and are activated by a unique proteolytic mechanism (Macfarlane et al., 2001; Vu et al., 1991a). That is, the amino-terminus of PAR is cleaved by a protease, such as thrombin or trypsin, and the newly formed amino-terminus serves as an intramolecular tethered ligand. The unmasked tethered ligand activates PAR by interacting with the second extracellular loop of the cleaved PAR. PAR-1 was the first PAR identified, and is the most extensively studied receptor of this family. PAR-1 is also known to evoke Ca^{2+} responses through the activation of phospholipase C (Chen et al., 1994; Hammes and Coughlin, 1999; Vu et al., 1991a,b).

Activation of PAR-1 induces a transient Ca^{2+} response that declines rapidly due to homologous desensitization (Brass, 1992). Since unmasking of the tethered ligand of PAR is an irreversible event, such desensitization might be important in terminating PAR-1-mediated signaling. Desensitization of PAR-1 appears to occur by the uncoupling of PAR-1 from the G-protein and subsequent internalization (Brass et al., 1994; Hoxie et al., 1993; Woolkalis et al., 1995). Interestingly, however, thrombin-desensitized receptors remain responsive to thrombin-receptor agonist peptides (TRAP) (Brass et al., 1994; Ishii et al., 1993; Mizuno et al., 2000; Molino et al., 1997). These observations suggest that thrombin-induced desensitization may also involve as yet undefined mechanisms that act upstream of the ligand-binding event.

To elucidate the mechanism by which thrombin induces PAR-1 desensitization, we compared the effects of thrombin and TRAP on Ca^{2+} responses in the human parotid cell line HSY-EA1. Our results suggest that the thrombin-induced desensitization of PAR-1 involves both the inactivation of the tethered ligand and the uncoupling of downstream signaling processes.

* Corresponding author. Tel.: +81-1332-3-1211; fax: +81-1332-3-1399

E-mail address: tanimura@hoku-iryo-u.ac.jp (A. Tanimura).

Abbreviations: BSA, Bovine serum albumin; $[Ca^{2+}]_i$, cytosolic Ca^{2+} concentrations; GPCR, G-protein-coupled receptor; PAR, protease-activated receptor; RT–PCR, reverse transcriptase–polymerase chain reaction; TRAP, thrombin-receptor agonist peptides.

1065-6995/03/$ - see front matter © 2003 Elsevier Ltd. All rights reserved.
doi:10.1016/j.cellbi.2003.10.001

2. Materials and methods

2.1. Reagents

The agonist peptides SFLLRN, SLIGKV and GYPGQV were obtained from Bachem (Bubendorf, Switzerland) and TFLLR-NH2 from Tocris Cookson Ltd (Bristol, UK). Bovine plasma thrombin was purchased from Itoham Foods Inc. (Hyogo, Japan). Bovine serum albumin (BSA) was from Sigma. Fura-2 acetoxymethyl ester (fura-2/AM) was purchased from Dojin Chemicals (Kumamoto, Japan). All other reagents used were of analytical grade.

2.2. Cell culture

The HSY human parotid cell line, a generous gift from Dr Mitsunobu Sato (Tokushima University, Japan), was subcloned by a dilution plating technique, and six clones were obtained. One of these subclones, HSY-EA1, was used in this study. The cells were cultured in Dulbecco's Eagle's medium nutrient mixture F-12 Ham (Sigma) supplemented with 10% newborn calf serum, 2 mM glutamine, and 100 μg/ml each of penicillin and streptomycin, as previously described (Moran and Turner, 1993).

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

HSY-EA1 cells were plated in 100 mm diameter dishes at a concentration of 1×10^6 cells per dish or in 60 mm diameter dishes at a concentration of 4×10^5 cells per dish and cultured for 4–6 days. The cells were then detached with Ca^{2+}/Mg^{2+}-free phosphate buffered saline (Gibco) and incubated for 30 min at room temperature with 2 μM fura-2-AM in Hanks' balanced salt solution with HEPES (HBSS-H) containing 137 mM NaCl, 5.4 mM KCl, 1.3 mM $CaCl_2$, 0.41 mM $MgSO_4$, 0.49 mM $MgCl_2$, 0.34 mM Na_2HPO_4, 0.44 mM NaH_2PO_4, 5.5 mM glucose, 20 mM HEPES (pH 7.4), and 0.2% BSA. The fura-2-loaded cells were washed twice, resuspended in fresh HBSS-H and stored at room temperature until use. Fura-2 fluorescence was measured at 37 °C with a Hitachi F2000 spectrofluorometer (Hitachi, Tokyo, Japan) with excitation at 340 and 380 nm and emission at 510 nm. $[Ca^{2+}]_i$ was calculated from the fluorescence ratio (Grynkiewicz et al., 1985).

