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Proteinase-activated receptor 2 (PAR_2)-activating peptides: Identification of a receptor distinct from PAR_2 that regulates intestinal transport

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ABSTRACT **The effects of PAR_2-activating PAR_2-activating peptides, SLIGRL (SL)-NH_2, and *trans*-cinnamoyl-LIGRLO (tc)-NH_2 were compared with the action of trypsin, thrombin, and the PAR_1 selective-activating peptide: Ala-parafluoroPhe-Arg-cyclohexylAla-Citrulline-Tyr (Cit)-NH_2 for stimulating intestinal ion transport. These agonists were added to the serosa of stripped rat jejunum segments mounted in Ussing chambers, and short circuit current (Isc) was used to monitor active ion transport. The relative potencies of these agonists also were evaluated in two bioassays specific for the activation of rat PAR_2: a cloned rat PAR_2 cell calcium-signaling assay (PAR_2-KNRK cells) and an aorta ring relaxation (AR) assay. In the Isc assay, all agonists, except thrombin, induced an Isc increase. The SL-NH_2-induced Isc changes were blocked by indomethacin but not by tetrodotoxin. The relative potencies of the agonists in the Isc assay (trypsin≫SL-NH_2>tc-NH_2>Cit-NH_2) were strikingly different from their relative potencies in the cloned PAR_2-KNRK cell calcium assay (trypsin≫>tc-NH_2 ≅ SL-NH_2≫>Cit-NH_2) and in the AR assay (trypsin≫>tc-NH_2 ≅ SL-NH_2). Furthermore, all agonists were maximally active in the PAR_2-KNRK cell and AR assays at concentrations that were one (PAR_2 -activating peptides) or two (trypsin) orders of magnitude lower than those required to activate intestinal transport. Based on the distinct potency profile for these agonists and the considerable differences in the concentration ranges required to induce an Isc effect in the intestinal assay compared with the PAR_2-KNRK and AR assays, we conclude that a proteinase-activated receptor, pharmacologically distinct from PAR_2 and PAR_1, is present in rat jejunum and regulates intestinal transport via a prostanoid-mediated mechanism.**

The PAR_2, like the receptor for thrombin [proteinase-activated receptor-1 (PAR_1)], belongs to the large superfamily of G protein- coupled receptors (1–4). The unique mechanism whereby the proteinases thrombin (for PAR_1) and trypsin (for PAR_2) activate the PARs involves the proteolytic cleavage and unmasking of an N-terminal receptor sequence, that in turn acts as an anchored receptor-stimulating ligand (2, 5). Remarkably, short synthetic peptides based on the proteolytically revealed receptor sequences [PAR-activating peptides (APs)] can, in isolation, activate either PAR_1 (e.g., the peptide SFLLR-NH_2) or PAR_2 [e.g., SLIGRL (SL)-NH_2]. Studies of the structure–activity relationships for the activation of PAR_1 and PAR_2 by PAR-APs derived from either PAR_1 or PAR_2 have revealed that the PAR_2AP SL-NH_2 can selectively activate PAR_2 and not PAR_1; but the PAR_1-derived peptide SFLLR-NH_2 can activate both PAR_1 and PAR_2 (3, 6, 7). Thus, we have begun to design PAR_1APs that are selective for PAR_1 and that do not activate PAR_2 (and vice versa) (7). Such selective synthetic PAR-APs have become useful probes to assess the biological consequence of the activation of either PAR_1 or PAR_2 *in vivo*, and thus mimicking the actions of the proteinases, thrombin and trypsin.

Northern blot analysis has revealed the presence of PAR_2 in a variety of tissues, both in rodents and humans, with particularly prominent expression observed in the gastrointestinal tract (3, 8). Because PAR_2 was discovered fortuitously in the course of the screening of a mouse-genomic library for an unrelated receptor (3), the precise physiological role that PAR_2 plays is open to question. Functional studies with PAR_2APs have pointed to a role for PAR_2 in regulating the contractility of vascular and gastric smooth muscle (9–12). Recent work using immunofluorescence and confocal microscopy has localized PAR_2 in intestinal enterocytes, neuronal elements, and myocytes and also has demonstrated that both trypsin and the PAR_2AP SL-NH_2, when applied to the luminal side of rat jejunal tissue, can cause the secretion of prostaglandin E_2 (PGE_2) (13, 14). We were interested in the potential functional effects of activating PAR_2 on the serosal rather than the mucosal side of intestinal tissue, and we wondered whether PAR_2APs or trypsin, when applied to the serosal surface of jejunal strips, might modulate intestinal transport. To answer this question, we designed two new PAR-APs, one selective for PAR_1: Ala-parafluoroPhe-Arg-cyclohexylAla-Citrulline-Tyr-(Cit)-NH_2 and a second selective for PAR_2: *trans*-cinnamoyl-LIGRLO (tc)-NH_2. We used these two receptor-selective PAR-APs, along with the originally described PAR_2AP, SL-NH_2, to evaluate their structure–activity relationship for the regulation of active ion transport [by measuring short circuit current (Isc)], when applied to the serosal side of jejunal strips. Our data show not only that the PAR-APs can modulate intestinal transport by acting at the serosal surface, but the structure–activity relationships for this action of the PAR-APs point to the existence of a receptor, distinct from PAR_1 and PAR_2, that is responsible for regulating intestinal function.

