A potent and selective endogenous agonist for the μ-opiate receptor

James E. Zadina, Laszlo Hackler, Lin-Jun Ge & Abba J. Kastin

Veterans Affairs Medical Center and Tulane Unversity School of Medicine, New Orleans, Louisiana 70146, USA

Peptides have been identified in mammalian brain that are considered to be endogenous agonists for the δ (enkephalins) and κ (dynorphins) opiate receptors, but none has been found to have any preference for the μ receptor[1–3]. Because morphine and other compounds that are clinically useful and open to abuse act primarily at the μ receptor[4], it could be important to identify endogenous peptides specific for this site. Here we report the discovery and isolation from brain of such a peptide, endomorphin-1 (Tyr-Pro-Trp-Phe-NH_2), which has a high affinity (K_i = 360 pM) and selectivity (4,000- and 15,000-fold preference over the δ and κ receptors) for the μ receptor. This peptide is more effective than the μ-selective analogue DAMGO *in vitro* and it produces potent and prolonged analgesia in mice. A second peptide, endomorphin-2 (Tyr-Pro-Phe-Phe-NH_2), which differs by one amino acid, was also isolated. The new peptides have the highest specificity and affinity for the μ receptor of any endogenous substance so far described and they may be natural ligands for this receptor.

Endorphins, enkephalins and dynorphins, which all have the amino-terminal amino-acid sequence Tyr-Gly-Gly-Phe, bind with low to moderate specificity to the three opiate receptors[3] (Table 1). β-Endorphin binds to the μ and δ receptors with comparable affinity, but because of their selectivity, Met- and Leu-enkephalin are considered to be the endogenous ligands for the δ receptor and dynorphins for the κ receptor. No known mammalian peptide, however, has both high affinity and selectivity for the μ receptor; for example, Tyr-W-MIF-1 (Tyr-Pro-Trp-Gly-NH_2), a peptide in brain[5,6] with opiate-related activity[5–11], is highly selective for μ rather than δ and κ receptors (>200- and 300-fold), but its affinity for the μ receptor (K_i = 70 nM) is relatively low[12]. Natural amino-acid substitutions in the first three positions of Tyr-W-MIF-1 are not well tolerated for opiate binding, presumably because of a strict requirement for the amino and phenolic groups of Tyr at position 1, an appropriate spacer (Gly at position 2 and 3 or Pro at position 2) and an aromatic group (Phe at position 3 or 4 or Trp at position 3)[13]. With a view to identifying molecules that occur naturally in the brain, peptides containing all possible natural amino-acid substitutions at position 4 of Tyr-W-MIF-1 were synthesized by polyethylene pin technology[14] and screened for opiate-receptor binding. A high-affinity, selective and biologically potent sequence was discovered and then identified in brain.

Figure 1 (top) shows the relative affinity of each peptide for the μ receptor (black bars). The peptide with Phe in the fourth position (Phe-4-Tyr-W-MIF-1) had a greatly increased affinity compared with the other peptides: its affinity estimated from two peptide sets was >50-fold greater than that of the parent compound Tyr-W-MIF-1, with Gly in position 4.

The selectivity of the peptides for μ rather than δ receptors (Fig. 1, white bars) is compared with that of Tyr-W-MIF-1, which has a selectivity ratio of 240 in these assays, in agreement with our previous report[12]. This parent peptide proved to be one of the most selective in the set. The Phe-4 peptide, however, was far more selective for μ sites than the other peptides tested.

A more hydrophobic amino acid after the Trp 3 residue tended to increase μ receptor binding. The correlation between half-maximal inhibition (IC_{50}) of μ binding by synthetic peptide and the hydrophobicity[15] of uncharged amino acids in position 4 was significant ($P < 0.001$). Based on the r^2 value (0.56), however, only about half of the variance in binding affinity was attributable to hydrophobicity, and the binding of the Phe-4 analogue was >2 standard error units stronger than predicted from the regression. This indicates that physical chemical factors in addition to hydrophobicity are important for its high binding affinity.

