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EVIDENCE FOR DISTINCT DIBASIC PROCESSING ENDOPEPTIDASES WITH LYS-ARG AND ARG-ARG SPECIFICITIES IN NEUROHYPOPHYSIAL SECRETORY GRANULES

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SUMMARY: Two Ca^{2+}-dependent endopeptidases endowed with specificities for paired basic residues have been disclosed in rat and ox neurohypophysial secretory granules. Specificities investigated by using synthetic fluorogenic substrates showed the presence of a Lys-Arg endopeptidase with optimum pH close to the granule pH (5.5) and of an Arg-Arg endopeptidase more active at pH 7.0. Granule extracts have virtually no activity towards Lys-Lys-containing susbtrate or monobasic substrates. Pro-Gly-Lys-Arg-chloromethylketone appears a very efficient inhibitor for the Lys-Arg enzyme. Soluble and membrane-bound forms of both endopeptidases have been detected. pH-dependence of membrane binding and partitioning into Triton X-114 suggest that the membrane-bound form of Lys-Arg endopeptidase is associated through an amphiphilic α-helix. It is proposed that the enzyme Lys-Arg cleaves prooxytocin and provasopressin at their signal sequence Gly-Lys-Arg when these precursors arrive in the neurosecretory granules. The processing proceeds in the granules through carboxypeptidase E and α-amidating enzyme complex for giving mature pharmacologically active nonapeptide hormones.

Recent research on the prohormone processing along the intracellular secretory pathway has revealed the occurrence of endopeptidases cleaving on the carboxyl side of a pair of basic residues and located either in trans-Golgi apparatus or in secretory vesicles. In yeast, the endopeptidase Kex2 has been shown to be a calcium-dependent serine protease with a subtilisin-like catalytic domain displaying a cleavage specificity on both Lys-Arg and Arg-Arg containing substrates (1). This enzyme has a pH optimum of 7.0 - 7.5 with little activity at pH 5.0 and would be mainly located in the Golgi complex as a transmembrane enzyme (2). In purified insulinoma granules, two distinct Ca^{2+}-dependent acid endoproteases have been detected and separated by ion-exchange chromatography ; one (type I) cleaves exclusively on the C-terminal side of Arg-Arg in proinsulin, the other (type II) splits preferentially after the pair Lys-Arg but

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also after the pair Arg-Arg (3). Because type I has a pH optimum of 5.5 and negligible activity at pH 7.5 whereas type II has at pH 7.5 an activity about 30-40% of that at pH 5.5, it has been assumed that type I is active only in secretory granules (internal pH about 5.5) whereas type II could already act in the Golgi apparatus (internal pH about 7.0) "en route" for the secretory granule compartment (3,4).

Two mammalian gene products, termed PC2 and PC3, have been shown to display a structural similarity to the yeast endopeptidase Kex2, in particular to possess the subtilisin-like catalytic domain (5-9). From co-expression of proopiomelanocortin, PC2 and PC3 in cultured cells and identification of the processing products, it has been deduced that PC2 and PC3 have dibasic specificities (7). Two glycoproteins displaying sequence similarities with PC2 and PC3 have been detected in adrenal chromaffin granules and regarded as N-terminal processed forms of PC2 and PC3 (10). In contrast to Kex2, PC2 and PC3 lack a hydrophobic transmembrane sequence but have a potential C-terminal amphipathic helical segment known as putative membrane anchor (6).

We have investigated neurohypophysial secretory granules from rat and ox posterior pituitary glands in order to identify the dibasic endopeptidases involved in the processing of prooxytocin and provasopressin and their possible membrane association.

MATERIALS AND METHODS

Purification of neurohypophysial granules : Posterior pituitary glands from rats (average wet weight : 1.9 mg) or from oxen (average wet weight : 320 mg) are homogenized in a potter Thomas A with 10 mM Tris-HCl buffer pH 7.4 (0.05 ml or 1.7 ml per gland for rat or ox, respectively) containing 0.27 M sucrose, 1 mM EDTA, 2.5 mM PMSF, 10 μM E64, 10 μM pepstatin, 50 μM TPCK and 50 μM TLCK. A first centrifugation is carried out at 600 g for 10 min and the supernatant is saved. The pellet is homogenized with fresh buffer and after centrifugation the supernatant is added to the previous one.
The combined supernatant is layered on 0.6 M sucrose and centrifugation is performed at 10.000 g for 1 h at 4°C. Four fractions can be distinguished : 0.27 M sucrose, an interface, 0.6 M sucrose and a pellet containing the most part of secretory granules. The yield in granules at this stage, determined by the neurophysin granule content compared with the tissue content, reaches approximately 50%.

