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ENDOPEPTIDASE-24.11 CLEAVES A CHEMOTACTIC FACTOR FROM α-CALCITONIN GENE-RELATED PEPTIDE

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Abstract—The sequence of rat α-calcitonin gene-related peptide (CGRP-α) contains the tetrapeptide eosinophil granulocyte chemotactic factor Val^{32}-Gly-Ser-Glu^{35}. Peptide fragments formed following hydrolysis of rat CGRP-α *in vitro* by endopeptidase-24.11 were identified. The tetrapeptide fragment was generated following cleavage at a substrate recognition site unusual for this enzyme (-Glu-Ala-). Chemotactic activity of rat CGRP-α was increased following hydrolysis. Furthermore, rat CGRP-β, which lacks the tetrapeptide sequence and is completely devoid of chemotactic activity, displayed low but measurable activity after hydrolysis. Val-Gly-Ser-Glu was identified as the principle fragment with chemotactic activity in rat CGRP-α. The results show that the chemotactic activity of the neuropeptide rat CGRP-α towards eosinophil polymorphonuclear leukocytes is increased following its hydrolysis *in vitro* by endopeptidase 24.11 through the formation of a previously identified eosinophil chemotactic tetrapeptide.

Neuropeptides released at peripheral terminals of small-calibre sensory nerves during the axon reflex are implicated in the local inflammatory response [1]. CGRP§, present in cutaneous and visceral afferent nerves [2, 3], may contribute to the reflex by increasing local blood flow [4] and activating leukocytes [5]. Amino acid residues 32–35 of rat CGRP-α (Val-Gly-Ser-Glu) are identical to a tetrapeptide eosinophil chemotactic factor of anaphylaxis [6]. Rat CGRP-α elicits a mild chemotactic response in guinea-pig eosinophil polymorphonuclear leukocytes *in vitro*, but limited proteolysis of the peptide with trypsin results in an increase in the activity of its products towards eosinophils [7]. In this report we demonstrate that CGRP-α is a substrate for endopeptidase-24.11 (EC 3.4.24.11; neutral endopeptidase), a widely distributed cell-surface ectoenzyme which has been implicated in the biological inactivation of enkephalins, atrial peptides and tachykinins [8, 9]. Endopeptidase-24.11 generates Val-Gly-Ser-Glu directly from CGRP-α *in vitro* reflecting a novel specificity for the enzyme. The chemotactic activity of rat CGRP-β is not increased by hydrolysis to a similar extent. This finding may have relevance for the biological functions of these two peptides.

MATERIALS AND METHODS

Materials. CGRP peptides (α and β) were from Peninsula Laboratories (St. Helens, U.K.). Other materials were from sources noted previously [9].

Enzyme preparation and assay. Endopeptidase-24.11 was purified to homogeneity from pig kidney cortex by using immunoaffinity chromatography with a monoclonal antibody as described previously [10]. The purified product was homogeneous by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (M_r, 90,000) and contained no detectable exopeptidase activity. The hydrolysis of (Leu^5)enkephalin by the purified enzyme was inhibited completely by 1 μM phosphoramidon. Rat CGRP-α (50 μM) was subjected to digestion by endopeptidase-24.11 (1.7 μg protein) for 4 hr in 0.1 M Tris–HCl buffer, pH 7.4 containing 0.15 M NaCl in a total volume of 100 μL and separation of peptide fragments was achieved by reverse-phase HPLC using a μBondapak C_{18} column [11]. The fractionation used a linear gradient of acetonitrile from 4.05 to 45% in 0.08% H_3PO_4, pH 2.5. The products were monitored at 214 nm.

Peptide sequencing. The sequences of the various peptide fragments (50–200 pmol) were determined by automated solid-phase Edman degradation [12] using the microsequencing facility of the Sequencing Unit, Department of Biochemistry and Molecular Biology, University of Leeds.