3. Results

Fig. 1A and B show the typical Ca^{2+} response elicited by thrombin (PAR-1 activating enzyme) and SFLLRN (PAR-1 agonist peptide) in HSY-EA1 cells. The addition of 20 U/ml thrombin rapidly elevated $[Ca^{2+}]_i$ levels, which returned to basal levels within 60 s after the maximal response (Fig. 1A). However, increased $[Ca^{2+}]_i$ levels induced by SFLLRN were sustained after the peak Ca^{2+} response (Fig. 1C). The magnitude of the peak Ca^{2+} response increased in a dose-dependent manner over the thrombin concentration range 0.2–20 U/ml, but the $[Ca^{2+}]_i$ level consistently returned to the basal level at 60 s after the peak, regardless of the thrombin concentration (Fig. 1B). Comparable peak responses were observed following treatment with SFLLRN over the concentration range 1–100 μM (Fig. 1D), however, the $[Ca^{2+}]_i$ level consistently remained ~150 nM above the basal level 60 s after the peak (Fig. 1D). Thus, the thrombin-induced Ca^{2+} response was more rapidly desensitized than the SFLLRN-induced Ca^{2+} response.

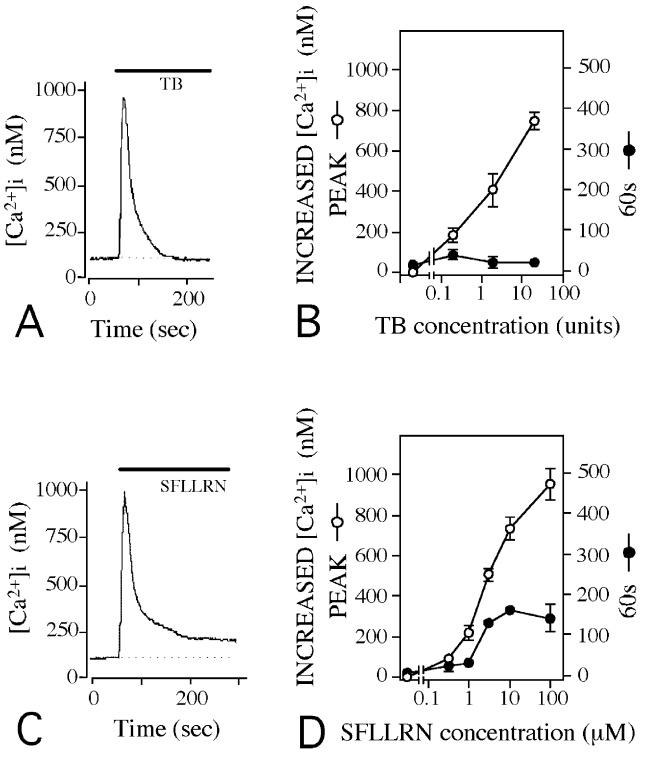

Fig. 1. Thrombin and SFLLRN induce different Ca^{2+} responses. (A) and (C): Fura-2-loaded HSY-EA1 cells were stimulated with 20 U/ml thrombin (A) or 100 μM SFLLRN (C). The presence of thrombin (TB) or SFLLRN in the medium is indicated by a horizontal bar. Results shown are typical representations of seven independent experiments. (B) and (D): Effect of different concentrations of thrombin (B) or SFLLRN (D) on $[Ca^{2+}]_i$ increases above basal level at the peak response (open circles) and at 60 s after the peak (closed circles). Values shown are the mean ± S.E. of four to seven independent experiments.

