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TWO MULTIGENE FAMILIES FOR MARSUPIAL NEUROHYPOPHYSIAL HORMONES ? IDENTIFICATION OF OXYTOCIN, MESOTOCIN, LYSIPRESSIN AND ARGININE VASOPRESSIN IN THE NORTH AMERICAN OPOSSUM (DIDELPHIS VIRGINIANA)

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Oxytocin, mesotocin ([Ile^8]-oxytocin), lysipressin ([Lys^8]-vasopressin) and arginine vasopressin have been identified in the North American opossum (Didelphis virginiana) by amino acid composition and high pressure liquid chromatography. The same peptides with the exception of mesotocin have previously been found in two South American opossums (Didelphis marsupialis and Philander opossum). Although a dual heterozygocity could also explain the simultaneous presence of oxytocin/mesotocin on one hand, lysipressin/arginine vasopressin on the other, it is assumed, from the results obtained with individual glands of Australian and South American marsupials, that distinct genes encode for the four peptides.

Metatherians (marsupials) appear particular among mammals because in contrast to Prototherians and Eutherians that have two neurohypophysial hormones, namely oxytocin and virtually always arginine vasopressin, they have often three neurohypophysial peptides. In Australian marsupials, 5 species belonging to the Macropodidae family have mesotocin ([Ile^8]-oxytocin), a peptide usually found in nonmammalian tetrapods (1), and two vasopressin-like peptides, lysipressin ([Lys^8]-vasopressin) and phenypressin ([Phe^2]-vasopressin) (2). Another Australian species, belonging to the Phalangeridae family, the brush-tailed possum (Trichosurus vulpecula), has mesotocin and arginine vasopressin (3). Among South American marsupials, in two species belonging to the Didelphidae family, oxytocin, lysipressin and arginine vasopressin have previously been characterized (4,5). We describe here the identification of four neurohypophysial peptides found in the North American opossum (Didelphis virginiana) and discuss a possible evolution of mammalian neurohypophysial hormones.

MATERIALS AND METHODS

Posterior pituitary glands : Nine entire pituitary glands from animals caught near Boston have been desiccated in cold

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acetone for several weeks and, after air-drying, the posterior lobes have approximately been dissected. From five glands, 25.7 mg of acetonic powder have been recovered. Two batchs of 12.8 and 12.9 mg have separately been homogeneized for 15 min with 0.1 M HCl (1 ml for 4.4 mg) and extracted for 4 h at 4°C. Oxytocic (6) and pressor (7) activities were measured on the clear supernatant solutions obtained by centrifugation.

Molecular sieving : The two solutions have been pooled and the mixture (5.6 ml) passed through a column (1 x 120 cm) of Bio-Gel P_4 equilibrated with 0.1 M acetic acid. The washing solution of the pellets (2 ml of 0.1 M HCl) has been added on the column and fractions (1 ml) have been collected. Bioassays have been performed and the tubes containing either oxytocic activity (81 to 85) or pressor activity (89 to 93 and 99 to 105) have been pooled. 7.6 U of oxytocic activity on one hand, 4.4 and 6.2 U of pressor activity on the other, have been recovered.

High pressure liquid chromatography : Purification of peptide hormones by HPLC has been carried out under conditions previously described (8) using a Waters liquid chromatograph (Model ALC/GPC 204) equipped with a WISP automatic injector (Model 710 B) and a Waters µ-Bondapak C-18 column. Absorbance has been measured at 254 and 280 nm and fractions (0.75 ml) have been collected every 30 sec with a collector LKB (Redirac 2112). Bioassays have been performed for locating the hormones. The three active fractions from Bio-Gel P_4 have been passed separately. From the oxytocic fraction, mesotocin (RT 49.08-49.56 min) and oxytocin (RT 50.76-51.24 min) have been isolated and subjected to amino acid analysis. From the first pressor fraction of Bio-Gel P_4, lysipressin (RT 44.26-45.80 min) has been purified and from the second, arginine vasopressin (RT 46.80-47.52 min) has been isolated.

Amino acid analysis : Peptides samples (2-10 nmol) have been hydrolyzed, either after performic acid oxidation, or dithiothreitol reduction, in sealed evacuated tubes (6 M HCl, 48 h, 100°C). Amino acid analyses have been carried out according to Spackman et al. (9) with a Spinco120B automatic analyzer fitted with a high-sensitivity cell.

RESULTS AND DISCUSSION

Purification has been carried out with 5 dry posterior pituitary glands (25.7 mg). The yields in activities at each step of the purification are given in Table I. The overall yields reach 33% for the oxytocic peptides and 48% for the pressor principles.

The amino acid compositions (Table II) and the retention times in HPLC permit the identification of mesotocin (RT = 49.32 min), oxytocin (RT = 51.00 min), lysipressin ([Lys^8]-vasopressin) (RT = 45.03 min) and arginine vasopressin (RT = 47.16 min). The last three peptides have previously been found in two South-American marsupials, Didelphis marsupialis and Philander opossum (4,5) but mesotocin has not been detected in these species. Mesotocin, however, is always present in

TABLE I

Purification of Didelphis virginiana neurohypophysial hormones
(5 dry glands : 25.7 mg)

	Step	Oxytocic activity			Pressor activity		
		Total	Step-yield	Overall yield	Total	Step-yield	Overall yield
		U	%	%	U	%	%
I	0.1 M HCl extract	10.2	100	100	15.3	100	100
II	Molecular sieving on Bio-Gel P_4						
	oxytocic peak	7.6	74	74			
	1st pressor peak				4.4	29	69
	2nd pressor peak				6.2	40	
III	High pressure liquid chromatography						
	mesotocin	1.1	15	45			
	oxytocin	2.3	30	33			
	lysipressin				3.3	75	48
	Arg-vasopressin				4.1	66	

Australian marsupials belonging to the families Macropodidae and Phalangeridae in contrast to oxytocin that is not found.

