Patents
Attorney Reference: 0225-LIC-0

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant in re: John Eng

Patent No.: 5,424,286

Serial No: 08/066,480

Issued: June 13, 1995

Filed: May 24, 1993

For: Exendin-3 and Exendin-4 Polypeptides, and Pharmaceutical Compositions Comprising the Same

PATENT TERM EXTENSION APPLICATION UNDER 35 U.S.C. 156

Mail Stop Patent Extension
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

Amylin Pharmaceuticals, Inc. respectfully requests a patent term extension for the U.S. Letters Patent No. 5,424,286. Submitted herewith is an Application for Patent Term Extension under 35 U.S.C. 156 based on the regulatory review period for BYETTA™ exenatide injection, in compliance with 37 CFR 1.710 - 1.740.

This application is being submitted by a registered practitioner on behalf of John Eng,

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CERTIFICATE OF EXPRESS MAILING
(37 C.F.R. §1.10)

I hereby certify that this paper (along with anything referred to as being attached or enclosed) is being deposited with the United States Postal Service as "Express Mail". Label No. EV 595441596 US on the date shown below with sufficient postage in an envelope addressed to Mail Stop Patent Extension, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

6/24/05
Date of Deposit

Deborah Wykes
Name of Person Mailing Paper

Deborah Wykes
Signature of Person Mailing Paper

who is the sole patentee and owner of U.S. Patent No. 5,424,286, and on behalf of Amylin Pharmaceuticals, Inc. ("Amylin" or "Applicant"), exclusive licensee of U.S. Patent No. 5,424,286 and the holder of NDA Number 21-773 for BYETTA exenatide injection. The referenced license to U.S. Patent No. 5,424,286 was recorded on September 27, 2004 at the U.S. Patent and Trademark Office at Reel 015177, Frame 0815. A Power of Attorney from John Eng granting power to Molly A. Holman is attached as Exhibit A.

Pursuant to the provisions of 37 C.F.R. §1.730, Applicant hereby applies for an extension of the term of U.S. Patent No. 5,424,286 under 35 U.S.C. §156 of 1286 days, to November 30, 2016, based on the materials set forth herein and in the accompanying papers.

The required information for this application is submitted in accordance with 35 USC 156(d) and 37 CFR 1.710 et seq., and follows the numerical format set forth in 37 CFR 1.740(a). A response to each requirement follows each numerical requirement. The required information starts on page 3 of this application.

1. **A complete identification of the approved product as by appropriate chemical and generic name, physical structure or characteristics.**

The approved product is exenatide, the active ingredient in BYETTA™ exenatide injection[1].

Exenatide (exendin-4) is a 39-amino acid peptide amide (elemental composition, $C_{184}H_{282}N_{50}O_{60}S$; molecular weight, 4186.6 Daltons). The CAS registry number for exenatide is 141732-76-5. The structure is shown below:

H-His-Gly-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Leu-Ser-Lys-Gln-Met-Glu-Glu-Glu-Ala-Val-Arg-Leu-Phe-Ile-Glu-Trp-Leu-Lys-Asn-Gly-Gly-Pro-Ser-Ser-Gly-Ala-Pro-Pro-Pro-Ser-NH_2

2. **A complete identification of the Federal statute including the applicable provision of law under which the regulatory review occurred.**

The regulatory review for BYETTA™ exenatide injection occurred under Section 505 of the Federal Food, Drug, and Cosmetic Act, 21 USC § 355.

3. **An identification of the date on which the product received permission for commercial marketing or use under the provision of law under which the applicable regulatory review period occurred.**

BYETTA™ exenatide injection received permission for commercial marketing or use under Section 505 of the Federal Food, Drug, and Cosmetic Act on April 28, 2005.

4. **In the case of a drug product, an identification of each active ingredient in the product and as to each active ingredient a statement that it has not been previously**

[1] We note that the term "product," for purposes of patent term extension for a drug product, is defined as "the active ingredient of a new drug, antibiotic drug, or human biological product...including any salt or ester of the active ingredient, as a single entity or in combination with another active ingredient." 35 U.S.C. § 156(f)(2). Nothing in this application should be construed as limiting the term "product" for purposes of the requested patent term extension to the specific form of exenatide approved in BYETTA™.

approved for commercial marketing or use under the Federal Food, Drug, and Cosmetic Act, the Public Health Service Act, or the Virus-Serum-Toxin Act, or a statement of when the active ingredient was approved for commercial marketing or use (either alone or in combination with other active ingredients), the use for which it was approved, and the provision of law under which it was approved.

The active ingredient in BYETTA™ exenatide injection is exenatide (exendin-4). That active ingredient has not been previously approved for commercial marketing or use under the Federal Food, Drug, and Cosmetic Act, the Public Health Service Act, or the Virus-Serum-Toxin Act.

5. A statement that the application is being submitted within the sixty day period permitted for submission pursuant to § 1.720(f) and an identification of the date of the last day on which the application could be submitted.

This application for patent term extension is being submitted within the sixty-day period permitted for submission pursuant to § 1.720(f). The product was approved on April 28, 2005. The last date on which this application can be submitted is June 27, 2005. As evident from the Certificate of Mailing by "Express Mail" pursuant to 37 C.F.R. 1.10, this application is timely filed.

6. A complete identification of the patent for which an extension is being sought by the name of the inventor, the patent number, the date of issue, and the date of expiration.

A complete identification of the patent for which an extension is being sought is as follows:

Inventor: John Eng

Patent Number: 5,424,286

Date of Issue: June 13, 1995

Date of Expiration: May 24, 2013

7. A copy of the patent for which an extension is being sought, including the entire

specification (including claims) and drawings.

A copy of U.S. Patent No. 5,424,286 for which an extension is being sought is attached as Exhibit B.

8. A copy of any disclaimer, certificate of correction, receipt of maintenance fee payment, or reexamination certificate issued in the patent.

Copies of the Maintenance Fee Statement receipts received for U.S. Patent No. 5,424,286 for years four and eight are attached as Exhibit C.

9. A statement that the patent claims the approved product, or a method of using or manufacturing the approved product, and a showing which lists each applicable patent claim and demonstrates the manner in which at least one such patent claim reads on:

(i) The approved product, if the listed claims include any claim to the approved product;

(ii) The method of using the approved product, if the listed claims include any claim to the method of using the approved product; and

(iii) The method of manufacturing the approved product, if the listed claims include any claim to the method of manufacturing the approved product.

Patent No. 5,424,286 claims a method of using the approved product. The approved product is exenatide (exendin-4), the active ingredient in BYETTA™ exenatide injection. Claim 6 reads on a method of using the approved product as follows.

> 6. A method of stimulating insulin release in a mammal comprising administering an effective insulinotropic amount of a substantially pure polypeptide, synthetic or purified from natural sources, having the amino acid sequence:
> HGEGTFTSDL SKQMEEEAVR LFIEWLKNGG PSSGAPPPS (SEQ ID NO: 2), wherein the resulting insulinotropic effect is greater than that attainable by administration of GLP-1.

BYETTA™ exenatide injection contains a substantially pure polypeptide having the sequence described in the claim. BYETTA™ exenatide injection improves glycemic control in

people with type 2 diabetes mellitus. BYETTA enhances glucose-dependent insulin secretion by the pancreatic beta-cell, suppresses inappropriately elevated glucagon secretion, and slows gastric emptying. BYETTA has acute effects on pancreatic beta-cell responsiveness to glucose and leads to insulin release only in the presence of elevated glucose concentrations. Administration of BYETTA at therapeutic plasma concentrations restored first-phase insulin response to an IV bolus of glucose in patients with type 2 diabetes. Both first-phase insulin secretion and second-phase insulin secretion were significantly increased in patients with type 2 diabetes treated with BYETTA compared with saline ($p<0.001$ for both). Thus, the approved use falls within the scope of claim 6.

10. **A statement beginning on a new page of the relevant dates and information pursuant to 35 U.S.C. 156(g) in order to enable the Secretary of Health and Human Services for the Secretary of Agriculture, as appropriate, to determine the applicable regulatory review period as follows:**

(i) For a patent claiming a human drug, antibiotic, or human biological product: (A) The effective date of the investigational new drug (IND) application and the IND number; (B) The date on which a new drug application (NDA) or a Product License Application (PLA) was initially submitted and the NDA or PLA number; and (C) The date on which the NDA was approved or the Product License Issued.

U.S. Patent No. 5,424,286 claims a human drug product. The relevant dates and information pursuant to 35 U.S.C. 156(g) to enable the Secretary of Health and Human Services to determine the applicable regulatory review period are as follows:

(A) The FDA accepted the IND for exenatide on January 13, 1999. The effective date of the IND application is February 10, 1999. The IND number is 57,725[2].

(B) The NDA was initially submitted to the FDA on June 29, 2004. The NDA number is 21-773.

(C) The date on which the NDA was approved is April 28, 2005.

11. A brief description beginning on a new page of the significant activities undertaken by the marketing applicant during the applicable regulatory review period with respect to the approved product and the significant dates applicable to such activities.

[2] Applicant acknowledges that this is two days prior to the normal 30-day review period, but as indicated in the attached letter (Exhibit D) , Amylin received permission from the FDA to proceed with its clinical study on February 10, 1999.

During the applicable regulatory review period, Amylin was diligently involved in obtaining FDA approval for exenatide. As stated in (10) above, the FDA accepted the IND for exenatide on January 13, 1999, the IND Exemption became effective on February 10, 1999, the NDA was submitted on June 29, 2004, and the NDA was approved on April 28, 2005. Amylin was in close consultation with the FDA during the clinical studies conducted under the IND. Similarly, subsequent to the submission of the NDA, Amylin had numerous contacts and meetings with the FDA with respect to the approval. A brief description of significant activities undertaken by Amylin during the applicable regulatory review period with respect to exenatide and the significant dates applicable to such activities follows in the table below.