METHODS

Animals. Male, Hooded Lister rats (150–200 g) were obtained from the Department of Biological Sciences at the University of Calgary (Calgary, Alberta, Canada) and were

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Abbreviations: AR, aorta ring relaxation; Cit, Ala-parafluoroPhe-Arg-cyclohexylAla-Citrulline-Tyr; COX, cycloxygenase; Isc, short circuit current; LS, LSIGRL; PAR, proteinase-activated receptor; PAR-APs, PAR-activating peptides; PAR_2-KNRK cells, rat KNRK cells expressing cloned rat PAR_2; PGE_2, prostaglandin E_2; SL, SLIGRL; tc-, *trans*-cinnamoyl-LIGRLO; TFA, trifluoroacetic acid; TTX, tetrodotoxin.

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used for all tissue bioassay experiments. Animals had free access to food and water and were housed under constant temperature (22°C) and photoperiod (12-hr light–dark cycle). All experimental procedures were approved by the Animal Care Committee of the University of Calgary and were performed in accordance with the guidelines established by the Canadian Council on Animal Care.

Ussing Chamber Assay. Rats were killed by cervical dislocation, and immediately, segments of jejunum 10-cm distal to the ligament of Treitz were removed and flushed with ice-cold Krebs solution. The segments were stripped of external muscle by blunt dissection and were mounted between halves of standard Ussing chambers. Once mounted, the tissues were bathed with Krebs buffer (37°C; pH 7.4). Krebs buffer contained (mM): NaCl (115), KH_2PO_4 (2.0), $MgCl_2$ (2.4), $NaHCO_3$ (25.0), KCl (8.0), and $CaCl_2$ (1.3). The serosal Krebs buffer contained 10 mmol/liter glucose, and the mucosal Krebs buffer contained 10 mmol/liter mannitol. Buffers were aerated and mixed by using a gas lift system (5% CO_2 and 95% O_2). Tissue responses were measured by clamping the potential difference (PD) to 0 mV by applying an Isc with a voltage-clamp apparatus (EVC-4000, World Precision Instruments, Sarasota, FL). Isc was monitored throughout the experiment as the indicator of net active electrolyte transport across the tissue (15). After a 20-min equilibration period, the viability of the tissues was assessed by delivering an electrical field stimulation (100 V, pulse duration 500 μs, 25 Hz, 3 s) with a dual-impedance stimulator (Harvard Apparatus). Drugs were then added to the serosal side of the tissues, and the changes in Isc were determined. At the end of all experiments, carbachol (1 μM) was applied to the serosal side of the tissues. Changes in Isc induced by the drugs were expressed as a percentage of the carbachol response. Concentration-effect curves [$n = 6$ for each point ± SEM. (bars)] were established for SL-NH_2, tc-NH_2, Cit-NH_2, and trypsin. The effects of the addition of LSIRGL (LS)-NH_2: an inactive form of the SL-NH_2 peptide), PGE_2 (1 μM), and thrombin (5 units/ml; 50 nM) to the serosal side of the tissues also were observed.

The effect of a previous addition of a proteinase-inhibitor mixture for mammalian cells (Sigma P 8340, containing 4–2-aminoethyl benzenesulfonyl fluoride/pepstatin A/*trans*-epoxysuccinyl-L-leucylamino-4-guanidino butane/bestatin/leupeptin/aprotinin) on PAR-AP-induced Isc response was observed. Paired tissues from the same animal were matched on the basis of basal conductance. The serosal side of one member of each pair was exposed to the proteinase inhibitor mixture (P 8340, 1 μl/20 mg of tissue) and the other member of the pair was exposed to vehicle. Ten minutes later, SL-NH_2 (15 and 30 μM), tc-NH_2 (40 and 60 μM), or Cit-NH_2 (80 and 100 μM) were added to the serosal side of both tissues ($n = 6$ for each concentration used) and the Isc response to the peptides were compared, in the presence or in the absence of proteinase inhibitors.

To determine whether the SL-NH_2 responses were desensitized by trypsin (and vice versa), the intestinal tissue was first exposed to either trypsin (100 units/ml; 200 nM) or SL-NH_2 (80 μM). The serosal side of the chamber was then washed twice and either SL-NH_2 (80 μM) or trypsin (100 units/ml; 200 nM), respectively, was then added to the serosal side of the tissues.

The effects of various inhibitors on the Isc response to SL-NH_2 were studied: indomethacin (10 μM), a nonselective cycloxygenase (COX) inhibitor, SC-58125 (3 μM), a COX-2 inhibitor, and the neural blocker tetrodotoxin (TTX; 1 μM). These inhibitors were added to the serosal side of jejunal tissue 20 min before the addition of SL-NH_2 (80 and 40 μM).

To confirm the chloride dependency of the Isc response to SL-NH_2, experiments were conducted with chloride-free Krebs buffer, as described (16). In this experiment, after a 20-min equilibration period, SL-NH_2 was added to the serosal side of the chamber and the Isc response was recorded.