The granular fraction, suspended in 5 mM acetate pH 5.5 containing the inhibitors indicated above, is subjected to five freezings and thawings in order to lyse the vesicles. Membranes, separated by centrifugation at 100.000 g for 30 min, are taken up in the same buffer and centrifugation is repeated under the same conditions. The two supernatants are pooled and referred to as granule content. The membranes are suspended in the acetate buffer and both materials are kept at -18°C.

Enzymatic assays : Activities are measured on a series of synthetic fluorogenic substrates containing 7-amino-4-methylcoumarin (AMC). The following dibasic substrates have been used : Z-Ala-Lys-Arg-AMC (Novabiochem), Z-Arg-Arg-AMC (Bachem), N-t-Boc-Gln-Arg-Arg-AMC (Peninsula), N-t-Boc-Glu-Lys-Lys-AMC (Bachem). The following monobasic substrates have been employed : Z-Phe-Arg-AMC (Sigma), N-t-Boc-Val-Pro-Arg-AMC (Novabiochem). Aminopeptidase activities have been assayed on Arg-AMC and Leu-AMC. A model 3 Jobin and Yvon fluorometer is used for measuring AMC release. The excitating wave is 383 nm and the emitting light is 465 nm. Measures are made in triplicate in 50 mM acetate buffer pH 5.0 containing the above inhibitors, at 37°C. The reaction is stopped with 1 mM $CuSO_4$ in the same buffer. Assays are made with and without addition of 2 mM $CaCl_2$ and values without Ca^{2+} are substracted. Hydrolysis is linear during 3 h. Endopeptidase activities are expressed in pmol AMC released per hour and per µl. Carboxypeptidase B-like activity is measured on hippuryl-L-arginine (Mann Res) in 50 mM acetate pH 5.0 containing 1 mM $CoCl_2$. Released arginine is determined after 1, 2 and 3 h using the fluorescamine procedure (11).

Enzyme inhibitors : Covalent inhibitors for serine endopeptidases, namely DFP, PMSF (Merck), TLCK (Calbiochem), TPCK (Sigma) have been tested. A specific inhibitor for dibasic endopeptidase Pro-Gly-Lys-Arg-CK, kindly given by Dr E. Shaw, has been employed. Covalent inhibitors for cysteine endopeptidases, namely iodoacetate (Fluka) and trans-epoxy-succinyl-leucylamido(4-guanido)butane (E-64, Sigma) have been examined. Inhibitors of metalloendopeptidases, EDTA (Prolabo), EGTA (Sigma), 1-10 phenanthroline (Fluka) have been used.
Peptide inhibitors of serine proteases, namely trypsin inhibitors such as aprotinin (Choay), ovomucoide, Kunitz soja bean inhibitor, or subtilisin inhibitors such as chick pea subtilisin inhibitor and barley subtilisin inhibitor, and peptide inhibitors for aspartyl proteases such as pepstatin (Sigma) have been investigated. Leupeptin (Sigma) and antipain (Sigma) have also been used.

pH-dependence of membrane binding and partitioning into Triton X-114.
The partitioning of membrane-bound dibasic endopeptidases into Triton X-114 has been investigated in order to detect a possible membrane association through an amphiphilic α-helix (12,13). A pre-condensation of Triton X-114 is carried out according to Bordier (12) : Triton X-114 (20 g) is added to 980 ml of 10 mM tris-HCl pH 7.4 150 mM NaCl and the mixture is stirred for 2 h at 0°C. The clear solution is incubated at 35°C overnight and a separation of a large aqueous phase and a smaller phase enriched in detergent occurs. The aqueous phase is discarded and replaced by the same volume of 10 mM Tris-HCl pH 7.4 150 mM NaCl. The mixture gives a single phase at 0°C and condensation is repeated twice at 35°C under the same conditions. The Triton concentration of the third lower phase is 11.4 % as determined at 275 nm.
162 µl of membranes or granule content are mixed with 20 µl of 100 mM Tris-acetate pH 5.0 or 7.5, 150 mM NaCl and 18 µl of 11.4% Triton X-114. The mixture is stirred for 1 h at 0°C. 90 µl of this solution is layered on 200 µl of 10 mM Tris-acetate pH 5.0 or 7.5 containing 150 mM NaCl, 6% sucrose, 0.06% Triton X-114 in an Eppendorf microfuge tube. After incubation 3 min at 30°C, the tube is centrifuged for 3 min at 300 g at 30°C (Hettich centrifuge). The upper phase (aqueous) is removed, measured and each phase is completed with either the detergent solution or the buffer-sucrose solution in order to obtain equal volumes and approximately the same salt and surfactant content for both samples (12). Activities on Z-Ala-Lys-Arg-AMC are measured in each phase. Activities on hippuryl-Arg, substrate for granule carboxypeptidase E known to be membrane-associated through an