Date	**Regulatory Activity**
1/12/1999	Amylin submitted IND for AC2993 (exenatide), including Protocol 2993-102
1/22/1999	FDA acknowledged receipt of Amylin's IND submission dated January 12, 1999 and assigned number 57,725 to the file. FDA's 30-day review began on January 13, 1999.
2/10/1999	FDA provided Amylin authorization to proceed with Protocol 2993-102
4/18/1999	Amylin submitted all non-clinical pharmacology and toxicology research reports referenced in Section 8 of the original IND.
5/21/1999	Amylin submitted Protocol AC2993-103
6/11/1999	Amylin submitted final clinical study report for Protocol 2993-101
8/11/1999	Amylin submitted briefing package for discussion on 8/24/99 relating to the preclinical development of AC2993
8/24/1999	Teleconference between Amylin and FDA regarding preclinical issues
10/22/1999	Amylin submitted Protocol AC2993-104
1/17/2000	Amylin submitted Protocol 2993-105
4/27/2000	Amylin submitted Protocol 2993-106
9/29/2000	Amylin submitted Protocol 2993-107
12/15/2000	Amylin submitted Protocol 2993-108
4/11/2001	Teleconference between Amylin and FDA/CAC regarding Executive CAC's Final Report and recommendations
4/24/2001	Amylin submitted Protocol 2993-109 with Amendment 1; and Protocol 2993-107
5/25/2001	Amylin submitted Protocol 2993-110; and results of reproduction studies
7/27/2001	Amylin submitted an End of Phase 2 meeting request.
10/10/2001	End of Phase 2 Meeting between Amylin and FDA

12/19/2001	Amylin submitted Request for Type A Meeting/Teleconference regarding Response to 2993-112 Special Protocol Assessment
1/8/2002	Teleconference between Amylin and FDA regarding 2993-112/113 Special Protocol Assessment
1/10/2002	Amylin submitted Request for Type C Meeting/Teleconference: Chemistry, Manufacturing, and Controls.
2/1/2002	Amylin submitted Protocol 2993-115
3/28/2002	Amylin submitted Protocol 2993-112E.
4/23/2002	Amylin submitted CMC Briefing Document
5/8/2002	Teleconference between Amylin and FDA regarding statistical plan for 2993-112.
7/1/2002	Amylin submitted Protocol 2993-113E
7/22/2002	Amylin submitted Protocol 2993-111; Protocol 2993-116.
8/6/2002	Amylin submitted Protocol 2993-117.
8/26/2002	Amylin submitted final reports for the Segment III reproduction toxicity study.
9/5/2002	Amylin submitted Protocols 2993-118 & 2993-119.
12/4/2002	Amylin submitted Protocol 2993-115E
1/23/2003	Amylin submitted Request For Type A Meeting/Teleconference: Pharmacology/Toxicology
2/11/2003	Type A teleconference between Amylin and FDA regarding carcinogenicity studies.
4/29/2003	Amylin submitted Protocol H8O-EW- GWAB(a).
5/7/2003	Amylin submitted Protocol 2993-121
5/8/2003	Amylin submitted Protocol H80-EW-GWAG
5/14/2003	Amylin submitted Protocol H8O-MC-GWAA(a)
5/23/2003	Amylin submitted Protocol H8O-FW-GWAF.
6/24/2003	Amylin submitted Protocol H8O-EW-GWAE(a).
7/1/2003	Amylin submitted Protocol 2993-120
7/3/2003	Amylin submitted Request for Type C Meeting
7/7/2003	Amylin submitted Protocols H8O-MC-GWAD and H8O-EW-GWAJ
7/18/2003	Amylin submitted Protocol 2993-122
8/7/2003	Amylin submitted Briefing Document for Type C Teleconference
9/3/2003	Type C teleconference between Amylin, Lilly, and FDA
9/10/2003	Amylin submitted Request for Type B Meeting regarding chemistry, manufacturing, controls,

and nonclinical and clinical topics.

9/24/2003	Teleconference between Amylin and FDA regarding CMC Pre-NDA Meeting
11/19/2003	Amylin submitted Protocols H8O-MC-GWAK and Protocol H8O-MC-GWAN
11/24/2003	Amylin requested a Type B (Pre-NDA) meeting between Amylin, Eli Lilly and Company, and FDA
12/30/2003	Amylin submitted Briefing Document for Type B Meeting (Pre-NDA Meeting)
2/2/2004	Pre-NDA Meeting between Amylin, Lilly, and FDA
3/18/2004	Amylin submitted Protocol H8O-MC-GWAP(a)
6/29/2004	Amylin submitted Drug Application NDA 21-773 for exenatide injection
7/1/2004	Amylin submitted Protocol 2993-114(1).
8/4/2004	Amylin submitted Protocol Amendment: New Protocol, Change in Protocol, and New Investigator Information for H8O-MC-GWAO.
9/13/2004	Amylin received notice from the FDA that its NDA was filed under section 505(b) of the Act on August 29, 2004 in accordance with 21 CRF 314.101(a).
10/28/2004	Amendment submitted by Amylin including Four-Month Safety Update, CM&C Stability Update
11/4/2004	Amendment submitted by Amylin including response to FDA request for information regarding delivery device.
12/8/2004	Amylin submitted Protocol H8O-FW-GWAC.
12/17/2004	Amendment submitted by Amylin regarding cartridge stability data
2/22/2005	Teleconference between FDA and Amylin regarding pen-injector device
4/28/2005	NDA 021-773 Approval Letter dated 28 April 2005 sent by FDA to Amylin

12. A statement beginning on a new page that in the opinion of the applicant the patent is eligible for the extension and a statement as the length of the extension claimed, including how the length of the extension was determined.

It is the opinion of the Applicant that U.S. Patent No. 5,424,286 is eligible for a patent term extension under 35 USC 156.

The length of extension claimed is 1286 days, for an extension to November 30, 2016. The period of this extension was calculated under 35 USC 156(c) and 37 CFR 1.775(d), using 35 U.S.C 156(g) and 37 CFR 1.775(c) to calculate the regulatory review period, as described below.

Calculation of Regulatory Review Period: Under 37 CFR 1.775(c) (bolded text), the length of the regulatory review period for a human drug is the sum of:

(1) The number of days in the period beginning on the date an exemption under subsection (i) of section 505 or subsection (d) of section 507 of the Federal Food, Drug, and Cosmetic Act became effective for the approved product and ending on the date an application was initially submitted for such product under these sections or under section 351 or the Public Health Service Act; and

(2) The number of days in the period beginning on the date the application was initially submitted for the approved product under section 351 or the Public Health Service Act, subsection (b) of section 505 or section 507 of the Federal Food, Drug, and Cosmetic Act, and ending on the date such application was approved under such section.

The period under 37 CFR 1.775(c)(1) is 1966 days. An Investigational New Drug exemption under subsection 505 of the Federal Food, Drug, and Cosmetic Act became effective for exenatide on February 10, 1999. A New Drug Application for exenatide was initially submitted for exenatide on June 29, 2004. The period under 37 CFR 1.775(c)(2) is 303 days. This application was approved on April 28, 2005.

The regulatory review period is the sum of the number of days above listed sections (c)(1) and (c) (2), which is 2269 days.

Calculation of Patent Term Extension: Under 37 CFR(d) (bolded text), the term of U.S. Patent 5,424,286 as extended for a human drug product has been calculated to be until November 30, 2016, which is 1286 days from the original expiration of the patent as shown below:

(1) Subtracting from the number of days ...[in] the Regulatory Review Period:

(i) The number of days in the periods of paragraphs (c)(1) and (c)(2) of this section which were on and before the date on which the patent

issued;

(ii) The number of days in the periods of paragraphs (c)(1) and (c)(2) of this section during which it is determined under 35 U.S.C. 156(d)(2) (B) by the Secretary of Health and Human Services that applicant did not act with due diligence;

(iii) One-half the number of days remaining in the period defined by paragraph (c)(1) of this section after that period is reduced in accordance with paragraphs (d)(1)(i) and (ii) of this section; half days will be ignored for this section;

(i) U.S. Patent No. 5,424,286 issued on June 13, 1995. The number of days in the periods of paragraphs (c)(1) and (c)(2) of this section which were on and before the date on which the patent issued is zero days.

(ii) The Secretary of Health and Human Services has not determined that Applicant did not act with due diligence, as defined in U.S.C. 156(d)(3), and in fact Applicant did act with the degree of attention, continuous directed effort, and timeliness reasonably expected from and exercised by a person during the regulatory review period. Therefore, no reduction in the number of days of the regulatory review period is appropriate.

(iii) The number of days remaining in the period defined by paragraph (c)(1) of this section after that period is reduced in accordance with paragraphs (d)(1)(i) and (ii) is 1966 days. One-half of this number of days is 983 days.

Thus, the number of days to be subtracted under 37 CFR 1.775(d)(1) (i) – (iii) is 983 days. By subtracting this number of days from the number of days determined to be the Regulatory Review Period in accordance with paragraphs (c)(1) and (c)(2) of this section, 1286 days remain.

(2) By adding the number of days determined in paragraph (d)(1) of this section to the original term of the patent as shortened by any terminal disclaimer;

The number of days determined in paragraph (d)(1) of this section is 1286 days. There is no terminal disclaimer for this patent and therefore no reduction in patent term extension under this subpart. With a term extension of 1286 days, US 5,424,286 would expire November 30, 2016.

(3) By adding 14 years to the date of the approval of the application under subsection 505 of the Federal Food, Drug, and Cosmetic Act;

When 14 years is added to the date of the approval of the application under subsection 505 of the Federal Food, Drug, and Cosmetic Act, the date is April 28, 2019.

(4) By comparing the dates for the ends of the periods obtained pursuant to paragraphs (d)(2) and (d)(3) of this section with each other and selecting the earlier date;

The earlier date calculated under paragraphs (d)(2) and (d)(3) of section 1.775 is November 30, 2016, under subsection (d)(3).

(5) If the original patent issued after September 24, 1984,

(i) By adding 5 years to the original expiration date of the patent or any earlier date set by terminal disclaimer; and

(ii) By comparing the dates obtained pursuant to paragraphs (d)(4) and (d)(5)(i) of this section with each other and selecting the earlier date;

U.S. Patent No. 5,424,286 issued after September 24, 1984. When 5 years is added to the original expiration date of the patent, May 24, 2013, the date is May 24, 2018. By comparing this date to the dates obtained pursuant to paragraphs (d)(4) and (d)(5)(i) of this section, the earlier date is November 30, 2016.

U.S. Patent No. 5,424,286 issued after September 24, 1984. Therefore, section (d)(6) does not apply.

In summary, Applicant concludes that it is entitled to a period of extension for this patent to November 30, 2016.

13. A statement that applicant acknowledges a duty to disclose to the Commissioner of Patents and Trademarks and the Secretary of Health and Human Services or the Secretary of Agriculture any information which is material to the determination of entitlement to the extension sought.

Applicant acknowledges a duty toward the Commissioner of Patent and Trademarks and the Secretary of Health and Human Services to disclose any information which is material to the determination of entitlement to the patent term extension sought.

14. The prescribed fee for receiving and acting upon the application for extension.

The Commissioner is hereby authorized to charge the $1,120.00 under 37 CFR 1.20(j)(1) fee for receiving and acting upon the application for patent term extension to Applicant's Deposit Account, No. 01-0535. Additionally, the Commissioner is authorized to charge any other fees related to this filing or credit any overpayment to Applicant's Deposit Account. A Fee Transmittal Form (PTO/SB/17) is enclosed for this purpose.

15. The name, address, and telephone number of the person to whom inquiries and correspondence relating to the application for patent term extension are to be directed.

