A specific binding of the cholecystokinin-releasing peptide (monitor peptide) to isolated rat small-intestinal cells

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A specific binding of the cholecystokinin (CCK)-releasing peptide (monitor peptide) to isolated rat jejunal mucosal cells was investigated. The ^{125}I -labelled purified monitor peptide bound to the rat jejunal cells, and a large excess amount of the non-labelled monitor peptide inhibited the binding. The binding was completed within 60 min at 37 °C. The optimum pH for the binding was 8–9. A Scatchard plot of the specific binding was linear, and the dissociation constant was 50 nM. The density of the monitor-peptide-binding sites was high in duodenum but low in ileal and absent in colonic mucosa. A recombinant monitor peptide and four kinds of point mutants of it were prepared. The binding of the mutant monitor peptides to the cells indicated that only a trypsin inhibitor of the mutants could bind to the mucosal cells.

Human pancreatic secretory trypsin inhibitor inhibited the specific binding, but other trypsin inhibitors, i.e. bovine basic pancreatic trypsin inhibitor, soybean trypsin inhibitor, egg-white trypsin inhibitor, leupeptin, antipain and FOY-305, did not affect the specific binding at all. These findings suggested that the specific binding site for the monitor peptide on the jejunal mucosal cells has a trypsin-like specificity, exhibiting an especial specificity for the pancreatic-secretory-trypsin-inhibitor family. Autoradiography of an affinity-cross-linked complex of the ^{125}I -labelled intact monitor peptide and the binding site suggested that its molecular mass was 33 kDa or 53 kDa in the presence or absence of 2-mercaptoethanol respectively.

INTRODUCTION

It is well established that dietary trypsin inhibitors evoke increased pancreatic enzyme secretion, via cholecystokinin (CCK) secretion into the blood (Louie et al., 1986). Green and co-workers (Green and Lyman, 1972; Green et al., 1973) first proposed that the presence of trypsin, chymotrypsin or a mixture of bile and pancreatic juice in the small intestine controls enzyme secretion from the pancreas. Removal of proteolytic activity from the intestine by intestinal infusion of trypsin inhibitors produced a large increase in pancreatic-enzyme output. It was suggested that this negative-feedback control of pancreatic-enzyme secretion could be the mechanism by which dietary trypsin inhibitors induce excessive pancreatic-enzyme secretion in rats.

Recently, trypsin-sensitive CCK-releasing peptides have been hypothesized independently: that is, a peptide spontaneously secreted from the small intestine into the small-intestinal lumen (Miyasaka and Green, 1983; Lu et al., 1989; Miyasaka et al., 1989a) or, alternatively one secreted from the pancreas into the lumen (Fushiki et al., 1984; Iwai et al., 1987; Fushiki and Iwai, 1989). The monitor peptide, a good candidate for a trypsin-sensitive, CCK-releasing peptide, was found in rat pancreatic juice and purified, and its amino acid sequence was determined (Iwai et al., 1987). This peptide is proposed as an essential factor for CCK secretion, because when it was absorbed with a specific antibody, no further pancreatic enzyme secretion was observed in response to intraduodenal infusion of a trypsin inhibitor (Fushiki et al., 1989). However, no evidence has been obtained that the peptide interacts with the small-intestinal cells for CCK release.

In the present study we determined the specific binding site for

the monitor peptide on isolated jejunal mucosal cells. We investigated some properties of the specific binding site and detected the binding protein by means of autoradiography.

MATERIALS AND METHODS

Materials

Male Wistar rats weighing 250 g were purchased from Oriental Bio (Kyoto, Japan). TCM-199 medium was purchased from Nakarai Tesque (Kyoto, Japan). BSA, soybean trypsin inhibitor (SBTI), egg-white trypsin inhibitor, bovine basic pancreatic trypsin inhibitor (BPTI), leupeptin, antipain, bestatin, chymostatin, diprotin A and elastinal were obtained from the Peptide Institute (Osaka, Japan). Human pancreatic secretory trypsin inhibitor (hPSTI) and FOY-305 were supplied by Mochida Pharmaceutical Co. and Ono Pharmaceutical Co. respectively. Other biochemical reagents were purchased from Wako Pure Chemical Co. (Tokyo, Japan). Na^{125}I and $^{45}\text{CaCl}_2$ were purchased from du Pont–New England Nuclear.

Recombinant and point mutants of the monitor peptide

The production of a recombinant monitor peptide and point mutants of it was performed as follows. Briefly, oligonucleotide fragments synthesized by the phosphoramidite method were ligated to obtain double-stranded DNA, as shown in Figure 1(a). The DNA (0.18 kb *NcoI*–*HindIII* fragment) was ligated with the 4.4 kb *NcoI*–*HindIII* fragment from pKK233-2 (Pharmacia) to yield an expression plasmid for the monitor peptide. Site-directed mutagenesis was carried out by the Gapped duplex method with a commercial mutagenesis kit (Takara Shuzo, Kyoto, Japan) according to the manufacturer's instructions. The single-stranded

Abbreviations used: CCK, cholecystokinin; (h) PSTI, (human) pancreatic secretory trypsin inhibitor; SBTI, soybean trypsin inhibitor; EWTI, egg-white trypsin inhibitor; BPTI, bovine basic pancreatic trypsin inhibitor; TFA, trifluoroacetic acid; CM-MP, carboxymethylated monitor protein.

