$k_0 = (16 \pm 4)$ nmol Phe/mol tunichrome d^{-1}. Since the amount of tunichrome in the circulating blood cells of *A. ceratodes* has been shown to be roughly 1–3 mM, this rate would appear to be inadequate to account for all the native biosynthesized tunichrome. We note, however, that the radiolabel was observed to be present in several TLC bands in addition to the band identified with pure tunichrome An-1. We do not know if these other bands are either precursors or degradation products of tunichrome. For example, Phe is probably not the only tunichrome precursor, and tunichrome synthesis need not occur exclusively in circulating blood cells. It is therefore probable that the models used in the dynamical analysis are oversimplified and do not account for the presence of two or more competing processes in the mechanism.

These results confirm our preliminary experiments in which the radiolabel from both ^{14}C-Phe and ^{14}C-tyrosine was shown to be incorporated into *A. ceratodes* tunichrome using autoradiographic procedures. Identical TLC procedures were utilized previously but, instead of extracting tunichrome from TLC plates and measuring tunichrome concentrations by HPLC as we have done here, the TLC plates were mated with X-ray film to afford a qualitative detection of radiolabel. In the autoradiography experiments weak radioactivity was observed in the tunichrome TLC band 3 days post-exposure, while more activity was recorded for both the 5-day and 10-day groups of animals. Labeled Phe was incorporated into tunichrome in both seawater-exposed *A. ceratodes* (as in the present experiments) as well as in *A. ceratodes* injected with the label.

The major finding of these ^{14}C-Phe uptake studies is that for *A. ceratodes* L-phenylalanine is a precursor of endogenously synthesized (biosynthesized) tunichrome. The incorporation of ^{14}C-Phe within 24 h of exposure indicates that new tunichrome is rapidly synthesized by one or more types of tunicate blood cells. Free tunichrome has been identified primarily in the morula cells of *A. ceratodes*[9]. It is hypothesized that vanadium-bound tunichrome is present in the signet ring cells of this species. Since these two blood cell types probably could not have developed from the lymphocyte-like precursor cells that are released from the hemopoietic tissues in the short period of time our experiments covered[10], we conclude that ongoing tunichrome synthesis occurs in fully differentiated blood cells.

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Dynorphin-degrading cysteine protease is highly specific for paired arginine residues

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Abstract. The cleavage of dynorphin and three analogs containing paired basic residues by several proteases was investigated. The cysteine protease of neuroblastoma cells cleaved only the bond between Arg-Arg residues. Submandibular arginylendopeptidase, however, cleaved bonds between both Arg-Arg and Arg-Lys residues, and pancreatic trypsin at the carboxyl sides of both arginine and lysine residues. This shows that the cysteine protease is highly specific for paired arginine residues.
Key words. Neuropeptide; dynorphin; paired basic residues; neuropeptidase; arginylendopeptidase.

It has been believed that endopeptidases bound to the cell surface membrane play essential roles in the termination of the biological actions of neuropeptides[1]. For example, endopeptidase-24. 11 (enkephalinase, EC 3.4.24. 11), together with an aminopeptidase, is the principal enzyme in the case of the degradation of enkephalin[2,3].

In the course of studies on the proteases functioning in the degradation of dynorphin, an endogenous Leu-

	1	2	3	4	5	6	7	8	9	10	11	12	13
A. Dyn (1 - 13)	Tyr	Gly	Gly	Phe	Leu	Arg	Arg	Ile	Arg	Pro	Lys	Leu	Lys
B. [^{6}Arg,^{7}Lys] Dyn	Tyr	Gly	Gly	Phe	Leu	Arg	Lys	Ile	Arg	Pro	Lys	Leu	Lys
C. [^{6}Lys,^{7}Arg] Dyn	Tyr	Gly	Gly	Phe	Leu	Lys	Arg	Ile	Arg	Pro	Lys	Leu	Lys
D. [^{6}Lys,^{7}Lys] Dyn	Tyr	Gly	Gly	Phe	Leu	Lys	Lys	Ile	Arg	Pro	Lys	Leu	Lys

