Isolation and Characterization of Exendin-4, an Exendin-3 Analogue, from Heloderma suspectum Venom

FURTHER EVIDENCE FOR AN EXENDIN RECEPTOR ON DISPERSED ACINI FROM GUINEA PIG PANCREAS

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The recent identification in Heloderma horridum venom of exendin-3, a new member of the glucagon superfamily that acts as a pancreatic secretagogue, prompted a search for a similar peptide in Heloderma suspectum venom. An amino acid sequencing assay for peptides containing an amino-terminal histidine residue (His3) was used to isolate a 39-amino acid peptide, exendin-4, from H. suspectum venom. Exendin-4 differs from exendin-3 by two amino acid substitutions, Gly2-Glu6 in place of Ser2-Asp6, but is otherwise identical. The structural differences make exendin-4 distinct from exendin-3 in its bioactivity. In dispersed acini from guinea pig pancreas, natural and synthetic exendin-4 stimulate a monophasic increase in cAMP beginning at 100 pm that plateaus at 10 nm. The exendin-4-induced increase in cAMP is inhibited progressively by increasing concentrations of the exendin receptor antagonist, exendin-(9-39) amide. Unlike exendin-3, exendin-4 does not stimulate a second rise in acinar cAMP at concentrations >100 nm, does not stimulate amylase release, and does not inhibit the binding of radiolabeled vasoactive intestinal peptide to acini. This indicates that in dispersed pancreatic acini, exendin-4 interacts only with the recently described exendin receptor.

An assay for His5 peptides was recently used to identify the presence of helospectin and a new, previously unrecognized His5-Phe8 peptide in Heloderma horridum venom (1). This new peptide, designated exendin-3, is a pancreatic secretagogue. At concentrations greater than 100 nm, exendin-3 interacts with VIP3 receptors on guinea pig pancreatic acini to stimulate an increase in cellular cAMP and amylase release (2). At lower concentrations (0.1-10 nm), however, exendin-3 interacts with a putative exendin receptor that causes an increase in acinar cAMP but not amylase release. This conclusion is based on the observation that increasing concentrations of a specific antagonist, exendin-3-(9-39) amide, progressively inhibit exendin-3-induced increases in cAMP (2). Because venom from a closely related lizard, Heloderma suspectum, has been shown to contain helodermin (3), a peptide closely related in structure to helospectin (4), a search was undertaken for a His5 peptide in H. suspectum venom that might be analogous to exendin-3. We report the isolation from H. suspectum venom of such an analogue that has been named exendin-4. Exendin-4, unlike exendin-3, is not a pancreatic secretagogue. Instead, it interacts exclusively with the newly described exendin receptor (2) to increase pancreatic acinar cAMP.

MATERIALS AND METHODS

H. suspectum venom (lots HS19SZ and HS20SZ) was purchased from Miami Serpentarium Laboratories (Salt Lake City, UT). Diphenylcarbamyl chloride-treated trypsin was purchased from Sigma. Endoproteinase Asp-N was purchased from Boehringer Mannheim. This Assay and Amino Acid Sequencing—Amino-terminal amino acid analysis was performed by a single cycle of Edman degradation using an automated gas phase protein sequenator in combination with an on-line PTH-amino acid analyzer (Applied Biosystems, Foster City, CA). PTH-His was positioned to elute between PTH-Ala and PTH-dehydro-Ser. Purified peptides and peptide fragments were sequenced with the gas phase sequenator.

Isolation of His6 Peptides from Heloderma Venom—Venom (25 mg) was dissolved in distilled water (10 mg/ml) and passed through a C18 Sep-Pak cartridge (Waters Associates, Milford, MA). The C18 cartridge was washed with 5 ml of water and eluted with 2 ml of 0.1% trifluoroacetic acid, 60% acetonitrile. Peptides in the eluate were separated by HPLC on an 8 mm × 10-cm Radial-Pak column (Waters Associates). The column was eluted with a linear gradient (20-60%) of acetonitrile in 0.13% heptfluorobutyric acid at a flow rate of 1 ml/min. One-minute fractions were collected, and aliquots were assayed for His5 content.