Figure 1 (bottom) and Table 1 show that, in comparison with one of the most potent and μ-selective analogues of enkephalin available (DAMGO), the Phe-4 peptide has an equal affinity and greater

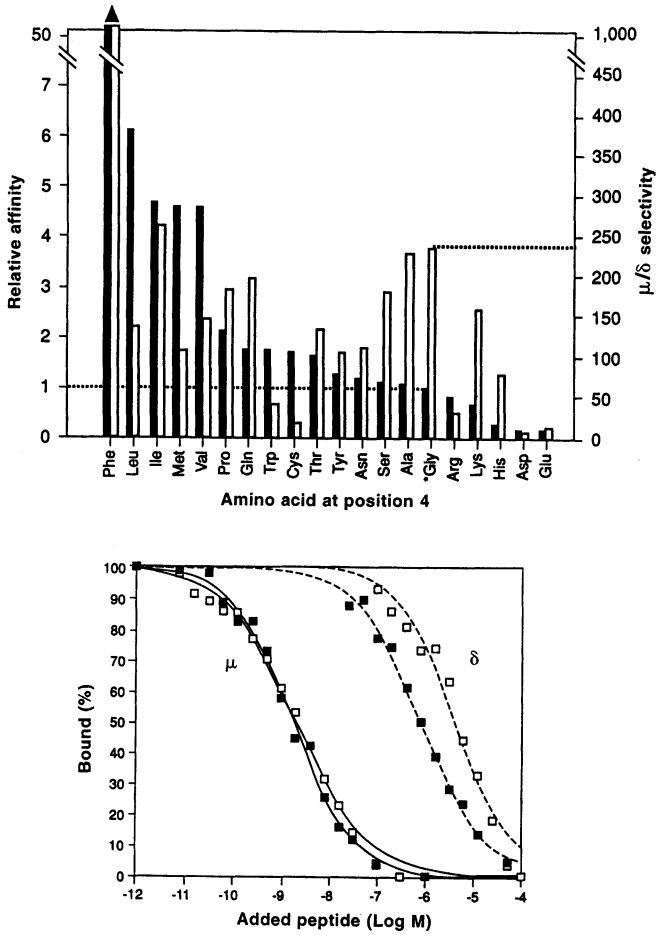

Figure 1 Binding of analogues to opiate receptors. Top, affinity and selectivity of 20 Tyr-Pro-Trp-X-NH_2 analogues. The K_i for the μ opiate receptor (black bars) is expressed relative to that of Tyr-W-MIF-1 on the left axis. The dashed line from the left axis to the bar for Tyr-W-MIF-1 (Gly at position 4; asterisk) indicates an affinity ratio of 1. The selectivity for μ over δ receptors ($\delta K_i/\mu K_i$) is shown on the right axis and indicated by white bars. The dashed line from the right axis represents the 240-fold selectivity of Tyr-W-MIF-1 for μ receptors. The arrow above the Phe analogue indicates that its extraordinary affinity and selectivity were well outside the range of the other compounds. Binding of [^{3}H]-DAMGO (Tyr-D-Ala-Gly-N-Me-Phe-Gly-ol) to μ receptors or of ^{3}H-pCl-DPDPE (D-Pen2, pCl-Phe4, D-Pen5-enkephalin) to δ receptors was assayed as described[12]. Values represent the mean of two separate assays with each of two separate syntheses of the set of 20 peptides. Bottom, comparative binding of the Phe-4 peptide and DAMGO to μ and δ receptors. The Phe-4 peptide (white squares) inhibited μ (^{3}H-DAMGO, solid line) binding with an IC_{50} comparable to that of DAMGO (black squares), but it inhibited δ (^{3}H-pCl-DPDPE, dotted line) binding with a higher IC_{50}, reflecting its greater selectivity for the μ receptor. K_i values are given in Table 1.

