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Processing Endopeptidase Deficiency in Neurohypophysial Secretory Granules of the Diabetes Insipidus (Brattleboro) Rat

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The homozygote Brattleboro rat exhibits a hereditary diabetes insipidus due to a deficiency of vasopressin, the antidiuretic hormone. It has previously been shown that in this animal a single nucleotide deletion in the provasopressin gene leads to a mutant precursor with a C-terminal amino acid sequence different from that of the wild-type. However the N-terminal region including the hormone moiety, the processing signal as well as the first two-thirds of the neurophysin is entirely preserved and absence of maturation has to be explained by an additional cause. We show here that the neurohypophysis of the homozygote Brattleboro rat, in contrast to the adenohypophysis, displays a significant decrease in the Lys–Arg processing endopeptidase activity when compared to the heterozygote or the wild-type Wistar. It is suggested that hypothalamic vasopressinergic neurons of the homozygote Brattleboro rat display a deficiency in the processing enzyme in contrast to the oxytocinergic neurons in which processing of prooxytocin is normal.

KEY WORDS: Diabetes insipidus (Brattleboro) rat; dibasic processing endopeptidases; neurosecretory granules; neurohypophysial hormones; provasopressin processing.

INTRODUCTION

The Battleboro rat is a mutant form of the Long Evans strain displaying a hereditary diabetes insipidus for the homozygote phenotype (di/di) (1). The pathology is due to a lack of vasopressin production and the subsequent deficiency of water resorption in the distal kidney tubuli (2). Normal rat vasopressin precursor is a 147-residue protein comprising three domains: the hormone (residues 1 to 9) connected by a processing signal sequence Gly-Lys-Arg to MSEL-neurophysin (residues 13 to 105) itself followed by a second processing signal, an arginine and finally a glycopeptide, copeptin (residues 107 to 147) (ref. 3–5). Comparison of the normal rat provasopressin gene (6) with the Brattleboro gene (7) has revealed in the latter a single base deletion leading to a reading frame shift. As a result the amino acid sequence of the precursor should change

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from residue 76 up to the C-terminal. The N-terminal sequence including the hormonal moiety, the processing signal Gly–Lys–Arg and the first 2/3 of neurophysin is entirely preserved so that normal processing should occur provided that the precursor be actually expressed and able to follow the compartmental secretory pathway for meeting processing enzymes. Possible anomalies about these enzymes have therefore been searched for and a deficiency in Lys–Arg endoproteolytic activity has been detected in the homozygote Brattleboro rat.

For explaining the lack of mature vasopressin several hypotheses have been proposed. Deficiency in the translation has first been suspected (8). However occurrence of a mutant provasopressin displaying immunoreactivity with an antibody raised against a synthetic 14-residue peptide derived from the predicted mutated region of the precursor has been shown (9, 10). Deficiency in the routage, the precursor remaining blocked in the membrane of the rough endoplasmic reticulum, has also been suggested (11). Possible variation in the conformation of the "pathological" precursor preventing the access of the processing endopeptidase has been discussed (12). Paradoxically mature vasopressin, identified by its immunological, physical and biological properties compared with those of synthetic vasopressin, has been found in the adrenal gland (13) and the ovary (14) of the homozygote Brattleboro rat. Futhermore, a few "solitary" hypothalmic neurons of this animal display all the immuno-reactivities of the wild-type vasopressin precursor (vasopressin, neurophysin, copeptin) (14). Somatic intrachromosomal gene conversion between the homologous exons of the vasopressin and the related oxytocin genes generating the heterozygote di/+ phenotype has been proposed for explaining occurrence of the normal precursor in the solitary neurons (15).

Another explanation for the provasopressin processing deficiency could be a default in the processing enzymes themselves either in their biosynthesis or in their action. Because neither vasopressinyl-Gly–Lys–Arg, nor vasopressinyl-Gly have been detected in the homozygote Brattleboro rat, the defect should affect the processing endopeptidase rather than carboxypeptidase E or the α-amidating enzymes involved in the maturation. Calcium-dependent "dibasic" endopeptidases have recently been studied in the secretory granules of neuroendocrine cells of human (17), ox, and rat (18). One type seems more specific for the pair Lys–Arg and the other type for the pair Arg–Arg; these enzymes are apparently devoid of monobasic endoproteolytic activity and have a pH optimum around 5.5 (17, 18).