We also compared the effect of thrombin and SFLLRN on PAR-1 desensitization by exposing HSY-EA1 cells to various concentrations of thrombin or SFLLRN for 3 min and then stimulating them again with the thrombin concentration that elicited a maximal primary response (20 U/ml) (Figs. 2 and 3). Pretreatment with 0.2 U/ml thrombin (Fig. 2B and E) reduced the subsequent Ca^{2+} response to ~34% of control cells (Fig. 2A), even though 0.2 U/ml thrombin itself elicited only a small Ca^{2+} response (Fig. 2B). When cells were

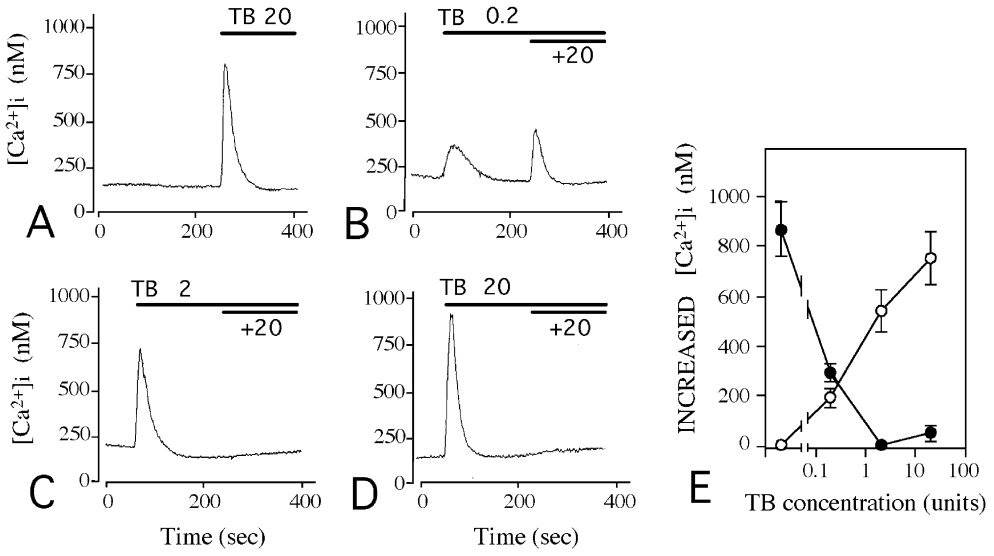

Fig. 2. Desensitization of PAR-1 by thrombin. (A): Cells were stimulated with 20 U/ml thrombin in the absence of any pretreatment. (B)–(D): Cells were stimulated with 0.2 U/ml (B), 2 U/ml (C) or 20 U/ml (D) thrombin for 3 min, after which 20 U/ml thrombin was added. Results shown are typical representations of six or seven independent experiments. The presence of thrombin (TB) is indicated by a horizontal bar. (E): The increase in $[Ca^{2+}]_i$ above basal level after the first stimulation with various concentrations of thrombin (open circles) and after the second stimulation with 20 U/ml thrombin (closed circles). Values shown are the mean ± S.E. of six or seven independent experiments.