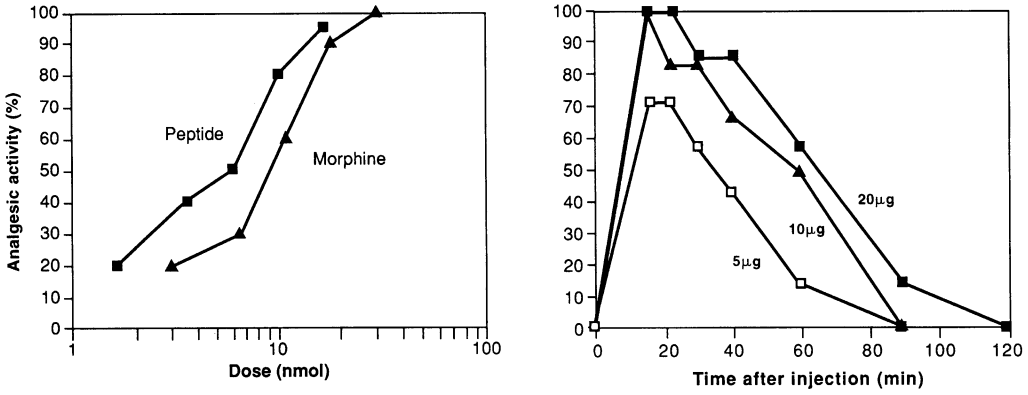

Figure 2 Left, dose-response curves for the analgesic effects of morphine and the Phe-4 peptide 15 min after i.c.v. administration to mice ($n = 10$), where dosage is in nmol per animal. Analgesia was defined as a doubling from baseline of the tail-flick latency in response to a focused light. Data were analysed quantally[9,30] with the BLISS program. The half-maximal effective dose (ED_{50}) and 95% confidence intervals for the peptide were 4.7 nmol (3.1–6.7) and for morphine 7.5 nmol (4.8–10.5). Right, time course of the analgesic effects of the Phe-4 peptide. Mice were given 5, 10 or 20 μg Phe-4 peptide i.c.v. and tested at various times after injection.

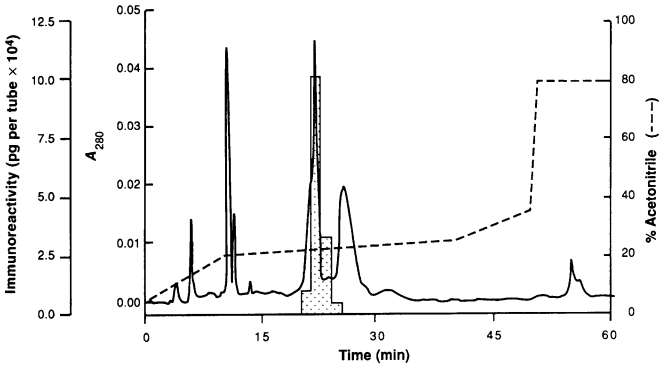

Figure 3 Isolation of Tyr-Pro-Trp-Phe-NH_2 from bovine frontal cortex. The immunoreactive (dotted bar) and ultraviolet absorbance profiles are shown from the final reversed-phase HPLC purification step. The peak of immunoreactive material isolated at fraction 24 was subjected to Edman degradation for sequencing.

selectivity for the μ receptor. Table 1 also shows that the Phe-4 peptide has higher affinity and selectivity for the μ receptor than the three principal endogenous mammalian peptides with opiate activity.

The analogue with Phe at position 4 had potent μ-selective activity *in vitro*, inhibiting electrically induced contractions of the guinea-pig ileum[16] with an IC_{50} significantly more potent than that of DAMGO (3.6 ± 0.3 nM compared with 6.8 ± 0.8 nM, mean ± s.e.m. of four assays; $F_{1,6} = 13.4$, $P < 0.05$). The effect of the Phe-4 peptide was blocked and reversed by the antagonists naloxone (0.5 μM) and the μ-selective CTOP (D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2, 0.5 μM), but not by the κ-selective norbinaltorphimine (20 nM), when these antagonists were applied to the assay 1 min before or after the peptide. These results confirm the μ-selective bioactivity of the peptide.

The *in vivo* activity of the peptide as an analgesic was tested after intracerebroventricular (i.c.v.)[17] and intrathecal[18] injection to mice. Figure 2 (right) shows that after i.c.v. injection, the Phe-4 peptide, with a half-maximal effective dose (ED_{50}) of 4.7 nmol ((95% confidence interval (CI) = 3.1–6.7) = 2.9 μg (1.9–4.1 μg)) was at least as potent as morphine in producing analgesia (7.5 nmol (95% CI = 4.8–10.5) = 2.5 μg (1.6–3.5)). Figure 2 (left) shows that prolonged analgesia can be induced by the Phe-4 peptide. About half the animals were still analgesic 1 hour after 10–20 μg of the peptide. By contrast, the ED_{50} for the endogenous pentapeptide Met-enkephalin is 75 μg and its effects last for less than 10 min[19]. The analgesia induced by 10 μg of the Phe-4 peptide was reversed by a low dose of naloxone (1 mg kg^{-1} injected subcutaneously) and by pretreatment for 24 h with the irreversible μ-selective antagonist β-funaltrexamine (40 mg kg^{-1}), indicating that its action is mediated by μ receptors. The peptide was even more effective when given by intrathecal injection rather than by the i.c.v. route, with an ED_{50} of 0.8 ± 0.4 μg. Thus, the Phe-4 peptide, an unmodified, all-natural amino-acid tetrapeptide, is a very potent analgesic.

