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PROTEOLYTIC INACTIVATION OF SUBSTANCE P IN THE EPITHELIAL LAYER OF THE INTESTINE

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Abstract—Metabolites of substance P, produced by incubation with isolated epithelial cells and with purified brush border and basolateral membrane from pig small intestine, were isolated by high performance liquid chromatography and identified by amino acid analysis. Rapid cleavages between Gln^6-Phe^7, Phe^7-Phe^8 and Gly^9-Leu^{10} and oxidation of the methionine residue at position 11 were observed with cells and with both membrane fractions. Formation of substance P_{3-11}, indicative of the action of dipeptidylaminopeptidase IV (EC 3.4.14.5), was observed only at high substrate concentration. Proteolytic degradation was inhibited by phosphoramidon and by EDTA but was insensitive to chloride ion concentration and to captopril. These observations suggest that inactivation of substance P in the epithelial layer of the gut is mediated through endopeptidase-24.11 (EC 3.4.24.11) in the cell-surface membrane and that degradation by angiotensin-converting enzyme (EC 3.4.15.1), although present in high concentration in the mucosa, is unimportant.

The gut wall of all mammalian species yet studied is innervated by extrinsic and intrinsic neurones containing substance P [1–3]. In rodents, immunohistochemical evidence for a dual location in nerves and in some mucosal enterochromaffin cells has been obtained [4]. In humans [3], the intrinsic nerves emanate from cell bodies in the myenteric and submucous ganglionic plexuses and, in addition to locations in smooth muscle and around the blood vessels, project into the mucosa. Release of substance P in the gastrointestinal lumen in response to electrical stimulation of the vagus nerve has been described [5, 6] and the peptide has been detected by radioimmunoassay in mesenteric vein blood [7]. A role for substance P in the local regulation of intestinal secretion, motility, blood flow or nutrient absorbtion is thus a possibility.

The catabolism of substance P within the central nervous system has been studied extensively and investigations in the human [8], bovine [9], rat [10] and rabbit [11] brain and in human cerebrospinal fluid [12] have been described. Studies *in vitro* using purified enzymes have shown that substance P is a good substrate for the thermolysin-like metallopeptidase (EC 3.4.24.11) (previously described as "enkephalinase") giving the (1–6), (1–7) and (1–9) fragments [13, 14] and also for angiotensin-converting enzyme (EC 3.4.15.1) giving the (1–8) and (1–9) fragments in a 4:1 ratio [14]. These plasma membrane-bound enzymes have a wide distribution in mammalian tissues including the intestinal microvilli [15, 16]. In this study, the mechanism of proteolytic inactivation of substance P in the epithelial layer of the pig small intestine was investigated and the relative importance of these enzymes in the degradation pathway is assessed.

MATERIALS AND METHODS

Materials

Synthetic peptides were purchased from Peninsula Laboratories Inc., Belmont CA and other reagents from Sigma Chemie GmbH, Taufkirchen, FRG. Captopril (D-3-mercapto-2-methylpropanol-L-proline) was a gift from Sanol Schwartz GmbH, Monheim, FRG.

Preparation of isolated epithelial cells and cell membranes

Enterocytes were prepared from a section (40–50 cm) of porcine mid-jejunum by a modification of the non-enzymatic dissociation procedure of Weiser [17]. The lumen of the gut was filled with buffer (sodium chloride 140 mmole/l, disodium hydrogen phosphate 16 mmole/l, EDTA 1.5 mmole/l, dithiothreitol 0.5 mmole/l; pH 7.3) and incubated at 37° for 9 min. The tissue was palpated gently with the fingers for 1 min and the luminal contents were filtered through nylon gauze (pore size 0.25 mm). After centrifugation (50 × *g* for 10 min at 4°), cell pellets were washed with Hanks Minimum Essential Medium (Seromed, Berlin, FRG) containing 10 mmole/l Hepes and 200 mmole/l glutamine; pH 7.35. The incubation procedure was repeated three times and the washed cell pellets were combined. Cell concentrations were determined using a Neubauer hemocytometer and cell viability was assessed from the ability of the cells to exclude eosine red. Only cell preparations in which the viability was > 85% were used.