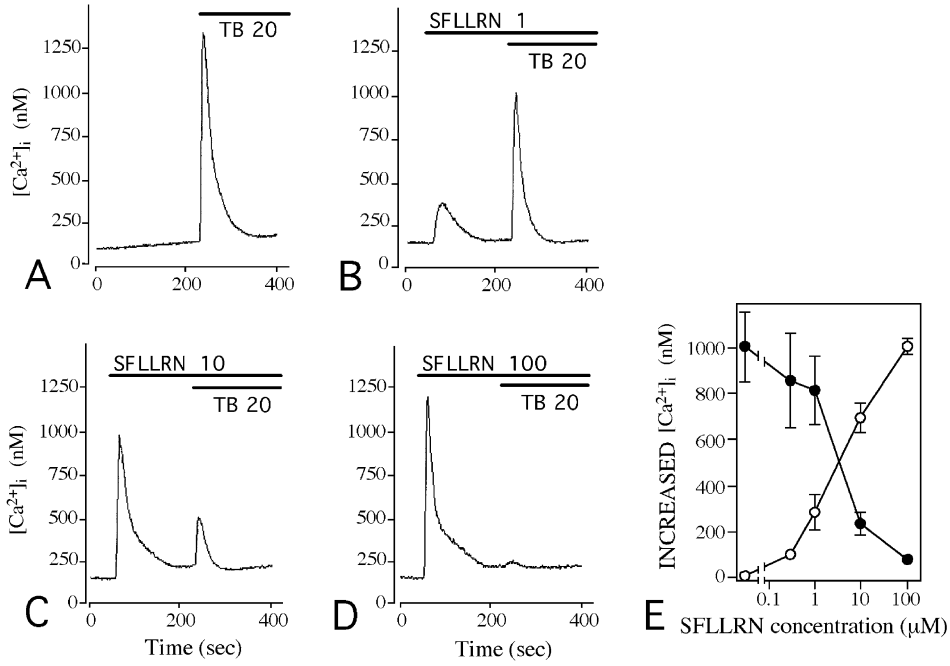

Fig. 3. Desensitization of PAR-1 by SFLLRN. (A): Cells were stimulated with 20 U/ml thrombin in the absence of any pretreatment. (B)–(D): Cells were stimulated with 1 μM (B), 10 μM (C) or 100 μM (D) SFLLRN for 3 min, after which 20 U/ml thrombin was added. Results shown are typical representations of six or seven independent experiments. The presence of thrombin (TB) and SFLLRN in the medium are indicated by a horizontal bar. (E): The increase in $[Ca^{2+}]_i$ above basal level after the first stimulation with various concentrations of SFLLRN (open circles) and after the second stimulation with 20 U/ml thrombin (closed circles). Values shown are the mean ± S.E. of six or seven independent experiments.

pretreated with the suboptimal 2 U/ml or the optimal 20 U/ml thrombin concentrations, the subsequent Ca^{2+} response was completely abolished (Fig. 2C–E).

The Ca^{2+} response to treatment with 1 μM SFLLRN was comparable to the response elicited by 0.2 U/ml thrombin (compare Fig. 2B and Fig. 3B). However, pretreatment with 1 μM SFLLRN reduced the subsequent Ca^{2+} response to treatment with 20 U/ml thrombin to ~81% (Fig. 3B and E) of the control response (Fig. 3A). The suboptimal concentration of

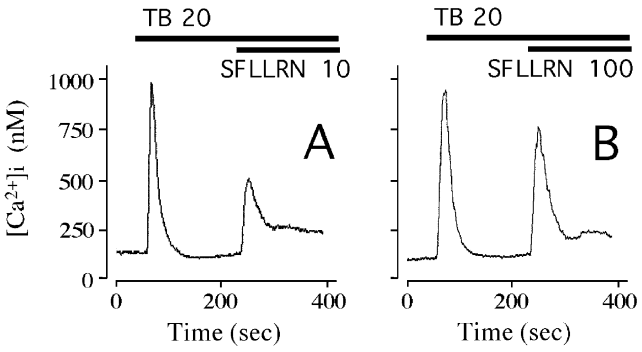

Fig. 4. SLFFRN-induced Ca^{2+} responses in thrombin-pretreated cells. Cells were pretreated with 20 U/ml thrombin for 3 min, after which 10 μM SFLLRN (A) or 100 μM SFLLRN (B) was added. The presence of thrombin (TB) or SFLLRN in the medium is indicated by a horizontal bar. Results shown are typical representations of six independent experiments.

Table 1
Desensitization of PAR-1 and PAR-2 by pretreatment with SLIGKV and thrombin

Stimulating peptides	Pretreatment with SLIGKV	Pretreatment with thrombin	
		−	+
SFLLRN	−	1061.7 ± 126.3 (6)	630.2 ± 91.7 (6)
(100 μM)	+	723.9 ± 103.4 (3)	532.0 ± 36.1 (3)
SFLLRN	−	714.0 ± 179.3 (6)	366.4 ± 30.3 (6)
(10 μM)	+	363.9 ± 86.9 (5)	154.9 ± 12.0 (3)
TFLLRN	−	673.2 ± 157.7 (7)	123.9 ± 33.1 (5)
(100 μM)	+	390.5 ± 100.4 (4)	116.5 ± 8.4 (4)
SLIGKV	−	724.9 ± 64.9 (4)	590.4 ± 28.7 (4)
(100 μM)	+	ND	ND
SLIGKV	−	443.6 ± 12.2 (5)	232.0 ± 43.2 (3)
(20 μM)	+	88.7 ± 23.1 (4)	43.5 ± 10.7 (3)

Results shown are stimulating peptide-induced increases in $[Ca^{2+}]_i$ above basal level with (+) or without (−) pretreatment with 100 μM SLIGKV and/or 20 U/ml thrombin. Values shown are the mean ± S.E. from three to seven independent experiments.

