Formation of metabolites of [Arg^8]vasopressin (AVP) by brain peptidases

Conversion of the intermediate [Cyt^6]AVP-(3–9)

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[Cyt^6]AVP-(3–9), an intermediate in the metabolism of AVP-(1–9) in vitro, was used to investigate the mechanism of formation of centrally active AVP metabolites. Exposure of [Cyt^6]AVP-(3–9) to rat brain membranes resulted in formation of [Cyt^6]AVP-(4–9), -(5–9), [pGlu^4,Cyt^6]AVP-(4–9) and AVP-(3–5), which were isolated and chemically identified. Products derived from cleavage of the C-terminus of the substrate were absent. Time-course experiments further indicated that the conversion process is predominantly mediby an aminopeptidase-like mechanism. The conversion of [Cyt^6]AVP-(3–9) by membranes from hippocampus, amygdala and septum was quantitatively and qualitatively similar. The results point to a major role of aminopeptidase activity in the metabolic conversion of AVP and the formation of centrally active AVP metabolites.

Vasopressin *Neuropeptide* *Peptidase* *Peptide metabolism*

1. INTRODUCTION

The proteolytic conversion of the neuropeptide [Arg^8]vasopressin [AVP-(1-9); AVP] by brain synaptic membranes in vitro, like that of oxytocin and vasotocin, follows an aminopeptidase-like pathway [1-3]. C-terminal fragments, including [Cyt^6]AVP-(2-9), (3-9), -(5-9) and [pGlu^4,Cyt^6] AVP-(4-9), have been identified as metabolities [1,3,4]. This conversion is of biological relevance since C-terminal fragments have central activities and are far more potent than AVP in their behavioral activities [4,5]. The presence of such AVP metabolites in the brain and the existence of binding sites for [pGlu^4,^{35}S-Cyt^6]AVP-(4-9), which are different from [^{3}H]AVP binding sites, have been demonstrated [6,7]. These findings suggest that C-terminal AVP metabolites are a class of endogenous neuropeptides with distinct biological properties.

The cyclization of the Gln^4 residue to form [pGlu^4,Cyt^6]AVP-(4-9) is a significant event in the pathway of AVP conversion [1,4]. This modification protects the peptide against further aminopeptidase conversion. Since the potency of behavioral activities of AVP metabolites resides in [pGlu^4, Cyt^6]-AVP-(4-9), [Cyt^6]AVP-(5-9) and their des-Gly-NH$_2$ derivatives, but not in [Cyt^6]AVP-(3-9) [5], the enzymatic formation of the potent peptides and Gln cyclization were investigated in more detail by using [Cyt^6]AVP-(3-9) as substrate. This paper reports the formation and identification of peptides derived from [Cyt^6]AVP-(3-9) by in vitro processing by brain membranes.

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Abbreviation: AVP, [Arg^8]vasopressin (H-Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Leu-Glu-NH$_2$)

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2. MATERIALS AND METHODS

2.1. *Peptides*

The AVP fragments [Cyt^6]AVP-(3–9), -(5–9), -(5–8), [pGlu^4,Cyt^6]AVP-(4–9) and -(4–8) were synthesized by Drs J.W. Van Nispen and H.M. Greven, Organon International BV, Oss, The Netherlands [8,9].

2.2. *Incubations*

[Cyt^6]AVP-(3–9) was incubated at 50 μM with brain membranes in 40 mM sodium phosphate buffer, pH 7.0, at 37°C for various periods of time. Different protein concentrations were used as indicated in the legends to the figures. Incubations were stopped by addition of HCl to 0.5 M (final concentration) and boiling for 10 min. Samples were centrifuged to remove membranes and subjected to HPLC by direct injection (sample volume $\leqq$ 1 ml) or freeze-drying and reconstitution in 50 mM HOAc.