HPLC Analysis of PAR-APs Recovered from the Bioassay Systems. Previous work had established that peptide hydrolysis does not occur during the course of vascular assays of PAR-APs (18, 26). We used a comparable HPLC analysis to assess possible peptide hydrolysis in the Ussing chamber assay. Immediately after observing a jejunal response, the peptide-containing medium was withdrawn from the tissue and quick frozen for subsequent HPLC analysis. Tissue-exposed solutions were analyzed by HPLC by using a microbondpak C-18 analytical column (Waters, Mississauga, ON, Canada) with a 0–50% gradient of acetonitrile in 0.1% vol/vol aqueous trifluoroacetic acid (TFA), begun 5 min after the sample application to the column (flow rate of 1 ml/min of the 0.1% TFA eluant). The linear acetonitrile gradient, run over the course of 60 min, resulted in the elution of standard peptide samples at reproducible times for each peptide, ranging from 20 to 50 min. Peptide elution was monitored by measuring absorption at 215 nm.

Aorta Relaxation Assay. Immediately after killing, animals were anticoagulated by the injection of heparin (1,000 units in 2 ml of isotonic saline) into the left ventricular circulation. Clot-free samples derived from the aorta were dissected free from adhering tissue and ring preparations (≈2 mm × 2 mm) were cut for use in the bioassay. Aorta ring tissue was equilibrated for 1 h at 37°C in a gassed (5% CO_2, 95% O_2) Krebs–Henseleit buffer, pH 7.4, of the following composition (mM): NaCl (118), KCl (4.7), $CaCl_2$ (2.5), $MgCl_2$ (1.2), $NaHCO_3$ (25), KH_2PO_4 (1.2), and glucose (10). As described (10), the relaxant actions of the PAR_2APs and trypsin were measured in endothelium-intact rings that were precontracted with 1 μM phenylephrine. For the construction of concentration–relaxation curves, the relaxant responses to increasing concentrations of PAR_2APs and trypsin were expressed as a percentage of the relaxation caused by 1 μM acetylcholine (% Ach) and monitored both before and after the exposure of the tissue to all test concentrations of the peptides. Agonists were added directly to the organ bath (4 ml) and ring tension was monitored by using either Grass- or Statham force-displacement transducers.

Calcium-Signaling Assay by Using PAR_2-Transfected KNRK Cells. The rat PAR_2 receptor (10) was subcloned into pcDNA3 (Invitrogen) and transfected into Kirsten Sarcoma virus-transformed rat kidney epithelial cells (KNRK; American Tissue Type Culture Collection) by using the Lipofectamine method, according to the manufacturer's instructions (GIBCO/BRL). Transfected cells were subcloned in geneticin-containing medium (0.6 mg/ml) to yield a permanent cell line (PAR_2-$KNRK_s$) expressing ≈75,000 receptors/cell (B.A.-A., S. Mokashi, and M.D.H., unpublished data). A comparable cell line (PAR_2-$KNRK_b$) also was obtained by using the viral LNCX vector (17). Routinely, PAR_2-KNRK cells were grown in a geneticin-containing (0.6 mg/ml) DMEM supplemented with 5% (vol/vol) fetal calf serum by using 80-cm^2 plastic T-flasks; cells were propagated without the use of trypsin. Background KNRK cells were similarly grown in the absence of cytocidal antibiotic. For the calcium-signaling assay, cells grown just to the point of confluence were harvested by suspension in EDTA-containing calcium-free isotonic PBS. Disaggregated cells were pelleted by centrifugation and were resuspended in 1 ml of DMEM/10% fetal calf serum for loading with the calcium indicator (Molecular Probes) at a final concentration of 22 μM (25 μg/ml) fluo-3AM ester (Molecular Probes). Indicator uptake was achieved over a 20- to 25-min incubation period at room temperature in the presence of 0.25 mM sulfinpyrazone, at which time cells were washed twice by centrifugation and resuspension (10^6 cells/ml stock suspension) in assay buffer, pH 7.4, of the following composition (mM): NaCl (150), KCl (3), $CaCl_2$ (1.5),

Hepes (20), Glucose (10), and sulfinpyrazone (0.25). Fluorescence measurements, reflecting elevations of intracellular calcium were done at 24°C, using a Perkin–Elmer fluorescence spectrometer with an excitation wavelength of 480 nm and emission recorded at 530 nm. Cells (2 ml of $\approx 3 \times 10^5$ cells/ml) were stirred in a thermostated plastic cuvette to which agonist stock solutions were added directly for monitoring trypsin- or peptide-induced changes in fluorescence. To construct fluorescence-yield concentration-effect curves, the signals elicited by agonists in replicate cell suspensions, were expressed as percentage of the fluorescence peak height caused by 2 μM ionophore A23187 (Sigma). This concentration of ionophore was at the plateau of its concentration-effect curve for a fluorescence response. Measurements of PAR-APs-stimulated calcium signals were done both in the absence and presence of either amastatin (10 μM) or the same mixture of proteinase inhibitors (P8340) used in the Ussing chamber assay (above).