Figure 1. Dynorphin (1–13), and analogs in which paired basic residues replace the Arg-Arg residues.

enkephalin-containing opioid peptide[4–6], we found a novel enzyme in neuroblastoma cell membranes catalyzing the cleavage of the Arg6-Arg7 bond in dynorphin[7]. The enzyme is characterized as a cysteine protease on the basis of its inhibitor-susceptibility. Studies using several peptides that contain paired basic residues suggested that the enzyme has strict specificity toward the Arg-Arg doublet.

To obtain definitive evidence for its strict substrate specificity, we investigated whether the cysteine protease can hydrolyze three analogs of dynorphin (1–13) in which only paired basic residues are replaced by residues other than Arg-Arg (fig. 1). The present paper also compares the activity of the cysteine protease towards dynorphin and its analogs with that of other enzymes which recognize arginine residues: mouse submandibular arginine-specific endopeptidase (EC 3.4.21. 99) and bovine pancreatic trypsin (EC 3.4.21. 4).

Materials and methods

Dynorphin (1–6) and dynorphin (1–13) were purchased from Sigma Chemical Co. and the Peptide Institute, Inc., Osaka, respectively. Mouse submandibular arginine-specific endopeptidase, which is called arginylendopeptidase in this paper, and bovine pancreatic trypsin were obtained from Takara Shuzo Co., Ltd., Kyoto, and Sigma, respectively. Three dynorphin analogs (fig. 1) were synthesized by the solid phase method on an Applied Biosystems 430A peptide synthesizer using t-butyloxycarbonylated derivatives of amino acids. Synthesized peptides were leaved from the resin using trifluoromethane sulfonic acid, and purified by reversed-phase HPLC on a column (3.9 × 300 mm) of μBondapak C18 (Waters). The accuracy of the synthesis was confirmed by sequencing the purified peptides on an Applied Biosystems 477A automatic protein sequencer and a 120A PTH analyzer.

The activity of dynorphin-degrading cysteine protease was assayed at 37 °C in 75 mM Tris-HCl, pH 8.0, containing 5 mM $CaCl_2$, using 0.02 mM dynorphin (1–13) as a substrate, on the basis of the appearance of a newly-formed dynorphin fragment (1–6) monitored by reversed-phase HPLC as described previously[7].

The cysteine protease was purified from mouse neuroblastoma N-18 cells, as described previously[7], except that the plasma membrane-rich fraction was used as the starting material for purification of the enzyme and that Mono Q (Pharmacia) ion-exchange chromatography and Superose 12 (Pharmacia) gel filtration using the FPLC system (Pharmacia) were utilized. Briefly, the enzyme was solubilized with 0.2% Brij 35 from the plasma membrane-rich fraction which had been prepared from the neuroblastoma cells according to the method of Jones and Matus[8]. The Brij extract was subjected to *p*-mercuribenzoate-Sepharose chromatography as described previously[7]. The 2-mercaptoethanol-eluted fraction was then applied to a column (0.5 × 5.0 cm) of Mono Q previously equilibrated with 50 mM Tris-HCl, pH 7.5, containing 0.01% Brij 35 and 1 mM 2-mercaptoethanol and the enzyme was eluted with a 0–0.5 M linear gradient of NaCl in the same buffer. Active fractions eluted at about 0.5 M NaCl were concentrated and subjected to gel filtration on a column (1.0 × 30 cm) of Superose 12 previously equilibrated with 50 mM Tris-HCl buffer, pH 7.5, containing 0.01% Brij 35, 1 mM 2-mercaptoethanol and 0.2 M NaCl. Active fractions were subjected to the second Superose 12 gel filtration under the same conditions as described above. The activity generating dynorphin fragment (1–6) was eluted as a single peak superimposable on the protein peak. Active fractions were used as the purified cysteine protease. Since the amount of the purified enzyme was not adequate for the detection of a silver-stained band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, we could not ascertain the purity of the enzyme preparation.

