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MEASUREMENT AND DISTRIBUTION OF VASOPRESSIN-CONVERTING AMINOPEPTIDASE ACTIVITY IN RAT BRAIN

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The aminopeptidase activity in the brain which converts vasopressin into centrally active metabolites, was quantitated on basis of the release of ^{3}H-Phe from the substate [^{3}H-Phe^3]vasopressin and separation by hydrophobic interaction chromatography on mini-columns. After subcellular fractionation of whole rat brain homogenates the highest specific activity of the peptidase was recovered in membrane fractions, in particular microsomes and the P_3 fraction, and the cytosol. The peptidase activity was present in all brain areas. Highest activity was measured in membranes of the bulbus olfactorius, preoptical area and cerebellum. Lowest activity was found in the medulla oblongata and striatum. The peptidase activity is not restricted to the vasopressin system per se, but may have a more general role in neuropeptide metabolism.

Aminopeptidase activity is responsible for the conversion of VP into C-terminal fragments with central activity (1-4). These metabolites are formed by stepwise cleavage of VP in vitro upon incubation with brain membranes (1). Endogenous forms of similar peptides and binding sites for one of the VP fragments, [pGlu^4,Cyt^6]VP-(4-9), have been found in the brain (5,6). The potency and selectivity of the VP fragments with respect to their central actions suggest that VP metabolites are a class of neuropeptides in itself. These findings prompt to investigate the properties and significance of the VP-converting aminopeptidase in the regulation of central VP metabolism. Using a rapid method for quantitation, the subcellular and regional distribution of the peptidase activity in the brain is reported here.

MATERIALS AND METHODS

Peptides: Synthetic VP and fragments were gifts of Drs J.W. Van Nispen and H.M. Greven (Organon International BV, Oss, The Netherlands). [^{3}H-Phe^3]VP (^{3}H-VP; 50 Ci/mmol) was purchased from New England Nuclear (Boston, MT, USA). ^{3}H-Phe (50 Ci/mmol) was from The Radiochemical Centre Amersham (UK).

Peptidase assay: ^{3}H-VP (0.01 µCi, 2.5 nM) was incubated with subcellular fractions at different protein concentrations in 100 µl 40 mM Tris-HCl, 62 mM NaCl, pH 7.4, at 37°C. Before addition of the substrate, samples were

Abbreviations: VP, vasopressin; HPLC, high-pressure liquid chromatography.

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preincubated for 10 min. Incubations were performed for various lengths of time and terminated by addition of 100 µl 2 M acetic acid and boiling for 5 min. Separation of ^{3}H-Phe and intact ^{3}H-VP was achieved by a two-step elution from polystyrene beads (500 mg Amberlite XAD-2, BDH, Poole, UK) packed in 1 ml pipettips. Columns were equilibrated in 5% (v/v) ethanol in 100 mM Tris- base. Samples (200 µl) were applied and left on the columns for 30 min. The first elution step was 3 ml 5% ethanol in 100 mM Tris-base; the second was 3 ml 100% ethanol. The two fractions were separately collected and radioactivity was determined by scintillation counting. In each series of samples, the yields of ^{3}H-Phe and ^{3}H-VP were determined by elution of known amounts of these substances. The yield of ^{3}H-Phe was 75% in the first elution step; < 2% of ^{3}H-VP eluted in this fraction. The second step yielded 20% ^{3}H-Phe and 95% ^{3}H-VP. HPLC analyses of the 5% and 100% ethanol fractions from ^{3}H-VP digests of brain membranes showed similar yields.

Subcellular fractionation: Subcellular fractions were prepared from forebrains of male Wistar rats (160-200 g) as described previously (8). The P_3 was the sediment of the 17000 xg for 60 min step (9). Synaptic plasma membranes (SPM) were prepared according to (10). All pellets were washed in 155 mM NaCL. Low molecular weight components were removed from the soluble fraction by elution over a Sephadex G-10 column.

Regional distribution: Brain regions were obtained by dissection (11) and a 10% (w/v) homogenate was prepared in 20 mM Tris-HCl, pH 7.4. Brain membranes were obtained by centrifugation of the S_2 fraction (8) at 100,000 xg for 60 min. Pellets were washed in 155 mM NaCl.

