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Isolation and amino acid sequences of opossum vasoactive intestinal polypeptide and cholecystokinin octapeptide

(New World marsupial)

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ABSTRACT **Evolutionary history suggests that the marsupials entered South America from North America about 75 million years ago and subsequently dispersed into Australia before the separation between South America and Antarctica–Australia. A question of interest is whether marsupial peptides resemble the corresponding peptides of Old or New World mammals. Previous studies had shown that "little" gastrin of the North American marsupial, the opossum, is identical in length to that of the New World mammals, the guinea pig and chinchilla. In this report, we demonstrate that opossum cholecystokinin octapeptide, like that of the Australian marsupials, the Eastern quoll and the Tamar wallaby, is identical to the cholecystokinin octapeptide of Old World mammals and differs from that of the guinea pig and chinchilla. However, opossum vasoactive intestinal polypeptide differs from the usual Old World mammalian vasoactive intestinal polypeptide in five sites:**

Opossum: HSDAVFTDSYTRLLKQMAMRKYLDSILN
Pig: N R VK N

Evolutionary history suggests that the marsupials entered South America from North America about 75 million years ago (1). There was a subsequent dispersal into Australia before the separation soon thereafter between South America and Antarctica–Australia. Following the extinction of all North American marsupials about 15 million years ago, the Virginia opossum (*Didelphis virginiana*) is thought to have returned to North America when land bridges reconnected the two continents 2–5 million years ago (2). It is of interest therefore to determine whether marsupial peptides resemble the corresponding peptides of Old or New World mammals. Several studies have shown that in some cases marsupial peptides resemble more closely those of Old rather than New World mammals (3–5). However, opossum "little" gastrin like those of the New World hystricomorphs, guinea pig and chinchilla, is a 16-amino acid peptide because of deletion of a glutamic acid in the region 6–9 amino acids from the N terminus (6–8). Vasoactive intestinal peptide (VIP) is highly conserved among Old World mammals. Identical sequences have been reported for pig (9), cow (10), human (11), rat (12), rabbit (13), dog, and goat (14). However, guinea pig VIP differs in four sites from the usual peptide (14). Cholecystokinin octapeptide (CCK8) is also conserved among Old World mammals (15–18) but differs in the guinea pig (19) and chinchilla (20) by a substitution of valine for methionine in the third position from the N terminus. However, the CCK8 of the Australian marsupials, the Eastern quoll and the Tamar wallaby, is identical to that of Old World mammals (3). It is therefore of interest to purify and sequence opossum VIP and CCK8 to determine whether these peptides resemble the corresponding peptides of New or Old World mammals.

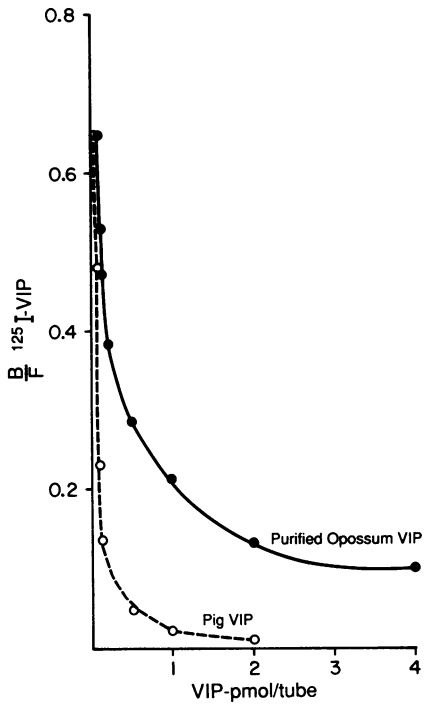

FIG. 1. Standard curves for RIA of pig and purified opossum VIP. B, bound; F, free.

MATERIALS AND METHODS

Purification of Intestinal VIP. Intestinal tissues including colon were freshly removed from five opossums and maintained frozen until extraction. Purification of VIP was monitored by RIA (21). Synthetic pig VIP, purchased from Peninsula Laboratories, was initially used as a standard. Subsequently, samples were reassayed with purified opossum VIP used as a standard.