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Figure 1 Structures of synthetic genes for the recombinant monitor peptide and mutants of it

The *E. coli* optimum codon was generally employed for designing the monitor peptide gene (a). Sequences replaced by other amino acids or bases are underlined. The two ends of the synthesized DNA fragment are the *Nco*I site and the *Hind*III site (underlined in the sequence). The primers used for mutagenesis in order to replace the amino acids at positions 23, 24, 47, 48, 57 and 58 are illustrated (b). The replaced bases are indicated by asterisks.

DNA template used for mutagenesis was an M13 tv-19 containing the *Eco*RI–*Hind*III fragment of the expression plasmid for the monitor peptide. The mutation points are summarized in Figure 1(b). *Escherichia coli* transformed with the expression plasmid was cultured in Luria–Bertani medium for 5 h at 37 °C. Cells harvested 3 h after the addition of isopropyl β -D-thiogalactopyranoside were sonicated in 6 M guanidinium chloride. Recombinant and mutant peptides were purified by h.p.l.c. Trypsin-inhibitory activities of these peptides were assayed by the procedures described previously (Fukuoka et al., 1987).

Purification and 125 I-labelling of the monitor peptide

The monitor peptide was purified as follows. Pancreatic juice obtained from conscious rats housed in a Ballman-type cage was collected in 0.05 M citrate buffer containing 0.5 M NaCl, pH 2.5. The pancreatic juice, in citrate buffer, was incubated at 80 °C for 30 min and then centrifuged at 3000 *g* for 30 min. The supernatant was applied to a reverse-phase column [0.8 cm $\times$ 3 cm; filled with Synchronprep RP-4 (SynChrom)] equilibrated with 0.05% trifluoroacetic acid (TFA), washed with 10% (v/v) acetonitrile/0.05% TFA and then eluted with a 50% acetonitrile/0.05% TFA solution. The eluate was further purified by h.p.l.c. as described previously (Iwai et al., 1987). The purity of the peptide was determined by reversed-phase h.p.l.c. (C4 column AP-823; YMC, Kyoto, Japan). 125 I-labelling of the purified monitor peptide was carried out by the chloramine-T method described by Greenwood et al. (1963). A 12 μ g portion of the purified monitor peptide was mixed with 1 mCi of carrier free Na 125 I and then oxidized with 1 mM chloramine-T for 30 s in 50 μ l of 0.4 M sodium phosphate buffer, pH 7.5, and sub-

sequently reduced with 6 mM sodium pyrosulphate. The reaction mixture was loaded on to a Sephadex G-10 column and the 125 I-labelled monitor peptide separated from free Na 125 I.

Carboxymethylation of the monitor peptide

The reduced and carboxymethylated monitor peptide was prepared by the method described by Crestfield et al. (1963). To the purified monitor peptide (100 μ g) were added 73 mg of urea, 6 μ l of 5% Na $_2$ EDTA, 60 μ l of 1.4 M Tris/HCl, pH 8.6, 2 μ l of 2-mercaptoethanol and 80 μ l of water successively, followed by reduction under anaerobic conditions for 4 h at room temperature. Then 20 μ l of iodoacetic acid (268 mg/ml in 0.1 M NaOH) was added to the reduced mixture; the reaction was allowed to proceed for 15 min at room temperature. The carboxymethylated monitor peptide (CM-MP) was purified on Sephadex G-10 followed by h.p.l.c. (C $_4$ reverse-phase column). The modified monitor peptide obtained had only 10% of the trypsin-inhibitory activity of the intact monitor peptide.

Preparation of intestinal-mucosal cells

The preparation of dispersed rat intestinal cells was performed according to the method used for the isolation of parietal cells from guinea-pig gastric mucosa (Jewell et al., 1975; Matsuo and Osada, 1981). Excised rat small intestines were washed with an ice-cold 0.155 M NaCl solution and then the mucosa of a proximal quarter was scraped off with a stainless-steel spatula. In the Scatchard-analysis experiments (Figure 5 below), the proximal and the distal quarters of the small intestine were used. The mucosa was gathered in TCM-199 medium (5 ml/small intestine) and digested with 0.1 mg/ml collagenase (Sigma), 2 mg/ml dispase (Godo Shusei Co., Tokyo, Japan) and 0.05 mg/ml deoxyribonuclease I (Sigma) for 30 min at 37 °C in a shaking water bath, bubbling with O $_2$ /CO $_2$ (19:1), and then centrifuged at 50 *g* for 10 min. The supernatant was stored; the pellet was once again subjected to the same treatment and the supernatant stored. The pellet was finally exposed to a chelating reagent (1 mM Na $_2$ EDTA/118 mM NaCl/5 mM KCl/1.2 mM KH $_2$ PO $_4$ /25 mM NaHCO $_3$ /25 mM Hepes buffer, pH 7.4) for 20 min at 37 °C, with bubbling, in a shaking water bath and then centrifuged at 50 *g* for 10 min. The supernatants were pooled and then filtered through a metal mesh (no. 200), followed by filtration through a no. 400 mesh. The filtrate was centrifuged at 1000 *g* for 30 min, and the pellet was washed twice with a buffer containing 128 mM NaCl, 5 mM KCl, 3 mM MgSO $_4$, 0.9 mM CaCl $_2$, 0.1 mM Na $_2$ HPO $_4$ and 10 mM Hepes, pH 7.4 (A-buffer). The prepared cell sample was used for the experiments freshly or after freezing at –70 °C in the washing buffer containing 10% glycerol. Isolation of cells from the stomach and colon was carried out by the same procedures as for the small-intestinal-mucosal cells described above, and for kidney and liver were carried out by the methods described by Nakajima et al. (1987).