Brush border membranes were prepared from pig mid-jejunum by the method of Lücke *et al.* [18]. The specific activity of the marker enzyme alkaline

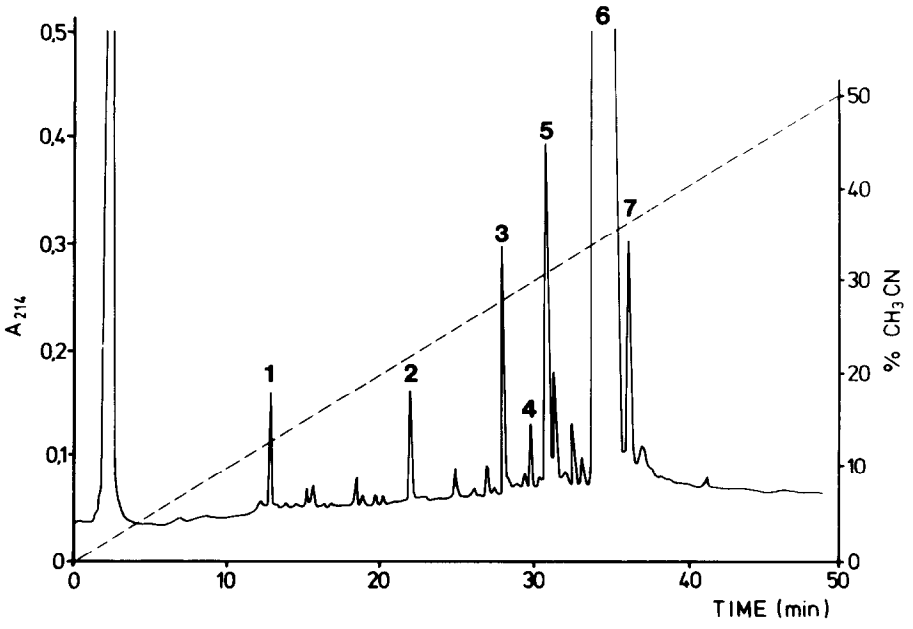

Fig. 1. Reverse phase HPLC of the metabolites of substance P formed during a 3 min incubation of substance P with basolateral membranes. Details of the incubation conditions and chromatography are described in the text. (---) refers to the concentration of acetonitrile in the eluting solvent. Peaks 1–7 are identified in Table 1.

phosphatase [19] was 13 ± 2 fold higher (N = 4; mean ± S.D.) in the membrane fraction than in the original homogenate but the specific activity of Na^+K^+-ATPase [19], a marker of the basolateral membrane, was enriched only 1.2 ± 0.2 fold, indicating that contamination was low. Basolateral membranes from the same tissue were prepared by the sorbitol density gradient method of Dharmsathaphorn *et al.* [20]. The enrichment factor for Na^+K^+ATPase was 9.2 ± 1.0 (N = 8) compared with an enrichment factor of 1.3 ± 0.3 for alkaline phosphatase. Electron microscopy indicated a vesicle size of 0.15–0.40 μm and absence of larger subcellular organelles.

Incubation conditions

Epithelial cells (15–20 × 10^6 cells/ml) and membranes (0.2–1.0 mg/ml protein concentration) were incubated at 37° in 300 mmole/l mannitol, 10 mmole/l Hepes buffer, pH 7.5 with substance P (25–150 nmole/ml). In order to identify initial sites of cleavage, reaction times were short (15 sec–2 min for cells and brush border membranes, 30 sec–5 min for basolateral membranes) such that less than 20% of the substrate was metabolized. Reactions were stopped by the addition of trifluoroacetic acid (TFA) and the reaction mixtures centrifuged (100,000 *g* for 30 min). Peptides were isolated using Sep-Pak C18 cartridges as previously described [21]. Incubations and extractions in the absence of substance P and in the presence of the inhibitors of proteolysis shown in Table 2 were carried out. The apparent K_m for the degradation process was determined by incubating epithelial cells (5–6 × 10^6 cells/ml) with substance P in the concentration range 3–192 μmole/l.