Enzyme Cleavages—Purified exendin-4 (5-20 nmol) was digested with 0.24 μg of trypsin or with 0.24 μg of endoproteinase Asp-N. The peptide fragment exendin-(9-39) amide was prepared as described previously (2). Although this fragment was previously referred to as exendin-3-(9-39) amide (2), the name has been shortened to exendin-(9-39) amide to indicate that the carboxyl-terminal SI amino acids of exendin-3 and exendin-4 are identical. Peptide fragments were purified by HPLC on a Nova C18 Radial-Pak column (Waters Associates).

Amino Acid Analysis—Peptides were dried and hydrolyzed with gas phase 6 M HCl at 150 °C for 60 min. Amino acids were analyzed with an automated amino acid derivatizer (Applied Biosystems) connected to an on-line phenylthiocarbamyl-derivative amino acid analyzer.

Mass Spectrometry—The mass of the COOH-terminal fragment generated by trypsin digestion of exendin-4 was determined by fast atom bombardment-mass spectrometry. Mass accuracy of greater than ±0.1 unit was achieved by peak matching to appropriate cesium chloride cluster ions. Fast atom bombardment-mass spectrometry was performed by the Laboratory for Macromolecular Analysis at the Department of Medicine, Division of Digestive Diseases, State University of New York-Health Science Center, Brooklyn, New York 11203-2089.

An assay for His5 peptides was recently used to identify the presence of helospectin and a new, previously unrecognized His5-Phe8 peptide in Heloderma horridum venom (1). This new peptide, designated exendin-3, is a pancreatic secretagogue. At concentrations greater than 100 nm, exendin-3 interacts with VIP3 receptors on guinea pig pancreatic acini to stimulate an increase in cellular cAMP and amylase release (2). At lower concentrations (0.1-10 nm), however, exendin-3 interacts with a putative exendin receptor that causes an increase in acinar cAMP but not amylase release. This conclusion is based on the observation that increasing concentrations of a specific antagonist, exendin-3-(9-39) amide, progressively inhibit exendin-3-induced increases in cAMP (2). Because venom from a closely related lizard, Heloderma suspectum, has been shown to contain helodermin (3), a peptide closely related in structure to helospectin (4), a search was undertaken for a His5 peptide in H. suspectum venom that might be analogous to exendin-3. We report the isolation from H. suspectum venom of such an analogue that has been named exendin-4. Exendin-4, unlike exendin-3, is not a pancreatic secretagogue. Instead, it interacts exclusively with the newly described exendin receptor (2) to increase pancreatic acinar cAMP.
Albert Einstein College of Medicine using a Finnigan MAT-90 mass spectrometer.

Dispersed Pancreatic Acini—Dispersed acini from guinea pig pancreas were prepared by digestion with collagenase and incubated with VIP, natural exendin-4, natural exendin-3, or synthetic exendin-4. The incubations were performed in the absence or presence of increasing concentrations of exendin-(9-39) amide. Amylase release, cellular cAMP, and binding of 125I-VIP were measured as described previously (2).

Peptide Synthesis—Exendin-4 and exendin-(9-39) amide were synthesized on solid phase support using a Finnigan MAT-90 mass spectrometer. The crude synthetic peptide mixtures were purified by preparative HPLC. Synthetic peptides were purified by preparative HPLC.

The new peptide has been named exendin-4. Its amino acid sequence was determined by direct sequencing of the intact peptide and by sequence analysis of overlapping peptide fragments generated by digestion with trypsin. The COOH-terminal trypsin fragment was further analyzed by mass spectrometry. Sequence analysis of exendin-4 showed that it contains a Gly at position 2 and a Glu at position 3, but is otherwise identical with exendin-3. The structure of the carboxy-terminal trypsin fragment of exendin-4 (T4), as determined by a combination of sequencing and mass spectrometry, was shown to be identical with that of exendin-3. The experimental monoisotopic mass of 1022.48 confirmed that the COOH terminus is amidated. Thus, exendin-4, like exendin-3, is a 39-amino acid peptide that has an amidated carboxyl terminus. Exendin-4 has a calculated mass of 4184 units. The full amino acid sequence of exendin-4 is shown in Fig. 2 with the sequences of related peptides for comparison.

