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Evolutionary specificity of hydrins, new hydroosmotic neuropeptides: occurrence of hydrin 2 (vasotocinyl-Gly) in the toad *Bufo marinus* but not in the viper *Vipera aspis*

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Hydrin 2 (vasotocinyl-Gly), a hydroosmotic peptide resulting from differential processing of provasotocin and recently identified in frog neurohypophysis, has been looked for in the pituitary gland of an exotic toad (*Bufo marinus*) and of a reptile (*Vipera aspis*). Hydrin 2 has been found in the amphibian but not in the reptile. This result confirms the evolutionary specificity of hydrin 2 that has been identified only in frogs and toads but not in birds and reptiles. Occurrence of hydrin 2 is explained by its regulatory function on the water permeability of the skin of anurans.

Hydrin; Neuropeptide; Provasotocin maturation; Differential processing; Neurohypophysial hormone; *Bufo marinus*; *Vipera aspis*

1. INTRODUCTION

Hydrins are neurohypophysial peptides discovered in amphibians and endowed with hydroosmotic activity on the skin and the urinary bladder of these animals [1,2]. They are physiologically produced by partial processing of the vasotocin-neurophysin precursor and have been found so far in anurans but not in birds such as geese [3]. Hydrin 1 (vasotocinyl-Gly-Lys-Arg) has been characterized in the aquatic *Xenopus laevis* (*Pipidae*) whereas hydrin 2 (vasotocinyl-Gly) has been identified in semi-aquatic or terrestrial anuran species such as *Rana esculenta* (*Ranidae*) [1,2]. *Bufo marinus* is an exotic toad imported on the American continent and it is of interest to assess whether hydrin 2 is also present in this animal despite its peculiar origin and habitat. On the other hand, because hydrins have been assumed to result from an adaptative processing proper to amphibians for facing a particular osmoregulation [1,2], we have examined a reptile, the viper (*Vipera aspis*) in order to check their absence in this class of vertebrates.

2. MATERIALS AND METHODS

2.1. Bufo marinus *pituitary extracts*

Bufo marinus pituitaries have been taken out by Dr Dores. They have been placed in cold and peroxide-free acetone immediately after removal from the animals. A dried pituitary gland (1.1 mg) has been dissected into posterior and anterior parts and extraction of each has been made with 0.2 ml 0.1 M HCl (4°C, 4 h with stirring). After centrifugation, the pellet is taken in 0.2 ml 0.1 M HCl, centrifuged, and the second supernatant, filtered on Millex GV4 Millipore, is added to the first. The filter is washed with 0.1 ml 0.1 M HCl and the washing added so that a final volume of 0.45 ml has been recovered and subjected to HPLC.

A Delta Pak C 18 column (3.9 × 140 mm, particle size 5 μm) has been used with a linear acetonitrile gradient (0–60%) containing 0.05% trifluoroacetic acid applied for 60 min (flow rate: 0.7 ml/min). Absorbance is monitored at 214 nm. Fractions of 0.5 ml have been collected in polypropylene tubes (Starsted) and stored frozen after addition of 0.02% Tween 20. Two experiments have been made with 30 μl and 420 μl of the hydrochloric acid extract of the posterior pituitary gland.

2.2. Vipera aspis *pituitary extracts*

Vipera aspis hypophyses have been removed in the laboratory less than 2 h after decapitation of the animals. They have been desiccated in pure acetone for 8 days at 0°C, then ground for giving a powder [4]. Acetone powder (0.285 mg, corresponding to two entire pituitaries) has been extracted by 0.1 M HCl (0.15 ml). From the supernatant after centrifugation, 10 μl have been taken for bioassays and two experiments with 60 and 70 μl have been performed on HPLC under the conditions described above.

3. RESULTS AND DISCUSSION

Fig. 1 shows separation of *Bufo marinus* neurohypophysial peptides by HPLC. Vasotocin has a retention time of 23.20 min and hydrin 2 a retention time of 23.40 min. The glycine extension present in hydrin 2 with regard to vasotocin and the C-terminal carboxyl group instead of the carboxamide group found in the latter make the molecule slightly less polar. Synthetic vasotocin and hydrin 2, subjected to HPLC under similar conditions, have retention times of 22.15 and 22.35 min, respectively (Fig. 2). In the case of *Rana esculenta*, the retention times found for

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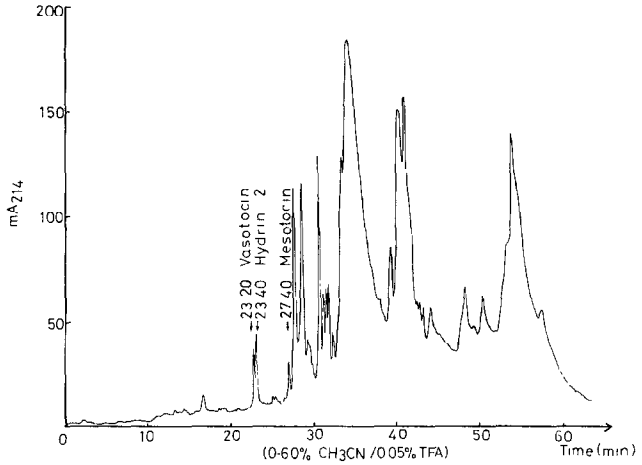

Fig. 1. Separation of *Bufo marinus* neurohypophysial hormones by HPLC (details in the text).