We generated an antibody against the Phe-4 peptide which we used to develop a sensitive and specific radioimmunoassay that detects 1 pg peptide and shows no crossreactivity for over 40 opioid and non-opioid peptides and compounds. This radioimmunoassay enabled us to screen fractions from high-performance liquid chromatography of bovine cortical brain extracts and to isolate the peptide as described[5,6], with modifications (see below). Figure 3 shows the final purification step. The isolated material was subjected to six successive cycles of automated Edman degradation. The most prevalent phenylthiohydantoin (PTH)-amino acids were easily distinguishable from background amino acids, and the first four successive cycles yielded (in pmol): Tyr, 310; Pro, 154; Trp, 72; and Phe, 143. After the fourth cycle, no further PTH-amino acid was detected above background. The peptide structure was identified as Tyr-Pro-Trp-Phe-NH_2 and confirmed by comparison with synthetic Tyr-Pro-Trp-Phe-NH_2 and its free acid, Tyr-Pro-Trp-Phe-OH. The elution time of the synthetic peptide amide (21.9 min) was the same as that of the isolated peptide and was clearly separated from that of the peptide acid (25.9 min).

The only other amino acid that increased in the third cycle was Phe, with a value of 51 pmol, indicating the presence of the sequence

Table 1 Binding of agonist peptides to μ, δ and κ opiate receptors

	Receptor binding K_i (nM)			Binding selectivity	
	μ	δ	κ	δ/μ	κ/μ
DAMGO	0.34 ± 0.07	190 ± 16	1,300*	559	3,824
Endomorphin-1 (Tyr-Pro-Trp-Phe-NH_2)	0.36 ± 0.08	1,506 ± 174	5,428 ± 474	4,183	15,077
Endomorphin-2 (Tyr-Pro-Phe-Phe-NH_2)	0.69 ± 0.16	9,233 ± 201	5,240 ± 460	13,381	7,594
β-Endorphin	4.4 ± 0.41				
	2.1†	2.4†	96†	1.1	46
Met-enkephalin	5.9 ± 0.9				
	9.5†	0.91†	4,442†	0.01	468
Dynorphin	2.0 ± 0.5				
	0.73†	2.4†	0.12†	3.3	0.16
Tyr-W-MIF-1 (Tyr-Pro-Trp-Gly-NH_2)*	70.9	15,520	22,300	219	314

Binding to μ (^{3}H-DAMGO), δ (^{3}H-pCl-DPDPE) or κ (^{3}H-ethylketocyclazocine with 100 nM DAMGO and DPDPE to quench μ and δ binding) opiate receptors was determined as described in ref. 12. Values are means ± s.e.m. for 3 separate assays with 5–10 different concentrations of peptide.
* Values taken from ref. 12.
† For β-endorphin, Met-enkephalin and dynorphin, the means ± s.e.m. of 2 separate assays are shown together with values taken from ref. 3.

Table 2 Concentrations of endomorphin-l-like immunoreactivity in different regions of bovine brain

Region	Immunoreactivity (pmol per g tissue)
Thalamus	16.1
Hypothalamus	12.4
Striatum	10.2
Frontal cortex	8.3

Tyr-Pro-Phe-Phe-NH_2. This second peptide was synthesized and the retention time of the two synthetic peptides was similar (Tyr-Pro-Trp-Phe-NH_2, 21.9 min; Tyr-Pro-Phe-Phe-NH_2, 21.2 min). Again, the amidated peptide was clearly separated from that of the peptide acid (25.3 min). Like the two ligands for the δ receptor (Met- and Leu-enkephalin), the two new peptides differ by a single amino acid. The Trp-3 and Phe-3 in the new peptides represent the only two natural amino acids that maintain binding and activity in this position of the opioid pharmacophore[20,21], indicating the limit of similar natural sequences. From the 157 g of starting material, the isolated material from the Edman degradation steps was estimated to be ~200 ng, which consisted of ~75–85% Tyr-Pro-Trp-Phe-NH_2 and 15–25% Tyr-Pro-Phe-Phe-NH_2. At 2.1 pmol g^{-1}, this value indicates that minimal concentrations (after losses during isolation) may be below those of enkephalin, but similar to the less abundant opioids β-endorphin and dynorphin[2].