2.3. *High-pressure liquid chromatography*

AVP fragments were separated by paired-ion reverse-phase HPLC on a μBondapak C18 column (Waters Associates, Millford, USA). The aqueous solvent (A) was 0.2% (v/v) heptanesulfonic acid (PIC-B7 Reagent, Waters Associates); the organic solvent (B) was 0.1% (v/v) heptanesulfonic acid in methanol/H_2O (50:50). The concave gradient (curve 7 on Waters' model 660 programmer) ran from 0 to 70% B in 30 min at a flow rate of 2.0 ml/min. UV detection at 210 nm was used.

2.4. *Amino acid analyses*

The amino acid composition of samples was determined by HPLC of *o*-phthaldialdehyde derivatives of amino acids according to [10]. Peptides were oxidized by performic acid treatment and hydrolyzed in 6 M HCl at 110°C for 18 h [1,10].

3. RESULTS

Development of a reverse-phase HPLC system based on ion pairing of peptides with heptane sulfonic acid enabled one to resolve various hydrophilic AVP fragments, which had little or no retention in an ammonium acetate/methanol HPLC solvent system [1,2]. Employing the paired-ion HPLC system several products of [Cyt^6]AVP-(3–9), which were generated during in-

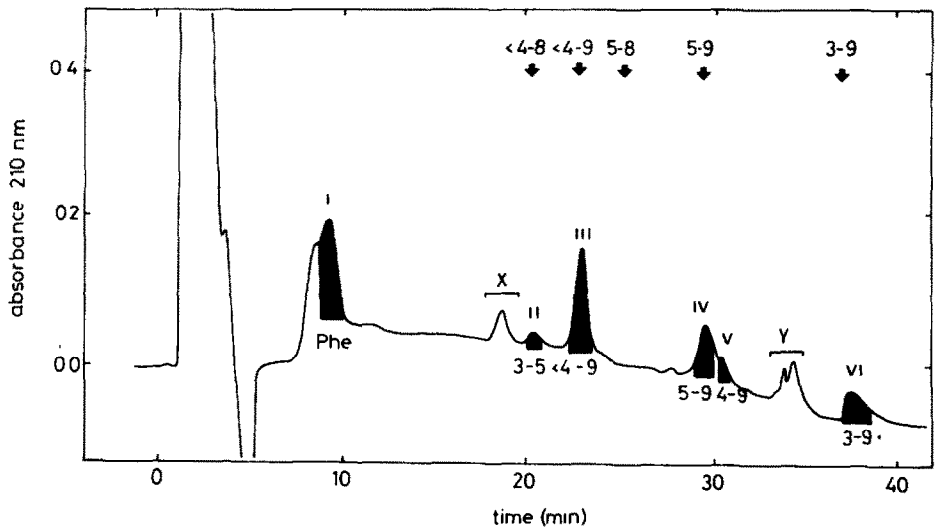

Fig.1. Products of [Cyt^6]AVP-(3–9) separated by paired-iron reverse-phase HPLC. [Cyt^6]AVP-(3–9) (5×10^{-5} M) was incubated with brain membranes (2.5 mg protein/ml) in 5 ml of 40 mM sodium phosphate buffer, pH 7.0, at 37°C for 4 h. The total sample was subjected to HPLC as described in section 2. The fractions coded I–VI (in black) were purified in a second HPLC step and analyzed (see table 1). The identity of products is given below the fractions. Component X is derived from the membrane preparation; components Y are contaminants of the mobile phase. The elution position of synthetic reference peptides is marked by arrows shown at the top.

Table 1

Amino acid composition[a] of peptides purified from digests of [Cyt^6]AVP-(3-9)

Amino acid[b]	I[c]	II	III	IV	V	VI
Cys-acid	–	–	2.12	1.96	2.08	2.06
Asp	–	0.98	1.03	0.94	1.03	0.99
Ser	–	–	–	–	–	–
Glu	–	1.01	1.00	0.04	0.96	0.98
His	–	–	–	–	–	–
Arg	–	–	1.05	1.00	1.00	1.00
Gly	–	–	0.83	0.91	0.96	0.87
Thr	–	–	–	–	–	–
Ala	–	–	–	–	–	–
Tyr	–	–	–	–	–	–
Met	–	–	–	–	–	–
Val	–	–	–	–	–	–
Phe	1.00	1.00	0.02	0.03	–	0.85
Ile	–	–	–	–	–	–
Leu	–	–	–	–	–	–
Lys	–	–	–	–	–	–
AVP peptide	Phe	(3-5)	[pGlu^4,Cyt^6]-(4-9)	[Cyt^6]-(5-9)	[Cyt^6]-(4-9)	[Cyt^6]-(3-9)