Preparation of BIND-buffer and binding assay

The binding buffer (BIND-buffer) which was used in the experiments illustrated in Figures 4 and 5 and Tables 1 and 2 (below) was a modification of A-buffer, i.e. it had the same composition as A-buffer described above, but the pH was adjusted to the binding optimum, 8.0. The binding assay was carried out as follows. 125 I-monitor peptide (0.3 μ Ci/pmol) was added to the cell solution (total volume, 100 μ l) in a microcentrifuge tube and then incubated for certain periods. After the incubation, 1 ml of ice-cold BIND-buffer was added to the microcentrifuge tube and then the cells were precipitated by

centrifugation at 1500 *g* for 5 min at 4 °C. After washing twice with ice-cold BIND-buffer, the radioactivity of the pellet was measured with a γ -radiation counter. Total and non-specific binding of 125 I-monitor peptide to cells were measured in the presence or absence of a 1000-fold excess amount of unlabelled monitor peptide respectively. Specific binding was obtained by subtraction of the non-specific binding from the total binding.

Cell-surface binding, which was studied only to determine the cell-surface, not the internalized ligand, was carried out as illustrated in Figure 2 (below). Cells were incubated with 125 I-labelled monitor peptide in A-buffer, described above, for 1 h in the presence or absence of a 1000-fold excess amount of unlabelled monitor peptide and then centrifuged at 1500 *g* for 5 min. The pellets were then washed with buffer (50 mM glycine/HCl, pH 3.0, containing 0.15 M NaCl) and centrifuged again at 1500 *g* for 5 min at 4 °C. The radioactivity that moved to the supernatant was defined as cell-surface-bound. Remaining binding was determined after centrifugation for certain periods.

Pretreatment of the mucosal cells with the mixture of trypsin, chymotrypsin and Pronase E

The dispersed mucosal cells were preincubated with the mixture of 1 mg/ml porcine trypsin (Type II; Sigma), 1 ml/mg bovine chymotrypsin (Type II, Sigma) and 1 ml/mg Pronase E (Kaken kagaku Co., Tokyo, Japan) in a buffer containing 10 mM CaCl_2 , 145 mM NaCl and 10 mM Hepes, pH 8.0 for 5 min and 1 h. The cells were then washed twice with the same buffer without proteinases and 125 I-monitor-peptide binding was determined by the method described above. The same treatment was performed without proteinases and the results were used as a control.

Ca^{2+} efflux assay

Ca^{2+} -release from the mucosal cells were determined by the procedures reported by Williams et al. (1988) with some modifications. Mucosal cells (10^8) were incubated in 1 ml of Hanks solution containing $^{45}\text{CaCl}_2$ (20 μCi) and 0.1% BSA for 2 h at 22 °C. After the incubation with $^{45}\text{CaCl}_2$, cells were washed three times with the Hanks balanced salt solution containing 1 mg/ml SBTI at room temperature and suspended in 0.3 ml of the incubation medium consisting of Hanks balanced salt solution and 0.1% BSA. The cells were then incubated at 37 °C and the medium was changed every 15 min for 75 min by centrifugation at 1500 *g* for 5 min. Then monitor peptide and trypsin inhibitors were added to the medium and incubated for 15 min at 37 °C. $^{45}\text{Ca}^{2+}$ release from the cells to the medium was determined by means of a liquid-scintillation counter. The amounts of trypsin inhibitors added were adjusted on the basis of their inhibitory activity towards rat trypsin, i.e. 30 μM monitor peptide (final concn.), 60 μM hPSTI, 13 μM SBTI, 342 μM EWTI and 429 μM FOY-305.