The frozen intestines were thawed in methanol and scraped free of mucosal tissues. The remaining muscle (200 g) was extracted with 5 vol of 0.1 M HCl/90% (vol/vol) ethanol. The acid-ethanol extract was centrifuged to remove insoluble material and then neutralized with NH_4OH to pH 7. The precipitate was removed by centrifugation. The clarified supernatant was mixed with 3 vol of acetone. After overnight

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Abbreviations: VIP, vasoactive intestinal polypeptide; CCK, cholecystokinin; IR, immunoreactivity; TFA, trifluoroacetic acid; ACN, acetonitrile.

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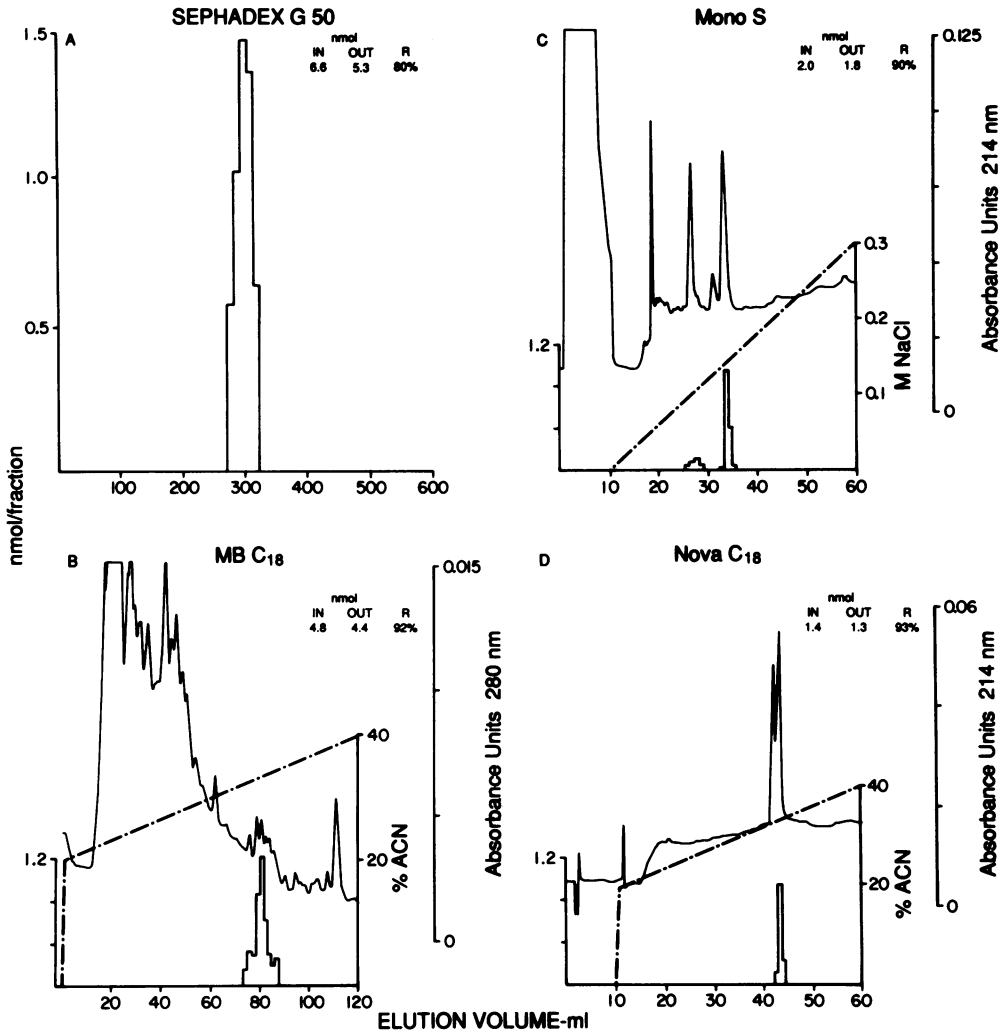