Peptides and Other Reagents. All peptides, prepared by solid phase synthesis, were obtained either from the peptide synthesis facility of the University of Calgary Faculty of Medicine (D. McMaster) or through the courtesy of L. Leblond via the peptide synthesis facility at BioChem Therapeutic (Laval, Quebec, Canada). The composition and purity of all peptides were confirmed by HPLC 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 concentration and purity. Porcine trypsin (14,900 units/mg, cat no. T7418), TTX, carbachol, indomethacin, and amastatin were from Sigma. A maximum specific activity of 20,000 units/mg was used to calculate the approximate molar concentrations of trypsin in the bioassay systems (upper axes in Fig. 3).

RESULTS

Isc Response of Jejunal Serosa to PAR-APs, Trypsin, and Thrombin. The serosal application of SL-NH_2, the PAR_2AP peptide based on the proteolytically revealed rat PAR_2 receptor sequence, evoked an increase in Isc in all preparations (n = 40). This response began within 3 min of the addition of SL-NH_2, quickly reached a peak, and fell to baseline during the subsequent 10 min (Fig. 1*A*). LS-NH_2, the inactive form of the SL-NH_2 peptide, had no effect on Isc (Fig. 1*A*). The Isc response to 80 μM of SL-NH_2 was abolished when chloride-free buffer was used (Fig. 1*A*). Another PAR_2AP, tc-NH_2, induced a similar Isc response, but much higher concentrations of this peptide were required (Fig. 1*B*). Low concentrations of trypsin (0.5, 1, and 10 units/ml; 1 to 20 nM) failed to reproduce the Isc response obtained with PAR_2AP (only 10 units/ml trypsin is shown in Fig. 1*B*). However, much higher concentrations (50 and 100 units/ml; 100 and 200 nM) of trypsin induced an Isc response (see Fig. 1*B* for 50 units/ml).

In contrast, the selective PAR_1AP Cit-NH_2 at concentrations more than sufficient to activate PAR_1 (20 μM; M.D.H., A. Kawabata, M.S., and L. Leblond, unpublished data) caused no effect on Isc (Fig. 1*C*). Nonetheless, an Isc increase was observed by using very high concentrations of Cit-NH_2 (50 and 100 μM) (shown in Fig. 1*C* for 100 μM). The high concentrations were, nonetheless, lower than those required to activate the cloned rat PAR_2 expressed in KNRK cells (see Fig. 3*A*). The addition of thrombin to the serosal side of the chambers did not provoke any increase in Isc, but a small decrease could be detected (Δ Isc = 11.37 $\pm$ 3.45 $\mu A/cm^2$, n = 6) (Fig. 1*C*).

Crossdesensitization of SL-NH_2-Induced Jejunal Isc Response by Trypsin. The repetitive and cumulative exposure of the tissues at short time intervals (<10 min) to high concentrations of either SL-NH_2 peptide (80 and 100 μM) or trypsin (100 units/ml; 200 nM) resulted in a desensitization of the Isc response (data not shown). To determine whether the SL-NH_2–Isc response could be desensitized by trypsin (and *vice versa*), a response of jejunal tissues was first obtained by the addition of 100 units/ml trypsin (200 nM). Then, after a return of Isc to the baseline, the tissues were washed and exposed to SL-NH_2 (80 μM). The tissues desensitized to trypsin were not able to respond further to SL-NH_2, even at high concentrations (80 μM) of the peptide (Fig. 1*D*). Pretreating the tissues with SL-NH_2 (80 μM) partially desensitized the subsequent increase in Isc in response to 100 units/ml trypsin (not shown). After desensitization of the tissues to either trypsin or SL-NH_2, the Isc response to carbachol was, nonetheless, intact (Fig. 1*D* and data not shown).