Autoradiography of cross-linked monitor peptide and binding site

Intestinal mucosal cells (5×10^7) were incubated with 125 I-labelled monitor peptide (2×10^6 c.p.m.) in the presence or absence of a 500-fold excess of unlabelled monitor peptide at 37 °C for 1 h in BIND-buffer, as used in the binding assay, in a total volume of 200 μl . A 1 ml portion of ice-cold BIND-buffer was added to the incubation mixture, followed by centrifugation at 1500 *g* for 5 min. The pellet was washed twice with BIND-buffer and then resuspended in 1 ml of BIND-buffer in which 0.4 mM disuccinimidyl suberate had been dissolved, followed by incubation at 4 °C for 30 min. After centrifugation at 1500 *g* for 5 min, the pellet was washed twice with 1 ml of 10 mM Tris/HCl buffer,

pH 7.1, containing 145 mM NaCl, and then incubated with 40 μl of a solubilizing solution (10 mM Tris/HCl, pH 7.1, containing 145 mM NaCl and 2% Triton X-100) for 1 h at room temperature. The supernatant of the cell lysate was mixed with 20 μl of SDS sample buffer with or without 2-mercaptoethanol and boiled for 5 min, and then subjected to SDS/PAGE. The resulting gel was autoradiographed using Fuji X-ray film RX after exposure at -70 °C for 48 h with an intensifying screen (Lightning Plus; du Pont).

RESULTS

125 I-monitor peptide binding to jejunal mucosal cells reached a plateau within 30 min at 37 °C at pH 7.4 (Figure 2). Throughout the incubation period, more than 95% of the mucosal cells were viable, as determined by Trypan Blue staining. Non-specific binding was determined in the presence of 1000-fold more unlabelled monitor peptide than labelled monitor peptide.

Figure 2 Time-dependence of the cell surface specific binding of the 125 I-monitor peptide (MP)

Rat intestinal mucosal cells (3×10^6) were incubated with 0.2 pmol of 125 I-labelled monitor peptide in 100 μl of buffer comprising 128 mM NaCl, 5 mM KCl, 3 mM MgSO_4 , 0.9 mM CaCl_2 , 0.1 mM NaH_2PO_4 and 10 mM Hepes, pH 7.4 (A-buffer in the text), at 37 °C in the presence (for non-specific binding) or absence (for total binding) of a 1000-fold excess amount of the unlabelled monitor peptide for the designated incubation time. After the incubation, 1 ml of ice-cold A-buffer was added, followed by centrifugation at 1500 *g* for 5 min and then washing with the same buffer twice. The washed pellet was mixed with 1 ml of 50 mM glycine/HCl buffer, pH 3.0, containing 0.155 M NaCl and then centrifuged at 1500 *g* for 5 min. The supernatants were pooled, and the pellet was then treated under the same conditions, the resultant supernatant being pooled with the first supernatant. The radioactivity which migrated to the supernatant fraction was assayed for the amount of 125 I-labelled monitor peptide bound. Results are means $\pm$ S.E.M. ($n = 3$) (125 I-labelled-monitor-peptide specific binding).

Figure 3 Effect of pH on 125 I-labelled monitor peptide (MP) specific binding to rat intestinal mucosal cells

The binding solution (128 mM NaCl/5 mM KCl/3 mM MgSO_4 /0.9 mM CaCl_2 /0.1 mM NaH_2PO_4 /5 mM Hepes/5 mM Tris/5 mM 3-cyclohexylamino-2-hydroxypropanesulphonic acid) was adjusted to various pH values with 1 M NaOH. Cells (1.5×10^6) were incubated with 0.4 pmol of the 125 I-labelled monitor peptide in 100 μl of the binding solution at 37 °C for 1 h in the presence (non-specific binding) or absence (total binding) of a 1000-fold excess of the unlabelled monitor peptide. After the incubation, 1 ml of ice-cold binding solution was added, followed by centrifugation at 1500 *g* for 5 min and then washing twice with the same solution. The radioactivity of the pellet was determined. Specific-binding data are means $\pm$ S.D. ($n = 3$).

Figure 4 Dissociation of ^{125}I -labelled monitor peptide binding from rat intestinal mucosal cells by the addition of unlabelled monitor peptide

Cells (1×10^6) from a proximal quarter of the small intestine were incubated with 0.2 pmol of ^{125}I -labelled monitor peptide in 75 μl of BIND-buffer (see the text) at 37 °C for 1 h and then mixed with 25 μl of the buffer containing a 1000-fold amount of the unlabelled monitor peptide (zero time); the incubation was then continued for the designated periods. After the incubation, cells were mixed with 1 ml of the same ice-cold buffer and then centrifuged at 1500 g for 5 min. The precipitates was washed twice with the same buffer and the radioactivity determined. Results are expressed as percentages of the radioactivity at zero time and are means $\pm$ S.D. for triplicate determinations at each time.

Figure 5 Scatchard analysis of ^{125}I -monitor-peptide binding to mucosal cells prepared from the proximal (●) and distal (○) quarters of the small intestine (1×10^6 cells)

Binding was determined by the same method as described in Figure 4. Values are means of triplicate determinations.