The second peptide showed a binding profile similar to the first, with about half the affinity for the μ site, but with a selectivity (>13,000-fold) for μ over δ sites of ~3-fold higher. Affinities at κ receptors were equally low for both peptides, making the first peptide about twice as selective (>15,000-fold) for μ over κ sites. The bioactivity of the second peptide approached that of the first *in vitro* (IC_{50} in the guinea-pig ileum assay was 4 ± 0.4 nM) and *in vivo* ((ED_{50} for analgesia after i.c.v. injection was 8.4 μg (CI = 5.5–11.7)). Thus, both peptides have high affinity and selectivity as well as potent bioactivity.

The peptides isolated here differ structurally from previously known endogenous opioids in their N-terminal (Tyr-Pro) sequence, C-terminal amidation and tetrapeptide length. In addition, one of them is the first high-affinity ligand to contain Trp rather than Phe as the second aromatic moiety. Although other peptides with this feature have been reported[22–24], their affinity is below that of even the parent compound (Tyr-W-MIF-1)[5], and the best known (hemorphin) is derived from enzymatic digests of blood[22]. β-Casomorphins[25] contain the Tyr-Pro-Phe sequence found in the second peptide, but contain the less active Pro in position 4, and are not found in neuronal tissue.

Because these are the first endogenous mammalian peptides that have a high affinity and a clear specificity for the opiate receptor preferred by morphine, we have named them endomorphin-1 (Tyr-Pro-Trp-Phe-NH_2) and endomorphin-2 (Tyr-Pro-Phe-Phe-NH_2).

Preliminary studies of the distribution of endomorphin-1 by radioimmunoassay indicate that it is found in thalamus, hypothalamus, cortex and striatum (Table 2). Thus, endomorphin-1-like immunoreactivity is present in several brain areas known to contain subregions of dense μ opiate receptors[26]. We also anticipate subregions of high peptide density, however, that may not match those of dense receptor localization. Only ~7% of opiate receptors are estimated to be associated with synaptic junctions[27], suggesting that there is considerable extrasynaptic transmission by opioids[28,29]. One advantage of selective, amidated peptides such as those described here would be to confer specificity and metabolic stability for such transmission.

In summary, we have discovered two new peptides in brain that have the highest affinity and specificity for the μ opiate receptor found so far in the mammalian nervous system. The peptides have potent μ-selective bioactivity, including analgesia. □

Methods

Isolation of endomorphins 1 and 2. Bovine frontal cortex was minced, boiled in 8 volumes of 0.08% $Na_2S_2O_5$ for 10 min, homogenized at 4 °C, adjusted to 25% acetonitrile in 1 M acetic acid, stirred for 4 h, and centrifuged at 29,000*g* for 20 min. Solid-phase extraction of the supernatants was achieved on Analytichem Mega Bond Elute C8 columns. Peptides were eluted with 70% acetonitrile, dried, and subjected to several sequential reversed-phase HPLC steps. Preparative separation on a Regis Prep-10D-60-ODS-FEC 25 × 2.1 cm column was done with an 8 ml min^{-1} acetonitrile gradient increasing from 10 to 20, 30 and 80% over 10, 50 and 5 min; immunoreactivity eluted at fractions 41–45. Further purification of these fractions on a Vydac 201HS54 25 × 0.47 cm analytical column was at 1 ml min^{-1} with acetonitrile increasing from 5 to 20, 25, 35, and 80% over 10, 30, 10 and 1 min. The immunoreactive fractions (23–25) were combined and chromatographed twice more using the same method and column; the final step is shown in Fig. 3.

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Correspondence and requests for materials should be addressed to J.E.Z.

Protease-activated receptor 3 is a second thrombin receptor in humans

Hiroaki Ishihara*, Andrew J. Connolly*†, Dewan Zeng*, Mark L. Kahn*, Yao Wu Zheng*, Courtney Timmons*, Tracy Tram* & Shaun R. Coughlin*‡

* *Cardiovascular Research Institute and Daiichi Research Center, Departments of † Pathology and ‡ Medicine, University of California, San Francisco, HSW-831, 505 Parnassus Avenue, San Francisco, California 94143-0130, USA*

Thrombin is a coagulation protease that activates platelets, leukocytes, endothelial and mesenchymal cells at sites of vascular injury, acting partly through an unusual proteolytically activated G-protein-coupled receptor[1–3]. Knockout of the gene encoding this receptor provided definitive evidence for a second thrombin receptor in mouse platelets and for tissue-specific roles for different thrombin receptors[4]. We now report the cloning and characterization of a new human thrombin receptor, designated protease-activated receptor 3 (PAR3). PAR3 can mediate thrombin-triggered phosphoinositide hydrolysis and is expressed in a variety of tissues, including human bone marrow and mouse megakaryocytes, making it a candidate for the sought-after second platelet thrombin receptor. PAR3 provides a new tool for understanding thrombin signalling and a possible target for therapeutics designed selectively to block thrombotic, inflammatory and proliferative responses to thrombin.

