Biochemical Pharmacology, Vol. 35, No. 21, pp. 3719–3725, 1986.
Printed in Great Britain.

0006-2952/86 $3.00 + 0.00
Pergamon Journals Ltd.

SPECIFIC BINDING AND PROTEOLYTIC INACTIVATION OF BRADYKININ BY MEMBRANE VESICLES FROM PIG INTESTINAL SMOOTH MUSCLE

GERTRUD SCHÄFER, ROLAND NAU, THOMAS COLE and J. MICHAEL CONLON*
Clinical Research Group for Gastrointestinal Endocrinology of the Max-Planck-Gesellschaft, University of Göttingen, Goßlerstraße 10d, D-3400 Göttingen and Max-Planck-Institut für Experimentelle Medizin, D-3400 Göttingen, Federal Republic of Germany

(*Received* 6 *February* 1986; *accepted* 5 *May* 1986)

Abstract—A preparation of closed membrane vesicles derived from the longitudinal and circular smooth muscle of pig small intestine was enriched eight-fold in the activity of 5′-nucleotidase and six-fold in the activity of peptidyl dipeptidase A relative to the tissue homogenate. The membrane vesicles specifically bound [^{3}H]bradykinin and the concentration of bradykinin required to inhibit 50% binding was 0.76 ± 0.05 nM. This concentration was not significantly different from the corresponding concentration of lysyl-bradykinin (0.45 ± 0.13 nM) but was less ($P < 0.05$) than the concentration of methionyl-lysyl-bradykinin (1.25 ± 0.10 nM). The concentration of des-Arg^9 bradykinin (7.5 μM) required for 50% inhibition was $>10^3$ times less than bradykinin indicating the presence of a B_2-type receptor. The membrane vesicles also degraded bradykinin and the principal metabolite was identified as bradykinin$_{1-7}$. Des-Arg^1 bradykinin, des-Arg^9 bradykinin and bradykinin$_{6-9}$ were also formed in low yield. Cleavage of the Pro^7–Phe^8 bond was inhibited by phosphoramidon but not by enalapril or captopril indicating that proteolytic inactivation of bradykinin in the muscle layer of the intestine is mediated through endopeptidase 24.11 ("enkephalinase") but not through peptidyl dipeptidase A ("angiotensin-converting enzyme").

Although synthesis and storage of bradykinin in neurones or endocrine cells of the gastrointestinal tract has not yet been demonstrated, homogenates of intestinal tissue are associated with kinin-forming and destroying activities [1]. Bradykinin, and the biosynthetically related lysyl-bradykinin, are potent contractors of intestinal smooth muscle [2], stimulate chloride secretion in the guinea pig ileum [3] and rat colon [4], enhance valine transport across the rat small intestine [5] and regulate blood flow to the gut [6]. Both stimulatory and inhibitory effects of bradykinin on trans-epithelial transfer of sodium and water in the rat jejunum have been observed [7]. The availability of [^{3}H]bradykinin of moderately high specific activity and of tyrosylated analogues of bradykinin labelled with [^{125}I] at the *N*-terminus has enabled demonstration of receptors for bradykinin in a variety of tissues, including guinea pig small and large intestine [8], rat uterus [9], isolated nephron segments of the rabbit [10] and cultured human fibroblasts [11]. Specific binding of bradykinin to a tissue preparation, as well as leading to a biological response, may also represent interaction with an acceptor site linked to a system of proteolytic inactivation [12]. This study has used [^{3}H]bradykinin to demonstrate the presence of specific binding sites for bradykinin and its analogues in a preparation of membrane vesicles derived from pig intestinal smooth muscle. The vesicle preparation also hydrolysed bradykinin and the mechanism of proteolytic inactivation has been elucidated.

* Address correspondence to: Dr J. M. Conlon, Klinische Arbeitsgruppe der MPG, Goßlerstraße 10d, D-3400 Göttingen, F.R.G.

EXPERIMENTAL

Materials. Synthetic peptides were supplied by Bachem Fine Chemicals, Bubendorf, Switzerland. Captopril (D-3-mercapto-2-methylpropanoyl-L-proline) was a gift from Sanol Schwarz GmbH, Monheim, F.R.G. and enalapril (*N*-[(*S*)-1-carboxy-3-phenylpropyl)]-L-alanyl-L-proline) was a gift from Merck, Sharp & Dohme, München, F.R.G. Phosphoramidon [*N*(α-rhamnopyranosyloxyhydroxyphosphinyl)-L-leucyl-L-tryptophan] and bestatin (3-amino-2-hydroxy-4-phenylbutanoyl-L-leucine) were from Sigma Chemie GmbH, Taufkirchen, F.R.G. [2,3-propyl-3,4-^{3}H(*N*)]bradykinin (specific activity 72 Ci/mmol) was supplied by New England Nuclear, Dreieich, F.R.G. and [phenyl-4(*n*)-^{3}H]hippuryl-glycylglycine (specific activity 0.5 Ci/mmol) was supplied by Amersham Buchler GmbH, Braunschweig, F.R.G.