Please direct all inquiries and correspondence relating to this application to:

Molly A. Holman, Ph.D., J.D.
Executive Director, Intellectual Property
Amylin Pharmaceuticals, Inc.
9360 Towne Centre Drive
San Diego, CA 92121
Telephone: (858) 552-2200
Facsimile: (858) 552-1936
e-mail: mholman@amylin.com

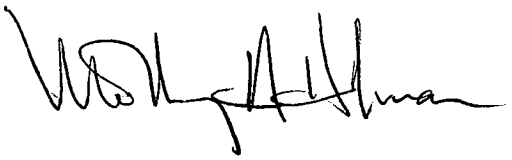
or

David R. Marsh, Ph.D.
Arnold & Porter LLP
555 Twelfth Street, NW
Washington, DC 20004-1206
Telephone: (202) 942-5068
Facsimile: (202) 942-5999

This Application is submitted together with two duplicate copies as required under 37 C.F.R.§ 1.740(b) and two additional copies of the application pursuant to M.P.E.P 2753, for a total of four copies and one original.

A Return Receipt Postcard is also attached so that the Office can notify Applicant that this complete application has been received.

Respectfully submitted,



Molly A. Holman, Ph.D., J.D.
Registration No. 40,022

AMYLIN PHARMACEUTICALS, INC.
9360 Towne Centre Drive
San Diego, CA 92121
(858) 642-7084

EXHIBIT A

Power of Attorney

Patents
Attorney Reference: 0225-LIC-0

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant in re: John Eng

Patent No.: 5,424,286

Serial No: 08/066,480

Issued: June 13, 1995

Filed: May 24, 1993

For: Exendin-3 and Exendin-4 Polypeptides, and Pharmaceutical Compositions Comprising the Same

Revocation of Prior Power of Attorney, Appointment of New Attorneys of Record and Change of Correspondence Address

Mail Stop Patent Extension
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

Dr. John Eng is the sole patentee and owner of U.S. Patent No. 5,424,286.

Dr. John Eng hereby revokes all powers of attorney heretofore given in the above-captioned application and appoints the attorneys listed below with full power of substitution, association, and revocation, to prosecute said application and to transact all business in the U.S. Patent and Trademark Office connected therewith.

Molly A. Holman, Reg. No. 40,022
James E. Butler, Reg. No. 40,931
Karen R. Zachow, Reg. No. 46,332

Timothy Torchia, Reg. No. 36,700
Susan J. Myers Fitch, Reg. No. 55,477
Joanna L. Moore, Reg. No. 44,950

of Amylin Pharmaceuticals, Inc.

and

Practitioners at Arnold & Porter, Customer No. 44638

Please address future correspondence to:

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Executive Director, Intellectual Property
Amylin Pharmaceuticals, Inc.
9360 Towne Centre Drive
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Telephone: (858) 642-7084
Facsimile: (858) 552-1936
E-mail: mholman@amylin.com

Or to:

David R. Marsh
ARNOLD & PORTER
Attn: IP Docketing Department
555 Twelfth Street, NW
Washington, DC 20004-1206
Telephone: (202) 942-5000
Facsimile: (202) 942-5999

6/10/05
Date

John Eng

EXHIBIT B

U.S. Patent Number 5,424,286

US005424286A

United States Patent [19]

Eng

[11] Patent Number: 5,424,286

[45] Date of Patent: Jun. 13, 1995

[54] **EXENDIN-3 AND EXENDIN-4 POLYPEPTIDES, AND PHARMACEUTICAL COMPOSITIONS COMPRISING SAME**

[76] Inventor: **John Eng**, 5427 Arlington Ave., Bronx, N.Y. 10471

[21] Appl. No.: **66,480**

[22] Filed: **May 24, 1993**

[51] **Int. Cl.**[6] **A61K 38/16**; C07K 14/46; C12N 15/63

[52] **U.S. Cl.** **514/2**; 514/866; 435/69.1; 530/324

[58] **Field of Search** 514/2, 866; 424/88; 435/69.1; 530/324

[56] **References Cited**

PUBLICATIONS

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Raufman, J. –P., Exendin–3 a Novel Peptide from *Heloderma horridum* Venom, Interacts with Vasoactive Intestinal Peptide Receptors and a Newly Described Receptor on Dispersed Acini from Guinea Pig Pancreas; J. Biol. Chem. 266:2897 (1991).

Eng, J. et al., Isolation and Characterization of Exendin–4, an Exendin–3 Analogue, from *Heloderma suspectum* Venom; J. Biol. Chem. 267:7402 (1992).

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John Eng, Exendin Peptides; The Mt. Sinai J. of Med. 59: 147 (1992).

Gutniak, M. et al., Antidiabetogenic Effect of Glucagon–Like Peptide–1 (7–36) Amide in Normal Subjects and Patients with Diabetes Mellitus; The New England J. Med. 326:1316 (1992).

Primary Examiner—Garnette D. Draper
Assistant Examiner—Elizabeth C. Kemmerer
Attorney, Agent, or Firm—Allegretti & Witcoff, Ltd.

[57] **ABSTRACT**

This invention encompasses pharmaceutical compositions containing exendin-3 or exendin-4, fragments thereof, or any combination thereof, and methods for the treatment of diabetes mellitus and the prevention of hyperglycemia.

7 Claims, 9 Drawing Sheets

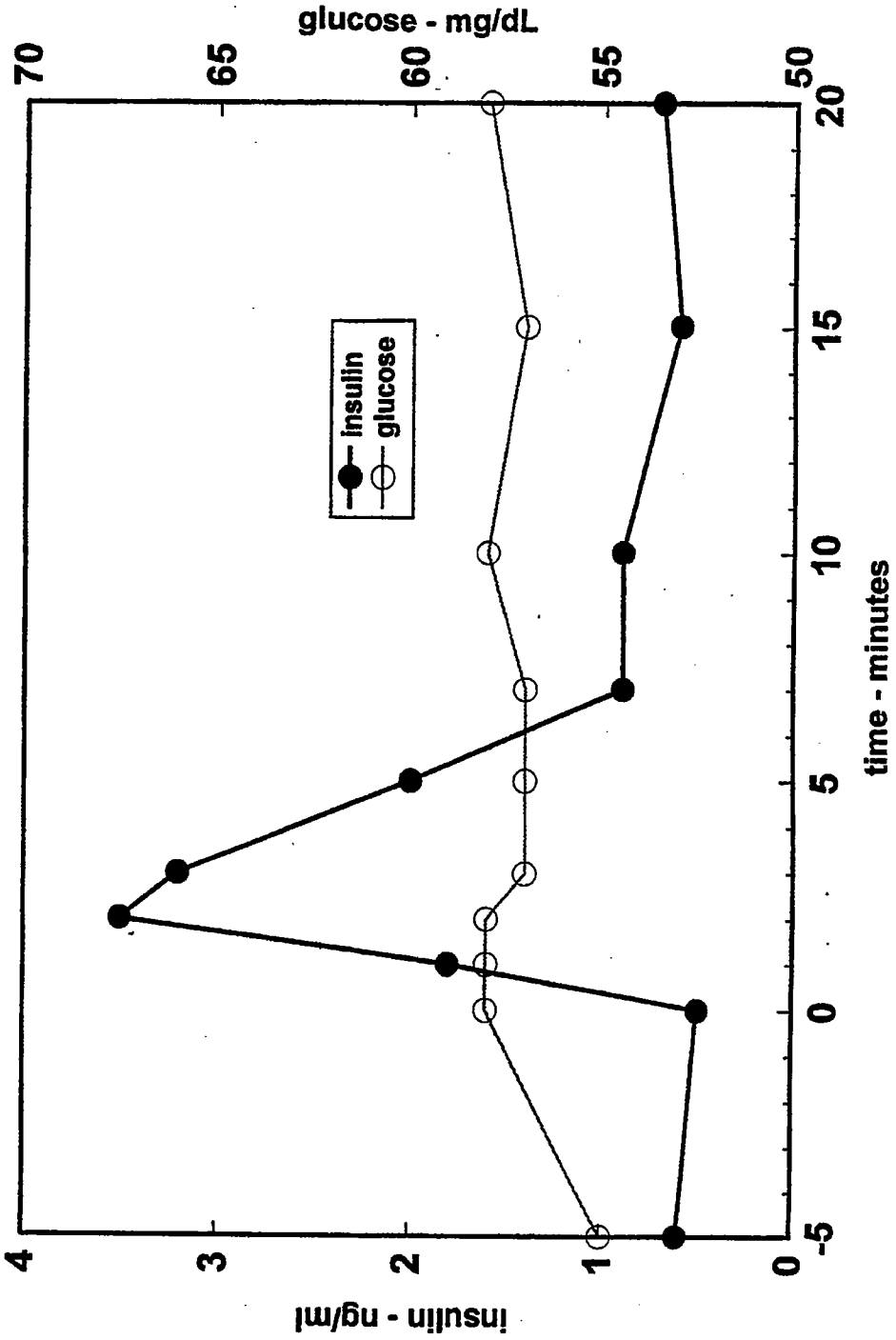
glucose - mg/dL
70
65
60
55
50
insulin
glucose
20
15
10
5
0
-5
time - minutes
4
3
2
1
0
insulin - ng/ml