The bioactivity of the new peptide was examined using dispersed acini from guinea pig pancreas. In Fig. 3, the effects of natural and synthetic exendin-4 on acinar cAMP are compared with those of exendin-3. As described previously (2), exendin-3 causes a biphasic increase in cAMP. In contrast, the increase in cAMP observed with increasing concentrations of natural or synthetic exendin-4 is monophasic (K<sub>d</sub> = 0.2–0.4 nM). In terms of potency and efficacy, this increase in cAMP corresponds to the first phase of exendin-3-induced increases in cAMP (exendin concentrations between 0.1 and 10 nM). As noted previously with exendin-3 (2), the first phase increase in cAMP is not associated with amylase release or inhibition of binding of radiolabeled VIP. It is the second phase increase in cellular cAMP that correlates with stimulation of VIP receptors. At concentrations up to 1 μM, exendin-4 does not alter binding of 125I-VIP to dispersed pancreatic acini (Table I) and thus does not interact with VIP receptors to stimulate further increases in cAMP (Fig. 3) or alter basal amylase release (Fig. 4).

Because the exendin fragment, exendin-(9-39) amide, is a specific exendin receptor antagonist (2), this fragment was used to determine whether exendin-4 interacts with the exendin receptor. As shown in Fig. 5 (top), increasing concent...
pared with natural exendin-4 provides further validation for its structural similarity to three peptides (helodermin, exendin-3) that were previously found to indicate its structural similarity to three peptides (helosby by the method of Schild (5) (Fig. 5, bottom) yields a single regression line having a slope of 1.3. By this method, the apparent affinity of the antagonist for the exendin receptor is 3.5 nM. These results indicate that, like exendin-3 at concentrations <100 nM (2), exendin-4 interacts with exendin receptors on dispersed pancreatic acini to stimulate an increase in cellular cAMP but not amylase release.

**DISCUSSION**

We report the discovery in H. suspectum venom of a new bioactive, 39-amino acid peptide that is designated exendin-4 to indicate its structural similarity to three peptides (helospectin, helodermin, and exendin-3) that were previously found in Helodermatidae venoms. The nearly superimposable dose-response curves for cAMP production with synthetic, as compared with natural exendin-4 provides further validation for the amino acid sequence of the new peptide.

Exendin-4 differs from exendin-3 by two amino acid substitutions near the amino terminus. This has a unique effect on the biological activity of the new peptide. Although exendin-4 retains the ability to interact with exendin receptors, its ability to interact with VIP receptors is abolished. Previous treatments of the antagonist cause a progressive rightward shift in the exendin-4 dose-response curve. Analysis of these data by the method of Schild (5) (Fig. 5, bottom) yields a single regression line having a slope of 1.3. By this method, the apparent affinity of the antagonist for the exendin receptor is 3.5 nM. These results indicate that, like exendin-3 at concentrations <100 nM (2), exendin-4 interacts with exendin receptors on dispersed pancreatic acini to stimulate an increase in cellular cAMP but not amylase release.

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**TABLE I**

**Effect of VIP, exendin-4, and exendin-(9-39) amide on binding of **

<table>
<thead>
<tr>
<th>Concentration (log M)</th>
<th>VIP bound</th>
<th>Exendin-4</th>
<th>Exendin-(9-39) amide</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 nM</td>
<td>99.8 ± 3.3</td>
<td>105.5 ± 2.6</td>
<td>97.8 ± 2.4</td>
</tr>
<tr>
<td>1 nM</td>
<td>82.5 ± 2.1</td>
<td>104.5 ± 2.9</td>
<td>100.5 ± 1.8</td>
</tr>
<tr>
<td>10 nM</td>
<td>22.0 ± 1.2</td>
<td>104.5 ± 0.6</td>
<td>100.0 ± 0.8</td>
</tr>
<tr>
<td>100 nM</td>
<td>5.6 ± 1.2</td>
<td>103.5 ± 2.8</td>
<td>101.7 ± 0.8</td>
</tr>
<tr>
<td>1 μM</td>
<td>3.2 ± 1.2</td>
<td>106.6 ± 2.0</td>
<td>106.7 ± 3.6</td>
</tr>
</tbody>
</table>

**FIG. 3. Effect of natural and synthetic exendin-4 and natural exendin-3 on cellular cAMP in dispersed pancreatic acini.** Acini were incubated with indicated concentrations of agents for 30 min at 37 °C. In each experiment, each value was determined in duplicate, and results given are means ± S.E. from four separate experiments.