Preparations of membranes. A section of porcine mid-jejunum (approximately 1 m long), removed from the animal as soon as possible after slaughter, was washed with isotonic saline. The gut was opened by longitudinal incision and the mucosa was removed by scraping with a glass slide. The circular and longitudinal smooth muscle layers were removed from the serosa using a glass slide. The muscle strips (40–50 g) were washed with ice-cold buffer A (20 mM

Mops* containing 0.25 M sucrose, pH 7.0) and membranes were prepared by a modification of the method of Grover *et al.* [13]. Tissue was homogenized at 4° in buffer A (10 ml/g) for 45 sec in a Waring blender followed by 7 passages at 1500 rpm in a loose-fitting Teflon–glass homogenizer. The homogenate was centrifuged at 10,000 *g* for 30 min) in a Beckman JA-20 rotor and the pellet discarded. The supernatant was centrifuged at 130,000 *g* for 30 min in a Beckman Ti45 rotor and the resulting pellet was resuspended in buffer A (12 ml). The suspension was layered onto a discontinuous gradient formed in six tubes from 40% w/v sucrose (11 ml), 28% w/v sucrose (7 ml), 22% w/v sucrose (7 ml) and 15% w/v sucrose (7 ml). The tubes were centrifuged at 4° at 100,000 *g* for 120 min in a Beckman SW-28 rotor. Six membrane fractions (F_1–F_6) were collected from the gradient using a Pasteur pipette. Fractions F_1, F_3 and F_5 represented diffuse bands in the 15%, 22% and 28% w/v sucrose layers and F_2, F_4 and F_6 were distinct bands at the interfaces between the 15% and 22%, 22% and 28%, and 28% and 40% w/v sucrose layers. The fractions were diluted with buffer B (20 mM Hepes containing 0.3 M mannitol, pH 7.4) and centrifuged at 130,000 *g* for 30 min. The pellets were resuspended in buffer B to give a protein concentration of 2–4 mg/ml and were stored in aliquots at −70°.

Characterization of the membrane fractions. Fractions F_1–F_6 were characterized by measurement of the activities of marker enzymes: plasma membrane, 5′-nucleotidase (EC 3.1.3.5) [14]; mitochondria, fumarase (EC 4.2.1.2) [15]; lysosomes, β-glucoronidase (EC 3.2.1.31) [16]; endoplasmic reticulum, NADPH-cytochrome *c* reductase (EC 1.6.2.4) [17]; brush borders, sucrase (EC 3.2.1.26) and alkaline phosphatase (EC 3.1.3.1) [18]. Peptidyl dipeptidase A (angiotensin-converting enzyme, EC 3.4.15.1) was determined according to ref. [19] using [^{3}H]hippurylglycylglycine as substrate. Protein concentration was measured by the method of Bradford [20]. The membrane fractions were further characterized by electron microscopy. The membranes were fixed with 3.0% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4 for 2 hr. After washing in cacodylate buffer, the membranes were post-fixed in 2% w/v OsO_4 in 0.1 M sodium cacodylate buffer, pH 7.4 for 90 min and embedded in Epon 812. The preparations were stained with 4% w/v uranyl acetate for 45 min at 50° followed by Sato lead stain for 45 min at 50°.

Binding studies. Muscle membrane (75 μg) was incubated for up to 60 min in the temperature range 4–37° in 25 mM Tes buffer containing 1 mM 1, 10 phenanthroline and 0.16% w/v human serum albumin, pH 6.8 (final volume 500 μl) in the presence of [^{3}H]bradykinin (15,000 dpm equivalent to 1.8×10^{-10} M) and unlabelled peptide. Bradykinin was studied in the concentration range 10^{-10}–10^{-6} M, lysyl-bradykinin and methionyl-lysyl-bradykinin in the range 10^{-11}–10^{-6} M and des-Arg^1 bradykinin and des-Arg^9 bradykinin in the range 10^{-7}–10^{-4} M. Reaction was stopped by the addition of 25 mM Tes buffer containing 1% w/v bovine serum albumin and 2% w/v bacitracin, pH 6.8 (1 ml). Membrane-bound radioactivity was separated on cellulose nitrate filters (pore size 0.1 μm; Sartorius GmbH, Göttingen, F.R.G.) as previously described [21]. Radioactivity was counted in an Isomess β-counter (efficiency 40%) using Aquasol scintillation fluid (New England Nuclear, Boston, MA) Specificity of binding was assessed by incubations in the presence of μmolar concentrations of somatostatin-14, somatostatin-28, gonadatropin-releasing hormone, physalaemin, bombesin and the head activator neuropeptide.