Specific binding was obtained by subtraction of the non-specific binding from the total binding. The specific binding after 1 h incubation at 37 °C showed a pH-dependent profile, the maximal binding occurring at pH 8–9 (Figure 3). Therefore, in subsequent experiments, the binding assay was performed with BIND-buffer, pH 8.0, at 37 °C for 1 h. The non-specific binding was within about 10 % of the total binding under the conditions used. The amount of ^{125}I -monitor peptide specifically bound to the jejunal mucosal cells increased when the cell number in the incubation medium was increased, and the non-specific binding was only 10 % of the total binding (results not shown). The ^{125}I -monitor peptide bound to the mucosal cells was dissociated in a time-dependent manner when a large amount of unlabelled monitor peptide was added (Figure 4). This indicated the ^{125}I -monitor-peptide binding to the cells was a reversible process. Unlabelled monitor peptide inhibited ^{125}I -monitor-peptide binding to the mucosal cells in a dose-dependent manner. Scatchard analysis indicated that the binding of the monitor peptide to the mucosal cells showed a dissociation constant of 50 nM. The density of the monitor-peptide-binding sites was very high in duodenum but low in ileum. The cell fraction obtained from the proximal small intestine showed maximal binding of about 350 fmol/ 10^6 cells, and only 100 fmol/ 10^6 of ileal cells (Figure 5).

Essentially the same profiles were obtained in the experiment involving monitor-peptide binding to stored mucosal cells, which were prepared and then stored at -70 °C with 10 % glycerol

Table 1 Effect of point mutation of the monitor peptide on its binding to rat intestinal epithelial cells

Cells (1×10^6) were incubated with the ^{125}I -labelled monitor peptide (1 ng) in the presence or absence of various monitor peptide-related peptides (1 μg). The monitor peptide mutant whose Arg²³-Ile²⁴ sequence was converted into Met-Asn was named MP1; that whose Arg⁴⁷-Lys⁴⁸ sequence was converted to His-Asn or Ser-Thr was named MP2 or MP3, and that whose Arg⁵⁷-Arg⁵⁸ sequence was converted into Ser-Ser was named MP4 and CM-(straight chain) MP is abbreviated as CM-MP in this Table. Trypsin-inhibitory activity was measured for rat pancreatic juice, and + indicates an equivalent inhibitory activity to that of purified MP and – indicates little inhibitory activity. —*, CM-MP showed slight inhibitory activity (about 10% of that of the intact peptide).

Substance	^{125}I -labelled MP bound (c.p.m.) (mean $\pm$ S.D.)	Inhibition (%)	Trypsin-inhibitory activity
No addition	4517 $\pm$ 455	—	—
Purified MP	1128 $\pm$ 110	75	+
Recombinant MP	824 $\pm$ 74	82	+
MP1	4979 $\pm$ 449	0	—
MP2	725 $\pm$ 50	84	+
MP3	760 $\pm$ 115	83	+
MP4	4772 $\pm$ 209	0	—
CM-MP	4538 $\pm$ 221	0	—*

Table 2 Specificity of the inhibition of ^{125}I -labelled monitor peptide binding to intestinal epithelial cells

Cells (1×10^6) were incubated with the ^{125}I -labelled monitor peptide (2.5 nM) and unlabelled trypsin inhibitors at 37 °C and pH 8.0 for 1 h. Soybean trypsin inhibitor is abbreviated as SBTI, egg white trypsin inhibitor as EWTI, bovine pancreatic trypsin inhibitor as BPTI, human pancreatic secretory trypsin inhibitor as h-PSTI and epidermal growth factor as EGF. Except in the case of epidermal growth factor (EGF), the administered concentrations of trypsin inhibitors were adjusted so as to be equivalent to 0.7 μM monitor peptide, based on the inhibitory activity for trypsin in rat pancreatic juice. Epidermal growth factor was also administered because of its amino acid sequence similarity to the monitor peptide.

Substance	Concn. (μM)	^{125}I -labelled MP bound (c.p.m.) (mean $\pm$ S.D.)	Inhibition (%)
No addition	—	4721 $\pm$ 213	—
MP	7.0	1010 $\pm$ 99	79
MP	0.7	2317 $\pm$ 94	51
SBTI	0.3	4413 $\pm$ 967	7
EWTI	8.0	5873 $\pm$ 243	0
BPTI	4.0	4132 $\pm$ 457	12
Leupeptin	3.0	4045 $\pm$ 327	14
Antipain	7.0	4566 $\pm$ 301	3
FOY-305	10.0	4174 $\pm$ 1214	12
hPSTI	1.4	1251 $\pm$ 139	74
EGF	0.7	4250 $\pm$ 266	10

before the experiment. Despite storing at -70 °C for a few days, the dissociation constant and the maximum number of binding sites of the cell preparation did not change, suggesting that the cell preparation could be stored by freezing until the binding assay.