Fig. 1

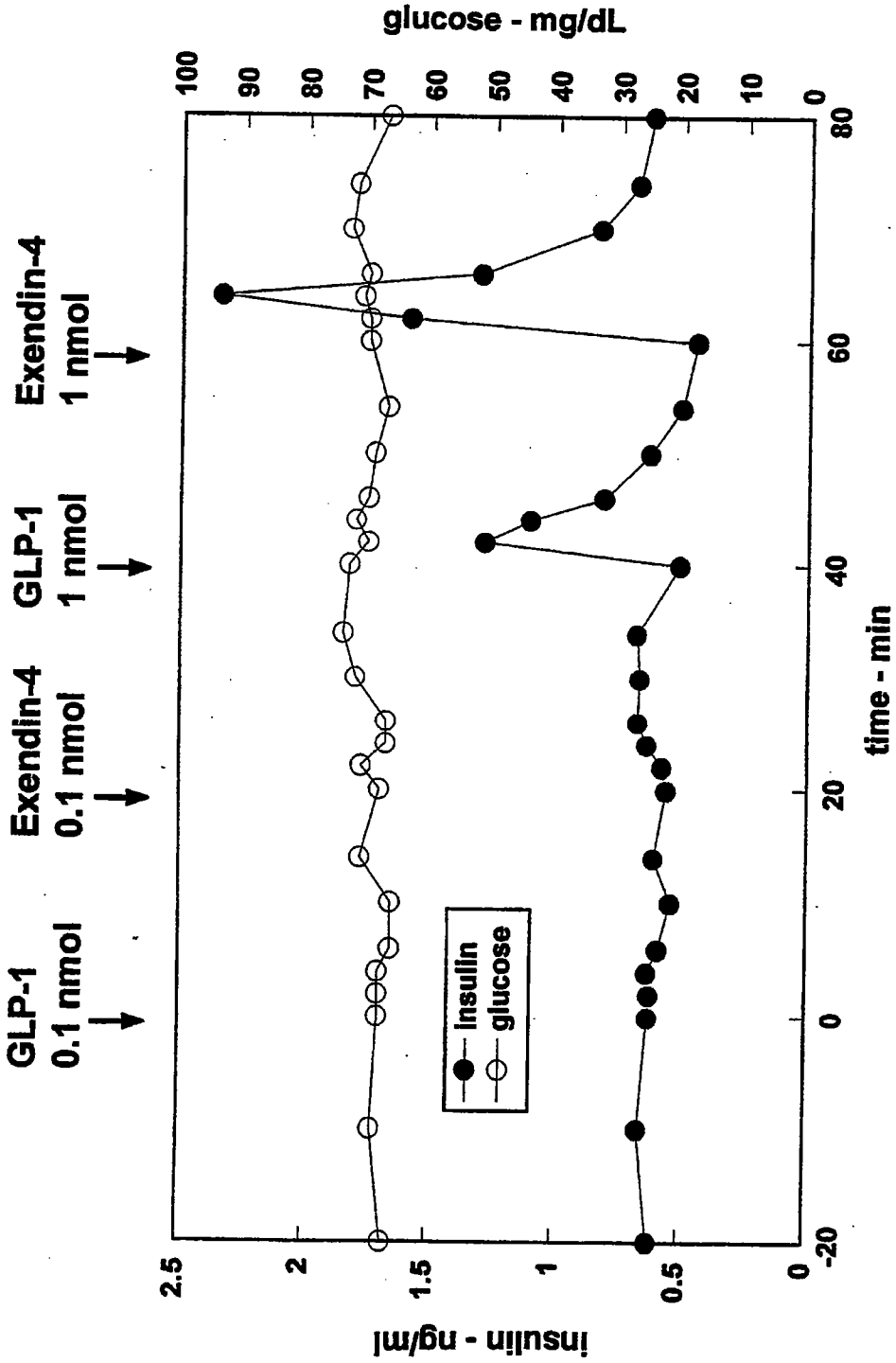
glucose - mg/dL
100
90
80
70
60
50
40
30
20
10
0
80
60
40
20
0
-20
time - min
insulin - ng/ml
2.5
2
1.5
1
0.5
0
insulin
glucose
GLP-1
0.1 nmol
Exendin-4
0.1 nmol
GLP-1
1 nmol
Exendin-4
1 nmol

Fig. 2A

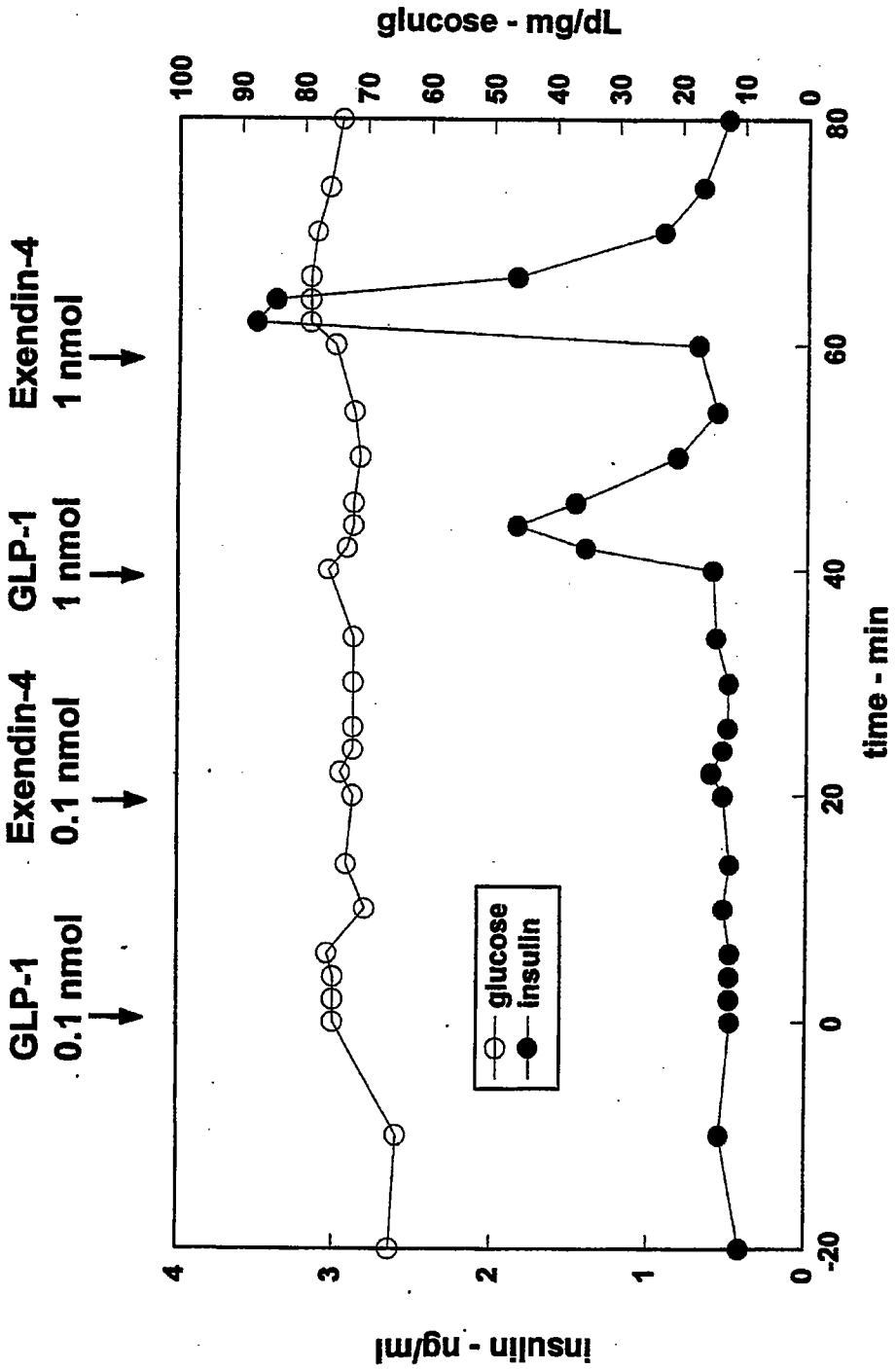
glucose - mg/dL
100
90
80
70
60
50
40
30
20
10
0
80
60
40
20
0
-20
time - min
insulin - ng/ml
4
3
2
1
0
glucose
insulin
GLP-1
0.1 nmol
Exendin-4
0.1 nmol
GLP-1
1 nmol
Exendin-4
1 nmol

Fig. 2B

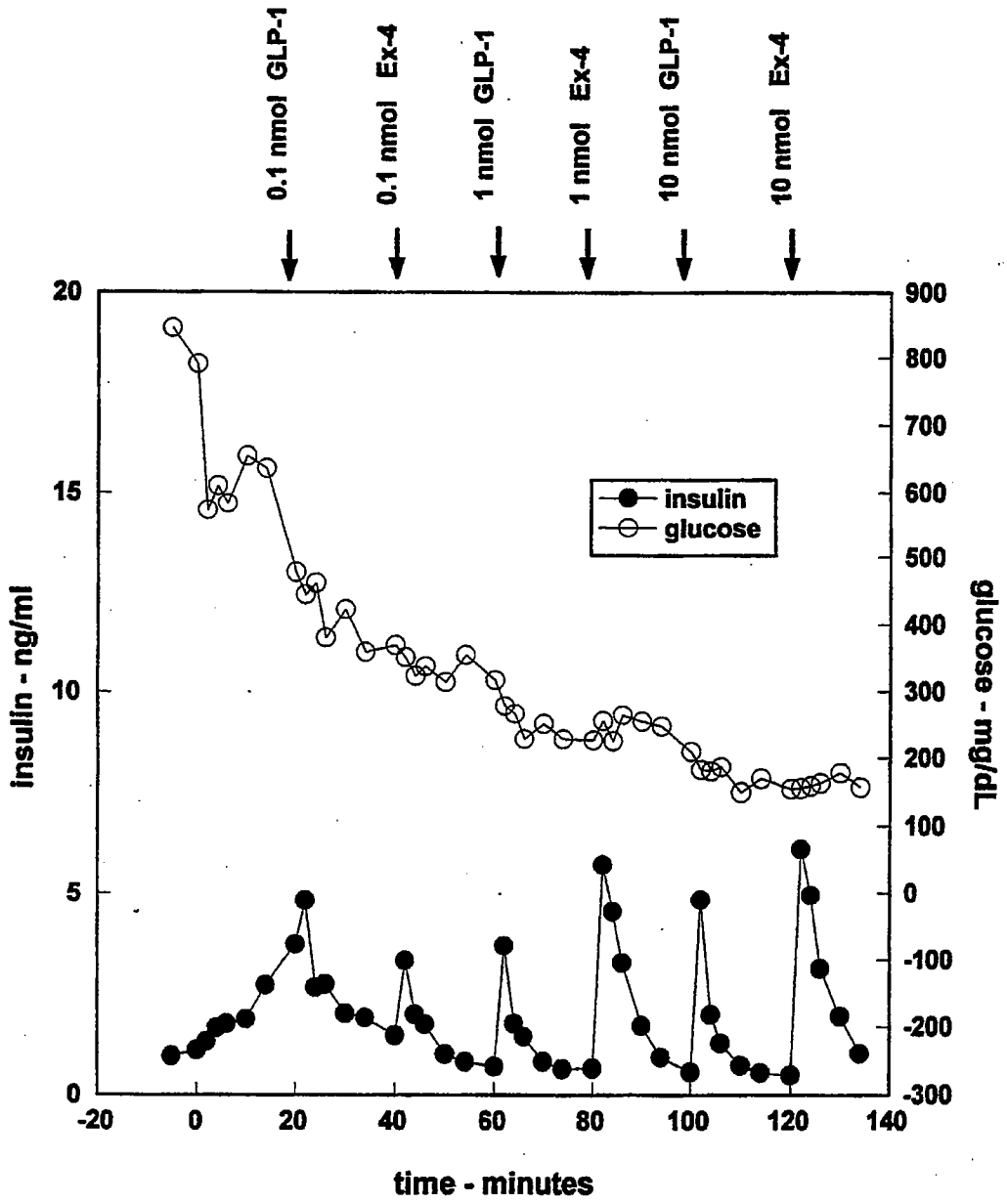
0.1 nmol GLP-1
0.1 nmol Ex-4
1 nmol GLP-1
1 nmol Ex-4
10 nmol GLP-1
10 nmol Ex-4
insulin
glucose
insulin - ng/ml
glucose - mg/dL
time - minutes
20
15
10
5
0
900
800
700
600
500
400
300
200
100
0
-100
-200
-300
-20
0
20
40
60
80
100
120
140