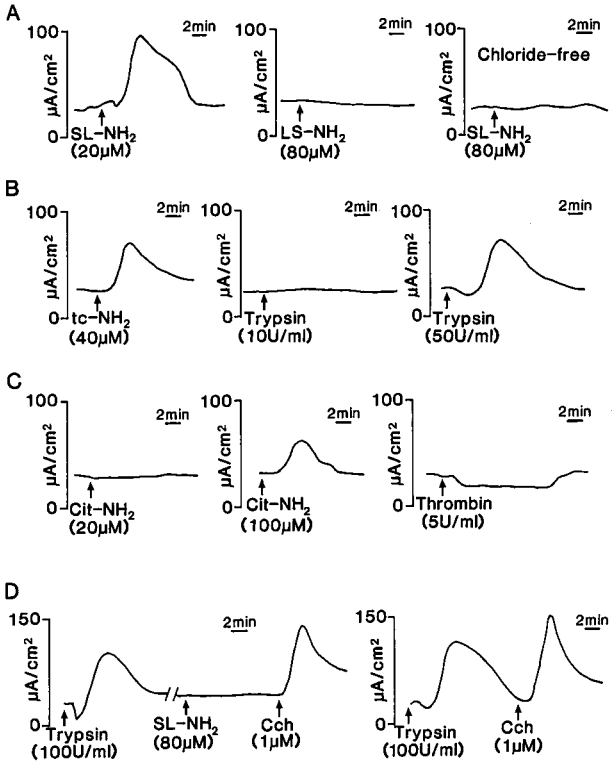

FIG. 1. Representative tracings of the Isc response of rat jejunum to the serosal addition of (*A*) SL-NH_2 (20 μM) and LS-NH_2 (80 μM), when tissues were bathed in normal Krebs buffer, or SL-NH2 (80 μM) in tissues bathed with chloride- (Cl^-) free Krebs buffer. (*B*) tc-NH_2 (40 μM) and trypsin (10 and 50 units/ml; 20 and 100 nM) in normal Krebs buffer. (*C*) Cit-NH_2 (20 and 100 μM) and thrombin (5 units/ml; 50 nM) in normal Krebs buffer. (*D*) SL-NH_2 (80 μM) after trypsin (100 units/ml; 200 nM), and trypsin alone (100 units/ml; 200 nM), followed by carbachol (Cch, 1 μM) in normal Krebs buffer. The scale for time and Δ Isc (in $\mu A/cm^2$) is shown respectively to the right and to the left of each tracing. All panels show a tracing for an individual tissue preparation that is representative of six independently conducted experiments.

Effects of Inhibitors on the Isc Response Induced by SL-NH_2. To determine whether the SL-NH_2-induced Isc response was mediated by the neuronal release of stored agonists, we preincubated the jejunal tissues with TTX, a sodium channel blocker that inhibits axonal conduction and action potentials. TTX (1 μM) completely inhibited the Isc response to electrical field stimulation but did not affect the SL-NH_2-induced Isc response (81.79 $\pm$ 12.9% of the carbachol response for an Isc response induced by 40 μM of SL-NH_2 in the presence of TTX vs. 78.57 $\pm$ 8.5% for the same Isc response in the absence of TTX) (mean $\pm$ SD for six independent experiments).

To determine whether the Isc response of the intestinal tissues to SL-NH_2 was mediated by the activation of cycloxygenase and the release of prostaglandins, we pretreated the

jejunal tissues either with indomethacin (10 μM), a nonselective COX inhibitor, or with SC-58125 (3 μM), a specific COX-2 inhibitor. The Isc response to 80 μM of SL-NH_2 was completely abolished in the presence of 10 μM indomethacin, but the SL-NH_2-induced response was unchanged in the presence of SC-58125 (85.15 ± 11.3% of the carbachol response with SC-58125 present vs. 92.67 ± 26.9% of the carbachol response in the absence of SC-58125) (Fig. 2 in which the tracings are representative of six independent experiments). The addition of PGE_2 (1 μM) to the serosal side of the Ussing chamber caused an increase in Isc, as did SL-NH_2. However, the Isc response to PGE_2 was sustained as opposed to the transient effect of SL-NH_2 (Fig. 2).

Concentration-Effect Curves for PAR-APs and Trypsin in the Three Bioassays. The concentration-effect curves for the changes in Isc induced by SL-NH_2, tc-NH_2, Cit-NH_2, and trypsin are shown in Fig. 3*A*. An increased Isc response was induced by SL-NH_2 at concentrations from 10 to 80 μM; tc-NH_2 was less potent than SL-NH_2 to induce the same response. Isc responses from 40 to 80% of the carbachol response were induced by 100–200 nM (50–100 units/ml) trypsin (the same magnitude of response caused by trypsin was obtained with 20–40 μM SL-NH_2; Fig. 3*A*). Concentrations of 50–100 μM of Cit-NH_2 were necessary to induce an Isc response in this bioassay. On a molar basis, the order of potencies of the agonists in the Ussing chamber Isc assay was: trypsin$\gg$SL-NH_2>tc-NH_2>Cit-NH_2 (as summarized in Table 1). These relative potencies were in stark contrast to the relative potencies of the same agonists in the PAR_2-KNRK cell calcium-signaling assay and in the AR assay (see below; Fig. 3 and Table 1).

The potency of tc-NH_2 to activate a calcium signal in rat KNRK cells expressing rat PAR_2 was equivalent to that of SL-NH_2 (Fig. 3*A*), and the inactive form of the SL-NH_2 peptide (LS-NH_2 peptide) had no effect (not shown). In the PAR_2-KNRK cell assay, 5–10 nM (2.5–5 units/ml) trypsin caused an activation of PAR_2 that was equivalent to the maximal activation caused by PAR_2APs. At comparatively high concentrations of Cit-NH_2 (100–200 μM), which were more than two orders of magnitude higher than those required for maximal activation of PAR_1 (not shown), the selective PAR_1AP Cit-NH_2 caused a low activation of the PAR_2-mediated calcium signal (≈20% of the maximal response caused by SL-NH_2; Fig. 3*A*, curve on far-right). In the nontransfected KNRK cells, none of the agonists at the concentrations shown in Fig. 3, caused a calcium signal (data not shown). Thus, in the PAR_2-KNRK cell calcium-signaling assay, the potency order of the agonists was: trypsin$\gg$>tc-NH_2 ≅ SL-$NH_2$$\gg$>Cit-$NH_2$ (as summarized in Table 1). Comparable

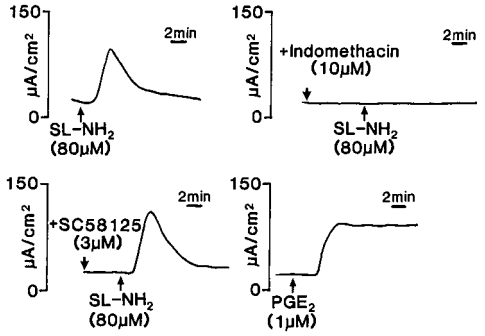

FIG. 2. Representative tracings of the Isc response of rat jejunum, to the serosal addition of SL-NH_2 (80 μM) in the presence or in the absence of indomethacin (10 μM), SC-58125 (3 μM) and to the serosal addition of PGE_2 (1 μM). The scale for time and Δ Isc (in $\mu A/cm^2$) is shown respectively to the right and to the left of each tracing. All panels show a tracing for an individual tissue preparation that is representative of six independently conducted experiments.