The specificity of the monitor-peptide binding to the cells was investigated by measuring ^{125}I -monitor-peptide binding in the presence of a recombinant monitor peptide and point mutants of it (Table 1). The intact monitor peptide has trypsin-inhibitory activity (Fushiki et al., 1984). The recombinant monitor peptide showed the same level of trypsin-inhibitory activity as the native

Table 3 Effect of proteinase digestion of the mucosal cells on ^{125}I -labelled-monitor-peptide binding

Dispersed mucosal cells were digested with the mixture of trypsin, chymotrypsin, and Pronase E (final concn. of each enzyme was 1 mg/ml) in a buffer containing 10 mM CaCl_2 , 155 mM NaCl and 10 mM HEPES, pH 8.0, for the indicated periods, and the cells were then washed twice with the same buffer without enzyme. The ^{125}I -labelled-monitor-peptide binding assay was performed by the same method as that described in Figure 5. Each value is the mean $\pm$ S.D. for three separate experiments (* $P < 0.01$ versus control).

Incubation time (min)	^{125}I -labelled-monitor-peptide specific binding (c.p.m.) (mean $\pm$ S.D.)	
	Control	Proteinase-treated
0	5400 $\pm$ 100	5330 $\pm$ 150
5	5326 $\pm$ 98	2811 $\pm$ 250*
60	4803 $\pm$ 363	620 $\pm$ 35*

Table 4 Ca^{2+} efflux from dispersed mucosal cells by the monitor peptide (MP) and other related trypsin inhibitors

Mucosal cells (10^6) were incubated with 30 μM monitor peptide, 13 μM SBTI, 342 μM EWTI and 429 μM FOY-305. Experimental conditions were described in the Materials and methods section. Data are expressed as means $\pm$ S.E.M. ($n = 3-6$). The values for groups not sharing a common superscript letter are significantly different ($P < 0.01$) on the basis of analysis of variance.

Substance	$^{45}\text{Ca}^{2+}$ efflux (c.p.m.) (mean $\pm$ S.E.M.)
Control	394 $\pm$ 31 ^a
MP	2090 $\pm$ 264 ^b
SBTI	555 $\pm$ 77 ^a
EWTI	713 $\pm$ 173 ^a
FOY-305	488 $\pm$ 161 ^a

one. These monitor peptides strongly blocked ^{125}I -monitor-peptide binding to the mucosal cells. By contrast, the reduced and CM-MP, which had only 10% of the trypsin-inhibitory activity of the intact monitor peptide, scarcely inhibited the ^{125}I -monitor-peptide specific binding. When Arg²³-Ile²⁴ was replaced by Met²³-Asn²⁴ (MP1) or Arg⁵⁷-Arg⁵⁸ was replaced by Ser⁵⁷-Ser⁵⁸ (MP4), the trypsin-inhibitory activity was abolished. On replacement of Arg⁴⁷-Lys⁴⁸ by His-Asn (MP2) or Ser-Thr (MP3), trypsin-inhibitory activity remained. As shown in Table 1, these non-trypsin-inhibitory monitor peptides (MP1 and MP4) did not inhibit the ^{125}I -monitor-peptide binding to the mucosal cells. But MP2 and MP3, which retained trypsin-inhibitory activity, strongly inhibited the binding of ^{125}I -monitor peptide to cells. The effects of various trypsin inhibitors on the monitor-peptide specific binding to the mucosal cell were determined (Table 2). hPSTI, which exhibits amino acid sequence homology with the monitor peptide, inhibited the monitor-peptide specific binding. However, SBTI, EWTI, BPTI, leupeptin, antipain and FOY-305 did not inhibit the ^{125}I -labelled-monitor-peptide binding at all when a 1000-fold excess amount of each of these inhibitors, as based on the trypsin-inhibitory activity, was added to the binding assay medium with ^{125}I -monitor peptide. Epidermal growth factor, which exhibits a partial amino acid sequence similarity to that of the monitor peptide (Fukuoka et al., 1986), did not show any interaction with the binding site. Neither bestatin, chymostatin, diprotin A nor elastinal inhibited the binding, even when they were each added at 1 mM to the binding-assay medium (results not shown).

Figure 6 Autoradiography of the affinity-cross-linked complex of the ^{125}I -labelled monitor peptide and the specific binding protein

Mucosal cells (5×10^7) from the proximal quarter of the small intestine were incubated with 2×10^6 c.p.m. of the ^{125}I labelled monitor peptide in 200 μl of BIND-buffer at 37 $^\circ\text{C}$ for 1 h in the presence (non-specific binding) or absence (total binding) of a 500-fold excess amount of the unlabelled monitor peptide and cross-linked with DSS. Experimental conditions were described in the Materials and methods section. Lane 1, without unlabelled monitor peptide but with 2-mercaptoethanol; lane 2, with unlabelled monitor peptide and 2-mercaptoethanol; lane 3, without unlabelled monitor peptide and without 2-mercaptoethanol; lane 4, with unlabelled monitor peptide but without 2-mercaptoethanol. The marker proteins shown at the left refer to electrophoresis with 2-mercaptoethanol (lanes 1 and 2) and at the right refer to without 2-mercaptoethanol (lanes 3 and 4). The differing mobilities of the marker proteins are due to the effect of 2-mercaptoethanol. Abbreviation: M, molecular mass.