10 μM SFLLRN also failed to abolish the subsequent Ca^{2+} response (Fig. 3C and E). The agonist peptide of PAR-4, GYPGQV (up to 1 mM), did not increase $[Ca^{2+}]_i$ following stimulation with SFLLRN (data not shown). Although the optimal concentration of 100 μM SFLLRN strongly attenuated the subsequent Ca^{2+} response, there was a detectable response to 20 U/ml thrombin (Fig. 3D and E). Pretreatment with 100 μM SFLLRN also attenuated the subsequent Ca^{2+} response to treatment with 100 μM SFLLRN to a similar extent (data not shown). These results suggest that either thrombin is more effective at desensitizing the receptor, or that thrombin and SFLLRN desensitize PAR-1 through different mechanisms.

When cells were pretreated with the optimal 20 U/ml thrombin concentration, the subsequent Ca^{2+} responses to 10 μM and 100 μM SFLLRN were reduced to only ∼52% and ∼59%, respectively (Fig. 4 and Table 1). Since SFLLRN is known to activate PAR-2, we examined the effects of TFLLR, a highly selective agonist peptide of PAR-1 (Hollenberg et al., 1997). Stimulation with 100 μM TFLLR induced a comparable Ca^{2+} response to that induced by 10 μM SFLLRN (Fig. 5A and B). Pretreatment with 20 U/ml thrombin reduced the TFLLR-induced Ca^{2+} response to ∼18% (Fig. 5C and Table 1). Thus, TRAP (SFLLRN and TFLLR) can reactivate PAR-1 after pretreatment with thrombin. On the other hand, pretreatment with thrombin more pronouncedly reduced the TFLLR-induced Ca^{2+} response than the SFLLRN-induced response, suggesting that the effect of SFLLRN is at least in part mediated by PAR-2.

To further determine whether PAR-2 is involved in the SFLLRN-induced Ca^{2+} response, we attempted to eliminate the PAR-2-mediated Ca^{2+} response. Stimulation with the PAR-2 selective agonist peptide SLIGKV rapidly elevated $[Ca^{2+}]_i$, which remained 100–200 nM above the basal level (Fig. 5D and E). Pretreatment with 20 U/ml thrombin reduced the 20 μM SLIGKV-induced Ca^{2+} response to ∼52% (Fig. 5F and Table 1). Thrombin appears to reduce the subsequent Ca^{2+} response to SLIGKV by heterologous desensitization, since SLIGKV is highly selective for PAR-2 (Hollenberg et al., 1993). Pretreatment with 100 μM SLIGKV reduced the subsequent Ca^{2+} response to 20 μM SLIGKV to ∼20% (Fig. 6A and Table 1) by homologous desensitization.

When cells were pretreated with 100 μM SLIGKV and thrombin, subsequent Ca^{2+} responses to 20 μM SLIGKV were attenuated almost completely (Fig. 6B), confirming that pretreatment with 100 μM SLIGKV eliminates the effect of PAR-2 in the subsequent Ca^{2+} response. We then examined the TRAP-induced Ca^{2+} response in PAR-2-desensitized cells (Fig. 6C–F). When cells were pretreated with 100 μM SLIGKV, the SFLLRN-induced Ca^{2+} response following the thrombin-induced response was further reduced to ∼58% (Fig. 6C, D and Table 1). On the other hand, ∼94% of the TFLLR-induced Ca^{2+} response following the thrombin-induced response was retained in SLIGKV-pretreated cells (Fig. 6E, F and Table 1). These results confirm that SFLLRN and TFLLR can reactivate thrombin-desensitized PAR-1.

4. Discussion

The present study demonstrated that thrombin elicited a transient Ca^{2+} response in HSY-EA1 cells, in which rapidly elevated $[Ca^{2+}]_i$ levels decreased to basal levels within 2 min. Although the agonist peptides SFLLRN and TFLLR are generally considered to mimic thrombin, these peptides induced more sustained Ca^{2+} responses. Since we have shown here that