Fig. 2C

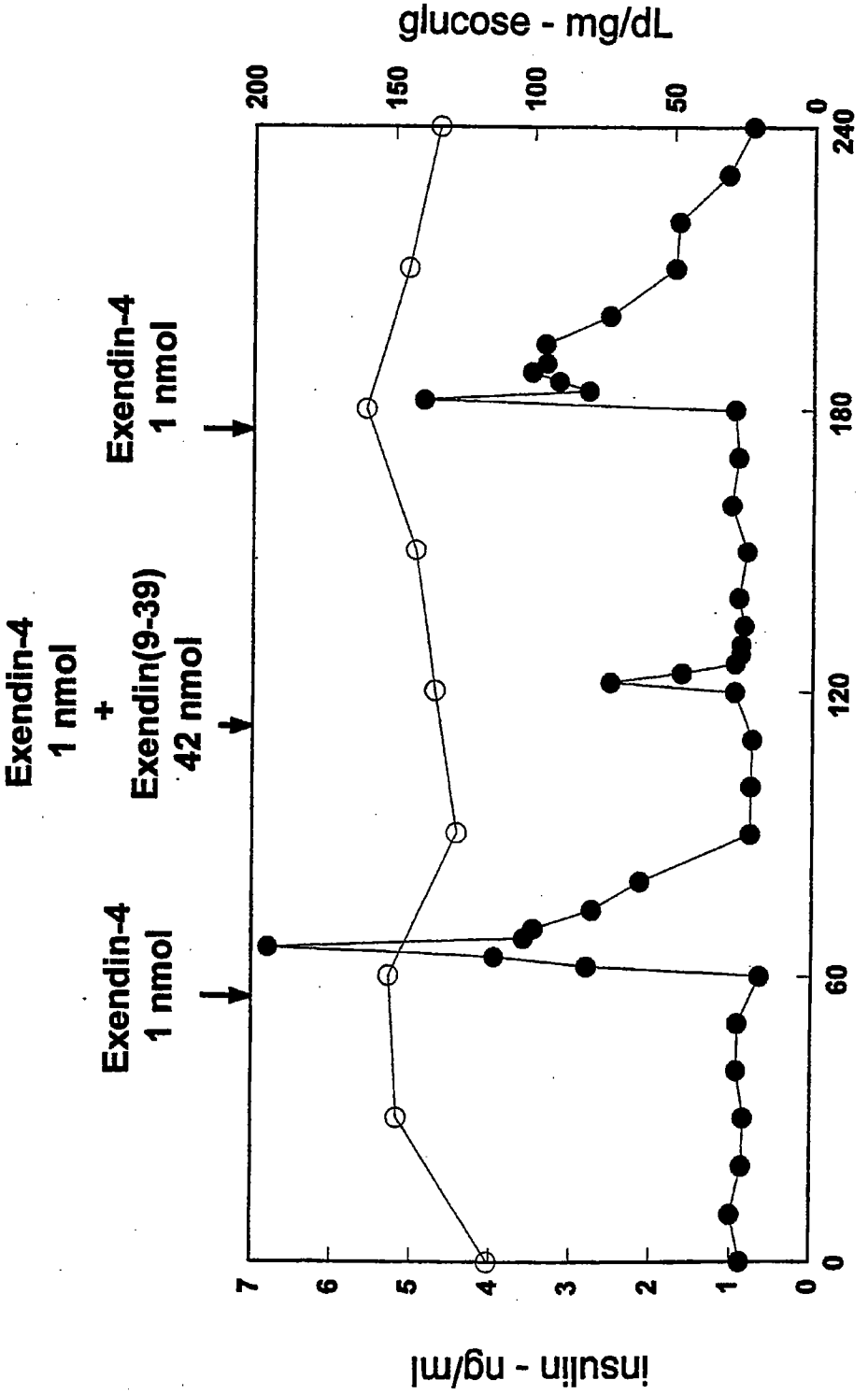
glucose - mg/dL
200
150
100
50
0
240
180
120
60
0
time - min
insulin - ng/ml
7
6
5
4
3
2
1
0
Exendin-4
1 nmol
Exendin-4
1 nmol
+
Exendin(9-39)
42 nmol
Exendin-4
1 nmol

Fig. 3

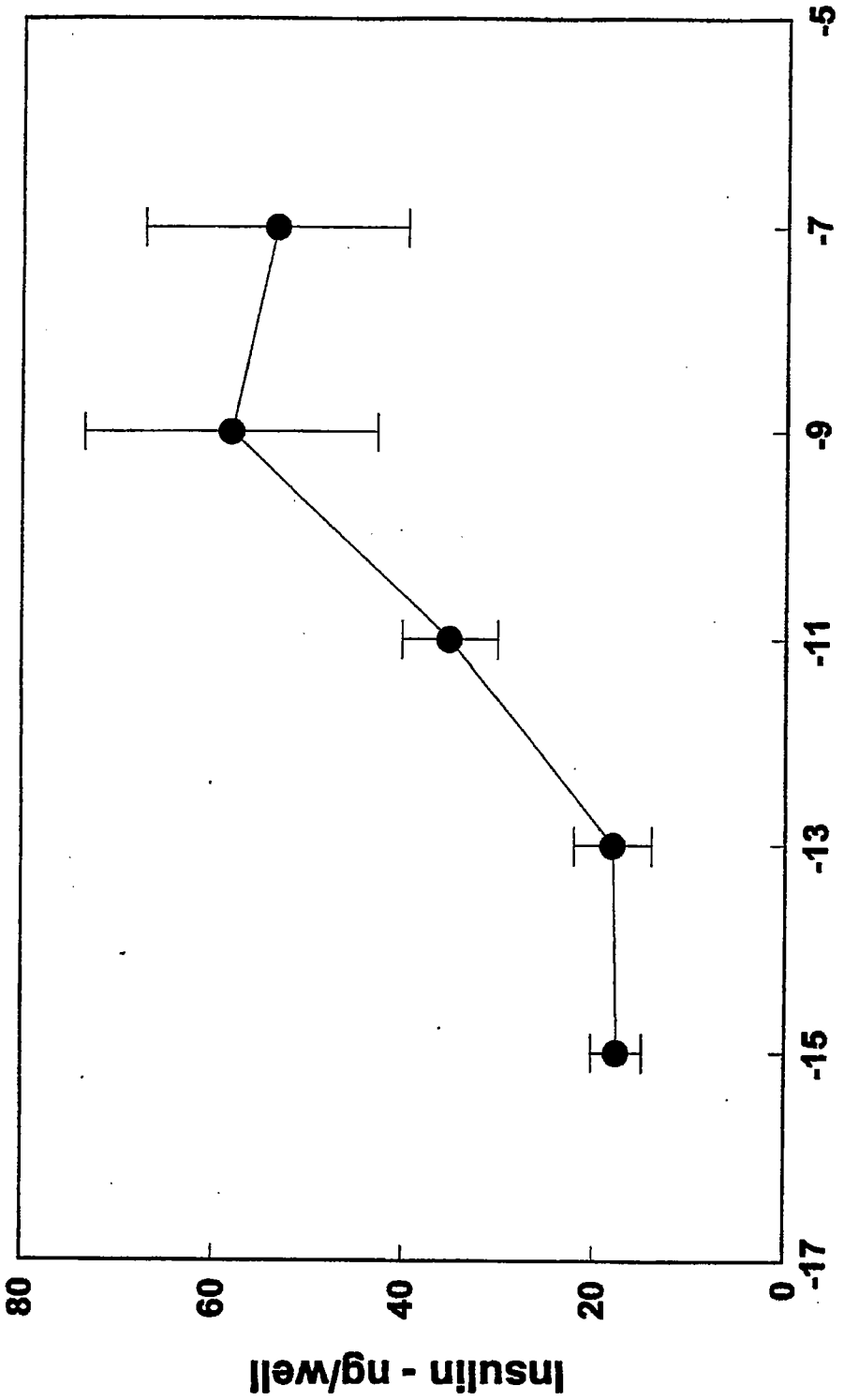

Fig. 4

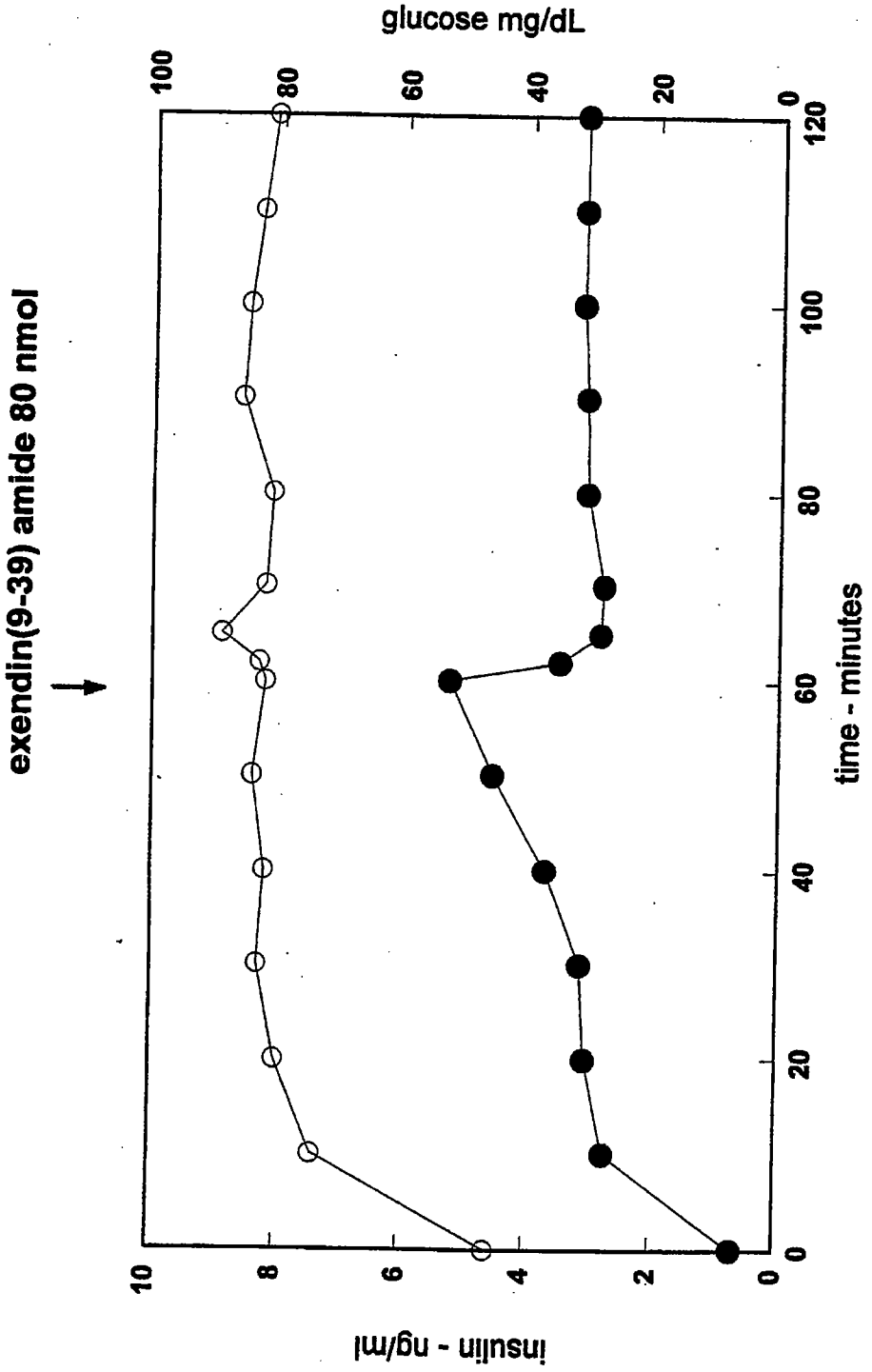
glucose mg/dL
100
80
60
40
20
0
120
100
80
60
40
20
0
time - minutes
exendin(9-39) amide 80 nmol
10
8
6
4
2
0
insulin - ng/ml