Leukocyte preparation and chemotactic assay. Preparation of peritoneal leukocytes from horse serum-challenged guinea-pigs was essentially as described previously [7], except that the cells were harvested in Ca^{2+}, Mg^{2+}-free Hank's buffered saline (pH 6.8) with 0.6% v/v glucose, 0.1 mg/L glycogen and 30 mg/L deoxyribonuclease 1 (Sigma Chemical Co., Poole, U.K.). Chemotaxis was performed in blind-well chambers. HPLC fractions were freeze-dried and reconstituted to 10 nM concentration in Gey's balanced salt solution (pH 7.3) for the assay. Chemotaxis was analysed by the leading front method in 6–8 microscopic fields on duplicate filters. Chemotactic index was determined using CGRP-α and Gey's balanced salt solution as positive

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§ Abbreviation: CGRP, calcitonin gene-related peptide.

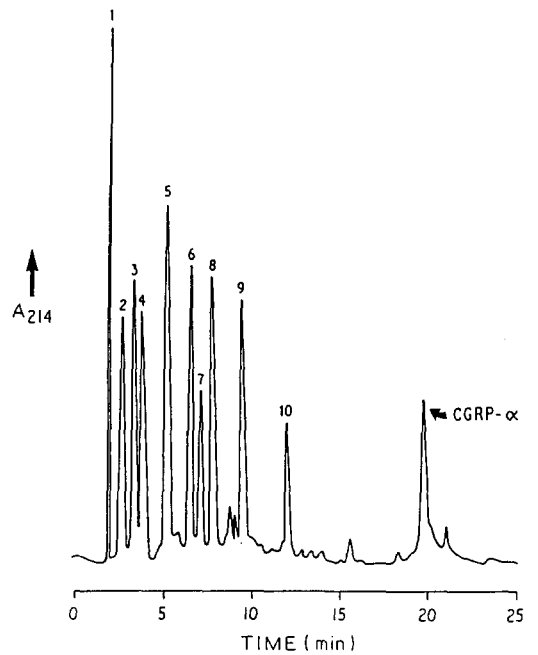

Fig. 1. Separation of the products of cleavage of CGRP-α by endopeptidase-24.11 by reverse-phase HPLC. See Materials and Methods for detail. The numbered product peaks refer to those used in the chemotaxis assay in Fig. 3.

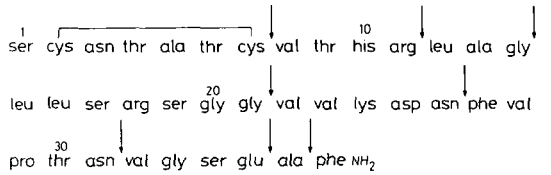

Fig. 2. Endopeptidase-24.11 cleavage sites in rat CGRP-α deduced from data of Fig. 1a.

and negative controls, respectively, within each experiment. The positive control level is normalized to 1.0.

RESULTS

Hydrolysis of CGRP by endopeptidase-24.11

CGRP-α was hydrolysed efficiently by endopeptidase-24.11 at multiple sites as revealed by HPLC analysis of the hydrolysate (Fig. 1). Hydrolysis was completely abolished by the endopeptidase-24.11 inhibitor, phosphoramidon (1 μM). Sequencing of the various cleavage products resolved in Fig. 1 indicates enzymic cleavage occurring at eight distinct sites in the peptide as shown in Fig. 2. Seven of the hydrolysis sites are to the amino side of a large hydrophobic residue (Val, Leu or Phe) consistent with the primary specificity of endopeptidase-24.11 [8]. An additional and unexpected cleavage of CGRP-α between residues Glu^{35} and Ala^{36} by endopeptidase-24.11 resulted in the formation of the chemotactic sequence Val^{32}-Gly-Ser-Glu^{35}. The identity of this peptide (fraction 4, Fig. 1) was confirmed both by microsequencing and by co-elution with authentic Val-Gly-Ser-Glu. The β form of rat CGRP, which differs only at position 35 (Lys for Glu), was also hydrolysed by endopeptidase-24.11 at equivalent sites (data not shown), but only rat CGRP-α contains the known chemotactic tetrapeptide sequence.