We have examined comparatively the neurohypophysial secretory granules of the heterozygote and homozygote Brattleboro rats for their Lys–Arg endoproteolytic activities using a fluorogenic synthetic substrate containing the dibasic sequence.

MATERIALS AND METHODS

Isolation of Neurosecretory Granules

Heterozygote and homozygote Brattleboro rats of both sexes were kindly provided by Dr. L. Bankir (Hôpital Necker, Paris) and Dr. A. Burlet (Lab.

d'Histologie, Nancy). These animals were 5–9 months old and their average weight was 240-370 g. Homozygotes urinated about 120 ml per day whereas heterozygotes gave one tenth of this volume. Parallel experiments were performed with normal Wistar rats (IFFA-Credo, L'Arbresle) in order to characterize the dibasic endopeptidases of the wild-type phenotype.

Rats were killed by decapitation and anterior and posterior pituitary lobes were dissected out. The wet weight of the neurointermediate pituitary varied between 1.2 and 2.1 mg and the wet weight of the anterior pituitary between 7.5 and 2.1 mg and the wet weight of the anterior pituitary between 7.5 and 10 mg. Rat neurohypophysial granules were isolated as described previously (18–21). Tissues were homogenized in a potter Thomas AA in a 10 mM Tris-HCl buffer pH 7.4 (50–100 μl/gland) containing 0.27 M sucrose and inhibitors: 1 mM EDTA, 2.5 mM PMSF, 10 μM E64, 10 μM pepstatin, 50 μM TPCK, 50 μM TLCK, in order to block cytosol proteolytic enzymes. After a first centrifugation at 600 g (10 min, 4°C, Sorvall centrifuge RC2B, rotor SS34), the supernatant was saved and the pellet was homogenized with fresh buffer and centrifuged under the same conditions. The combined supernatant (about 1 ml of 0.27 M sucrose) was layered on 1 ml of 0.6 M sucrose in a 3-ml tube. Centrifugation was carried out at 10.000 g for 1 h. Four phases could be distinguished: an upper layer (0.27 M sucrose), an interface, a lower layer and a pellet containing the most part of secretory granules. The granule yield at this stage, determined by comparing the neurophysin content of the recovered granules with the total amount, was roughly estimated about 50%.

The pellet of secretory granules was suspended in 5 mM acetate buffer pH 5.5 (50 μl/gland) containing the inhibitors described above except EDTA and lysed by five freezing-thawing cycles. A first centrifugation (10.000 g, 30 min, 4°C) gave a supernatant and a pellet that was retreated under the same conditions. The combined supernatant is referred to as the granule content and the pellet suspended in the buffer (100 μl/gland) as the granule membranes.

HPLC Investigation of Granule Components

15 μl from a total volume 500 μl (9 neurointermediate pituitaries in each case) were injected (Fig. 1). A Delta-pack Waters 300 A column (3 × 150 mm, particle size 5 μ) was employed and absorbance was monitored at 214 nm. An acetonitrile gradient 9–60% containing 0.05% trifluoroacetic acid was applied during 60 min. Retention times are indicated in min. Oxytocin-associated neurophysin and vasopressin-associated neurophysin are termed VLDV- and MSEL-neurophysins, respectively, using the one-letter symbols for amino acids in positions 2, 3, 6 and 7 (24). VLDV- and MSEL-neurophysins were identified by comparison with pure rat neurophysins (4).

Assay of Lys–Arg Endopeptidase Activity

The endoproteolytic activity on Z-Ala–Lys–Arg-7 amido-4-methyl-coumarin (AMC) (Novabiochem) was determined at final concentration 0.5 mM in 50 mM acetate buffer pH 5·0, with and without addition of 2 mM $CaCl_2$, as previously