amphiphilic helix (13), are measured in parallel. For the endopeptidase assay, 50 mM sodium acetate pH 5.0, 1 mM EDTA, 1 % Triton X-100 are used in order to obtain a homogeneous solution at the temperature of the assay (37°C).

RESULTS

Dibasic endopeptidase specificities. The dibasic specificities, particularly for the pair Lys-Arg, is apparent in Table I for both the ox and rat granular endopeptidases. At pH 5.0, the cleavage of the substrate Boc-Gln-Arg-Arg-AMC is about 10% of that of the substrate containing the pair Lys-Arg, and the percent falls to 0.2-2% for the substrate Z-Arg-Arg-AMC. There is virtually no activity on the dibasic substrate Boc-Glu-Lys-Lys-AMC. Monobasic endopeptidase activity, arginine aminopeptidase activity and leucine aminopeptidase activity appear negligible. Content and membrane of the neurohypophysial granules display the same enzymatic profile (Table I).

TABLE I

RELATIVE ACTIVITIES OF GRANULE ENZYMES ON SYNTHETIC SUBSTRATES, IN REGARD TO THE ACTIVITY ON Z-ALA-LYS-ARG-AMC

Substrate	Bovine granules (content)	Rat granules (membrane)
Z-Ala-Lys-Arg-AMC	100	100
Boc-Gln-Arg-Arg-AMC	9.3	2.5-15
Z-Arg-Arg-AMC	0.2	2.0
Boc-Glu-Lys-Lys-AMC	1.0	1.9
Boc-Val-Pro-Arg-AMC	2.8	0.3
Z-Phe-Arg-AMC	0.8	1.15
Arg-AMC	0	0.9
Leu-AMC	1.5	< 0.1

Activities are measured in 50 mM sodium acetate pH 5.0 containing 1 mM EDTA, 1 h at 37°C. Measures are made on samples with or without addition of 2 mM $CaCl_2$ and the latter values are subtracted.

The ratios of activities on Lys-Arg and Arg-Arg containing substrates are about 40 at pH 5.0 but 2 at pH 7.0 in buffers complemented with 2 mM $CaCl_2$, suggesting the occurrence in the granules of two endopeptidases with distinct specificities.
On the other hand the activity of the Lys-Arg enzyme is much higher at pH 5.0 than at pH 7.0 whereas the reverse is seen for the Arg-Arg enzyme (Table II).

Inhibition of dibasic endopeptidases. Table III summarizes the results obtained for covalent inhibitors, metals and peptide inhibitors of trypsin and subtilisin. The residual activities are measured on Z-Ala-Lys-Arg-AMC. In the case of covalent inhibitors, the endopeptidase activity is inhibited by DFP indicating serine proteinases. The inhibition by TLCK is moderate at the concentration of 20 mM whereas it is virtually complete by Pro-Gly-Lys-Arg-CK at the concentration 0.1 mM.
When the content of bovine granules is incubated for 1 h with Pro-Gly-Lys-Arg-CK at the concentration of 10, 25 and 50 μM in 0.05 M

TABLE II

COMPARATIVE ACTIVITIES AT pH 5.0 AND pH 7.0 OF DIBASIC ENDOPEPTIDASES FROM RAT NEUROHYPOPHYSIAL GRANULES

MEDIUM		SUBSTRATE	GRANULE	
pH	$CaCl_2$ (mM)		Content	Membrane
5.0	0	Z-AKR-AMC	0.95 ± 0.14	0.17 ± 0.03
5.0	0	b-QRR-AMC	0.06 ± 0.02	0.02 ± 0.05
5.0	2	Z-AKR-AMC	3.9 ± 0.2	0.48 ± 0.07
5.0	2	b-QRR-AMC	0.10 ± 0.02	0.0
5.0	10	Z-AKR-AMC	2.8 ± 0.1	0.36 ± 0.07
5.0	10	b-QRR-AMC	0.14 ± 0.00	0.03 ± 0.03
7.0	0	Z-AKR-AMC	0.34 ± 0.04	0.13 ± 0.03
7.0	0	b-QRR-AMC	0.34 ± 0.04	0.08 ± 0.01
7.0	2	Z-AKR-AMC	0.73 ± 0.03	1.15 ± 0.03
7.0	2	b-QRR-AMC	0.37 ± 0.02	0.08 ± 0.01
7.0	10	Z-AKR-AMC	0.78 ± 0.07	0.13 ± 0.03
7.0	10	b-QRR-AMC	0.32 ± 0.02	0.17 ± 0.08