[a] Molar ratios of residues are given

[b] Pro is not detected by the amino acid analysis system; Trp is destroyed upon performic acid oxydation and hydrolysis; –, below detection

[c] The sample numbers correspond to the fractions indicated in fig.1

cubation with brain membranes, were detected (fig.1). Each product was purified by rechromatography in the same HPLC solvent system with a slightly modified gradient and chemically analyzed. Based on amino acid composition and N-terminal residues [pGlu^4,Cyt^6]AVP-(4-9), [Cyt^6]AVP-(4-9), -(5-9), H-Phe-Gln-Asp-OH [AVP-(3-5)] and free Phe were identified as products (table 1).

In time-course experiments the peptide [Cyt^6]AVP-(4-9) appeared initially as the predominant product with lower amounts of the cyclized form [pGlu^4,Cyt^6]-AVP-(4-9) (fig.2). After prolonged incubation [pGlu^4, Cyt^6]AVP was the main metabolite (fig.1). The amounts of [Cyt^6]AVP-(5-9) always remained low, while the amount of H-Phe-Glu-Asn-OH was below detection in the time-course experiment.

Comparison of the conversion of [Cyt^6]AVP-(3-9) by membranes (1 mg protein/ml) from the hippocampus, amygdala and septum showed no significant difference in the disappearance rate of the peptide; after 60 min of incubation the disappearance of [Cyt^6]AVP-(3-9) was 71% for hippocampus, 74% for amygdala and 70% for septum. No differences were observed in the quality of products nor in the time course of product accumulation.

4. DISCUSSION

In this paper the formation of AVP metabolites was investigated using [Cyt^6]AVP-(3-9), an intermediate of AVP conversion, as substrate. The results show that the NH_2-terminal Phe is rapidly released from this peptide to generate the 4-9 sequence. This observation provides evidence for the interpretation of previous results that consecutive aminopeptidase-like cleavages occur during conversion of AVP-(1-9) [1]. The presence of [Cyt^6]-