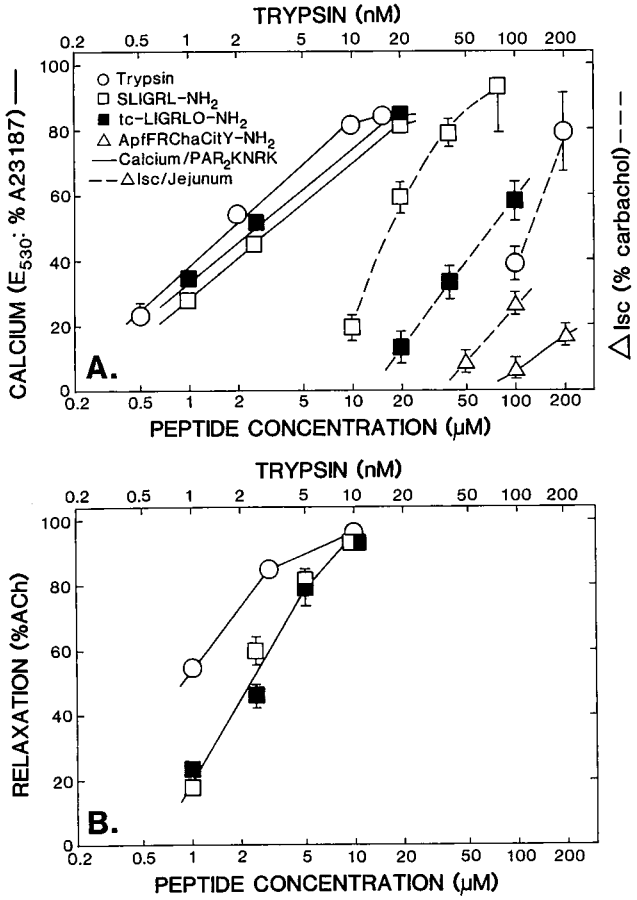

FIG. 3. Concentration-effect curves for PAR-APs and trypsin. (*A*). The PAR_2-KNRK calcium assay and the Isc assay and (*B*) the AR assay. The three bioassay responses: (*i*) Δ Isc, (*A*) (right-hand ordinate, Δ Isc in percentage of carbachol response) broken lines; (*ii*) PAR_2-KNRK cell calcium signaling, (*A*) (left-hand ordinate, E_{530} in percentage of A23187 response) solid lines; and (*iii*) AR, (*B*) (left-hand ordinate, in percentage of acetylcholine response; %ACh) were measured for increasing concentrations of PAR-APs (□, SL-NH_2, ■, tc-NH_2, △, Cit-NH_2; and ○, trypsin) as outlined in *Methods*. The concentrations of trypsin (nM) are shown on the upper abcissa. Each data point represents the average response (± SEM bars) observed at each concentration for three to six independently conducted experiments with tissues or cells derived from two to six different animals or cell culture preparations. Error bars smaller than the symbols are not shown.

results were obtained with a PAR_2-$KNRK_b$ cell line, obtained by using the viral LNCX vector, instead of the pcDNA3 expression vector (not shown).

With tissues derived from the same strain of rat (Hooded Lister) used for the Ussing chamber experiments, the AR assay showed that the potency of SL-NH_2 was the same as that for tc-NH_2 (Fig. 3*B*), and the LS-NH_2 peptide was inactive (not shown). Results comparable with those for the PAR_2-KNRK cell assay were obtained for the relative potency of the two PAR_2APs (SL-NH_2 ≅ tc-NH_2) in the AR assay, with tissues derived not only from the Hooded Lister strain of rats (Fig. 3*B*), but also from male Sprague–Dawley rats (not shown). Trypsin at concentrations of 5–10 nM (2.5–5 units/ml) caused a near-maximal relaxation of the tissue (Fig. 3*B*). Thus, in the relaxation assay, the relative potencies of the agonists were: trypsin$\gg$>SL-NH_2 ≅ tc-NH_2 (summarized in Table 1). Because of the complex action of Cit-NH_2, the selective PAR_1AP, in the aorta tissue that contains both PAR_1 and PAR_2 (PAR_1 activation causes both contraction and relaxation, ref. 19), this peptide was not evaluated in the Hooded Lister AR assay.