Hydrolyses of dynorphin and its three analogs were monitored by reversed-phase HPLC on a column (3.9 × 300 mm) of μBondapak C18 with a 50-min linear gradient of 1–65% acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 1 ml/min using a Waters 600 HPLC system. The elution profile was monitored at 210 nm. Assignment of the cleavage products separated by HPLC was carried out by amino acid analysis on a Hitachi 835 amino acid analyzer after a 24-h hydrolysis at 110 °C with 6 M HCl containing 1% phenol.

Results and discussion

Mouse neuroblastoma cysteine protease hydrolyzed dynorphin (1–13), but it scarcely hydrolyzed the three dynorphin analogs which contained Arg-Lys, Lys-Arg, or Lys-Lys residues in place of the Arg-Arg residues (fig. 2a). The cleavage site at the Arg-Arg bond is confirmed by amino acid analyses of newly-formed cleavage products (fig. 3a). These results are consistent with the previous observation[7] that the enzyme can also cleave peptide E at the bond between the two Arg residues, whereas all bonds around the Lys-Arg sequence in either peptide E or Met-Lys-bradykinin, around the Arg-Lys sequence in alpha-neoendorphin, or around the Lys-Lys sequence in mastoparan are resistant to the action of the enzyme. Therefore we propose that the enzyme is highly specific for the Arg-Arg doublet.

Several arginine-specific proteases have already been reported; among them, an arginylendopeptidase isolated from mouse submandibular gland is highly specific for arginine residues[9,10], and its highly-purified isozyme D