Present-day marsupials are supposed to have arisen from a single stem, Australian species branching from American ancestors which reached Australia either by the Antarctic sweepstake route or by the Bering route (10). Arginine vasotocin has been found in all the nonmammalian tetrapods examined to date whereas arginine vasopressin has been identified in Prototherian and Eutherian mammals (1). It seems reasonable to assume that the change of vasotocin into vasopressin, which involves the substitution of phenylalanine for isoleucine in position 3 and can be determined by a single nucleotide substitution in the gene, occurred very early, perhaps in mammal-like reptiles (11). Duplication of the arginine vasopressin gene in marsupial ancestors, which are supposed to have diverged from the placental stem some 130 million years ago (11), might explain the presence of two pressor peptides in all the individual macropodids and didelphids examined. In Macropodidae, each gene could have been subjected to a peculiar mutation giving either lysine vasopressin ([Lys^8]-vasopressin) or

TABLE II

AMINO ACID COMPOSITIONS OF THE DIDELPHIS VIRGINIANA NEUROHYPOPHYSIAL HORMONES [a]

Amino acid	Mesotocin			Oxytocin			Lysipressin			Arg-vasopressine		
	Reduced (4.4 nmol)	Oxidized (1.4 nmol)	Theor. values	Reduced (9.8 nmol)	Oxidized (1.9 nmol)	Theor. values	Reduced (2.2 nmol)	Oxidized (3.6 nmol)	Theor. values	Reduced (1.9 nmol)	Oxidized (4.4 nmol)	Theor. values
Lys							1.23	0.83	(1)			
Arg										0.95	1.00	(1)
Asp	1.00	1.00	(1)	1.00	1.00	(1)	1.00	1.00	(1)	1.00	1.00	(1)
Thr	0.20	-	-	0.47	-	-	-	0.19	-	-	-	-
Ser[b]	0.56	0.50	-	0.82	0.75	-	0.50	0.87	-	0.79	0.35	-
Glu	1.07	1.14	(1)	1.13	1.36	(1)	1.04	1.36	(1)	1.05	1.23	(1)
Pro	0.84	0.82	(1)	0.90	0.93	(1)	0.89	0.84	(1)	0.84	1.00	(1)
Gly	1.56	1.50	(1)	1.81	1.41	(1)	1.32	1.53	(1)	1.47	1.42	(1)
Ala	0.38	-	-	-	-	-	-	-	-	-	-	-
Val												
Met												
Ile	1.61	1.55	(2)	0.75	0.68	(1)						
Leu	0.35	-	-	1.01	0.73	(1)						
Tyr[c]	0.82	-	(1)	0.65	-	(1)	1.18	-	(1)	1.10	-	(1)
Phe							1.00	0.84	(1)	0.79	0.92	(1)
Cys[b]	-	1.50	(2)	-	1.10	(2)	-	1.16	(2)	-	1.55	(2)

[a] Values in molar ratios, aspartic acid taken as the reference

[b] Half-cystine is determined as cysteic acid on a separate performic acid-oxidized sample. A destruction, partial for cysteic acid and total for cysteine, is observed giving serine or alanine as by-products.

[c] Tyrosine, destroyed in oxidized sample, is protected in reduced sample.

phenypressin ([Phe^2]-vasopressin). In Didelphidae, a mutation in one of these genes could have given lysine vasopressin whereas the second continued to produce arginine vasopressin. In the family Phalangeridae, in which arginine vasopressin alone has been found (3), either the two genes could give arginine vasopressin (as in the case of the two genes encoding for the single human α-globin chain (12)) or one of them could have been transformed into an unexpressed pseudo-gene by a peculiar mutation (as in the case of the mutant Brattleboro rat (13)).

The passage of the reptilian mesotocin to the mammalian oxytocin is more puzzling to explain. Because a primitive egg-laying prototherian, echidna, has oxytocin (14), it has been assumed that the change, which involves the substitution of leucine for isoleucine in position 8 and can be determined by a single nucleotide substitution in the gene, occurred before the emergence of Therians. If Australian marsupials derived from American didelphids, as supposed by paleontologists (11), the presence of mesotocin in Australian species could be explained by a reverse mutation. The discovery of both oxytocin and mesotocin in the North American opossum, however, might suggest an early duplication of the mesotocin gene in mammalian ancestors. Although only two individual glands of Didelphis virginiana have been investigated up to now, and that the presence of the two oxytocic peptides in both cases could also be interpreted by heterozygocity, the co-existence of a mesotocin gene and an oxytocin gene in marsupial stem could explain oxytocin alone in South American opossums and mesotocin alone in Australian species by alternative non-expression of one of the two genes. Whatever the situation at the genome level, it seems that, in marsupials, mesotocin and oxytocin are functionally equivalent and that the structural variations are relevant to neutral drift rather than to selective evolution (15).

Oxytocin and vasopressin found in placental mammmals are fragments of protein precursors in which they are associated to VLDV-neurophysin and MSEL-neurophysin, respectively (16), as shown by direct isolation of the precursors (17) or deduced from the cDNA corresponding sequences (18,19) or from the genes((20,21). We can assume than the other neurohypophysial hormones are also associated with a neurophysin in a protein precursor and that duplication of the gene led to duplication of both hormone and

neurophysin. If it is so, characterization of marsupial neurophysins could give interesting informations about the evolutionary history of the relevant genes.

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