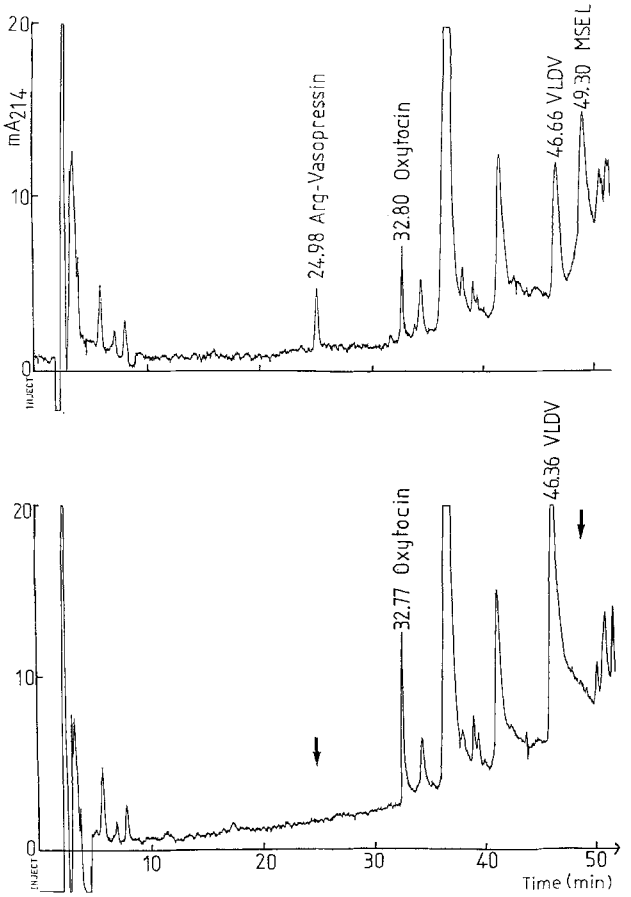

Fig. 1. Comparative HPLC of neurosecretory granule contents of heterozygote (top) and homozygote (bottom) Brattleboro rats (Experiment II, Table 2). The arrows show the positions of missing vasopressin and MSEL-neurophysin, respectively, in the homozygote Brattleboro rat.

described (18, 18a). The reaction is linear during at least 4 h. Values without addition of $CaCl_2$ were subtracted and fluorescence converted in AMC concentration using a standard curve. One provisional unit of enzymatic activity releases one pmol AMC per hour and per μl of content or membrane. Activities are calculated per mg of wet gland (18).

RESULTS

Components of Neurosecretory Granules of Homozygote and Heterozygote Brattleboro Rats

Components of granule contents, particularly neuro-hypophysial hormones and their associated neurophysins, were investigated by high-pressure liquid

chromatography as shown in Fig. 1. When secretory granules of posterior pituitary glands from heterozygote and homozygote Brattleboro rats are compared by HPLC, it is clear that in the homozygote vasopressin and its associated neurophysin, MSEL-neurophysin, are missing (Fig. 1). In contrast oxytocin and its associated neurophysin, VLDV-neurophysin, are present. It appears that prooxytocin is expressed and processed normally in the homozygote Brattleboro rat whereas provasopressin is not. An unprocessed "pathological" provasopressin has not been detected in this HPLC experiment but all the peaks were not systematically investigated.

Lys–Arg Endopeptidase Activity in the Anterior and Posterior Pituitary of the Wistar Rat

Secretory granules of neuroendocrine cells are known to possess two types of processing endopeptidases, one with a major specificity for the Lys–Arg sequence, the other displaying a preference for the Arg-Arg sequence (17, 18). In rat neurohypophysial secretory granules, Lys–Arg endoproteolytic activity has been shown to be largely predominant (18). Lys–Arg endopeptidase activities were measured on the fluorogenic substrate Z-Ala–Lys–Arg-AMC (Novabiochem) as previously described. Table 1 gives the dibasic proteolytic activities measured on posterior and anterior pituitary lobes of Wistar rats. Four batches have been used. Whereas there are variations from batch to batch, it appears that the neurointermediate lobe is much richer in dibasic endopeptidase than the anterior lobe when activities are calculated per mg/gland. Of the various cell types found in the anterior pituitary gland, only corticotrope cells are known to use dibasic endopeptidases for processing proopiomelanocortin (22).

Table 1. Ca^{2+}-dependent dibasic proteolytic activities in the posterior and anterior pituitaries of the Wistar Rat (expressed in U/mg gland)

	Membrane	Content	Total
Posterior			
1 (10[a]	—	3.2 ± 0.3	
2 (10)	6.0 ± 0.4	3.9 ± 0.6	9.6 ± 1.0
3 (12)	4.9 ± 0.2	5.2 ± 0.1	10.1 ± 0.3
4 (9)	2.7 ± 0.6	2.7 ± 0.3	5.4 ± 0.9
Anterior			
1 (10)	0.26 ± 0.04	0.08 ± 0.06	0.34 ± 0.11
2 (10)	0.17 ± 0.06	0.31 ± 0.12	0.48 ± 0.18
3 (12)	0.40 ± 0.01	0.48 ± 0.03	0.88 ± 0.04

Numbers of animals used for the four batches are indicated in parentheses.
Activities are measured in triplicate on Z–Ala–Lys–Arg-AMC (0.5 mM) in a 50 mM sodium acetate buffer pH 5.0 containing 1 mM EDTA with and without addition of 2 mM $CaCl_2$, the latter values being subtracted.
[a] Membrane lost.