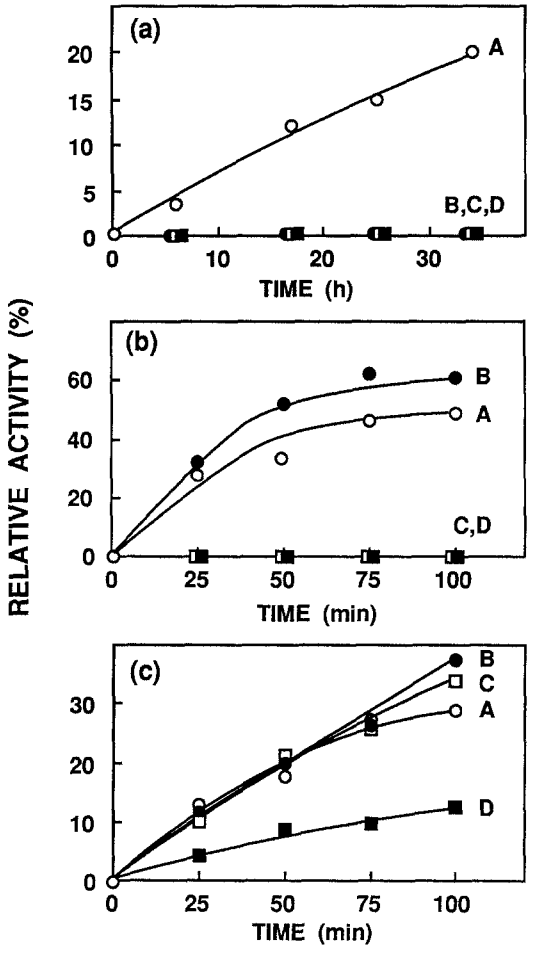

Figure 2. Hydrolyses of dynorphin and its three analogs by dynorphin-degrading cysteine protease (*a*), mouse submandibular arginylendopeptidase (*b*) and bovine pancreatic trypsin (*c*). The activity was assayed with 0.25 μg enzyme at 37 °C in 75 mM Tris-HCl, pH 8.0, containing 5 mM $CaCl_2$ (*a*), with 0.5 μg enzyme at 50 °C in 200 mM Tris-HCl, pH 8.0 (*b*) and with 0.1 μg enzyme at 25 °C in 200 mM Tris-HCl, pH 8.0, containing 10 mM $CaCl_2$ (*c*). The peptide substrate was used at a concentration of 0.02 mM. The cleavage products were analyzed by HPLC. The vertical coordinate indicates the ratio (%) of the sum of the chromatographic areas of newly-formed cleavage products to the area of the original peptide at zero time. The curves of A (o), B (●), C (□) and D (■) represent the hydrolyses of dynorphin (1–13), the analog containing Arg-Lys residues, that containing Lys-Arg residues and that containing Lys-Lys residues, respectively.

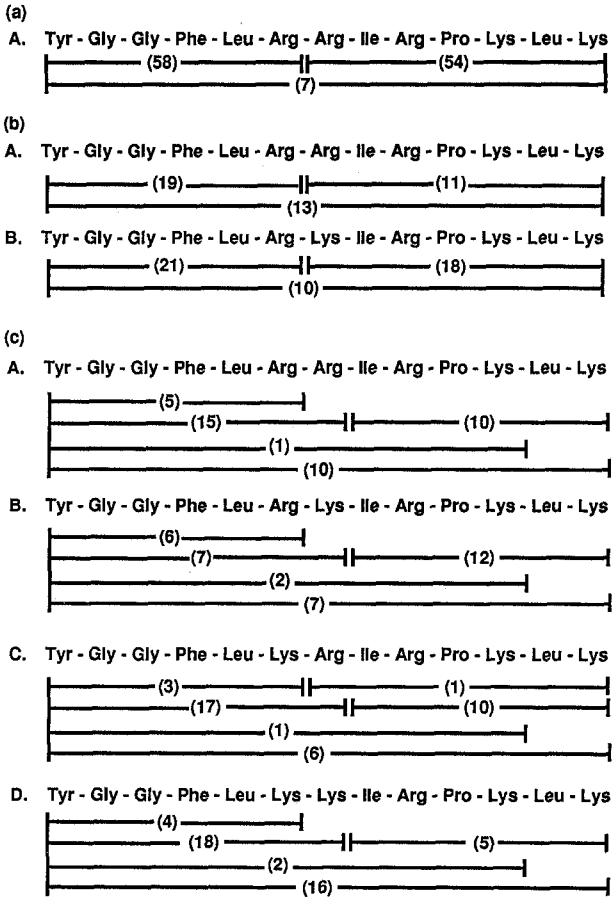

Figure 3. Summary of the cleavage products of dynorphin (1–13) and its three analogs produced by dynorphin-degrading cysteine protease (*a*), mouse submandibular arginylendopeptidase (*b*) and bovine pancreatic trypsin (*c*). Numbers in parentheses represent yields (%) of cleavage products determined on the basis of dynorphin (1–13) or its analogs which had been hydrolyzed.

is commercially available from Takara. The enzyme is characterized as a serine protease which hydrolyzes arginine-containing peptides at the carboxyl side of the arginine residue. We found that the submandibular arginylendopeptidase can hydrolyze both dynorphin (1–13) and the analog containing Arg-Lys residues, at almost similar rates (fig. 2b). The amino acid analyses of cleavage products indicated that the arginylendopeptidase cleaves the peptides between the Arg-Arg and the Arg-Lys residues (fig. 3b). It should be noted that the enzyme cannot cleave dynorphin (1–13) at the carboxyl side of the Arg-Arg doublet, or the analog containing Lys-Arg residues at the carboxyl side of the arginine residue. It seems that a basic amino acid as the P_2 residue in peptide substrates cannot be accommodated on the S_2 subsite of the arginylendopeptidase. As a control, we used bovine pancreatic trypsin and examined its activity toward dynorphin (1–13) and its analogs. The pancreatic trypsin can hydrolyze dynorphin (1–13) and its two arginine-containing analogs to an almost equal extent, and also the analog containing paired lysine residues, although to a lesser extent (fig. 2c). In all cases, trypsin could hydrolyze the peptides at the carboxyl sides of the arginine and the lysine residues (fig. 3c).