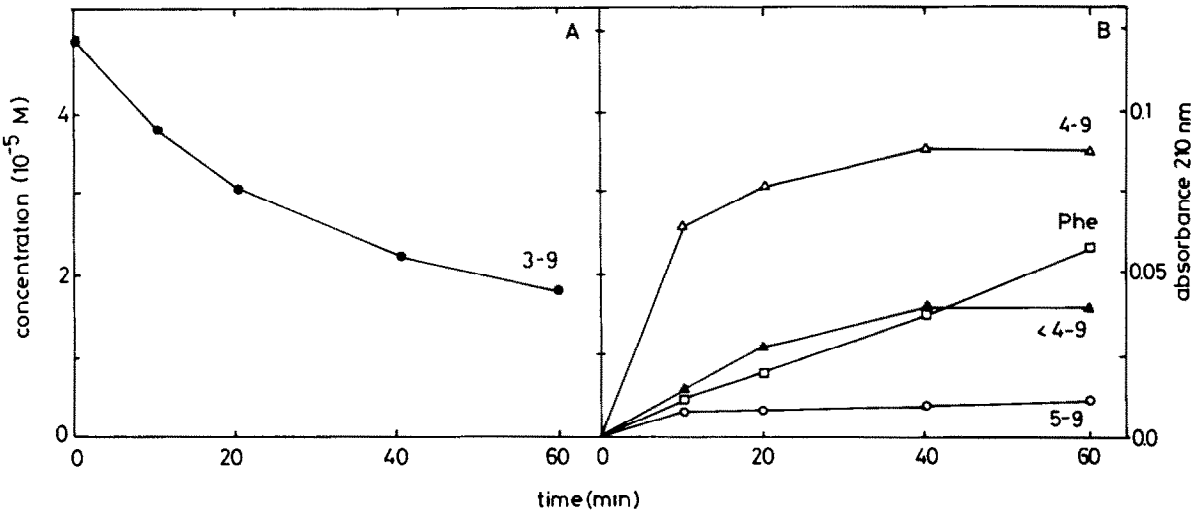

Fig.2. Time-course of conversion of [Cyt^6]AVP-(3-9) and formation of products. [Cyt^6]AVP-(3-9) (5×10^{-5} M) was incubated with 1 mg protein/ml of brain membranes as in section 2. The composition of samples obtained at various time intervals was analyzed by HPLC.

AVP-(5-9) in digests indicates that the aminopeptidase activity proceeds beyond residue 4.

Two forms of the 4-9 sequence were identified: [Cyt^6]AVP-(4-9) and the N-terminal cyclized form [pGlu^4,Cyt^6]AVP-(4-9). The ratio of 4-9 peptides with free and cyclized N-terminus differed between individual experiments indicating that pGlu formation is a spontaneous process not catalyzed by enzymes. In particular under acidic conditions a free N-terminal Gln is known to cyclize readily into the pGlu form [9]. The time-dependent accumulation of [pGlu^4,Cyt^6]AVP-(4-9) observed here and in previous experiments [4] indicates that cyclization occurs during incubation rather than during termination of enzyme reaction or preparation of samples for HPLC. In vitro pGlu^4 formation is an important structural modification which protects [pGlu^4,Cyt^6]AVP-(4-9) from further aminopeptidase degradation. The finding that [Cyt^6]AVP-(5-9) and -(5-8) are active in behavioral test situations and differ only in potency from [pGlu^4,Cyt^6]-AVP-(4-9) and -(4-8) [7] indicates that the pGlu^4 is not prerequisite for biological activity. The presence of this residue in endogenous forms of AVP metabolites [5] remains to be investigated.

The identification of the peptide H-Phe-Gln-Asn-OH points to cleavage of the Asn^5-Cys^6 bond in [Cyt^6]AVP-(3-9), probably by an endopeptidase. The complementary peptide [Cyt^6]AVP-(6-9) has not been identified in these experiments, however. The cleavage is of minor importance since the tripeptide appeared only in very small quantities. This cleavage has not been recognized before in experiments using AVP-(1-9) as substrate. It may be possible that this cleavage only occurs in linearized AVP metabolites like [Cyt^6]AVP-(2-9), -(3-9) or smaller, but not in AVP-(1-9) which has a rigid ring structure.

The absence of des-Gly-NH_2 fragments in the present study is noteworthy. [pGlu^4,Cyt^6]AVP-(4-8) and [Cyt^6]AVP-(5-8) have significant behavioral activities [4,5] and Gly-NH_2 is released during conversion of AVP-(1-9) at a rate which is 10% of the aminopeptidase mediated release of Tyr^2 [1,3]. Apparently, the activity of the C-terminal cleaving enzyme on [Cyt^6]AVP-(3-9) remains far behind the aminopeptidase activity and does not play a significant role in vitro in modifying the C-terminal AVP metabolites. In contrast to membranes of rat and chicken brain, high C-terminal cleaving activity is present in membranes of frog brain [2,12].

Different behavioral effects can be elicited by microinjection of AVP-(1-9) in limbic brain areas like hippocampus, amygdala and septum [11]. These differences may be due to differences in formation of behaviorally active fragments. However, the quantitative and qualitative similari-

ty of conversion of [Cyt^6]AVP-(3-9) by membranes from these areas indicates that such differences cannot be detected in vitro using isolated fractions or that other factors than metabolism cause the differences in behavioral response.

In conclusion, the formation of active AVP metabolites involves [Cyt^6]-AVP-(3-9) as an intermediate, and is based on the action of an aminopeptidase activity. The aminopeptidase action may be blocked by spontaneous pGlu formation.

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