Table 2. Comparison of dibasic endopeptidase activities in heterozygote and homozygote Brattleboro rat pituitaries (expressed in U/mg gland)

	Homozygote I[a] (3♂)	Heterozygote I[a] (3♂)	Homozygote II (9 = 5♀ + 4♂)	Heterozygote II (9♀)	Homozygote III (6 = 3♀ + 3♂)
Posterior					
Membrane	—	—	3.2 ± 0.5	5.5 ± 0.8	1.0 ± 0.1
Content	—	—	0.4 ± 0.2	1.6 ± 0.9	0.5 ± 0.1
Total	1.8 ± 0.7	3.0 ± 0.6	3.6 ± 0.7	7.1 ± 1.7	1.5 ± 0.2
Anterior					
Membrane	—	—	0.93 ± 0.25	0.25 ± 0.12	0.26 ± 0.04
Content	—	—	0.08 ± 0.05	0.05 ± 0.06	0.12 ± 0.03
Total	0.46 ± 0.03	0.66 ± 0.16	1.01 ± 0.30	0.30 ± 0.18	0.38 ± 0.07

Numbers of rats used in each experiment are indicated in parentheses.
In experiments I and II, heterozygotes and homozygotes were from the same breeding.
[a] In this experiment, membrane and content were not separated. Measures made after 16 h incubation.

Lys–Arg Endopeptidase Activity in Homozygote and Heterozygote Brattleboro Rats

Table 2 shows the comparison between posterior pituitary glands of homozygote and heterozygote Brattleboro rats. The amounts of activities found in the posterior and anterior pituitaries of the heterozygote Brattleboro rats are in the range of those found for the Wistar rats (6–10 U/mg gland for the posterior lobe and 0.3–0.9 U/mg gland for the anterior lobe). A significant decrease of dibasic endoproteolytic activity can be noted in the case of posterior lobes of homozygote Brattleboro rats when compared with values found for the heterozygote Brattleboro rats or Wistar rats (1.5–3·6 U/mg gland instead of 6–10 U/mg gland. This difference is not observed in the anterior lobe (0.4–1 U/mg gland instead of 0.3–0.8 U/mg gland).

It is clear that in the homozygote Brattleboro rat there is a significant decrease of the Lys–Arg endoproteolytic activity, routhly about 50% (Tables 1 and 2). This decrease could not be explained by a reduced biosynthesis in both vasopressinergic and oxytocinergic neurons since the processing of prooxytocin that involves the Lys–Arg sequence is normal. On the other hand the Lys–Arg endoproteolytic activity is not modified in the cells of the anterior pituitary gland of the homozygote Brattleboro rat. A more acceptable explanation would be that hypothalamic vasopressinergic neurons either lack the Lys–Arg endopeptidase or the amount is reduced at a level insufficient for processing the precursor. The defect should be at the cell-specific gene expression of the enzyme rather than in the gene itself. This expression would be normal in the solitary neurons as well as in the ovary and adrenal cells. The occurrence of a peculiar endopeptidase inhibitor or the lack of an essential cofactor could also explain but less plausibly the decrease of Lys–Arg endoproteolytic activity.

Mammalian furin (22) and amphibian furin (23) cleave proproteins following the consensus sequence Arg-X-(Lys/Arg)-Arg. Furin would be localized in the Golgi apparatus (22) and involved within the constitutive secretory pathway (23). In contrast the simple "dibasic" endo-peptidases are found in neuroendocrine

cells submitted to regulated secretion and mainly localized in secretory granules whose pH corresponds to their pH optimum (17, 18). It is possible that these latter enzymes exhibit more subtle substrate specificities that are not apparent from mere examination of the cleavage site. It remains to clarify the respective implications of the processing endo-peptidase deficiency and the vasopressin precursor defect in the lack of vasopressin in homozygote Brattleboro rat.

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