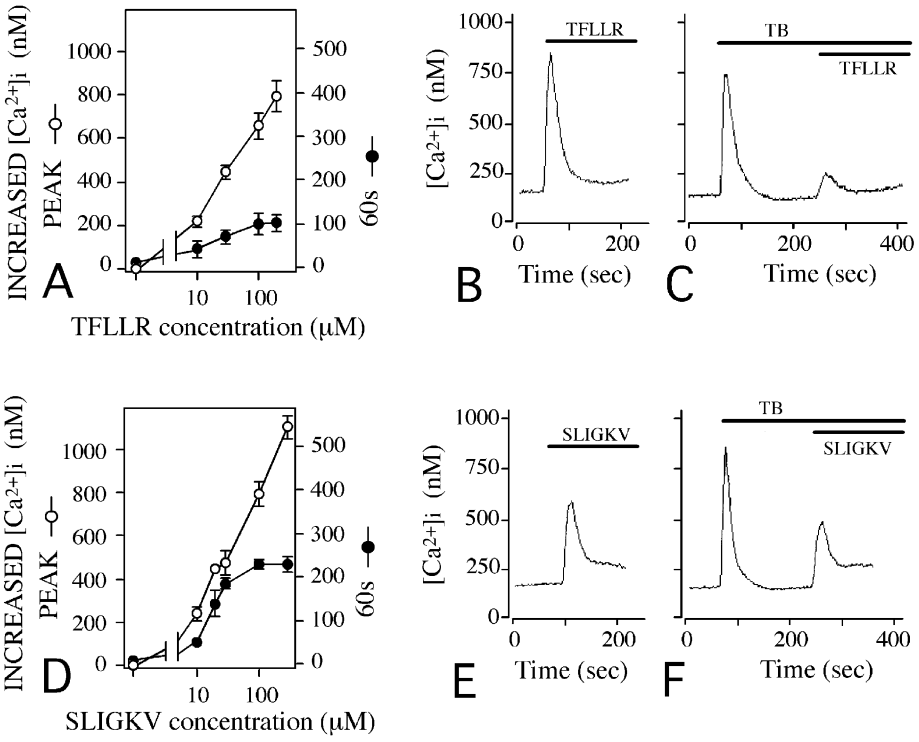

Fig. 5. TFLLR- and SLIGKV-induced Ca^{2+} responses with or without thrombin-pretreatment. (A) and (D): Effect of different concentrations of TFLLR (A) or SLIGKV (D) on $[Ca^{2+}]_i$ increases above basal level at the peak response (open circles) and at 60 s after the peak (closed circles). Values shown are the mean ± S.E. of four to seven independent experiments. (B) and (E): Cells were stimulated with 100 μM TFLLR (B) or 20 μM SLIGKV (E). (C) and (F): Cells were stimulated with 100 μM TFLLR (C) or 20 μM SLIGKV (F) after pretreatment with 20 U/ml thrombin. The presence of TFLLR, SLIGKV and thrombin (TB) in the medium is indicated by a horizontal bar. Results shown are typical representations of four independent experiments.

thrombin-desensitized HSY-EA1 cells retain responsiveness to SFLLRN and TFLLR, thrombin and agonist peptides appear to desensitize PAR-1 through diverse mechanisms. SFLLRN-induced Ca^{2+} responses after pretreatment with thrombin have been observed in many other cell types (Brass et al., 1994; Ishii et al., 1993; Mizuno et al., 2000; Molino et al., 1997).

SFLLRN-induced Ca^{2+} responses in thrombin-pretreated cells appear to be related to the activation of PAR-2 (Molino et al., 1997). However, we demonstrated here that the highly specific PAR-1 agonist peptide TFLLR induced Ca^{2+} responses after pretreatment with thrombin. In addition, PAR-2-desensitized cells responded to SFLLRN after pretreatment with thrombin. These observations clearly indicate that agonist peptides can activate thrombin-desensitized PAR-1. Therefore, thrombin may disarm PAR-1 from further proteolytic activation but leave the receptor responsive to TRAP. Consistent with this view, Hammes and Coughlin (1999) have reported that thrombin-desensitized PAR-1 mutants with altered cleavage sites are activated by the subsequent unmasking of the tethered ligand or the addition of TRAP.

As for other GPCRs, PAR-1 is known to be desensitized by a mechanism involving the uncoupling of the activated receptor from its G-protein through receptor phosphorylation (Hammes et al., 1999; Hoxie et al., 1993; Mizuno et al., 2000; Paing et al., 2002; Tiruppathi et al., 2000). Activated GPCRs are initially phosphorylated by G protein-coupled receptor kinases (GRKs), which uncouple the receptor from G proteins and internalize it (Böhm et al., 1997). This is probably the major pathway for TRAP-induced desensitization of PAR-1. In contrast, thrombin-induced desensitization of PAR-1 is not exclusively responsible for uncoupling or internalization. Because agonist peptides can induce the Ca^{2+} response via thrombin-desensitized PAR-1, proteolytically activated PAR-1 is desensitized by mechanisms situated upstream of the ligand binding event. The tethered ligand may be inactivated by proteolytic degradation (Chen et al., 1996; Coller et al., 1992) or sequestration by certain endogenous inhibitors (Hammes and Coughlin, 1999). The present study provides strong support for these hypotheses.