FIG. 2. Sequential steps (*A–D*) in purification of opossum VIP. The peak fractions of each step were applied to the subsequent step for further purification. R, recovery at each step.

storage at −30°C, the precipitate that formed was concentrated by centrifugation. The supernatant was discarded and the precipitate was redissolved in 18 ml of 1 M acetic acid. The solution was applied to a Sephadex G50 SF column (450 ml), which was equilibrated and eluted in 1 M acetic acid. Peak fractions of VIP immunoreactivity (IR) from the gel-filtration separation were concentrated on a C_{18} Sep-Pak cartridge (Waters), which was washed with 0.1% trifluoroacetic acid (TFA) and eluted with 2 ml of 0.1% TFA in 60% acetonitrile (ACN). The eluate was further purified by three HPLC steps. The peak fractions of VIP-IR were identified in each separation and further purified by the succeeding step: (*i*) MB C_{18} Radial-Pak column (Waters) eluted with a linear gradient from 20% to 40% ACN in 0.13% heptafluorobutyric acid. (*ii*) Mono S HR5/5 strong cation-exchange column (Pharmacia) eluted with a linear gradient from 0.0 to 0.3 M NaCl in 0.1% TFA/40% ACN. (*iii*) Nova C_{18} Radial-Pak column (Waters) eluted with a linear gradient from 20% to 40% ACN in 0.1% TFA.

Peptide fragments were generated by digestion of aliquots of the intact VIP with endoproteinase Asp-N (0.04 unit per 0.1 ml of NH_4HCO_3) and CNBr (1 mg per 0.1 ml of HCOOH). Purified opossum VIP and its peptide fragments were then sequenced on a gas-phase amino acid sequencer with an on-line phenylthiohydantoin amino acid analyzer (Applied Biosystems). The peptide fragments were aligned to obtain the full VIP sequence.

Purification of Brain CCK. Eleven opossum brains weighing 48 g were extracted with 5 vol of methanol. The methanol extract (240 ml) was stored at −30°C overnight and the precipitate that formed was removed by centrifugation. The extract contained 8 nmol of CCK as determined by RIA using antiserum R71, ^{125}I-labeled heptadecapeptide gastrin as tracer, and CCK8 as standard (22). The extract was pumped at a flow rate of 2 ml/min through four QMA anion-exchange Sep-Pak cartridges (Waters) connected in series. The QMA cartridges were washed with 0.05 M Tris·HCl (pH 7) (Tris) and eluted with 15 ml of Tris containing 1 M NaCl. The eluate contained 6.3 nmol of CCK-IR. The eluate was applied to a Sephadex G50SF column (500 ml), which was equilibrated and eluted with Tris. A single peak of CCK-IR eluting with

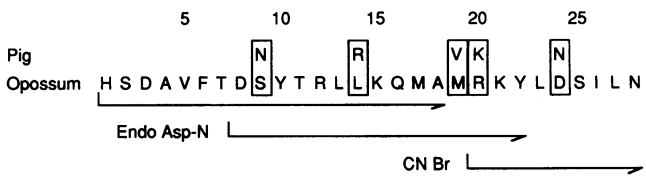

FIG. 3. Amino acid sequence of opossum VIP. One-letter notation for amino acids is used. The sites in the corresponding pig peptide that differ are shown in the line above the opossum sequence. Arrows indicate the extent of the sequencing run for the intact peptide or its fragments generated by endoproteinase Asp-N or CNBr digestion.