A polymerase chain reaction (PCR)-based strategy yielded a new human complementary DNA encoding a putative G-protein-coupled receptor with 27% amino-acid sequence similarity to the cloned human thrombin receptor[3] (henceforth called PAR1) and 28% similarity to PAR2 (Fig. 1a). PAR2, a possible trypsin receptor, is the only other known member of the protease-activated receptor family[5]. The amino-terminal exodomain of the new receptor, designated PAR3, contained a possible thrombin cleavage site at residues K38/T39, followed by a sequence strikingly identical to a thrombin-binding sequence in the leech anticoagulant hirudin[6] (Fig. 1b). Analogous sequences in PAR1 mediate recognition and efficient cleavage by thrombin[7–10] (Fig. 1b); this cleavage unmasks a new amino terminus which serves as a tethered peptide ligand, binding intramolecularly to the body of PAR1 to effect transmembrane signalling[3,11,12]. These observations indicated that PAR3 was a new thrombin receptor that should be activated by cleavage of the K38/T39 peptide bond.

We confirmed that PAR3 was a thrombin substrate in a reaction in which 20 nM thrombin cleaved 80% of PAR3 expressed on the surface of Cos 7 cells within 5 min, which is comparable to PAR1 cleavage (Fig. 2). PAR3 cleavage was prevented by substitution of proline for threonine at position 39 (Fig. 2), the P1′ residue in the putative K38/T39 cleavage site (Fig. 1). To confirm the location of the thrombin cleavage site on PAR3, the amino-terminal exodomain of PAR3 (residues 21–94) was expressed in *Escherichia coli* as a soluble polypeptide and cleaved in solution. Even when incubated with 50 nM thrombin for 1 hour at 37 °C, only two cleavage products were detected on SDS–PAGE. Their size was consistent with cleavage at the K38/T39 peptide bond, and amino-terminal sequencing revealed only the original amino terminus and a single new amino terminus with the sequence TFRGA (Fig. 1). Thus thrombin selectively cleaves the peptide bond between K38 and T39 in PAR3's amino-terminal exodomain.

Does PAR3 cleavage by thrombin trigger signalling? Cos 7 cells transfected with PAR3 cDNA gave robust thrombin-stimulated phosphoinositide hydrolysis (Fig. 3a). Co-transfection with α_{16}, a G-protein α-subunit expressed in haematopoietic cell lines[13], caused a 50–100% increase in the maximal PAR3-mediated response to thrombin in these cells (Fig. 3a). The half-maximal effective concentration (EC_{50}) for thrombin signalling by PAR3 in this system was ~0.2 nM, about twice that obtained with PAR1 and well within physiological thrombin concentrations (Fig. 3b). Mutation of the K38/T39 site to prevent receptor cleavage (Fig. 2) ablated PAR3 signalling (Fig. 3a), and thrombin rendered proteolytically inactive by the inhibitor PPACK[14] caused no signalling in PAR3-transfected cells at concentrations as high as 1 μM (not shown). Thus PAR3 mediates thrombin signalling and PAR3's K38/T39 peptide bond must be cleaved for activation of the receptor.

Several observations suggest that PAR3, like PAR1, uses thrombin's fibrinogen-binding exosite for receptor recognition[7–10]. γ-thrombin, which is defective in its fibrinogen-binding exosite[15], was 100 times less potent than α-thrombin (Fig. 3b). Similarly, incubation of α-thrombin with the fibrinogen-binding exosite blocker hirugen[16] right-shifted the dose–response curve by a factor of 100 (data not shown). Alanine substitutions at F48 and E49 in PAR3's hirudin-like sequence, residues predicted to dock with thrombin's fibrinogen-binding exosite by analogy with PAR1 (refs 7, 9) and hirudin[6] (Fig. 1), attenuated thrombin cleavage (Fig. 2) and right-shifted PAR3's concentration response to thrombin tenfold (data not shown).

How specific is PAR3 for thrombin compared with other proteases? We compared protease-triggered mobilization of radioactive calcium (^{45}Ca), which reflects phosphoinositide hydrolysis in