High performance liquid chromatography

Metabolites were isolated by HPLC using an Ultrasphere octadecasilyl-silica column (0.46 × 25 cm; Beckman Instruments) equilibrated with 0.1% TFA. The column was eluted at 30° and at a flow rate of 1.5 ml/min with a linear gradient of acetonitrile (1% per min) and peptides were identified by measurement of absorbance at 214 nm. Signals from the detector were quantitated using a Milton Roy Cl-10 integrator. Peaks that were not present in the chromatogram from a blank incubation (peaks 1–7, Fig. 1) were purified further by HPLC under isocratic conditions using the same column. The concentrations of acetonitrile in the eluting solvent were peak 1, 8.4%, peak 2, 17.5%, peak 3, 24.5%, peaks 4 and 5, 28.0% and peaks 6 and 7, 31.5%. Amino acid analyses were carried out using a Durrum D 500 automated analyser.

RESULTS

The HPLC elution profiles arising from incubations of substance P with epithelial cells and with brush border and basolateral membrane were qualitatively very similar. A representative chromatogram following incubation with basolateral membrane is shown in Fig. 1. The amino acid compositions of the components corresponding to peaks 1–7 are shown in Table 1. In all experiments, metabolites identified as the substance P_{1-6} (peak 1), substance P_{1-7} (peak 2), substance P_{1-9} (peak 3) and the oxidized form of substance P (peak 5) were observed. Peaks corresponding to the C-terminal fragments substance P_{7-11} (peak 7) and substance P_{8-11} (peak 4) were observed following incubations

Table 1. Identification by amino acid composition of the metabolites of substance P formed during incubations with basolateral membranes of pig small intestine.

Chromatography peak	Fragment	Amino acid residue Glx	Pro	Gly	Met	Leu	Phe	Lys	Arg
1	1–6	2.08	2.07	—	—	—	—	0.92	0.94
2	1–7	1.99	2.24	—	—	—	0.96	0.91	0.90
3	1–9	2.04	2.27	0.99	—	—	1.88	0.91	0.92
4	8–11	—	—	1.13	0.72	1.07	1.07	—	—
5	$1–11_{ox}$	2.13	1.01	1.01	—	0.97	1.98	0.90	0.86
6	1–11	2.22	1.03	1.03	0.81	0.98	1.97	0.97	0.99
7	7–11	—	1.12	1.12	0.64	1.09	2.16	—	—

with basolateral membranes but not after brush border or cellular incubations. Under isocratic elution conditions, peak 7 material (Phe-Phe-Gly-Leu-Met-NH_2), peak 4 (Phe-Gly-Leu-Met-NH_2) and peak 5 (Met(O)" substance P) coeluted with the corresponding synthetic peptides.

Incubation of substance P with brush border membranes for very short periods of time (Fig. 2) indicated that the initial rate of formation of the substance P_{1-9} fragment exceeded that of the substance P_{1-6} and substance P_{1-7} fragment and that this fragment underwent further rapid degradation. Consistent with this hypothesis a minor peak with amino acid composition (Phe 1.2, Gly 1.0) corresponding to substance P_{8-9} was observed in some chromatograms. The rate of degradation by purified brush borders exceeded the rate of degradation by an equivalent concentration of basolateral membrane protein by a factor of approximately 5 so that peptidases in the brush border membrane are probably responsible for the greater part of degrading activity of cellular suspensions. In support of this conclusion, an immunohistochemical study using a monoclonal antibody to endopeptidase-24.11 has demonstrated much stronger staining at the luminal surface of intestinal mucosal cells compared with the basolateral membrane [22].

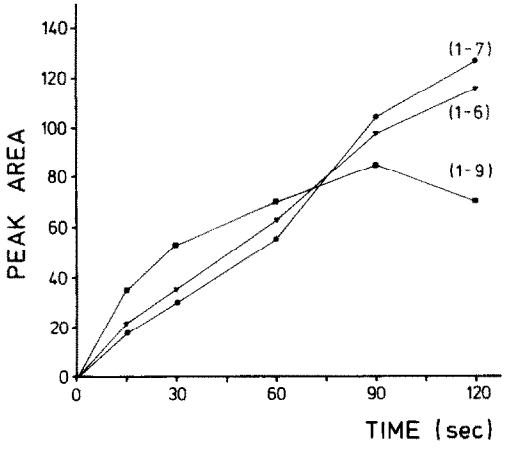

Fig. 2. Rate of formation of substance P_{1-9} (■—■), substance P_{1-6} (▼—▼) and substance P_{1-7} (●—●) during incubation of substance P with brush border membranes. The peak area is determined from the integral of the absorbance at 214 nm and is expressed in arbitrary units.