Thrombin-exposed cells become refractory to further thrombin stimulation, but can be reactivated by TRAP, suggesting that a homologous peptide may cause receptor reactivation in thrombin-desensitized cells. Interestingly, recent results have shown that a proteolytic product of the neuronal growth-associated protein B-50/

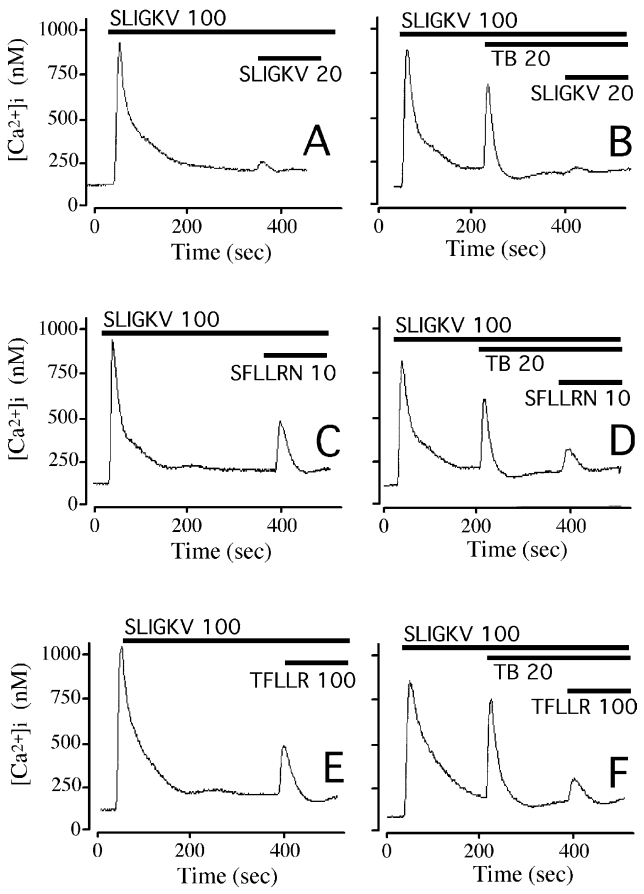

Fig. 6. Ca^{2+} responses in PAR-2-desensitized cells. (A) and (B): Cells were initially stimulated with 100 μM SLIGKV without (A) or with (B) 20 U/ml thrombin, after which 20 μM SLIGKV was added. (C) and (D): Cells were initially stimulated with 100 μM SLIGKV without (C) or with (D) 20 U/ml thrombin, after which 10 μM SFLLRN was added. (E) and (F): Cells were initially stimulated with 100 μM SLIGKV without (E) or with (F) 20 U/ml thrombin, after which 100 μM TFLLR was added. The presence of SLIGKV, SFLLRN, TFLLR and thrombin (TB) in the medium is indicated by a horizontal bar. Results shown are typical representations of three to six independent experiments.

GAP-43 acts as a soluble ligand for PAR-1 and PAR-2 (Hollenberg et al., 2000). The physiological relevance of this peptide fragment in vivo remains to be determined, however. Further studies are required to clarify all the mechanisms that lead to the desensitization of PAR, and their physiological importance.

Acknowledgements

This study was partially supported by the Academic Sciences Frontier Project and by the Grant-in-Aid for Scientific Research (14571770 to A.T.) from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

References

Böhm SK, Grady EF, Bunnett NW. Regulatory mechanisms that modulate signaling by G-protein-coupled receptors. Biochem J 1997;322:1–18.

Brass LF. Homologous desensitization of HEL cell thrombin receptors. J Biol Chem 1992;267:6044–50.

Brass LF, Pizarro S, Ahuja M, Belmonte E, Blanchard N, Stadel JM et al. Changes in the structure and function of the human thrombin receptor during receptor activation, internalization, and recycling. J Biol Chem 1994;269:2943–52.

Chen J, Ishii M, Wang L, Ishii K, Coughlin SR. Thrombin receptor activation. Confirmation of the intramolecular tethered liganding hypothesis and discovery of an alternative intermolecular liganding mode. J Biol Chem 1994;269:16041–5.