Fig. 5

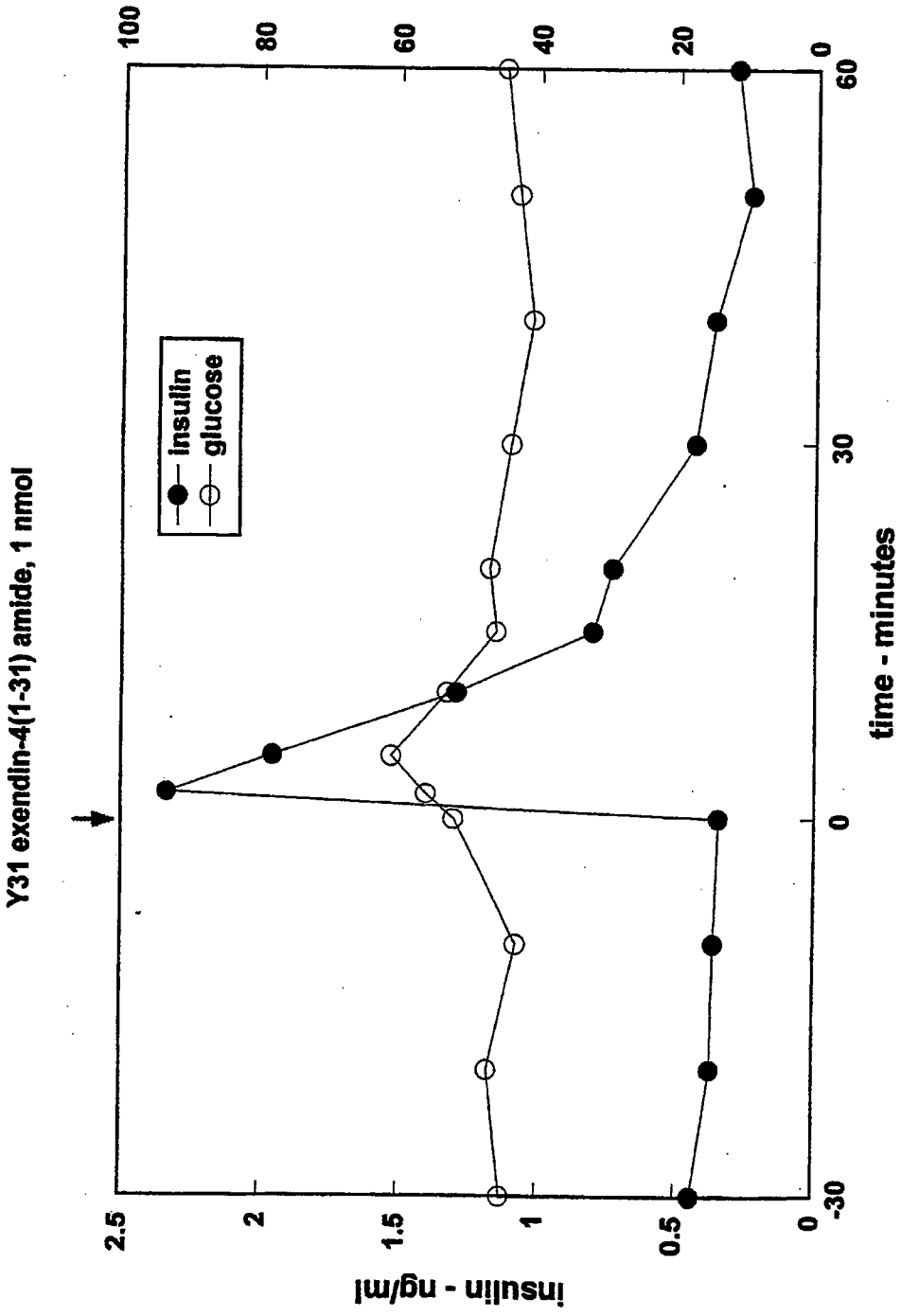
Y31 exendin-4(1-31) amide, 1 nmol
insulin
glucose
insulin - ng/ml
time - minutes
2.5
2
1.5
1
0.5
0
-30
0
30
60
100
80
60
40
20
0

Fig. 6

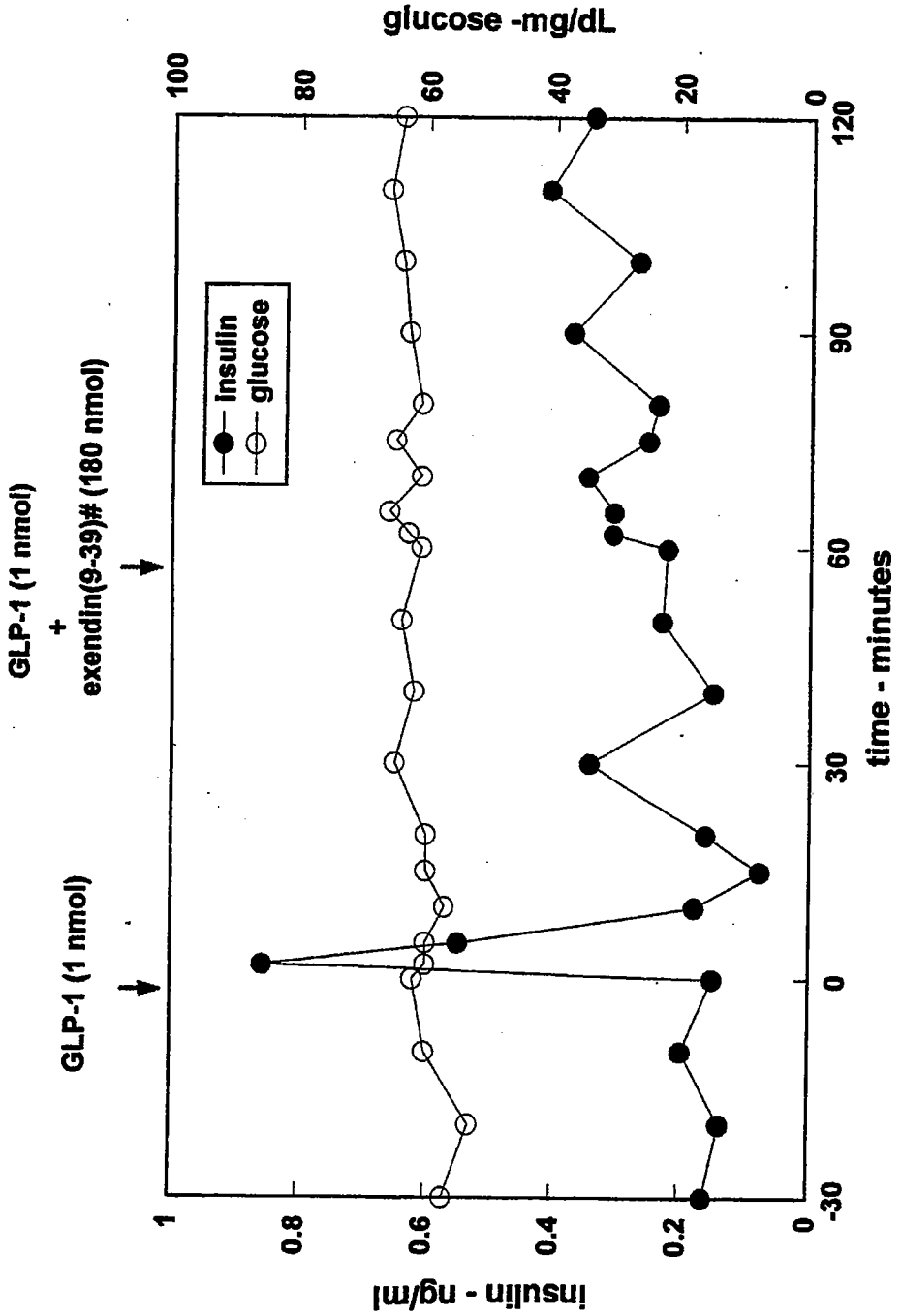
glucose -mg/dL
100
80
60
40
20
0
120
90
60
30
0
-30
time - minutes
insulin - ng/ml
1
0.8
0.6
0.4
0.2
0
insulin
glucose
GLP-1 (1 nmol)
GLP-1 (1 nmol)
+
exendin(9-39)# (180 nmol)

Fig. 7

EXENDIN-3 AND EXENDIN-4 POLYPEPTIDES, AND PHARMACEUTICAL COMPOSITIONS COMPRISING SAME

LICENSE RIGHTS

This U.S. Government has a paid-up license in this invention and the right in limited circumstances to require the patent owner to license others on reasonable terms as provided for by the terms of the agreement with the Department of Veterans Affairs, reference number 024I, GPB No. 20-560.

BACKGROUND OF THE INVENTION

1. Field of the Invention

This invention is in the field of the prevention and treatment of diabetes mellitus.

2. Description of the Prior Art

Diabetes mellitus (DM) is a major chronic illness found in humans with many consequences. Some complications arising from long-standing diabetes are blindness, kidney failure, and limb amputations. Insulin-dependent diabetes mellitus (IDDM) accounts for 10 to 15% of all cases of diabetes mellitus. The action of IDDM is to cause hyperglycemia (elevated blood glucose concentration) and a tendency towards diabetic ketoacidosis (DKA). Currently treatment requires chronic administration of insulin. Non-insulin dependent diabetes mellitus (NIDDM) is marked by hyperglycemia that is not linked with DKA. Sporadic or persistent incidence of hyperglycemia can be controlled by administering insulin. Uncontrolled hyperglycemia can damage the cells of the pancreas which produce insulin (the β-islet cells) and in the long term create greater insulin deficiencies. Currently, oral sulfonylureas and insulin are the only two therapeutic agents available in the United States. for treatment of Diabetes mellitus. Both agents have the potential for producing hypoglycemia as a side effect, reducing the blood glucose concentration to dangerous levels. There is no generally applicable and consistently effective means of maintaining an essentially normal fluctuation in glucose levels in DM. The resultant treatment attempts to minimize the risks of hypoglycemia while keeping the glucose levels below a target value. The drug regimen is combined with control of dietary intake of carbohydrates to keep glucose levels in control.