Table 1. Activity ratios (R_{EC}) for PAR-APs and trypsin in the I_{SC}, PAR_2-KNRK, and aorta relaxation assays

	Relative activity ratio (R_{EC})* assay		
Agonist	ΔI_{SC}	PAR_2KNRK	Aorta relaxation
SL-NH_2	*1*	*1*	*1*
tc-NH_2	3.4 ± 0.4	0.8 ± 0.01	1.0
Cit-NH_2	8.5 ± 0.4	460 ± 8	ND
Trypsin, $\times 10^3$	7.4 ± 0.1	0.5 ± 0.02	0.5 ± 0.02

*Activity ratios (R_{EC}) for each agonist, relative to the concentration of SL-NH_2 ($R_{EC} \equiv 1$) causing an equivalent biological response, were calculated as described (18) from the concentration-effect curves shown in Fig. 3, according to the formula: R_{EC} = (concentration of agonist for a given response in the linear portion of its concentration-effect curve)/(concentration of SL-NH_2 required to give the equivalent biological response). A value >1.0 designates an agonist with a potency lower than that of SL-NH_2. Values for trypsin were multiplied by 10^3. Each value represents an average (± SEM) obtained from measurements done at three to seven levels of response along the concentration-effect curves for each agonist shown in Fig. 3. ND, not determined.

The relative potencies of trypsin, SL-NH_2, and tc-NH_2 in the AR assay were, therefore, in good accord with the potency order in the PAR_2-KNRK cell calcium-signaling assay but were entirely out of keeping with the potency order observed in the intestinal Isc assay (Table 1). The concentration range for the activity of Cit-NH_2, relative to the concentration range for SL-NH_2 activity, also was very different in the PAR_2-KNRK cell assay (≈460:1, Table 1) compared with the activity relative to SL-NH_2 in the jejunal Isc assay (≈8.5:1, Table 1). Moreover, for all agonists, the concentration range of activity in the AR and the PAR_2-KNRK cell assays was approximately an order of magnitude lower than the concentration range necessary for the same agonists in the jejunal Isc assay.

Susceptibility to Degradation of PAR-APs. To determine that the different Isc responses of intestinal tissues to SL-NH_2, tc-NH_2, and Cit-NH_2 were not caused by differences in susceptibility to local degradation by cell surface proteinases, peptide degradation in Ussing chambers was assessed by HPLC analysis. In addition, the PAR-AP-induced Isc responses were observed in the presence or in the absence of a mammalian proteinase inhibitor cocktail. Even in the absence of proteinase inhibitors, the peptides (SL-NH_2, tc-NH_2, and Cit-NH_2) were recovered intact from the serosal buffer of Ussing chambers at the end of an experiment, and no other degradation product was detected by HPLC analysis. No difference was observed for SL-NH_2-, tc-NH_2-, or Cit-NH_2-induced Isc response in the presence or in the absence of proteinase inhibitors for two concentrations of the tested peptides. The same mixture of proteinase inhibitors (2.5 μl of P 8340/2 ml of cell suspension) as well as amastatin (10 μM) were added to the PAR_2-KNRK cells and the PAR_2-mediated calcium signal induced by SL-NH_2, tc-NH_2, or Cit-NH_2 measured. The fluorescence yield was not affected by the presence or the absence of these proteinase inhibitors in the assay cuvette.

DISCUSSION

One of the main findings of our study was that the administration of PAR-APs and trypsin to the serosal side of the rat jejunal preparation in Ussing chambers caused a prompt change in active ion transport, as reflected by the increase in Isc. The SL-NH_2-induced Isc effect was caused by active chloride transport because the response was abolished in chloride-free buffer. COX-1-derived prostaglandins appeared to act as mediators of this effect because indomethacin, but not a selective COX-2 inhibitor (SC58125), completely inhibited the SL-NH_2-induced response. PGE_2 caused an activation of transport that was sustained, as compared with the transient increase caused by SL-NH_2. The difference in the responses to PGE_2 and SL-NH_2 may suggest the production of distinct prostanoids in response to SL-NH_2. Alternatively, it could be that the high concentration (1 μM) or overall amount of PGE_2 in the bath results in sustained receptor activation and/or saturation of the mechanisms responsible for PGE_2 metabolism, events which are unlikely to occur after endogenous release in uninflamed tissue. That TTX did not block the Isc response to SL-NH_2, but did block the response caused by electrical field stimulation, indicated that SL-NH_2 was acting directly on the serosal side of the tissue and not via the neural release of stored neurotransmitters such as acetylcholine.

The peptide structure–activity data strongly suggest that the action of the PAR-APs to regulate transport was mediated via a receptor distinct from PAR_2. Clearly, neither of the two thrombin receptors (PAR_1 and PAR_3; refs. 1, 2, 20) were involved in the increase in Isc that we monitored because thrombin had an effect opposite to that obtained with PAR_2APs and because the PAR_1-selective agonist Cit-NH_2 failed to activate ion transport at concentrations well above those that are required to activate PAR_1. The low activity of Cit-NH_2 in stimulating changes in Isc at relatively high concentrations (50–100 μM) could possibly be ascribed to the low activity of this analog at the PAR_2 receptor, but its different potency relative to SL-NH_2 in the jejunal Isc assay, compared with that in the PAR_2-KNRK cell assay (Table 1), points to the activation of a receptor other than PAR_2.