**FIG. 4. Effect of natural exendin-3 and synthetic exendin-4 on amylase release from dispersed pancreatic acini.** Acini were incubated with indicated concentrations of agents for 30 min at 37 °C. In each experiment, each value was determined in duplicate, and results given are means ± S.E. from four separate experiments. In these experiments, amylase release with 30 μM carbachol, 1 μM VIP, and 1 μM secretin was 14.5 ± 1.5, 11.5 ± 0.6, and 11.2 ± 0.1%, respectively.

**FIG. 5. Effect of increasing concentrations of exendin-(9-39) amide on amylase release from dispersed pancreatic acini.** Acini were incubated with indicated concentrations of agents for 30 min at 37 °C. In each experiment, each value was determined in duplicate, and results given are means ± S.E. from three separate experiments. Bottom, data (solid symbols) are plotted by the method of Schild (5). Linear regression was calculated by least squares analysis (Minitab Inc., State College, PA). Dose ratio (DR) is the ratio of the concentration of exendin-4 required to cause a half-maximal increase in cAMP in the presence of the indicated concentration of exendin-(9-39) amide to that in the absence of the antagonist.
Purification, Structure, and Bioactivity of Exendin-4  

Studies using the same cell model have shown that carboxy-terminal fragments of VIP and secretin, such as VIP-(10-28) and secretin-(5-27), lose bioactivity but maintain their ability to interact with VIP receptors on pancreatic acini (6-10). Hence, these fragments function as VIP receptor antagonists. In contrast, structural modifications in the amino-terminal portion of exendin-3 (as in exendin-(9-39) and exendin-4) result in the loss of both secretagogue activity and the ability of these peptides to bind to VIP receptors. Several conclusions regarding structure-function relationships of these peptides can be drawn from these observations.

The following are true in terms of interactions with VIP receptors. (a) In contrast to VIP and secretin, removal of amino-terminal amino acids from exendin-3 (e.g., exendin-(9-39) amide) abolishes the ability of the peptide to interact with high affinity VIP-preferring receptors that mediate the major increase in amylase release from guinea pig pancreatic acini (2, 7). (b) Likewise, amino acid substitutions at positions 2 and 3 in the exendin-3 sequence (i.e., exendin-4) is sufficient to abolish interaction of the peptide with this same class of VIP receptors. (c) Thus, in contrast to VIP and secretin, the peptide regions of exendin-3 and -4 that determine their binding affinity for VIP receptors coincide with the peptide regions that activate VIP-dependent adenyl cyclase. Both regions localize to the amino terminus.

In terms of interactions with exendin receptors the following are true. (a) The amino-terminal regions of exendin-3 and -4 that activate exendin-dependent adenyl cyclase are distinct from the remaining middle and carboxy-terminal peptide sequences that influence the peptides' binding affinity for exendin receptors. (b) The intrinsic biological activity of exendin-3 and -4, in terms of increasing acinar cAMP, resides in the amino-terminal portion of the molecule (i.e., exendin-(9-39) amide does not stimulate an increase in cAMP). (c) The amino acid sequence of exendin-3 and -4 required for binding to exendin receptors resides in the middle and carboxyl-terminal portions of the molecule (i.e., exendin-(9-39) amide binds to exendin receptors, as evidenced by inhibition of the actions of exendin-3 (2) and exendin-4 (Fig. 5). (d) Differences at amino acid positions 2 and 3 between exendin-3 and -4 do not interfere with the interaction of the peptides with exendin receptors (Fig. 3).

In addition to the discovery of a new bioactive peptide, this report provides further evidence for the existence of a guinea pig pancreatic acini of an exendin receptor that mediates an increase in cellular cAMP. This increase in cAMP does not stimulate amylase release, and its function, if any, is at present unknown. Currently, the only peptides that are known to interact with this newly discovered receptor are exendin-3 and -4. Since the new exendin receptor predicts the existence of an endogenous mammalian ligand, we suggest that exendin-(9-39) amide will be useful in the search for mammalian peptides that bind to exendin receptors.

REFERENCES