High pressure liquid chromatography: HPLC was performed as described previously (1).

RESULTS

Incubation of [^{3}H-Phe^3]VP at low concentration (2.5 nM) with brain membranes resulted in the release of free ^{3}H-Phe without appreciable accumulation of ^{3}H-labeled peptide intermediates, as indicated by HPLC analyses (fig 1A). Chromatography on Amberlite XAD-2 polystyrene beads packed in mini-columns provided a rapid means to determine the released amounts of ^{3}H-Phe in digests. The enzyme activity showed a linear relationship with protein concentration and incubation time, provided that less than 50% of ^{3}H-VP was degraded (Fig 2).

VP-converting aminopeptidase activity was detected in all subcellular fractions of rat brain tissue (Fig 3). Highest activity was found in the microsomal and P_3 fractions. The nuclei and myeline fractions contained the lowest activity. Considerable activity was also detected in the soluble fraction. Analysis by HPLC confirmed the data from the assay and showed that in all membrane preparations, except mitochondria, ^{3}H-Phe was the only detectable product. In mitochondrial fractions small quantities (< 5% of initial ^{3}H-VP) of ^{3}H-labeled products co-eluting with [Cyt^6]VP-(2-9) and (3-9), were detected (not shown). In the soluble fraction the main product, co-eluted with VP-(1-7) (Fig 1B). Despite the formation of this product, the amounts of ^{3}H-Phe were accurately determined by the assay. The VP-converting aminopeptidase activity was determined in membrane preparations of various brain areas and pituitary lobes. A 2-fold difference was found between areas

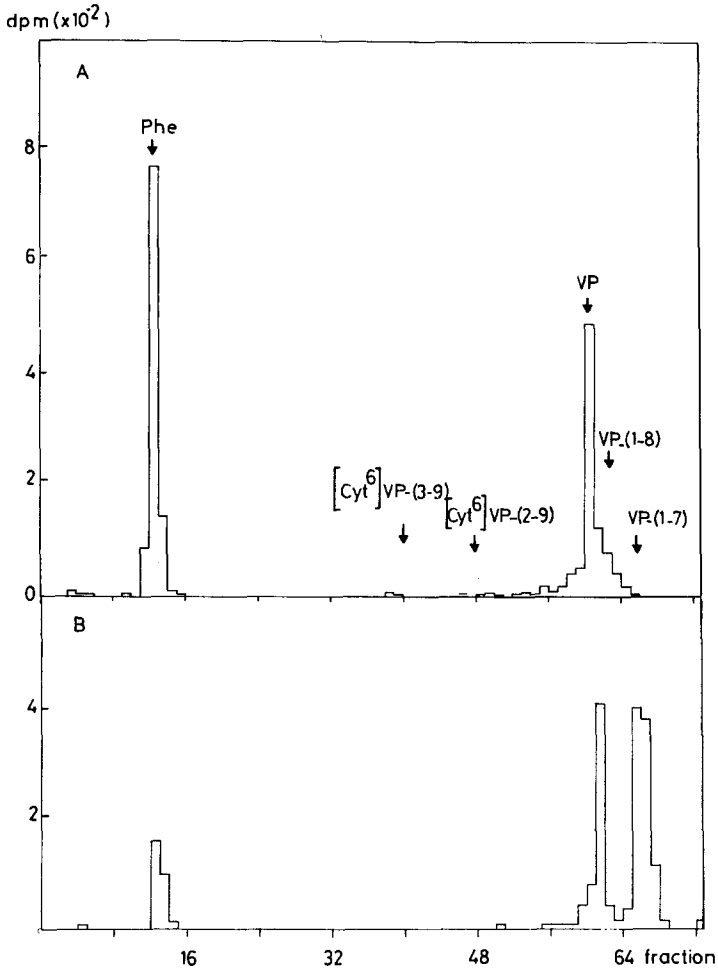

Figure 1. HPLC analysis of ^{3}H-labeled products formed from [^{3}H-Phe^3]VP by incubation with brain membranes (A) and cytosol (B). Incubations were performed at a protein concentration of 0.75 mg/ml of membranes and 0.25 mg/ml of soluble fraction for 40 min. Chromatography was performed on a μBondapak C_{18} column (Waters Ass.) using a concave gradient from 0% to 40% solvent B at a flow rate of 2 ml/min. Solvent A was 10 mM ammonium acetate, pH 4.15; solvent B was 0.15% (v/v) acetic acid in methanol (1). Fractions of 1 ml were collected for scintillation counting. The arrows indicate the elution position of synthetic VP metabolites.

with highest peptidase activity, bulbus olfactorius, preoptic area and cerebellum, and the region with lowest activity, striatum (Fig 4).