The lack of activity of the reverse peptide LS-NH_2 in the Isc assay and the ability of trypsin to stimulate intestinal transport might suggest that PAR_2 itself was involved in this tissue response. However, the relative potencies for trypsin and SL-NH_2 in the Isc assay, compared with the other two assays, pointed strongly to the activation of a receptor different from PAR_2. The clearest difference between PAR_2 and the receptor responsible for regulating jejunal ion transport can be seen in comparing the structure–activity profile for the PAR-APs and trypsin in the Isc assay, with the profiles observed in the PAR_2-KNRK cell assay and in the AR assay (Fig. 3 and Table 1). In the two quite distinct PAR_2 assays (PAR_2-KNRK cell and AR), one involving the cloned rat PAR_2 receptor expressed in a cultured cell system and the other using an intact tissue, tc-NH_2 was approximately equipotent (AR assay) or even slightly higher in potency (PAR_2-KNRK assay) than the parent PAR_2AP SL-NH_2. Yet, in the jejunal Isc assay, tc-NH_2 was only approximately one-third as potent as SL-NH_2 (Fig. 3*A* and Table 1). Moreover, as noted above, Cit-NH_2 also showed a distinct potency relative to SL-NH_2 in the Isc assay, compared with the PAR_2-KNRK cell assay. Furthermore, the concentration range over which the PAR_2APs and trypsin activated PAR_2 in the AR assay and the PAR_2-KNRK calcium-signaling assay was approximately one (PAR_2APs) or two (trypsin) orders of magnitude lower than the concentration required to activate the jejunal Isc response (Fig. 3). Classically, such differences in agonist potency profiles have been used to define receptor subfamilies, such as those for adrenoreceptor agonists (21) or histamine (22). Although the tc-NH_2 peptide was synthesized in the hope of generating a PAR_2 antagonist (comparable derivatives are antagonists of PAR_1; ref. 23), this analog proved to be a PAR_2 agonist. Thus, it has not yet been possible to obtain selective PAR_2 antagonists to complement the structure–activity data we have obtained with the PAR-AP agonists. Nonetheless, the low relative potency of tc-NH_2 compared with SL-NH_2 and the distinct relative potencies of trypsin and Cit-NH_2 in the Isc assay (Fig. 3 and Table 1), point clearly to a receptor different from PAR_2.

Although the requirement for high concentrations of trypsin in the Isc assay might possibly be accounted for by the presence of high levels of serpin-like trypsin inhibitors in the tissue, this possibility would not be in accord with our previous assays with

intact intestinal preparations (9, 10, 24) or vascular preparations (9, 10), in which trypsin was able to activate PAR_2 fully at concentrations in the same range as those shown in Fig. 3 (0.5–10 units/ml; 1–20 nM, for the PAR_2-KNRK cell and AR assays). Furthermore, our previous experience with tissue bioassays has shown that peptide degradation (e.g., by aminopeptidase action 25), as assessed by HPLC analysis, does not appear to be a factor in assigning relative potencies to PAR-APs (18, 26). In the Ussing chamber assay, no degradation of the peptides was detected by HPLC analysis. Moreover, both in the Ussing chamber assay and in the PAR_2-KNRK cell assay, the proteinase inhibitors did not affect the responses to different concentrations of the PAR-APs. Thus, the differences in the peptide potencies observed in these bioassays were unlikely to be caused by a local peptide degradation by cell surface proteinases. We therefore believe it to be unlikely that either elevated tissue serpins or tissue proteolysis of the PAR-APs could account for the distinct concentration range of activity for trypsin and the PAR-APs and for the clearly distinct relative potencies of SL-NH_2, tc-NH_2, and Cit-NH_2 in the jejunal Isc assay, compared with the PAR_2-KNRK cell calcium-signaling and AR assays.

We believe it is of significance: (*i*) that trypsin added at quite high concentrations (50–100 units/ml or 100–200 nM) did increase Isc upon serosal application and (*ii*) that this action of trypsin desensitized the tissue to SL-NH_2, but not to a second agonist (carbachol), that also acts via a G protein-coupled receptor (Fig. 1*D*). These data indicate that in the jejunal Isc assay, SL-NH_2 was working via a receptor that, like PAR_2 and other PARs, can be activated/desensitized by a serine proteinase. Possibly, mast cell tryptase may activate this receptor in the setting of intestinal inflammation. It is also significant that PAR_2AP-activated intestinal transport was blocked by indomethacin, in keeping with the ability of trypsin (10 μM; 5,000 units/ml) and SL-NH_2 (100 μM) to cause PGE_2 secretion when applied to the luminal side of everted sacs of rat jejunum (13). The very high concentrations of trypsin and SL-NH_2 that were required to stimulate luminal prostaglandin secretion would be more than sufficient to activate the "serosaside" receptor described here, that is responsible for modulating intestinal transport. We believe our data demonstrate, by a pharmacological approach, the presence in rat jejunal tissues of a receptor for PAR-APs, distinct from PARs 1, 2, and 3, that can be activated by a serine proteinase. Our findings add one more response, the regulation of intestinal transport, to the repertoire of physiological roles that PARs may play *in vivo*.

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