A fragment of human peptide molecule called, glucagon-like peptide-1 (GLP-1) has been found to be a glucose-dependent insulinotropic agent (Gutniak, M., et al. N. Engl. J. Bled. 1992; 326:1316–1322). GLP-1 is itself a fragment of the human proglucagon molecule. Another active fragment, glucagon-like insulinotropic peptide (GLIP), corresponds to GLP-1(7-36). It was reasoned that since GLIP is the naturally active form found in humans after a meal, this peptide may aid in glucose regulation in IDDM and NIDDM.

In normal subjects, the infusion of GLIP significantly lowered the meal-related increases in blood glucose concentration, and the plasma concentrations of insulin and glucagon. In patients with NIDDM, the treatment reduced the requirement for insulin by 8 fold. In patients with IDDM, the GLIP treatment lowered the insulin required by one half. This glucose-dependent activity is a very desirable characteristic for a therapeutic agent that can be used to treat DM avoiding tile complications of hypoglycemic side effects.

In 1981, it was discovered that Gila monster (Heloderma suspectum) venom stimulated pancreatic enzyme secretion in vitro (Raufman, J. P., et al., *Gastroenterology* 80:1257 abst. (1981); Raufman, J. P., et al., *Am. J. Physiol.* 242: G470-G474 (1982)). Several peptides have been isolated from the venom that can stimulate increased cAMP and amylase release from dispersed pancreatic acinar cells. These structural analogs to the mammalian peptides VIP (vasoactive intestinal peptide) and secretin include helospectin-I, helospectin-II (Parker, D. S. et al., *J. Biol. Chem.* 259:11751–11755 (1984)),and helodermin (Hoshino, M. et al., *FEBS Lett.* 178:233–239 (1984)). Recently, we discovered another peptide that increases cAMP and stimulated the release of amylase in dispersed acinar cells. This peptide was found in *Heloderma horridum* venom and was termed exendin-3 (Eng, J. et al., *J. BIol. Chem.* 265: 20259–20262 (1990). Exendin-3 shares homology with VIP, secretin, helospectin-I and -II, and helodermin. The venom of *Heloderma suspectum* was examined and another peptide was purified from it. This peptide called exendin-4 is an analogue of exendin-3 with an identical sequence except for substitutions in residues 2 and 3 from the amino terminus (Eng, J. et al.,*J. Biol. Chem.* 267:742–7405-(1992)). Experiments were done to establish that the exendins could stimulate cAMP activity in dispersed pancreatic acinar cells, and a specific antagonist, exendin(9-39) amide, which can inhibit the effects of the exendins, was identified. (Raufman, J. P. et al., *J. BIol. Chem.* 266: 2897–2902 (1991)) Experiments were performed to establish that GLP-1 could interact with possible exendin receptors in dispersed pancreatic acinar cells in vitro (Raufman, J. P. et al., *J. BIol. Chem.* 267:21432–21437 (1992)).

SUMMARY OF THE INVENTION

This invention encompasses pharmaceutical compositions containing exendin-3 or exendin-4, or any combination thereof, and methods for the treatment of diabetes mellitus and the prevention of hyperglycemia.

The compositions of the invention will normalize hyperglycemia through glucose-dependent, insulin-dependent and insulin-independent mechanisms. Therefore they will be useful as primary agents for the treatment of type II diabetes mellitus and as adjunctive agents for the treatment of type I diabetes mellitus. The invention specifically provides for exendin-4(1-39) as an insulinotropic agent.

The use of an effective amount of exendins as a treatment for diabetes mellitus has the advantage of being more potent than other insulinotropic peptides. The present invention is especially suited for the treatment of patients with diabetes, both type I and type II, in that the action of the peptide is dependent on the glucose concentration of the blood, and thus the risk of hypoglycemic side effects are greatly reduced over the risks in using current methods of treatment. Thus the use of insulinotropic peptides such as exendin-3 and exendin-4, has many advantages in the treatment of diabetes mellitus over current methods.

The present invention also provides for inhibitory agents derived from the exendins. In particular, exendin-4(9-39) as an inhibitor of exendin-4 and GLP-1 insulinotropic activity.

The present invention also provides for a method for treating diabetes mellitus in an individual, wherein said method comprises providing an amount of an insulinotropic composition sufficient to treat said diabetes; said

composition containing an insulinotropic molecule; wherein said molecule is selected from the group consisting of:

(a) a peptide having the amino acid sequence substantially identicle to the sequence of exendin-3 or exendin-4 or fragments thereof; and

(b) a derivative of said peptide (a), wherein said derivative is selected from the group consisting of:

(1) a pharmaceutically acceptable acid addition salt of said peptide;

(2) a pharmaceutically acceptable carboxylate salt of said peptide;

(3) a pharmaceutically acceptable lower alkyl ester of said peptide; and,

(4) a pharmaceutically acceptable amide of said peptide wherein said pharmaceutically acceptable amide is selected from the group consisting of amide, lower alkyl amide and lower dialkyl amide; wherein said molecule has an insulinotropic activity which exceeds the insulinotropic activity of exendin-3 or exendin-4 or fragments thereof.

Thus the invention provides for the peptides or peptide fragments, made synthetically or purified from natural sources, which embody the biological activity of the exendins, or fragments thereof, as described by the present specification.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a graph showing exendin stimulated insulin secretion in a dog. Endogenous insulin secretion stimulated by exendin-3 (200 nmol) in a conscious dog. Exendin-3 was given as a bolus injection into a leg vain at time 0. Plasma was measured by radioimmunoassay.

FIG. *2a* & *2b* & *2c* are graphs showing the serial injection of GLP-1 and Exendin-4. FIG. *2a*, Dog #1. FIG. *2b*, Dog #2. FIG. *2c*, Dog #3. Serial injections of GLP-1(7-36) amide alternating with exendin-4 into the left atrium via a chronically indwelling catheter. GLP-1(7-36) amide was given at time 0 (0.1 nmol) and at 40 min (1 nmol). Exendin-4 was given at 20 min (0.1 nmol) and at 60 min (1 nmol). In *2c*, the rise and fall in the baseline insulin between time 0 and 60 min is unexplained.

FIG. 3 is a graph illustrating the effect of exendin with and without antagonist. Insulin response in a normal dog to exendin-4 with or without exendin(9-39) amide. Glucose was infused at 100 mg/min. Exendin-4 (1 nmol) was given as an intravenous bolus at 60, 120 and 180 min. Exendin(9-39) amide, 42 nmol, was given together with exendin-4 at 120 min. The first phase of insulin release is greatly reduced and the second phase is abolished by this ratio of antagonist to agonist.

FIG. 4 is a graph illustrating the effect of exendin on cultured beta cells. βTC-3 cell insulin response to exendin-4, insulin mg/well vs. exendin-4 logM.

FIG. 5 is a graph demonstrating the effect of exendin antagonist on glucose -stimulated increase in insulin. Conscious dog infused with glucose at 200 mg/min beginning at time 0. A bolus injection of exendin(9-39) amide was made at 60 minutes.

FIG. 6 is a graph illustrating the effect of Y31 exendin-4(1-31) amide. Conscious, fasted dog injected with a bolus of Y31 exendin-4(1-31) amide at time 0.

FIG. 7 is a graph illustrating the effect of GLP-1 with and without antagonist. Insulin responses in a fasted dog to GLP-1 (1 nmol) injected either alone at time 0 or together with exendin(9-39) amide (180 nmol) at time 60 min. GLP-1's insulinotropic activity is inhibited by exendin (9-39) amide.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides for novel polypeptides which are unexpectedly useful as insulinotropic agents. Insulinotropic agents being agents which can stimulate, or cause the stimulation of, the synthesis or expression of the hormone insulin. The polypeptides of the present invention are termed exendin-3 and exendin-4. These peptides were originally isolated from the venom of *Heloderma horridum* and *Heloderma suspectum* respectively. In one embodiment of the invention, polypeptides corresponding to the amino acid sequence of exendin-3 and exendin-4 are synthesized by the solid phase method as previously described (Merrifield, J. M., *Chem. Soc.* 85: 2149 (1962); Stewart and Young, *Solid Phase Peptide Synthesis*, Freeman, San Francisco, 1969, pp. 27–66). In addition, it is also possible to isolate naturally occuring polypeptides from venom samples in a fashion similar to the original isolation of exendins 3 and 4. It is further possible to obtain the desired polypeptides by using recombinant DNA techniques (Maniatis, T. et al., *Molecular Biology: A Laboratory Manual*, Cold Spring Harbor, N.Y., 1982). The invention encompasses polypeptides which are insulinotropic and can be derived from naturally-occuring amino acid sequences. These proteins consist of the following amino acid sequences:

Exendin-3 [SEQ ID No:1] HSDGTFTSDL SKQMEEEAVR LFIEWLKNGG PSSGAPPPS

Exendin-4 [SEQ ID No:2] HGEGTFTSDL SKQMEEEAVR LFIEWLKNGG PSSGAPPPS

The invention also encompasses the insulinotropic fragments of exendin-4 comprising the amino acid sequentes:

Exendin-4(1-31) [SEQ ID No:3] HGEGTFTSDL SKQMEEAVR LFIEWLKNGG P

y^{31} Exendin-4(1-31) [SEQ ID No:4] HGEGTFTSDL SKQMEEEAVR LFIEWLKNGG Y

The invention also encompasses the inhibitory fragment of exendin-4 comprising the amino acid sequence: Exendin-4(9-39) [SEQ ID No:5] DL SKQMEEEAVR LFIEWLKNGG PSSGAPPPS

The invention further encompasses a method for the enhancement of insulin production or expression which comprises the steps of providing to a mammalian beta type pancreatic islet cell an effective amount of the insulinotropic peptides disclosed above.

Also provided for by the present invention are those amino acid sequences in the above peptides which are capable of functioning as insulinotropic hormones. In addition, the invention also provides for the addition of amino acids to enhance attachment to carrier molecules, or added to enhance the insulinotropic effect.

A material is said to be "substantially free of natural contaminants" if it has been substantially purified from materials with which it is normally and naturally found. Examples of natural contaminants of exendin-3 or exendin-4 are: other peptides, carbohydrates, glycosylated peptides, lipids, membrane, other venom components etc. A material is also said to be substantially free of natural contaminants if these contaminants are substantially absent of from a sample of the material.