DISCUSSION

Previous data have pointed to a role of C-terminal VP metabolites as neuropeptides of the brain (1-7) and prompted studies on the aminopeptidase activity involved in their formation. Such studies require methods for accurate determination of the enzyme activity. HPLC is suitable for separation of products (1), but is impractical for analysis of large series of samples.

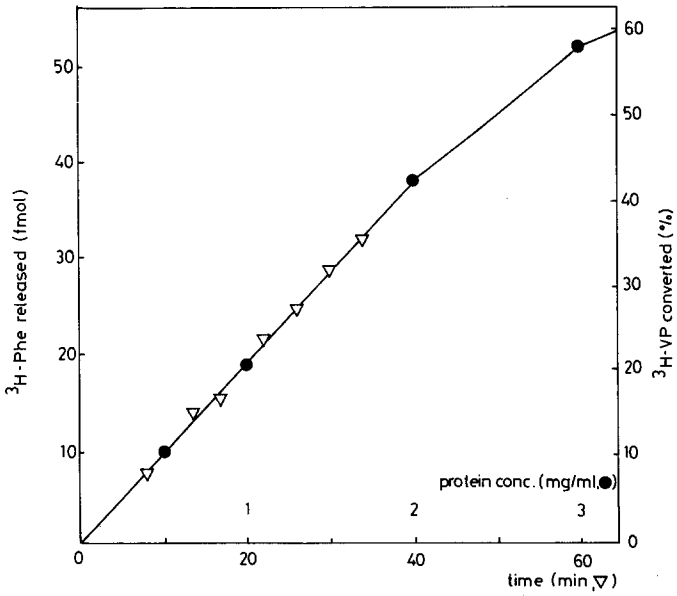

Figure 2. Measurement of VP-converting aminopeptidase activity as a function of membrane protein concentration (0) and incubation time (Δ). The incubation time was 20 min, when the protein concentration was varied. The protein concentration was fixed at 1 mg/ml in the time-course experiment. Enzyme activity is expressed as released amount of ^{3}H-Phe (fmol) as well as percentage of ^{3}H-VP converted (%).

In this paper a sensitive and rapid method for measurement of VP-converting activity has been evaluated. The principle of this method is the quantitation of ^{3}H-Phe by separation from [^{3}H-Phe^3] on polystyrene beads. This type of chromatography has been succesfully used before in peptidase assays (12,13).

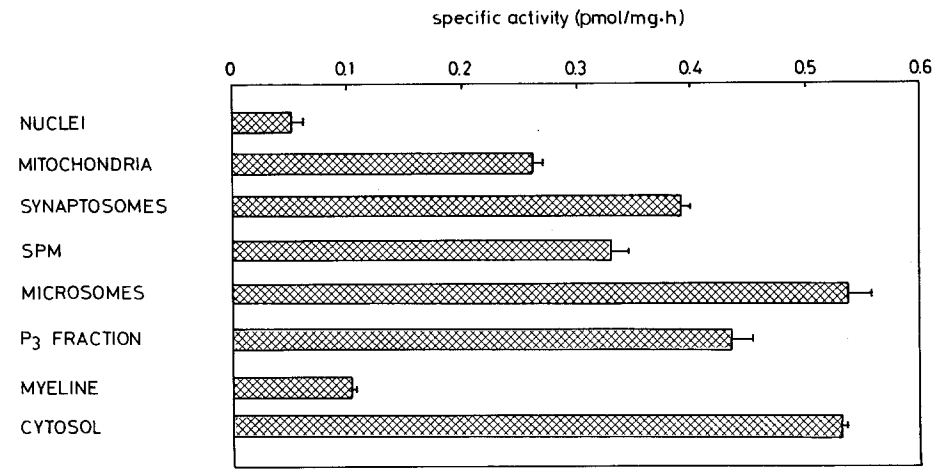

Figure 3. Subcellular distribution of VP-converting aminopeptidase activity in total rat forebrain tissue. Fractions were assayed at a protein concentration of 1 mg/ml; the incubation time was 40 min. Specific activity values are means ± SD of two determinations.