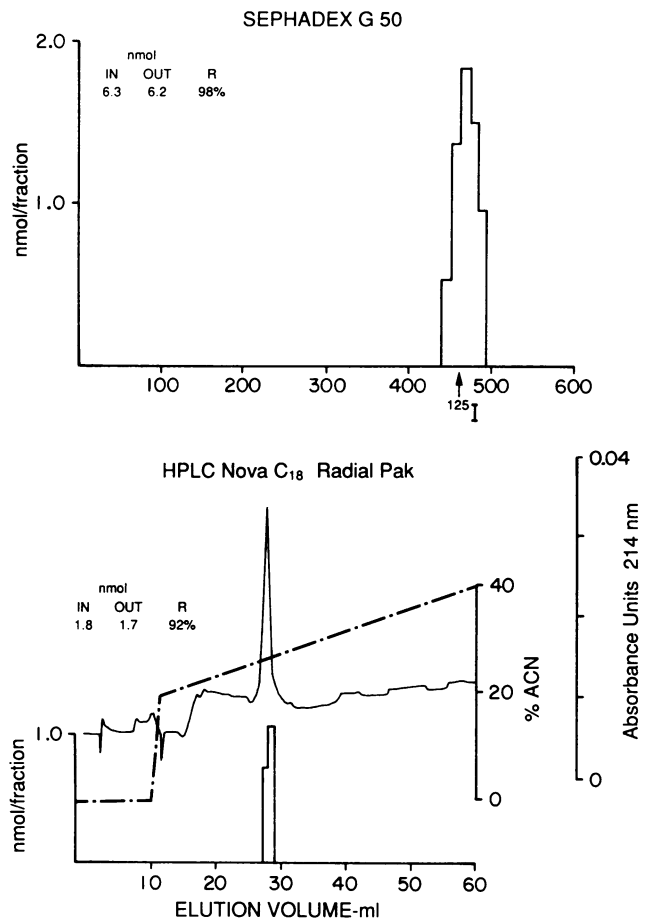

FIG. 4. Two-step purification of opossum brain CCK8. The peak fraction eluting from the Sephadex column was further purified on the Nova C_{18} column. R, recovery at each step.

the ^{125}I marker was observed. The main fraction in the peak was further purified on a Nova C_{18} Radial-Pak column and a portion of the purified peak was sequenced on the gas-phase amino acid sequencer.

RESULTS

The RIA for VIP initially used synthetic pig VIP as a standard during purification of the opossum peptide. The relative potency of pig and opossum VIP was determined after purification (Fig. 1). Shown in Fig. 2 are the steps in the four-stage purification of VIP. The concentrations shown were determined by using opossum VIP as a standard. The amino acid sequence of the purified opossum VIP and its overlapping fragments are compared to the sequence of the pig peptide in Fig. 3. The opossum peptide differs from the usual Old World mammalian VIP in five sites. These differences distributed through positions 9–24 from the N terminus probably account for the markedly decreased IR of opossum VIP compared to that of the pig peptide in the RIA system used.

The two-step purification of the CCK8 in the methanol extract of opossum brain is shown in Fig. 4. The sequence was shown to be identical to the usual brain CCK8 found in Old World mammals.

DISCUSSION

The striking improvements in peptide purification and sequencing have the potential for adding significant information concerning divergence of species, thus contributing to an improved understanding of evolutionary biology. Thus, our reports concerning the sequencing of marsupial and guinea pig peptides (3, 5, 6, 14) were referenced because of their particular relevance in the recent report asking whether the guinea pig is in fact a rodent (23). The demonstration that opossum CCK8 is identical to the corresponding peptide in Australian marsupials (3) as well as to the Old World mammalian peptide and that it differs from the corresponding peptide of the New World guinea pig (19) and chinchilla (20) suggests that sharing the same biospace does not necessarily lead to development of similar genetic alterations. However, it is particularly noteworthy that, in spite of the multimillion year separation of South America from Antarctica–Australia, kangaroo (4) and opossum (5) insulins have remained identical. Nonetheless, opossum "little" gastrin (6), like the corresponding peptide of the guinea pig (7) and chinchilla (8), is a hexadecapeptide rather than a heptadecapeptide, as is the case for Old World mammalian gastrin. Questions still to be answered are whether other Australian marsupial peptides such as gastrin or VIP are identical with those of the opossum or whether they have diverged over the past 70–75 million years.

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