Chen X, Earley K, Luo W, Lin S-H, Schilling WP. Functional expression of a human thrombin receptor in Sf9 insect cells: Evidence for an active tethered ligand. Biochem J 1996;314:603–11.

Coller BS, Ward P, Ceruso M, Scudder LE, Springer K, Kutok J et al. Thrombin receptor activating peptides: Importance of the N-terminal serine and its ionization state as judged by pH dependence, nuclear magnetic resonance spectroscopy, and cleavage by aminopeptidase M. Biochemistry 1992;31:11713–20.

Grynkiewicz G, Poenie M, Tsien RY. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J Biol Chem 1985;260:3440–50.

Hammes SR, Coughlin SR. Protease-activated receptor-1 can mediate responses to SFLLRN in thrombin-desensitized cells: Evidence for a novel mechanism for preventing or terminating signaling by PAR1's tethered ligand. Biochemistry 1999;38:2486–93.

Hammes SR, Shapiro MJ, Coughlin SR. Shutoff and agonist-triggered internalization of protease-activated receptor 1 can be separated by mutation of putative phosphorylation sites in the cytoplasmic tail. Biochemistry 1999;38:9308–16.

Hollenberg MD, Laniyonu AA, Saifeddine M, Moore GJ. Role of the amino- and carboxyl-terminal domains of thrombin receptor-derived polypeptides in biological activity in vascular endothelium and gastric smooth muscle: evidence for receptor subtypes. Mol Pharmacol 1993;43:921–30.

Hollenberg MD, Saifeddine M, Al-Ani B, Kawabata A. Proteinase-activated receptors: structural requirements for activity, receptor cross-reactivity, and receptor selectivity of receptor-activating peptides. Can J Physiol Pharmacol 1997;75:832–41.

Hollenberg MD, Saifeddine M, Zwiers H. Proteinase-activated receptors (PARs): activation of PAR_1 and PAR_2 by a proteolytic fragment of the neuronal growth associated protein B-50/GAP-43. Can J Physiol Pharmacol 2000;78:81–5.

Hoxie JA, Ahuja M, Belmonte E, Pizarro S, Parton R, Brass LF. Internalization and recycling of activated thrombin receptors. J Biol Chem 1993;268:13756–63.

Ishii K, Hein L, Kobilka B, Coughlin SR. Kinetics of thrombin receptor cleavage on intact cells. Relation to signaling. J Biol Chem 1993;268:9780–6.

Macfarlane SR, Seatter MJ, Kanke T, Hunter GD, Plevin R. Proteinase-activated receptors. Pharmacol Rev 2001;53:245–82.

Mizuno O, Hirano K, Nishimura J, Kubo C, Kanaide H. Proteolysis and phosphorylation-mediated regulation of thrombin receptor activity in *in situ* endothelial cells. Eur J Pharmacol 2000; 389:13–23.

Molino M, Woolkalis MJ, Reavey-Cantwell J, Praticó D, Andrade-Gordon P, Barnathan ES et al. Endothelial cell thrombin receptors and PAR-2. Two protease-activated receptors located in a single cellular environment. J Biol Chem 1997;272:11133–41.

Moran A, Turner RJ. Secretagogue-induced RVD in HSY cells is due to K^+ channels activated by Ca^{2+} and protein kinase C. Am J Physiol 1993;265:C1405.

Paing MM, Stutts AB, Kohout TA, Lefkowitz RJ, Trejo J. β-Arrestins regulate protease-activated receptor-1 desensitization but not internalization or Down-regulation. J Biol Chem 2002;277:1292–300.

Tiruppathi C, Yan W, Sandoval R, Naqvi T, Pronin AN, Benovic JL et al. G protein-coupled receptor kinase-5 regulates thrombin-activated signaling in endothelial cells. Proc Natl Acad Sci U S A 2000;97:7440–5.

Vu T-KH, Hung DT, Wheaton VI, Coughlin SR. Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. Cell 1991a;64:1057–68.

Vu T-KH, Wheaton VI, Hung DT, Charo I, Coughlin SR. Domains specifying thrombin-receptor interaction. Nature 1991b;353:674–7.

Woolkalis MJ, DeMelfi TM Jr, Blanchard N, Hoxie JA, Brass LF. Regulation of thrombin receptors on human umbilical vein endothelial cells. J Biol Chem 1995;270:9868–75.