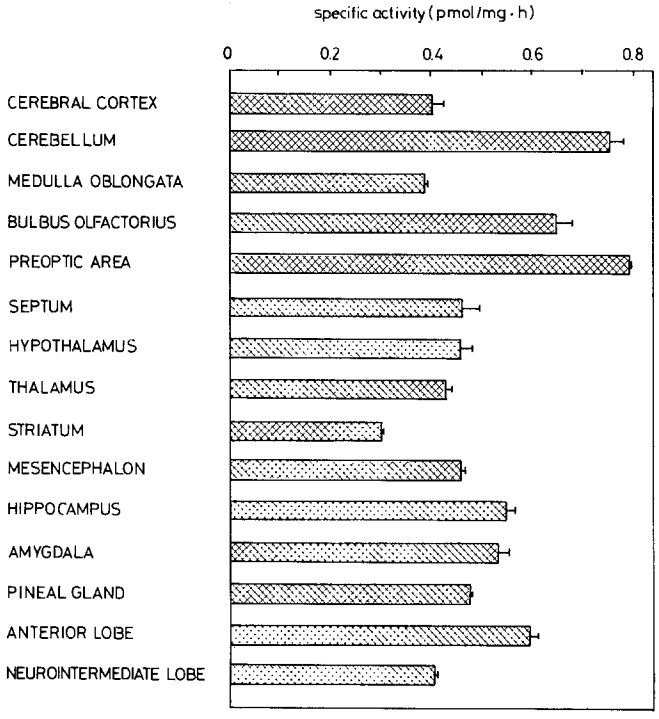

Figure 4. Regional distribution of VP-converting aminopeptidase activity in the rat brain. Measurements were conducted on membrane preparations incubated at a protein concentration of 0.5 mg/ml for 40 min. Specific activity values are means ± SD of two determinations.

The VP-converting aminopeptidase cleaves the Cys^1-Tyr^2 bond of intact VP, thereby opening the ring portion of the peptide. Subsequently, the linear metabolite [Cyt^6]VP-(2-9) is converted by further aminopeptidase action to fragments as small as [Cyt^6]VP-(5-9) (1,3,4). Using [^{3}H-Phe]VP in the nM range as substrate, ^{3}H-Phe appeared as the only significantly accumulating product. Only at higher substrate concentrations (> µM) intermediate ^{3}H-VP fragments accumulate (1). This indicates that under conditions of the assay the first cleavage of VP is the rate limiting step in the proteolytic processing of VP. It is followed by rapid degradation of intermediates, releasing Phe. Preliminary data indicate that various general aminopeptidases are responsible for this rapid degradation, while the initial cleavage involves a separate type of aminopeptidase, which we have tentatively termed here "VP-converting aminopeptidase". This aminopeptidase has been classified as an amastatin-sensitive metallopeptidase with requirement for Zn^{++} (4,14).

VP-converting aminopeptidase activity has originally been found in brain synaptic membranes (1,14). From the present experiments the aminopeptidase activity can also be assigned to other membrane preparations of the brain, in particular microsomes and the so-called P_3 membranes of unknown composition (9). These preparations mainly contain aminopeptidase activity and lit-

tle enzyme activity degrading the C-terminus of VP, as shown by HPLC analysis. In the soluble fraction C-terminal VP-degrading activity predominates (Fig 1B). A similar distribution has been found for oxytocin-degrading enzymes (15). The soluble fraction also contains VP-converting aminopeptidase activity, but it remains to be investigated whether this activity is the same enzyme as the membrane-associated activity. The finding that VP-converting aminopeptidase activity generally occurs in the brain with only a 2-fold difference between areas with highest and lowest activity indicates that the peptidase is a cellular component of brain tissue, rather than an enzyme restricted to the VP system. This suggests that all brain areas can potentially convert VP into active metabolites. In addition, this aminopeptidase may also serve additional proteolytic functions such as the metabolism of other neuropeptides.

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