Activities are measured in pmol/h/µl. Measures are made at 37°C for 3h in 50 mM sodium acetate pH 5.0 or 50 mM Tris-HCl pH 7.0 containing 1 mM EDTA. One-letter symbols are used for amino acids.

TABLE III

SUSCEPTIBILITY TO VARIOUS INHIBITORS

OF GRANULE ENDOPEPTIDASE ACTIVITY ON Z-ALA-LYS-ARG-AMC

INHIBITOR	Concentration (mM)	Residual activity (%) Rat	Ox
CONTROL		100	100
I - Covalent Inhibitors			
DFP	1.0	160	97
	10.0	36	93
	50.0	0	0
NPGB	0.5		35
	1.0	115	
TLCK	0.1	69	
	1.0	77	
Pro-Gly-Lys-Arg-CK	0.001	95	
	0.01	14	
	0.1	2.5	
Iodoacetate	1.0	68	
II - Metal Chelators			
EGTA	1.0	25	
1-10-phenanthroline	2.0	128	
III - Metals			
$HgCl_2$	1.0	0	
$CuSO_4$	1.0	17	
$ZnSO_4$	1.0	29	
$MgSO_4$	1.0	48	
$CoCl_2$	1.0	22	
IV - Peptides			
Ovomucoid	0.005	84	106
	0.050	116	
Aprotinin	0.016	115	
Kunitz soja trypsin inhibitor	0.005	86	
Chick pea subtilisin inhibitor	0.015	109	80
Barley subtilisin inhibitor	0.025		100
Leupeptin	0.1		48
	0.2	83	
	1.0		7.6
	2.0	11	
Antipain	1.0	3.5	3.6

Measures are made in 50 mM sodium acetate pH 5.0 containing 1 mM EDTA, 2 mM $CaCl_2$. Inhibitors are incubated with granule content for 1 h at room temperature prior addition of 0.5 mM substrate.

acetate pH 5.0 then activities measured on Z-Ala-Lys-Arg-AMC and Boc-Gln-Arg-Arg-AMC, residual activities are 25, 11 and 9% for the first substrate but 44, 22 and 13% for the second, suggesting again the presence of two endopeptidases, the Lys-Arg enzyme being more specifically inhibited. The sequence Gly-Lys-Arg is the cleavage signal for processing endopeptidases acting on prooxytocin and provasopressin.

Lys-Arg endopeptidase activity is greatly decreased by metals such as Cu^{2+}, Hg^{2+}, Zn^{2+} and Co^{2+} at the concentration of 1 mM. Peptide trypsin inhibitors are inactive as well as subtilisin inhibitors (Table II). Benzamidine, a competitive inhibitor of classical trypsin-like enzymes, is poorly active at the concentration of 10 mM. In contrast the tripeptide Gly-Lys-Arg displays an inhibitory action on the Lys-Arg endopeptidase activity (data not shown).

Soluble and membrane-bound forms of the endopeptidases. Table I shows the activities on the one hand with the content of bovine granules, on the other with the membranes of rat granules. The general enzymatic profiles are virtually identical, suggesting that the same enzymes are involved. A pH-dependent association of endopeptidases with the membrane through a C-terminal amphiphilic α-helix, such as the one found for granule carboxypeptidase E (6), could explain the similarity between the two forms (Table IV). Another explanation could be that soluble forms are enzymatic domains of membrane-bound enzymes detached by proteolysis.

DISCUSSION

Results obtained with rat and ox neurohypophysial granules suggest that dibasic endopeptidases involved in the prooxytocin and the provasopressin processing are located in the granules. In these precursors, oxytocin and vasopressin are linked to their specific neurophysins, VLDV-neurophysin and MSEL-neurophysin respectively (14,15), by a sequence Gly-Lys-Arg. This sequence is present in the best substrate Z-Ala-Lys-Arg-AMC and the efficient inhibitor Pro-Gly-Lys-Arg-CK. Furthermore the optimum pH for Z-Ala-Lys-Arg-AMC is around 5.0 and compatible with the pH 5.5 found within the granules (16). Comparative measures at pH 5.0 and pH 7 made on Z-Ala-Lys-Arg-AMC and Boc-Gln-Arg-Arg-AMC suggest the occurrence of two endopeptidases, one more specific for the first substrate with an optimum pH near 5.0, the other more specific for the second substrate and more active at pH 7.0.