Proteolytic degradation of bradykinin. Membrane protein (75 μg) was incubated with bradykinin (100 μg) at 37° for 10 min in 25 mM Tes buffer, pH 6.8 (500 μl) in the presence and absence of 1,10 phenanthroline (1 mM), captopril (10 μM), enalapril (10 μM), phosphoramidon (10 μM) and bestatin (100 μM). Reaction was stopped by the addition of trifluoroacetic acid (50 μl) and membranes were removed by centrifugation at 85,000 *g* for 30 min. Metabolites were isolated using Sep-Pak C18 cartridges as previously described [21]. The eluate from the cartridge (80% methanol) was lyophilized and redissolved in 0.1% v/v trifluoroacetic acid (100 μl). The solution was chromatographed on a Supelcosil LC-18-DB column (25 × 0.46 cm; Supelco, Inc., Bellefonte, PA, U.S.A.) equilibrated with water/trifluoroacetic acid (99.9:0.1). The column was eluted at room temperature and at a flow rate of 1.5 ml/min with a linear gradient (total volume 52.5 ml) formed from starting solvent and acetonitrile/water/trifluoroacetic acid (34.9:65.0:0.1). Absorbance was monitored at 214 nm and signals from the detector were quantitated using a LDC-Milton Roy C1-10 integrator. Major peaks of u.v. absorbance were purified to homogeneity by rechromatography under appropriate isocratic elution conditions. The amino acid composition of the metabolites was determined by the method of Bidlingmeyer *et al.* [22].

RESULTS

Characterization of membrane vesicles

The relative specific activities of marker enzymes of the muscle membrane fractions F_1–F_6 compared to the tissue homogenate are shown in Table 1. Maximum enrichment of 5′-nucleotidase, a reliable marker enzyme for the plasma membrane of muscle [23] was seen in the F_3 and F_4 fractions and, as shown in Fig. 1, these fractions also showed maximum binding capacity for [^{3}H]bradykinin. Consequently, all subsequent studies were carried out using a pool of these fractions. Contamination of the F_3 and F_4 fractions by mitochondria was low but the contribution of membranes derived from the endoplasmic reticulum, lysosomes and brush borders was appreciable. The enrichment factor for 5′-nucleotidase in the F_3 and F_4 preparations compares well with the corresponding values for membranes prepared from rabbit intestinal circular smooth muscle (3.2-fold) and longtitudinal smooth muscle (5.1-fold)

* Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid; Tes, *N*-tris[hydroxymethyl]methyl-2-aminoethanesulphonic acid; Mops: 3-[*N*-morpholino]propanesulphonic acid.

Table 1. Relative specific activities of marker enzymes compared with tissue homogenate (= 1) in membrane fractions (F_1–F_6) prepared from pig intestinal smooth muscle by density gradient centrifugation. N is the number of determination and data are presented as mean ± S.E.M.

Enzyme	Membrane fraction F_1	F_2	F_3	F_4	F_5	F_6
5′-Nucleotidase (N = 8)	2.9 ± 0.3	6.4 ± 0.7	8.9 ± 1.0	8.1 ± 0.8	5.6 ± 0.4	3.3 ± 0.1
Fumarase (N = 3)	0.22 ± 0.02	0.28 ± 0.01	0.22 ± 0.03	0.24 ± 0.01	0.58 ± 0.02	0.98 ± 0.06
NADPH-Cytochrome *c* reductase (N = 5)	3.1 ± 0.8	3.4 ± 0.5	5.1 ± 1.0	4.5 ± 0.7	4.8 ± 0.9	3.8 ± 0.8
β-Glucoronidase (N = 5)	1.6 ± 0.2	1.8 ± 0.5	3.7 ± 0.2	4.2 ± 0.5	3.9 ± 0.5	3.7 ± 0.5
Alkaline phosphatase (N = 3)	0.9 ± 0.2	2.5 ± 0.6	3.8 ± 0.3	4.4 ± 0.7	4.8 ± 0.5	6.5 ± 0.8
Sucrase (N = 4)	0.3 ± 0.1	0.8 ± 0.3	2.5 ± 0.7	4.9 ± 1.1	4.6 ± 1.4	6.6 ± 2.6
Peptidyl dipeptidase A (N = 4)	1.8 ± 0.2	2.8 ± 1.0	5.0 ± 1.2	6.1 ± 1.4	5.4 ± 1.6	4.2 ± 1.2
Protein % of homogenate (N = 6)	0.40 ± 0.04	0.41 ± 0.06	0.44 ± 0.06	0.32 ± 0.04	0.41 ± 0.11	0.64 ± 0.05

and the ratios of the relative specific activities of 5′-nucleotidase and NADPH-cytochrome *c* reductase are similar in the preparations from pig and rabbit [13]. The specific activity of peptidyl dipeptidase A was also highest in the F_4 fraction and the activity of the enzyme was completely inhibited by 10 μM enalapril and 10 μM captopril. Electron microscopy of the F_4 membrane fraction (Fig. 2) showed that the preparation consisted predominantly of a mixed population of closed vesicles. Contamination by free ribosomes was apparent but the membranes were free from mitochondria and larger sub-cellular organelles.