In conclusion, we found that the dynorphin-degrading cysteine protease isolated from the neuroblastoma cell membranes is highly specific for paired arginine residues, compared with an endopeptidase that is highly specific for arginine residue. To our knowledge, this is the first report describing an enzyme showing high specificity for paired arginine residues. The protease specific for paired arginine residues has been isolated as the principal enzyme functioning in the initial stage of the degradation of dynorphin at the cell surface membrane[7]. The enzyme may also play a role in the metabolism of other physio-

logically important peptides at the cell membrane surface. Alternatively, the enzyme may function as a processing enzyme, acting on a putative precursor containing the Arg-Arg doublet.

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Purification of platelet-derived endothelial cell growth inhibitor and its characterization as transforming growth factor-β type 1

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Abstract. In 1986, Brown and Clemmons (Proc. natl Acad. Sci. USA *83* (1986) 3321) showed that platelets contain a substance, platelet-derived growth inhibitor (PDGI), that inhibits in vitro endothelial cell replication. Although platelets are rich in transforming growth factor β (TGF-β), PDGI was considered not to be related to TGF-β, on the basis of its reported properties (extraction from platelets at neutral pH, binding to heparin-Sepharose). However, we purified PDGI to near homogeneity and showed that on the basis of HPLC retention behavior, in vitro growth inhibitory activities with several cell types, receptor binding, and immunoneutralization of growth inhibitory activity with specific anti-TGF-β type 1 antibodies, PDGI is most probably identical with TGF-β type 1.
Key words. Platelet-derived growth inhibitor; transforming growth factor-β type 1; bovine aortic endothelial cells.

The growth of cells in culture and in vivo is modulated by various effectors, some of which are growth factors and others growth inhibitors. The equilibrium between stimulatory and inhibitory signals is essential for normal control of cell proliferation and cell differentiation. The significance of growth factors in these processes is well established, but the role of growth inhibitors is less clear. The regulation of vascular endothelial cell proliferation by growth factors and inhibitors is likely to be physiologically important in new capillary blood vessel formation. This process, often termed angiogenesis or neovascularization, is a complex and tightly regulated sequence of events involving migration, proliferation and maturation of endothelial cells[1]. Endothelial cell proliferation and angiogenesis are virtually absent in healthy adult tissue, but occur in controlled manner during tissue growth (embryogenesis, menstrual cycle, placenta formation) and repair (wound healing). Furthermore, unregulated neovascularization is a hallmark of pathological states (tumor growth, chronic inflammations, retinopathies, etc.)[2,3].

Many factors are known which stimulate endothelial cell proliferation and/or angiogenesis. They include the fibroblast growth factors basic and acidic FGF[4,5], platelet-derived endothelial cell growth factor[6], and vascular permeability factor/endothelial cell growth factor VPF/VEGF[7,8]. These mitogens also stimulate angiogenesis, but there are additional angiogenic factors that stimulate neovascularization without inducing endothelial cell proliferation[9]. Likewise, a number of molecules with potent growth inhibitory activity for endothelial cells have been characterized, i.e. TGF-β[10-12], tumor necrosis factor-α[13-15], interleukin-1[16,17], interferons[15,18,19] and heparin[20,21]. In addition, there are numerous reports describing less well-characterized endothelial cell and angiogenesis inhibitors[22-34]. In view of the potential importance of negative regulatory factors in the biological control of neovascularization, it would be of considerable interest to determine the chemical nature of novel endothelial cell growth or angiogenesis inhibitors. Brown and Clemmons[22] described an apparently novel endothelial cell growth inhibitory factor from platelets, which they named platelet-derived growth inhibitor (PDGI). The reported properties (extraction at neutral pH, binding to heparin) suggested that this factor was not related to the major endothelial cell growth inhibitor