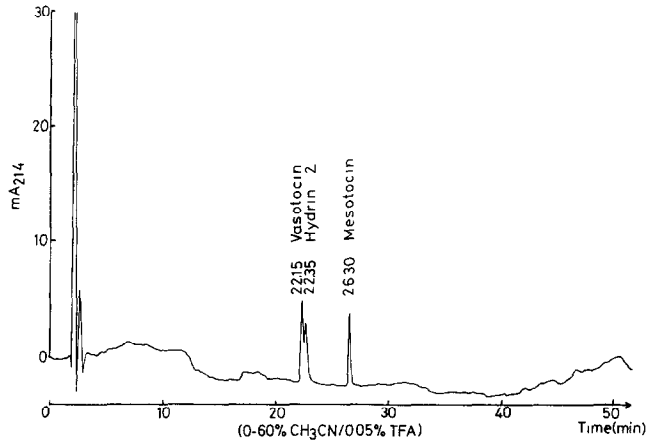

Fig. 2. Separation of synthetic vasotocin, hydrin 2 and mesotocin by HPLC (conditions similar to those used for *Bufo marinus* neurohypophysial hormones).

chemically identified vasotocin and hydrin 2, using a linear acetonitrile gradient 5–60% [1], were 20.95 and 21.30 min, respectively, so that the relative positions were virtually the same. In the same way, the amount of hydrin 2 detected in *B. marinus* is somewhat higher than that of vasotocin, as found for *R. esculenta* either in neurointermediate pituitary extracts [1] or in neurosecretory granules [2].

Mesotocin is detected with a retention time 27.40 min compared with 26.30 for the synthetic peptide and 26.05 for *R. esculenta* mesotocin in the acetonitrile gradient 5–60% [1]. Mesotocin is more hydrophobic than vasotocin and hydrin 2 because of the presence of an isoleucine in position 8 instead of an arginine.

In the viper, only vasotocin and mesotocin are detected with retention times 24.0 and 28.5 min, respectively. No trace of hydrin 1 or 2 can be detected (Fig. 3). The chromatographic profile resembles the one found for goose neurohypophysial peptides [3]. Among tetrapods, only amphibians seem to be endowed with hydrins, these peptides being absent in birds and reptiles.

Hydrin 2 has been detected by HPLC in several *Ranidae* (such as *R. esculenta, R. temporaria, R. pipiens*) but not in the permanently freshwater-dwelling *Xenopus laevis* in which hydrin 1 (vasotocinyl-Gly-Lys-Arg) is found along with vasotocin [1]. Hydrin 1 is approximately as active as hydrin 2 on the water permeability of the frog skin measured in vivo [5] and of the frog urinary bladder in vitro [6]. *Xenopus* skin and bladder have, however, been reported to be not sensitive to vasotocin and their derivatives [7]. Apparently adaptation has acted both on prohormone processing and receptor sensitivity.

The down-regulation of provasotocin processing implies a decreased activity of the granule carboxypeptidase β-like enzyme (carboxypeptidase H or E) [8–10] in the case of hydrin 1, and of the α-amidating enzyme (peptidyl-glycine α-monooxygenase) in the case of hydrin 2 [11,12]. The successive actions of an endopeptidase with specificity for a pair of basic residues, a carboxypeptidase β-like enzyme, and the α-amidating enzyme are usually complete in non-amphibian vertebrates so that only α-amidated nonapeptide hormones are found, C-terminally extended intermediates being very rarely detected [13]. The down-regulation of these processing enzymes could be performed in the secretory neuron by decreasing enzyme biosynthesis or by synthesizing specific inhibitors. In the case of anurans, differential processing of the same precursor has allowed to manufacture two hormones, apparently acting on different osmoregulatory target cells (hydrin 2 on skin and vasotocin on kidney). The relative proportions of hydrin 2 and vasotocin are similar in all anurans investigated so that processing regulation does not seem to depend upon particular species or habitat. It is not known whether the two peptides are present in distinct neurons or in different granules of the same neuron. Neurons producing mesotocin in the same animal are not subjected to the down-regulated processing since extended intermediates are not detected.

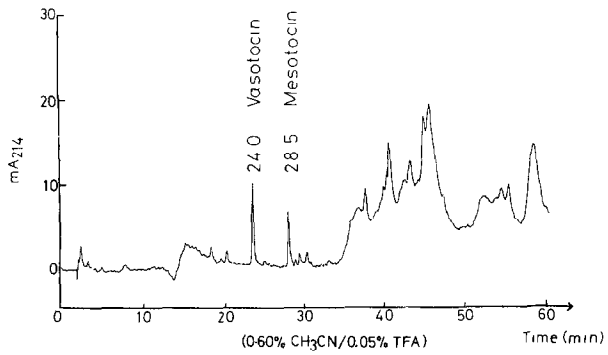

Fig. 3. Separation of *Vipera aspis* neurohypophysial hormones by HPLC (details in the text).

The water permeability of the skin and of the urinary bladder is specific to amphibians and related to their evolutionary adaptation to terrestrial conditions. It would be of interest to investigate differential processing for other prohormones in respect to differentiation and adaptation. Differential processing of pro-opiomelanocortin has been observed, this precursor being processed into corticotropin (39 residues) in corticotropic cells of the anterior pituitary gland and α-melanocyte stimulating hormone (13 residues) in melanotropic cells of the intermediate lobe [14].

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