The effect of inhibitors of proteolysis upon the formation of substance P metabolites during incubation with brush border membranes is shown in Table 2. Degradation is inhibited in a concentration-dependent manner by the metal chelator EDTA and by phosphoramidon (*N*(α-rhamnopyranoslyoxyhydroxyphosphinyl)-L-leucyl-L-tryptophan), an inhibitor of endopeptidase-24.11. The rate of degradation of substance P by purified angiotensin-converting enzyme from human kidney [14] is enhanced by the presence of chloride ion increasing eleven-fold in 20 mM Cl^- and completely inhibited by captopril. As shown in Table 2, degradation of substance P by the intestinal brush border membranes was insensitive to Cl^- concentration and was not inhibited by captopril at a concentration of 20 μM. Using hippurylglycylglycine as substrate, a K_i of 3 nM for the inhibition of pig intestinal angiotensin-converting enzyme by captopril has been reported [16].

The hydrolysis of substance P by endopeptidase-24.11 involves cleavage of more than one bond in the substrate so that the reaction is not amenable to simple kinetic analysis. However, the rate of formation of the substance P_{1-9} fragment as a function of substance P concentration could be expressed as a linear Lineweaver–Burk plot ($r = 0.99$) so that

Table 2. Effects of Cl^- ions and inhibitors of proteolysis upon the formation of substance P metabolites following incubations with brush border membranes

Reagent	Relative amount of metabolite formed 1–6	1–7	1–9
Control	1.00	1.00	1.00
NaCl (60 mM)	0.68	1.01	1.14
NaCl + captopril (60 mM + 20 μM)	0.75	1.06	1.26
EDTA 1 mM	0.62	0.51	1.02
EDTA 10 mM	0.10	0.11	0.24
EDTA 100 mM	0.00	0.00	0.04
Phosphoramidon 1 nM	0.79	1.03	0.93
Phosphoramidon 10 nM	0.60	0.65	0.62
Phosphoramidon 100 nM	0.36	0.45	0.17
Phosphoramidon 1 μM	0.34	0.43	0.10

The values represent the mean of two experiments.

it was possible to compare the apparent K_m for degradation by epithelial cells with previously reported values for degradation by purified enzymes. The apparent K_m for the formation of substance P_{1-9} in the presence of isolated epithelial cells was 55 μM (N = 2). This value compares well with that of 32 μM for the degradation of substance P by endopeptidase-24.11 isolated from pig kidney [13]. During incubitions at the higher concentrations of substance P (48–192 μmole/l), an additional peak was observed in the HPLC chromatogram, eluting as an incompletely resolved peak immediately before substance P, that was absent from the chromatograms following incubations at the lower substrate concentrations. Formation of this metabolite was unaffected by phosphoramidon. The amino acid composition and retention time of this metabolite indicated identity with substance P_{3-11} and suggested the action of dipeptidylaminopeptidase IV [21, 23]. The apparent K_m for the formation of this metabolite was estimated to be > 1 mM. Formation of substance P_{3-11} was not observed in chromatograms following incubations with basolateral membranes.