Binding of bradykinin and analogues to muscle membranes

Maximum binding of [^{3}H]bradykinin to the F_3 and F_4 vesicles occurred at temperatures between 15° and 21° and in the pH range 6.5–7.0. In the presence of 1,10 phenanthroline (1 mM), binding reached a maximum value after 30 min and remained relatively constant up to 60 min (Fig. 3). At 21° and at protein concentrations up to 150 μg/ml, the amount of radioactivity that was not displaced from the membranes by 1 μM bradykinin represented <10% of the total radioactivity bound (Fig. 3). At 37°, binding of [^{3}H]bradykinin reached a maximum after 10 min but thereafter decreased appreciably. Incubations at this temperature were associated with proteolytic inactivation of the tracer. Consequently, assays were routinely carried out with an incubation time of 45 min at 21° and at pH 6.8 with a protein concentration of 75 μg/500 μl. Under these conditions, total binding of [^{3}H]bradykinin to the membranes represented between 4.5 and 8.0% of the total radioactivity in the incubation mixture. Binding of [^{3}H]bradykinin was inhibited in a concentration-dependent manner by bradykinin, lysyl-bradykinin and methionyl-lysyl-bradykinin (Fig. 4). The concentration of peptides required to inhibit 50% of the binding of [^{3}H]bradykinin were: bradykinin 0.76 ± 0.05 nM (N = 7), lysyl-bradykinin 0.46 ± 0.13 nM (N = 8) and methionyl-lysyl-bradykinin 1.25 ± 0.10 nM (N = 5). Transformation of the binding data by the method of Scatchard gave rise to non-linear plots suggesting, as one possibility, the existence of multiple classes of binding site. Binding characteristics were consistent with the presence of two classes of site for each ligand with the following dissociation constants: bradykinin 0.34 nM and 2.2 nM, lysyl-bradykinin 0.61 nM and 6.5 nM and methionyl-lysyl-bradykinin 1.2 nM and 3.5 nM. The concentrations of the metabolites des-Arg^1 bradykinin and des-Arg^9 bradykinin required for 50% inhibition of binding were >10^3-fold higher: des-Arg^1 bradykinin 8.3 μM and des-Arg^9 bradykinin 7.5 μM. The structurally unrelated peptides listed in the Methods section did not significantly inhibit binding of [^{3}H]bradykinin to the membranes. Similarly, the head activator peptide (pGlu-Pro-Pro-Gly-

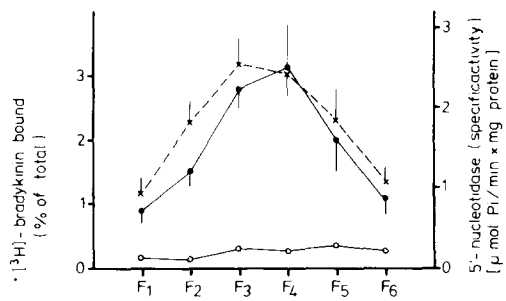

Fig. 1. The activity of 5′-nucleotidase (×—×) and the binding of [^{3}H]bradykinin in the presence (○—○) and absence (●—●) of 10^{-6} M bradykinin to membrane fractions (F_1–F_6) prepared from porcine intestinal smooth muscle as described in the text. Binding studies were carried out at 21° using 50 μg membrane protein and data are expressed as mean ± SEM (N = 8).