Table 3 shows that the pretreatment of the mucosal cells with proteinases decreased in the ^{125}I -monitor-peptide binding to the cells, suggesting that easily digestible protein is involved in the specific binding site of the ^{125}I -monitor peptide. The decrease in the binding by proteinase digestion was not due to cell lysis, since less than 5% of DNA leaking from the mucosal cells was observed throughout the digestion period.

Ca^{2+} efflux from dispersed mucosal cells after addition of monitor peptide was determined. As shown in Table 4, monitor peptide stimulated Ca^{2+} mobilization, but other trypsin inhibitors, i.e. SBTI, EWTI and FOY-305, did not induce Ca^{2+} release.

Autoradiography of the affinity-cross-linked complex of the ^{125}I -monitor peptide and the specific binding protein on the mucosal cells showed a molecular mass of about 53 kDa after SDS/PAGE without mercaptoethanol (Figure 6, lane 3). Pretreatment of the ^{125}I -monitor peptide and binding-protein complex with mercaptoethanol decreased its molecular mass to ~ 33 kDa (Figure 6, lane 1). This complex of the ^{125}I -monitor peptide and binding protein was not detected when the ^{125}I -monitor peptide was incubated with mucosal cells in the presence of a large excess of the non-labelled monitor peptide (Figure 6, lanes 2 and 4). SBTI inhibitor did not inhibit the formation of the ^{125}I monitor peptide-binding protein complex (results not shown). Little specific binding to the ^{125}I -labelled monitor peptide was observed in the dispersed cells obtained from the stomach, colon, liver and kidney. The relative specific binding on autoradiography were 1.8, 12, 2.0 and 1.8 respectively with respect to binding to the small-intestinal cells (= 100%).

DISCUSSION

The monitor peptide is a peptide of 6.5 kDa that was first isolated from the bile pancreatic juice of rats (Fushiki et al., 1984), and its amino acid sequence (Iwai et al., 1987) and cDNA sequence (Tsuzuki et al., 1991) have been determined. Its role has been proposed to be the stimulation of CCK release in the jejunum in response to protein intake (Fushiki et al., 1984; Fukuoka et al., 1986; Iwai et al., 1986, 1987).

The monitor peptide exhibits trypsin-inhibitory activity and amino acid sequence similarity to PSTI, which was first described by Kazal et al. (1948). The trypsin-inhibitory activity of the monitor peptide seemed to be relevant to the binding activity; that is, point mutants, in which certain amino acids were replaced by other amino acids, clearly indicated that only the trypsin inhibitor of the mutants could competitively inhibit the ^{125}I -monitor-peptide binding to the mucosal cells (Table 1). These data led us to speculate that a binding protein which has trypsin-like specificity may be involved in the present monitor-peptide-specific-binding site.

It should be noted that human PSTI, which exhibits sequence similarity to the monitor peptide, strongly inhibited the monitor-peptide-specific binding, suggesting that the common amino acid sequence between human PSTI and the monitor peptide may be responsible for the interaction between the monitor peptide and its binding site. Miyasaka et al. (1989b,c) reported that the monitor peptide stimulated CCK release and protein output from the pancreas, whereas human PSTI and rat PSTI did not produce elevation of either CCK release or protein output. The discrepancy between PSTI binding to the monitor-peptide-specific-binding site and the low CCK-releasing activity of PSTI is not explicable so far. A possible explanation is that human PSTI and rat PSTI bind to the monitor peptide specific binding site without stimulation of CCK release as receptor antagonists or bind as very weak agonists. Further studies are required.

In any event, the trypsin-sensitive amino acid sequences in the common regions between the monitor peptide and PSTIs should be important for identifying the specific binding sites for the monitor peptide on mucosal cells. Such sequences are Arg²³-Ile²⁴ (Lys-Ile in human, porcine and canine PSTIs), Arg⁵⁷-Arg⁵⁸ (Lys-Ser in all PSTIs, except that of rat) and Arg⁴⁷-Lys⁴⁸ (Lys-Lys in porcine and canine PSTIs, and Lys-Glu in bovine and ovine PSTIs). The present study, which employed point mutants, indicated that the first two conservative regions were essential for the trypsin-inhibitory activity of the monitor peptide and, concurrently, they were essential sites for monitor-peptide binding to mucosal cells. Arg⁴⁷-Lys⁴⁸ was relevant to neither trypsin-inhibitory activity nor specific binding to mucosal cells. These findings suggested that two sites, Arg²³-Ile²⁴ and Arg⁵⁷-Arg⁵⁸, are involved in the interaction with a trypsin-like protein at the monitor-peptide-specific binding site on mucosal cells. When an Arg residue of the monitor peptide was chemically modified with cyclohexanedione, pancreatic-enzyme secretion caused by the peptide was abolished (M. Tsujikawa, unpublished work). It should also be noted that one of the essential regions, Arg²³-Ile²⁴, is included in the region, Cys²¹-Pro²²-Arg²³-Ile²⁴-Tyr²⁵-Asp²⁶-Pro²⁷-Val²⁸-Cys²⁹ (designated as the C--Y--C module), which is conserved in all PSTI family proteins, and shows high sequence identity with the epidermal-growth-factor family (Hunt et al., 1974; Doolittle, 1985) and laminin (Sasaki et al., 1987). This C--Y--C module is speculated to be the functional unit responsible for molecular recognition between the ligand and its receptor, or for protein-protein interactions. In particular, it was demonstrated that the corresponding region in the laminin B1 chain acts as a binding site for the cell-surface receptor (Graf et al., 1987). Therefore, the monitor peptide binding to the binding site may be one of the protein recognition systems mediated by the C--Y--C module.