Chemotactic activity of peptide products

To investigate the biological activity of products of endopeptidase-24.11 digestion of CGRP-α, the principle peptide fractions were collected. Their chemotactic activity towards guinea-pig peritoneal eosinophils was determined *in vitro*. The results of the assays of undigested CGRP-α and unresolved endopeptidase-24.11 digestion fragments are shown in Table 1. CGRP-α itself possessed significant chemotactic activity (typical mean migration distance = 40.42 ± 1.96 μm; significantly different from negative control $P < 0.01$). The chemotactic activity of intact CGRP-α was not altered significantly by inclusion of the endopeptidase-24.11 inhibitor, phosphoramidon (1 μM), in the chemotactic assay indicating that the activity of the intact peptide does not result from exposure to endopeptidase-24.11 or a similar peptidase on peritoneal leukocytes. Unfractionated endopeptidase-24.11 hydrolysates of CGRP-α elicited chemotactic responses significantly greater than those of the intact peptide. Intact rat CGRP-β exhibited no chemotactic activity but low activity was detected after digestion with endopeptidase-24.11. Since CGRP-β lacks the Val-Gly-Ser-Glu sequence, this is consistent with the occurrence of modest chemotactic activity among CGRP-α peptide fragments apart from Val-Gly-Ser-Glu (Table 1).

Following fractionation of hydrolysis products by HPLC the chemotactic assay revealed significant activity of some fractions towards eosinophils (Fig. 3). Fraction 4, which contained the tetrapeptide Val-Gly-Ser-Glu as determined by microsequencing and by co-elution with authentic peptide, was the only fraction that contained activity greater than that of intact CGRP-α. The results in Figs 1 and 3 strongly support the hypothesis that the increase in chemotactic activity of rat CGRP-α following digestion by endopeptidase-24.11 *in vitro* is due to the formation of Val-Gly-Ser-Glu as a product. One other peptide fragment, fraction 8 (Ala-Phe-NH_2), possessed low but significant chemotactic activity.

DISCUSSION

Endopeptidase-24.11 has been shown to inactivate peptide neurotransmitters and hormones both *in vitro* and *in vivo* in brain and peripheral tissues, cleaving them at internal peptide bonds to the amino side of hydrophobic amino acid residues [8]. Our findings show that rat CGRP-α, like human CGRP [13], is a substrate for this enzyme, and that it cleaves it at eight recognition sites, most of which are typical of the specificity of this enzyme. The peptide is

Table 1. Activity of undigested CGRP peptide and unfractionated products of endopeptidase-24.11 digestion

Treatment (10 nM)	Chemotactic index ± SEM	P
CGRP-α	1.00 ± 0.06	< 0.01
CGRP-α, chemotaxis control*	0.835 ± 0.6	0.01
CGRP-α, digested†	1.53 ± 0.09	< 0.01
CGRP-α, control incubation‡	0.93 ± 0.04	< 0.1
CGRP-β, digested†	0.38 ± 0.02	0.01
CGRP-β	0.20 ± 0.02	NS
CGRP-β, control incubation‡	0.22 ± 0.02	NS

* Eosinophils were preincubated with 1 μM phosphoramidon prior to the chemotaxis assay to control for the possible activity of products of CGRP-α following hydrolysis by leukocyte endopeptidase-24.11 in the filter.

† Peptides were hydrolysed exhaustively with endopeptidase-24.11 (see Materials and Methods) and the product mixture assayed for chemotactic activity.

‡ As for digestion experiment, except that 1 μM phosphoranidon was included to inhibit hydrolysis of CGRP peptides by endopeptidase-24.11 prior to the chemotaxis assay. Incubation of the peptides with boiled endopeptidase-24.11 gave essentially similar results.

Values shown means ± SEM; significant difference from Gey's balanced salt solution calculated through ANOVA; NS, not significant.