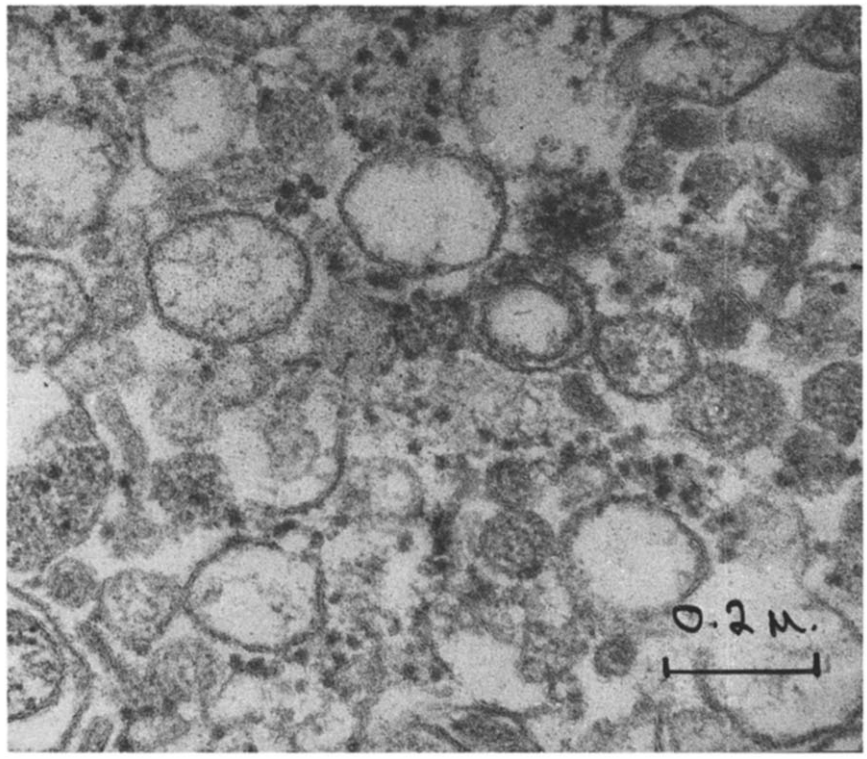

Fig. 2. Electron micrograph of vesicles derived predominantly from the plasma membrane of pig intestinal smooth muscle cells (magnification ×97,600).

Gly-Ser-Lys-Val-Ile-Leu-Phe), a neuropeptide present in mammalian intestine [24] and bearing some structural homology to bradykinin, also did not inhibit binding of [^{3}H]bradykinin at a concentration of 1 μM.

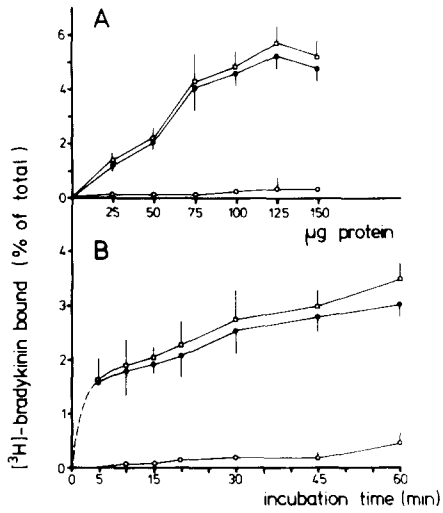

Fig. 3. Effect of (A) membrane protein concentration and (B) time on the binding of [^{3}H]bradykinin to smooth muscle membrane vesicles. In (A) incubations were carried out at 21° for 30 min and in (B) at 21° at a protein concentration of 50 μg/ml. Data points represent mean ± SEM (N = 6). Total binding (□—□), binding in the presence of 1 μM bradykinin (○—○) and specific binding (●—●) are shown.

Hydrolysis of bradykinin by intestinal smooth muscle membranes

A representative elution profile on reverse phase HPLC of the metabolites formed by incubation of bradykinin with the F_3 and F_4 membrane fractions is shown in Fig. 5. The major metabolites were identified by amino acid analysis (Table 2). Peak 5 represented undegraded bradykinin. The principle metabolite (peak 4) represented bradykinin$_{1-7}$ arising from a cleavage of the Pro^7-Phe^8 bond. The corresponding C-terminal fragment Phe^8-Arg^9 was identified as

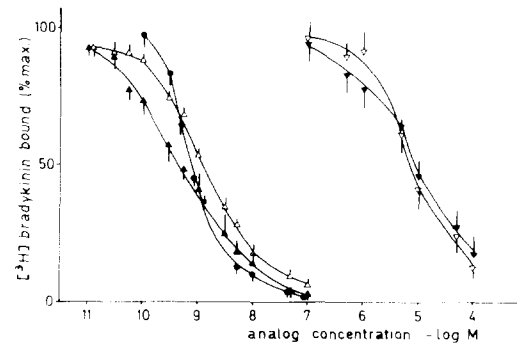

Fig. 4. Inhibition of binding of [^{3}H]bradykinin to membrane vesicles by (●—●) bradykinin, (▲—▲) lysyl-bradykinin, (△—△) methionyl-lysyl-bradykinin, (▼—▼) des-Arg^1-bradykinin and (▽—▽) des-Arg^9 bradykinin. Data points represent mean ± SEM and incubations were carried out as described in the text using 15,000 dpm (1.8×10^{-10} M) [^{3}H]bradykinin.

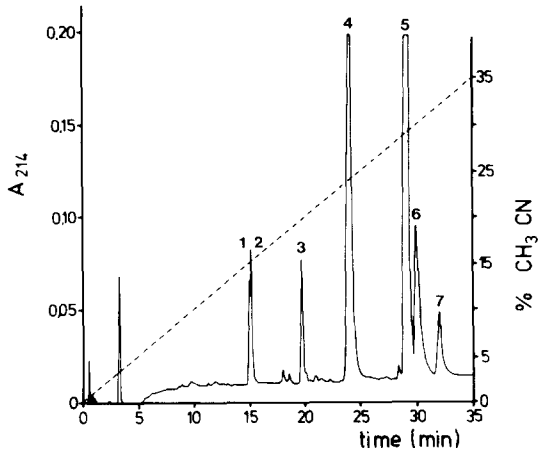

Fig. 5. Reverse phase HPLC of metabolites of bradykinin produced during incubation with membrane vesicles from pig small intestine. Elution conditions are described in the text and - - - shows the concentration of acetonitrile in the eluting solvent. Peaks 1–6 were identified by amino acid analysis (Table 2). The chromatogram is representative of at least six experiments.