DISCUSSIONS

The present study has demonstrated that substance P is rapidly metabolized by proteolytic enzyme(s) in the cell-surface membrane of gut epithelial cells. Previous structure–function studies have shown that the amino acids at the C-terminus (residues 5–11) interact with the receptor and are responsible for the ability to contract intestinal smooth muscle [24]. It follows, therefore, that the proteolytic cleavages observed in this study, between Gln^6-Phe^7, Phe^7-Phe^8 and Gly^9-Leu^{10}, will result in termination of the biological activity of the substance P released from enteric neurones. The sites of proteolytic cleavage, apparent K_m and sensitivity to phosphoramidon suggest that endopeptidase-24.11 is important in the degradation process. This enzyme cleaves preferentially at the amino-terminal site of hydrophobic residues and the purified enzyme from pig kidney has been shown to degrade enkephalins, bradykinin, neurotensin, luliberin and the octapeptide of cholecystokinin primarily at this site [13]. In a related study (M. Weber and J. M. Conlon, unpublished data), incubation of somatostatin-14 with purified basolateral membranes from pig gut resulted in cleavages between Phe^6-Phe^7, Phe^7-Trp^8 and Thr^{10}-Phe^{11} indicative of the action of endopeptidase-24.11. The fact that degradation of substance P is not completely inhibited by phosphoramidon, even at a concentration of 1 μM, suggests that a phosphoramidon-insensitive form of the enzyme may be present in the gut. The K_i for the inhibition of pig intestinal endopeptidase-24.11 by phosphoramidon is 10 nM [15].

In view of the high concentration of angiotensin-converting enzyme in the brush border membrane of pig intestine [16], the lack of hydrolysis of substance P by this enzyme during incubations with isolated enterocytes was unexpected. Although the K_m for the degradation of substance P by purified angiotensin-converting enzyme (25 μM) is similar to the K_m for the degradation by endopeptidase-24.11 (32 μM), the K_{cat} for the latter enzyme (5062 min^{-1}) is considerably greater than for angiotensin-converting enzyme (225 min^{-1}) [13, 25]. Thus, angiotensin-converting enzyme in the gut epithelial layer is unable to compete successfully with endopeptidase-24.11 for available substrate.

The sites of cleavage of substance P by cell membranes from gut epithelial cells are not identical to those during incubations with membranes from the central nervous system. A membrane-bound metallo-endopeptidase from human brain inactivated substance P by cleavage between Gln^6-Phe^7, Phe^7-Phe^8 and Phe^8-Gly^9 [8] and, in a rat hypothalamic slice system, the major site of degradation of $pGlu^6$ substance P_{6-11} was between Phe^8-Gly^9 with minor cleavage sites between Phe^7-Phe^8 and Gly^9-Leu^{10} [25]. A specific enzyme cleaving between Pro^4-Gln^5 of substance P has been reported to be present in bovine brain [9] but no evidence for the formation of substance P_{1-4} was obtained in this study. The enzyme dipeptidylpeptidase IV, present in human plasma [21] and in vascular surface membranes [23], converts substance P to the SP_{3-11} and SP_{5-11} fragments which undergo further rapid degradation by aminopeptidases. This study has demonstrated that this pathway does not represent a major mechanism of inactivation in the gut epithelial layer as the rate of reaction becomes significant only at high substrate concentration and degradation at the amino terminus does not lead to loss of biological activity [24]. Our observation is consistent with the report that the K_m for the degradation of substance P by dipeptidylaminopeptidase IV (purified from human submaxillary gland) is 2.0 mM [27]. In addition, it has been shown that dipeptidylpeptidase IV, although present in the cryptic epithelium, is absent from the superficial epithelium of guinea pig enterocytes [28]. Endopeptidase-24.11 is absent from the rat stomach and in this tissue dipeptidylpeptidase IV and angiotensin-converting enzyme are important in the catabolism of substance P [29].

The oxidation of the methionine residue at position 11 of substance P was mediated in part by enzyme(s) in the cell-surface membrane. Although the formation of [(Met(O)")] substance P was observed in control incubations in the absence of membranes or cells, the amount of this metabolite formed was consistently less than the amount formed during equivalent incubation with membranes and isolated epithelial cells. As oxidation of the methionine residue results in reduced biological activity [30], this observation may be of physiological relevance. This study has provided support for the general concept [13] that the catabolism of neuropeptides is effected by relatively few membrane-associated peptidases with broad specificity and tissue distribution rather than by specific "neuropeptidases". Enzyme-resistent analogues of substance P may have a therapeutic application in gastroenterology, e.g. stimulation of peristalsis. In view of the important role of endopeptidase-24.11 in the inactivation of substance P in the gut, structural modifications to the hydrophobic residues at positions 7, 8 and 10 may produce compounds with increased stability.

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