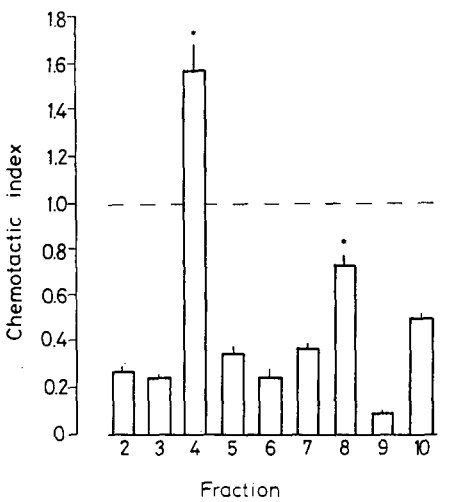

Fig. 3. Eosinophil chemotaxis in response to fractions containing CGRP peptide fragments after hydrolysis. The positive control level is normalized to 1.0 (dashed line). Bars show mean relative migration distances expressed as chemotactic indices ± SEM. Significance of differences from negative controls: *P < 0.001.

cleaved at one atypical site (Glu-Ala) between residues 35 and 36. Anomalous cleavages of human atrial natriuretic peptide by endopeptidase-24.11 (Gly-Ala and Arg-Ser bonds) have also been reported recently [11, 14] suggesting a more complex specificity for the enzyme than recognised previously. Our most important observation is that, as the result of cleavage at this site, a tetrapeptide (Val-Gly-Ser-Glu) with previously established biological activity [6] is cleaved from rat CGRP-α. Moreover, this peptide has both greater biological activity and potency in an eosinophil chemotaxis assay compared with its precursor.

A search of the protein data bases revealed that, among regulatory peptides and growth factors, the only other peptide containing the sequence Val-Gly-Ser-Glu was the platelet-derived growth factor A-chain precursor (residues 53–56) [15]. Our results indicate also that at least one other fragment of rat CGRP-α possesses chemotactic activity, suggesting that the chemotactic response to the intact peptide may depend on multiple active sequences. This hypothesis is strengthened by the observation that an endopeptidase-24.11 hydrolysate of rat CGRP-β, which lacks the eosinophil chemotactic factor of anaphylaxis tetrapeptide sequence, also possessed some biological activity (Table 1). These results provide evidence that endopeptidase-24.11 may play a physiological role in processing neuropeptides to produce new biological activities in addition to its role in peptide inactivation. This hypothesis is supported by the report that C-terminal fragments of substance P possess biological activity towards leukocytes greater than that of the intact peptide, but no direct evidence for conversion of substance P to such fragments by endopeptidase hydrolysis was provided [16]. Furthermore the N-terminal heptapeptide fragment of substance P generated by endopeptidase-24.11 in the nucleus of the solitary tract has been implicated in mediating baroreceptor reflex activity [17]. The processing and activation of the endothelin-1 precursor peptide ("big endothelin") has also been shown recently to be accomplished both *in vitro* and *in vivo* by a phosphoramidon-sensitive metalloendopeptidase [18], but this is probably by an enzyme closely related to but distinct from endopeptidase-24.11.

Previous studies had shown that the chemotactic activity of rat CGRP-α can be increased following

exposure to an endopeptidase activity present in a particulate fraction of guinea-pig lung [7]. Future experiments will be designed to show whether the presence of endopeptidase-24.11 in lung membranes or elsewhere accounts for at least part of this activity and whether phosphoramidon can inhibit its generation. Endopeptidase-24.11 is known to be present in the lung, especially in airway epithelia, where, among other actions, it may play a role in tachykinin and vasoactive intestinal polypeptide metabolism [19, 20]. An additional role in CGRP metabolism must now also be considered. Endopeptidase-24.11, which is also localized on the surface of Schwann cells in the peripheral nervous system [21], may function additionally in terminating the mitogenic actions of CGRP on these cells.

The migration of eosinophils into inflamed tissues is characteristic of asthma and other hyperreactive disorders in which the nervous system may be implicated [22]. Our findings strengthen the hypothesis that sensory neuropeptides, whose peripheral release is elicited by an axon reflex mechanism, may be involved in long-term tissue reactions. Cleavage of α-CGRP peptides by endopeptidase-24.11 or a related endopeptidase in peripheral tissues may simultaneously achieve the destruction of activity of the peptide at smooth muscle and vascular endothelial sites and the potentiation of a local mediator action within the extracellular space. The existence of more than one peptide fragment with chemotactic activity amongst the endopeptidase digestion products is suggestive of a mechanism for the avoidance of rapid desensitization of eosinophils to a neurally mediated chemotactic stimulus.

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