peak 1, present in a much lower amount, and the presence of free phenylalanine in the chromatogram (peak 2) suggested that the Phe^8-Arg^9 bond was susceptible to further hydrolysis. Peak 3 was identified as Ser-Pro-Phe-Arg (bradykinin$_{6-9}$) but no peak corresponding to the (1–5) fragment of bradykinin was observed in the chromatograms. The peak 6 peptide coeluted with synthetic des-Arg^1 bradykinin on HPLC under elution conditions in which des-Arg^1 bradykinin and des-Arg^9 bradykinin could be completely resolved. The peak 7 peptide was obtained in too small an amount for a reliable amino acid analysis but the retention time of the peptide was identical to that of des-Arg^9 bradykinin.

The effect of inhibitors of proteolysis upon the formation of the bradykinin metabolites is shown in Table 3. Phosphoramidon, a relatively specific inhibitor of intestinal endopeptidase-24.11 [25] inhibited strongly, but not completely, the formation of bradykinin$_{1-7}$ and bradykinin$_{8-9}$. At the concentration used (10 μM), phosphoramidon inhibits completely the activity of purified endopeptidase-24.11 from pig kidney [26] and in a human synaptic membrane preparation [27] but the lack of complete inhibition observed in this study is consistent with the observation that the epithelial layer of pig intestine is associated with a phosphoramidon-insensitive form of the enzyme when substance P was used as substrate [28]. The metal chelator, 1,10 phenanthroline, completely inhibited the formation of the bradykinin$_{1-7}$ metabolite, consistent with the reported sensitivity of intestinal endopeptidase-24.11 to this agent [25]. Enalapril and captopril, specific inhibitors of peptidyl dipeptidase A, had no significant effect upon the formation of any metabolite despite the fact that, at the concentration used, the activity of the enzyme

Table 2. Identification by amino acid composition of the metabolites of bradykinin formed during incubations with muscle membranes from pig intestine

Chromatography peak	Bradykinin fragment	Amino acid residue				
		Arg	Pro	Gly	Phe	Ser
1	8–9	1.08	—	—	0.97	—
2	Phe	—	—	—	+ve	—
3	6–9	0.97	1.06	—	0.92	1.05
4	1–7	1.03	2.96	1.05	1.00	0.95
5	1–9	1.91	3.23	1.13	1.79	0.94
6	2–9	1.07	3.14	1.08	1.67	1.00

Table 3. Effect of inhibitors on the hydrolysis of bradykinin by pig intestinal smooth muscle membranes. Data are expressed as mean ± SEM and represent the results of at least six experiments

Chromatography peak	Bradykinin fragment	Effects of inhibitors on peak area (no inhibitor = 100)				
		1,10 phenanthroline (1 mM)	Phosphoramidon (1 μM)	Captopril (10 μM)	Enalapril (10 μM)	Bestatin (100 μM)
1	8–9	n.d.	15 ± 1	96 ± 4	96 ± 3	237 ± 18
2	Phe	n.d.	n.d.	n.d.	n.d.	30 ± 5
3	6–9	n.d.	91 ± 5	82 ± 5	96 ± 6	65 ± 8
4	1–7	2 ± 1	23 ± 5	89 ± 3	93 ± 3	95 ± 7
6	2–9	n.d.	102 ± 6	88 ± 6	97 ± 4	118 ± 8

n.d. = not determined.

was completely inhibited when [^{3}H]hippurylglycylglycine was used as substrate. Bestatin, a broad spectrum inhibitor of several aminopeptidases, inhibited cleavage of the Phe^8-Arg^9 bond resulting in an increase in the area of the Phe-Arg peak and decrease in the amount of free phenylalanine in the chromatograms. The formation of des-Arg^1 bradykinin was, however, bestatin insensitive.