The trypsin-like binding site of the monitor peptide showed very high specificity, because other trypsin inhibitors, i.e., Kunitz type SBTI, EWTI, BPTI, leupeptin, antipain and camostate, did not interact with this binding site (Table 2), clearly indicating that the binding was not due to contamination by pancreatic proteinases adhering to the mucosal membrane, because such

proteinases should interact with the trypsin inhibitors described above. This accords well with the results of previous physiological studies on CCK release caused by the monitor peptide. Iwai et al. (1986) reported that only intraduodenal infusion of the monitor peptide, i.e. not that of SBTI, increased the blood CCK level in anaesthetized and atropine-treated rats. Miyasaka et al. (1989a,b) reported similar results in the conscious-rat model, that is, the monitor peptide, but not Trasylol, stimulated protein output.

In the present cell-dispersion system, CCK release by monitor peptide could not be detected, mainly due to insufficiency of the sensitivity of the CCK assay system established for plasma CCK. However, changes of intracellular events were observed; that is, Ca^{2+} efflux from the mucosal cells was induced by the monitor peptide, but not by other trypsin inhibitors (Table 4). The Ca^{2+} efflux is thought to be one of the intracellular events for CCK release (Kanno et al., 1979, 1980) and a convenient indicator for endocrine processes, including CCK release. Quite recently, Liddle et al. (1992) and Bouras et al. (1992) reported the importance of increases in intracellular Ca^{2+} when purified monitor peptide induces CCK release. They indicated that CCK-releasing cells purified by fluorescence-activated cell sorting respond to monitor peptide with an increase in intracellular Ca^{2+} . The coincidence between the characteristic binding of monitor peptide and the Ca^{2+} -releasing activity of the monitor peptide is consistent with our speculation that this monitor-peptide binding has at least some physiological function in the CCK release.

It is not unlikely that a combination of a trypsin-like protein and a tryptic peptide is responsible for the signal transmission in cells. Nakamura et al. (1984) reported that a trypsin-like proteinase on the surface of the plasma membrane of rat hepatocytes is involved in the cell survival. Such a trypsin-like proteinase on the plasma membrane may act on the signal receptor for regulation of the metabolism of the cell.

Direct evidence could not be obtained that the present specific binding site is located on CCK-releasing endocrine cells. This will be supplied by experiments involving purified CCK-releasing endocrine cells and many receptor antagonists. There have been many obstacles, however, in such experiments to date. CCK-releasing endocrine cells have not yet been purified from the rat. Barber et al. (1986) obtained partially purified CCK-releasing cells from canine small-intestinal mucosa. However, the dog does not have feedback regulation of pancreatic enzyme secretion, indicating that the CCK-releasing peptide would contribute little to CCK release in the dog. The high density of the monitor-peptide-specific-binding sites in the proximal small intestinal, but not ileal and colonic, mucosa and those in other organs supported the notion that these binding sites may exist on the CCK-releasing cells, which are known to exist mainly in the duodenum. Kobayashi et al. (1980) reported that the CCK-releasing cells in the duodenum were 3–4-fold more prevalent than in distal small intestine. Our data accord well with this observation.

Autoradiography of an affinity-cross-linked complex of the ^{125}I -monitor peptide and the specific binding protein indicated that a 53 kDa complex was involved in the binding. The molecular mass of the binding protein was decreased to 33 kDa in the presence of mercaptoethanol. This binding was highly specific to the monitor peptide, because a large excess of the unlabelled monitor peptide completely abolished the formation of the complex. SBTI did not affect the formation of the ^{125}I -monitor peptide and binding-protein complex, which was consistent with the results of the binding assay described above and the CCK-releasing experiment involving the peptide (Iwai et al., 1986).

Overall, the monitor-peptide-binding site was found on isolated rat jejunal mucosal cells by using the ^{125}I -monitor peptide and some recombinant point mutants of the monitor peptide,

suggesting that the binding site may be a PSTI-family-specific proteinase-like protein. The coincidence of specific binding to mucosal cells with characteristic CCK release and the Ca^{2+} efflux from mucosal cells suggests that this binding could be involved in CCK-releasing mechanisms. The density of the monitor-peptide receptor was very high in proximal intestinal mucosa, consistent with our speculation that the binding site is expressed mainly in the CCK-releasing endocrine cells. The molecular mass of the complex of the monitor peptide and the binding site was about 53 kDa. Further studies are required to prove directly that the binding site is involved in CCK release from the small intestine.

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