TABLE IV

PARTITIONING OF GRANULAR LYS-ARG ENDOPEPTIDASE INTO AQUEOUS AND TRITON X-114 PHASES AT pH 5.0 AND 7.5

	ACTIVITIES (%)		
	Dibasic Endopeptidase	Carboxy-peptidase E	Serum albumin*
MEMBRANE			
pH 5.0			
Control	100	100	100
Aqueous phase	38	29	94
Triton phase	61	64	6
pH 7.5			
Control	100	100	100
Aqueous phase	78	69	97
Triton phase	8	29	4
CONTENT			
pH 5.0			
Control	100	100	
Aqueous phase	55	54	
Triton phase	36	43	
pH 7.5			
Control	100	100	
Aqueous phase	84	80	
Triton phase	9	18	

* Serum albumin is used for comparison as a soluble hydrophilic protein.

These features can be brought together to those of the two dibasic Ca^{2+}-dependent endopeptidases studied by Hutton and his co-workers in the human insulinoma granules (3,4). The specificities of these endopeptidases have been determined using ^{125}I-labelled human proinsulin as substrate. The type I cleaves exclusively on the C-terminal side of Arg31-Arg32 at pH 5.5 with negligible activity at pH 7.0. The type II splits preferentially on the C-terminal side of Lys64-Arg65 and its activity at pH 7.5 is still about 30-40% of that at pH 5.5. Although the results in the case of neurohypophysial

granule endopeptidases on synthetic substrates are in agreement with those for insulinoma granule endopeptidases on proinsulin with regard to the presence of two distinct enzymes specific for Lys-Arg and Arg-Arg, respectively, the Lys-Arg enzyme of neurohypophysis is much less active at pH 7.0 than at pH 5.0, whereas the corresponding enzyme of insulinoma has still at pH 7.5 30-40% of its activity at pH 5.5. This neurohypophysial endopeptidase would not be active in the Golgi apparatus (pH close to neutrality (17)) through which it presumably passes "en route" for the secretory granule compartment. In contrast the Arg-Arg enzyme should already be able to split in the Golgi apparatus. However despite most mammalian provasopressins possess a pair Arg-Arg in their MSEL-neurophysins (positions 93-94 in bovine protein), no cleavage occurs at this level. There is a processing split between neurophysin and copeptin at a single arginine two residues down-stream (18).

On the other hand the specificities of neurohypophysial endopeptidases can be compared with those of endopeptidases PC2 and PC3 expressed in cultured cells. PC2 and PC3 expressed in processing-deficient BSC-40 cells cleave mouse proopiomelanocortin at the Lys-Arg pairs but the efficiency depends on the location in the sequence (6). Furthermore the pairs Lys-Lys and Arg-Lys appear virtually not cleaved in a series of mutant mouse proopio-melanocortins containing all possible pairs of basic residues at certain sites (6). In this respect, neurohypophysial dibasic endo-peptidases are not active on the Lys-Lys-containing synthetic substrate (Table I).

Dibasic endopeptidase activities have been found in both the content and the membrane of the neurohypophysial granules (Table I). Furthermore the partition of membrane-bound Lys-Arg endopeptidase activity during phase separation in solutions of Triton X-114 at pH 5.0 and 7.5 (Table IV) is consistent with a binding through an amphiphilic α-helix (12,13). The behaviour is similar to that of carboxypeptidase E (13). This conclusion is in agreement with the expected anchorage of PC2 an PC3 deduced from C-terminal amino acid sequences (6,13). C-terminal amphipathic helical segment (6) could explain the co-presence of soluble and membrane-bound forms in the secretory granule compartment (13).

Neurohypophysial secretory granules are also endowed with a carboxypeptidase B-like enzyme (carboxypeptidase E) similar to the one found in several endocrine tissues (13,19), and an α-amidating enzymatic system (20-22). Conversion of amphibian hydrin 2 (vaso-

tocinyl-Gly) into mature amidated vasotocin (22) and conversion of vasopressinyl-Gly-Lys-Arg into active vasopressin (unpublished data) have been carried out using neurohypophysial secretory granules purified as described above. The four enzymes involved in the processing cascade have optimum pHs compatible with the granule pH so that the entire processing could develop in the granule compartment of most neuroendocrine cell types.

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