DISCUSSION

The present study has described the preparation and characterization of membrane vesicles from pig intestinal smooth muscle that specifically bind and hydrolyse bradykinin. The total preparation time of the membranes was relatively short (approximately 7 hr) and preliminary experiments indicate that the method is suitable for the preparation of membranes from surgically-resected human intestinal tissue. The eight-fold enrichment of 5′-nucleotidase in the F3 and F4 fractions indicated that the vesicles were derived, at least in part, from the plasma membrane. As bradykinin and lysyl-bradykinin are highly charged peptides it is unlikely that they would cross the cell membrane (other than by a mechanism of receptor-mediated endocytosis) and so would not have access to intracellular binding sites or proteolytic enzymes. The concentration of bradykinin required to inhibit 50% of the binding of [^{3}H]bradykinin to the vesicles (0.76 nM) is comparable to the corresponding values in preparations from guinea pig ileum (0.2 nM) [8], bovine uterine myometrum (0.17 nM) [29] but lower than in human fibroblasts (4.6 nM) [11] and rabbit nephron segments (12 nM) [10]. In agreement with the observations of Innis *et al.* [8], the ability of lysyl-bradykinin to inhibit binding was not significantly different from that of bradykinin but the lower binding affinity of methionyl-lysyl-bradykinin is consistent with the lower potency of the analogue in contracting the guinea pig ileum. The greatly reduced ability of des-Arg^9 bradykinin to inhibit the binding of [^{3}H]bradykinin is indicative of the presence of a B_2 type receptor in pig intestinal smooth muscle [30].

The interaction of [^{3}H]bradykinin with human fibroblasts could be represented by a linear Scatchard plot with a Hill coefficient close to unity, consistent with the presence of a single class of binding site with no significant cooperativity effects [11]. In contrast, analysis of the binding of bradykinin, lysyl-bradykinin and methionyl-lysyl-bradykinin to the intestinal membranes in this study gave rise to non-linear Scatchard plots which were consistent with the presence of two classes of binding site. A previous study [9] has demonstrated that the binding characteristics of [^{125}I]Tyr^1 Kallidin to subcellular particles from bovine myometrium are consistent with the presence of two classes of site with dissociation constants of 0.1 and 5 nM. Several recent studies [12, 31], however, have identified possible artifactual causes of deviation from linearity and caution against ascribing such deviation to the presence of receptor subtypes or negative cooperativity effects. Further studies are required using a [^{3}H]bradykinin analogue of higher specific activity before an unambiguous interpretation of the binding data can be made.

Incubation of bradykinin with the membrane vesicles resulted in rapid hydrolysis of the Pro^7-Phe^8 bond. Formation of both bradykinin$_{1-7}$ and bradykinin$_{8-9}$ fragment was inhibited by phosphoramidon but not by captopril and enalapril. This result indicates that hydrolysis is mediated through the action of endopeptidase-24.11 and not through peptidyl dipeptidase A. Purified endopeptidase-24.11 from pig kidney [26] cleaves bradykinin at the Pro^7-Phe^8 bond with further cleavage at the Gly^4-Phe^5 bond on prolonged incubation. Although the kinetic parameters of the intestinal enzymes have not been reported, the K_{cat} for the hydrolysis of bradykinin by endopeptidase-24.11 from pig kidney (6364 min^{-1}) [26] is much higher than the K_{cat} for hydrolysis by peptidyl dipeptidase A from human kidney (500 min^{-1}) [19] suggesting that, in the membrane vesicles, the latter enzyme is unable to compete successfully with endopeptidase-24.11 for available substrate. This conclusion is supported by earlier observations that endopeptidase-24.11, but not peptidyl dipeptidase A, will hydrolyse substance P in the epithelial layer of pig intestine [28] and neurokinin A [32] and neurokinin B [33] in synaptic membranes from pig striatum.

Minor metabolites identified following incubation of bradykinin with the membrane vesicles were des-Arg^1 bradykinin and des-Arg^9 bradykinin. The formation of the des-Arg^1 metabolite was not inhibited by bestatin, demonstrating that its formation was not mediated through the action of aminopeptidase N (EC 3.4.11.2), an integral membrane protein that may be involved in the degradation of neuropeptides in the brain [34] and has been shown to be present in the cell surface membrane of vascular smooth muscle [35]. Bestatin did, however, inhibit the cleavage of the Phe^8-Arg^9 bond in the bradykinin$_{8-9}$ fragment. A further minor metabolite was identified as Ser-Pro-Phe-Arg (bradykinin$_{6-9}$). Formation of this component during incubation of bradykinin with pulmonary artery endothelial cells in culture [36] and during perfusion of bradykinin through the rat liver [37] has been reported. The fact that formation of this metabolite was partially inhibited by bestatin suggests that it may have arisen from the rapid cleavage by aminopeptidase(s) of the endopeptidase-24.11 derived metabolite Phe-Ser-Pro-Phe-Arg. The present study has demonstrated that metabolism of bradykinin within the muscle layer of the intestine follows a different pathway than in plasma where carboxypeptidase N is responsible for 90% of kinin destruction and in the lung where degradation is mediated primarily through peptidyl dipeptidase A [38].

Acknowledgements—This work was supported by the Stiftung Volkswagenwerk.

REFERENCES

1. E. Amundsen and K. Nustad, *J. Physiol.* **179**, 479 (1965).
2. J. Barabe, J. N. Drouin, D. Regoli and W. K. Park, *Can. J. Pharmac.* **55**, 1270 (1977).
3. D. C. Manning, S. H. Snyder, J. E. Kachur, R. J. Miller and M. Field, *Nature, Lond.* **299**, 256 (1982).

4. A. W. Cuthbert and H. S. Margolius, *Br. J. Pharmac.* **75**, 578 (1982).
5. C. Moriwaki, H. Fujimori, H. Moriya, K. Kizuki and K. Kizuki, *Chem. pharmac. Bull. Tokyo* **25**, 1174 (1977).
6. F. J. Haddy, T. E. Emerson, J. B. Scott and R. M. Daugherty, *Handbk. exp. Pharmac.* **25**, 362 (1970).
7. A. D. Crocker and S. P. Willavoys, *J. Physiol.* **253**, 401 (1975).
8. R. B. Innis, D. C. Manning, J. M. Stewart and S. H. Snyder, *Proc. natn. Acad. Sci. U.S.A.* **78**, 2630 (1981).
9. C. E. Odya, T. L. Goodfriend and C. Pena, *Biochem. Pharmac.* **29**, 175 (1980).
10. K. Tomita and J. J. Pisano, *Am. J. Physiol.* **246**, F732 (1984).
11. A. A. Roscher, V. C. Manganiello, C. L. Jelsema and J. Moss, *J. clin. Invest.* **72**, 626 (1983).
12. P. M. Laduron, *Biochem. Pharmac.* **33**, 833 (1984).
13. A. K. Grover, C. Y. Kwan, R. E. Garfield, J. McLean, J. E. T. Fox and E. E. Daniel, *Can. J. Physiol. Pharmac.* **58**, 1102 (1980).
14. N. N. Aronson and O. Touster, *Meth. Enzym.* **31**, 90 (1974).
15. V. P. Whittaker and L. A. Barker, *Meth. Neurochem.* **2**, 1 (1972).
16. G. Szasz, *Clin. chim. Acta* **15**, 275 (1967).
17. G. L. Sottocasa, B. Kuylenstierna, L. Eruster and A. Bergstrand, *J. cell. Biol.* **32**, 415 (1967).
18. H. Lücke, W. Berner, H. Menge and H. Murer, *Pflüger's Arch.* **373**, 243 (1978).
19. T. A. Stewart, J. A. Weare and E. G. Erdös, *Meth. Enzym.* **80**, 450 (1981).
20. M. Bradford, *Analyt. Biochem.* **72**, 248 (1976).
21. M. Weber, T. Cole and J. M. Conlon, *Am. J. Physiol.* **250**, G679 (1986).
22. B. A. Bidlingmeyer, S. A. Cohen and T. L. Tawin, *J. Chromat.* **336**, 93 (1984).
23. C. Y. Kwan, Y. Sakai, A. K. Grover and R. M. K. W. Lee, *Molec. Physiol.* **2**, 107 (1982).
24. H. Bodenmüller and H. C. Schaller, *Nature, Lond.* **293**, 579 (1981).
25. E. M. Danielsen, J. P. Vyas and A. J. Kenny, *Biochem. J.* **191**, 645 (1980).
26. R. Matsas, A. J. Kenny and A. J. Turner, *Biochem. J.* **223**, 433 (1984).
27. R. Matsas, M. Rattray, A. J. Kenny and A. J. Turner, *Biochem. J.* **228**, 487 (1985).
28. R. Nau, G. Schäfer and J. M. Conlon, *Biochem. Pharmac.* **34**, 4019 (1985).
29. M. J. Fredrick, R. J. Vavrek, J. M. Stewart and C. E. Odya, *Biochem. Pharmac.* **33**, 2887 (1984).
30. D. Regoli, J. Barabé and W. K. Park, *Can. J. Physiol. Pharmac.* **55**, 855 (1977).
31. C. M. Mendel and D. B. Mendel, *Biochem. J.* **228**, 269 (1985).
32. N. M. Hooper and A. J. Turner, *Fedn. Eur. Biochem. Soc. Lett.* **190**, 133 (1985).
33. N. M. Hooper, A. J. Kenny and A. J. Turner, *Biochem. J.* **231**, 357 (1985).
34. R. Matsas, S. L. Stephenson, J. Hrysko, A. J. Kenny and A. J. Turner, *Biochem. J.* **231**, 445 (1985).
35. F. E. Palmieri, J. J. Petrelli and P. E. Ward, *Biochem. Pharmac.* **34**, 2309 (1985).
36. J. W. Ryan, U. S. Ryan, A. Chung and G. H. Fisher, *Adv. exp. med. Biol.* **156**, 775 (1983).
37. D. R. Borges, J. A. Guimaraes, E. A. Limaos, J. L. Prado and A. C. M. Carmargo, *Naunyn-Schmiedeberg's Arch. Pharmac.* **309**, 197 (1979).
38. D. Regoli and J. Barabé, *Pharmac. Rev.* **32**, 1 (1980).