The compounds of the present invention can be formulated according to known methods to prepare pharmaceutically useful compositions. In these composi-

tions, exendin-3 and or exendin-4, or their functional derivatives are combined in admixture with a pharmaceutically acceptable carrier vehicle. Suitable vehicles and their formulations, inclusive of other human proteins, e.g. human serum albumin, are well known. In order to form an effective pharmaceutical composition, the composition will contain an effective amount of the exendin-3 or exendin-4, or functional derivatives together with a suitable amount of carrier vehicle. Other compositions may combine exendin-3 and exendin-4, or their functional derivatives with other effective drugs that may treat other symptoms, or the same symptoms.

The use of exendin-3 and 4 in compositions that may be injected intravenously, intramuscularly, subcutaneously, or intraperitoneally, would call for dosages of about 0. 1 pg/kg to 1,000 mg/kg body weight depending on many individual factors such as age, severity of disease, total body weight, sex and other mitigating factors.

The insulinotropic properties of a compound may be determined by in vitro or in vivo assay. The compound in question may be administered to animals and monitoring the release of insulin. It is possible to monitor the increase in insulin production in cell culture as well.

The sequences of the invention also provide a means for identifying any specific mamalian analogs to the exendins. This can be accomplished by direct comparison of amino acid sequences, or by the translation of RNA or DNA sequences which may encode for the amino acid sequences of the invention, or by inhibition of activity by the specific exendin inhibitor, exendin (9-39) amide.

The sequences of the invention also provides a means for generating antibodies specific for the exendins, and further for the production of monoclonal antibodies for the exendins and fragments thereof. Thus the invention provides a means for purifying mammalian or other analogs to the exendins by the method of affinity chromatography.

Specific Examples

Testing was done to establish if exendin-3 or exendin-4 could stimulate pancreatic insulin secretion in mammals. Since both exendin-3 and exendin-4 peptides have about 50% homology with glucagon and GLP-1(7-36) (glucagon-like peptide-1), and GLP-1(7-36) was found to bind to exendin receptors, it was thought possible that exendins could act in similar fashion as GLP-1 on other receptors.

TABLE 1

Exendin-3	HSDGTFTSDL	SKQMEEEAVR	LFIEWLKNGG	PSSGAPPPS
Exendin-4	HGEGTFTSDL	SKQMEEEAVR	LFIEWLKNGG	PSSGAPPPS
GLP-1	HAEGTFTSDV	SSYLEGQAAK	EFIAWLVKGR	
Glucagon	HSQGTFTSDY	SKYLDSRRAQ	DFVQWLMNT	

Polypeptides corresponding to the amino acid sequence of exendin-3 and exendin-4 were synthesised by the solid phase method as previously described (Merrifield, J. M., *Chem. Soc.* 85:2149 (19625; Stewart and Young, *Solid Phase Peptide Synthesis*, Freeman, San Francisco, 1969, pp. 27–66). It is also possible to isolate naturally occuring polypeptides from venom samples in a fashion similar to the original isolation of exendins 3 and 4. It is further possible to obtain the desired polypeptides by using recombinant DNA techniques (Maniatis,T. et al., *Molecular Biology: A Laboratory Manual*, Cold Spring Harbor, N.Y., 19825).

The examples which follow are illustrative of specific embodiments of the invention, and various uses thereof. They are set forth for explanatory purposes only, and are not to be taken as limiting the invention.

Example 1

The exendins are insulinotropins

Naural or synthetic exendin-3 and exendin-4 were tested in several biological systems, including conscious dog, anesthetized dog with chronic indwelling left atrial catheters, and beta TC-3 insulinoma cell line (described in D'Ambra et al., *Endocrinology* 126:2815–2822 (1990)) in cell culture. FIG. 1 shows an insulin secretory response to bolus injection of exendin-3 in a conscious dog with a seven-fold increase in insulin concentration above basal levels. Similar results are obtained using exendin-4. Since exendin-4 does not interact with VIP receptors and acts solely on exendin receptors, it has been used for subsequent studies.

Example 2

Exendin-4 insulin secretagogue activity is glucose dependent

Dogs with glucose concentrations clamped at graded levels show a glucose-dependent insulinotropic response to exendin-4. Dosages of exendin-4 which do not stimulate insulin release at fasting glucose concentrations of 50–75 mg/dL (such as 0.1 nmol exendin-4 given as a bolus) are able to produce a peak insulin response of one-fold above basal when given to dogs in a clamped, hyperglycemic state.

Exendin-4 stimulates a greater insulin secretory response than GLP-1

Synthetic exendin-4 was compared with GLP-1 (purchased from Peninsula Labs, Belmont, Calif.) by alternating injections of bolus doses into dogs with chronic indwelling left atrial catheters. Since GLP-1 and exendin-4 are glucose dependent in their insulinotropic response, paired equimolar doses of GLP-1 and exendin-4 were given with GLP-1 administered first to avoid the possibility that falling glucose levels in the animals cause a diminished insulinotropic response to GLP-1 relative to exendin-4. Dogs #1 and #2 in FIGS. 2*a* and 2*b* maintained constant fasting glucose concentrations throughout the experiments in a range between 60 and 80 mg/dL. FIGS. 2*a*, 2*b* and 2*c* show a comparison of insulinotropic responses to alternating bolus injections of GLP-1 and exendin-4 at 20 minute intervals and at increasing doses ranging from 0.1 nmol to 10 nmol administered through chronic indwelling left atrial catheters into anesthetized dogs.

In contrast to the euglycemia present in the first two dogs, the third dog in FIG. 3*c* was exceptionally hyperglycemic, probably as a result of an infected catheter. Several points are illustrated by this experiment. First, euglycemic dogs normally do not respond to 0.1 nmol of either GLP-1 or exendin-4 with an insulin secretory resonse as illustrated by the first two dogs, whereas the hyperglycemic dog had clear insulinotropic responses to this lower dose of peptide. Second, the rapid normalization of hyperglycemia to euglycemic levels follow-

ing modest doses of the two peptides reflects the great potential for use of these peptides in treatment of diabetic states. Third, despite the rapid normalization of the hyperglycemia, hypoglycemia does not occur. This class of therapeutic agents might be termed "euglycemic" agents. The potential for hypoglycemia caused by overdosages of these agents is minimized. Hypoglycemia is avoided even when the agents are given in the euglycemic state. Fourth, despite the administration of exendin-4 following an equivilent dose of GLP-1 in the setting of decreasing glucose levels, the insulin response as defined by area under the curve, is consistently 2–3 fold greater for exendin-4 compared to GLP-1. The greater response to exendin-4 holds true for the two euglycemic animals as well. A summary of the insulin responses is shown in Table 2.

TABLE 2

Dog	Dose	AUC (GLP-1)	AUC (EX-4)	EX-4/GLP-1
#1	1 nmol	2.0	4.1	2.1
#2	1 nmol	2.8	7.3	2.6
#3	1 nmol	5.0	12.9	2.6
	10 nmol	5.7	14.2	2.5

Table 2 shows the relative ratio of insulin secretion stimulated by serial injections of GLP-1 (7-36) amide and exendin-4 expressed as area under the curve (AUC). AUC=T-B where T=total insulin secreted (sum of concentrations at times 2,4,6, 10 mad 14 min. and B=baseline insulin=average of insulin concentrations at times 0 and 20 min. Multiplied by a factor of 5.

Example 3

Exendin(9-39) amide inhibits endogenous, exendin-4, and GLP-1 insulinotropic activity.

Antagonistic peptides can arise by a number of mechanisms. The gene encoding the exendins may also encode for related peptides which have antagonistic activity. The production of antagonistic peptides may then be either initiated or suppressed by differential cleavage of the pre-propeptide. The antagonistic peptides may also arise through post-translational modification of the agonist peptide, specifically through differential cleavage to produce extended or truncated forms of the agonist peptide. In our studies of the structure-function relationship of exendin peptide sequences, the NH_2-terminally truncated exendin analog, exendin(9-39) amide, was shown to have potent antagonistic activity against exendin-3 and exendin-4 in a pancreatic acinar cell system measuring cAMP activity. (Raufman et al., *J. Biol. Chem.* 266:2897–2902 (1991); Eng et al., *J. Biol. Chem.* 267:7402–7505 (1992)). FIG. 5 shows the effect of exendin(9-39) amide when administered alone on circulating insulin levels while glucose levels are clamped at approximately 100% above fasting levels. Following injection of the antagonist there is a rapid decrease in circulating insulin levels to 60% of the maximum concentration. This result indicates that the antagonist inhibited an endogenous insulinotropin that accounted for a substantial portion of the insulin secretory response to hyperglycemia.

When exendin(9-39) amide is given together with exendin-4 at a molar ratio of 40:1, there is substantial inhibition of the insulin secretory response, as shown in FIG. 3. The second phase of insulin release is completely inhibited while the first phase is more resistant to complete inhibition. This finding suggests a differential sensitivity to inhibition between the first and second phases of insulin release. A pathological condition which may correlate to this phenomenon is a loss of first phase insulin secretion in type 2 diabetes.

When exendin(9-39) amide is given together with GLP-1 at molar ratio of 180:1 there is substantial inhibition of the insulin secretory response, as shown in FIG. 7.

Example 4

Exendin-4 acts directly on the beta cell

Beta TC-3 cells were obtained through Norman Fleischer (Diabetes Research and Training Center, Albert Einstein College of Medicine, N.Y.) and cultured in serum-containing media in 48-well culture dishes to confluency. Fresh media was added 24 hours before the cells were tested. The cells were tested in Earle's balanced salt solution containing IBMX, BSA and 16.7 mM glucose with graded concentrations of exendin-4 for 1 hour at 37° C. before collection of media supernate and assay for insulin concentrations. FIG. 4 shows a dose response curve to exendin-4 indicating that exendin-4 acts directly on beta cells to stimulate insulin secretion.

Example 5

Exendin-4 reduces the hyperglycemic state in a diabetic animal model

The db/db mouse is a genetically obese and diabetic strain of mouse. The db/db mouse develops hyperglycemia and hyperinsulinemia concomitant with its development of obesity and thus serves as a model of obese type 2 diabetes (NIDDM). Five 11-week old db/db mice purchased from The Jackson Laboratories (Bar Harbor, Me.) had sub-orbital sinus blood samples taken before and 60 minutes after intraperitoneal injection of exendin-4 at 10 nmol each animal (1 microgram/gram body weight). Blood glucose measurements were made with a glucose meter (YSI 1500 glucose analyzer, Yellow Springs, Ohio). The blood glucose levels in the animals were (average±standard error, in mg/dL glucose) 310±37 before and 181±37 one hour after administration of exendin-4. Thus, exendin-4 was able to reduce the diabetic levels of blood glucose by 40% in these animals.

Example 6

We have compared the effect of COOH-terminal truncations on the insulinotropic activity of exendin-4. The Y^{31} mutation of exendin-4(1-31) amide has a TYR for PRO substitution at position 31 from the amino terminus. This mutant was shown to have insulinotropic activity when infused into dog. FIG. 6 shows this result. This result indicates that the amino acids in the exendin-4 sequence located between residues 1 and 31 are important for the insulinotropic activity.

This invention thus provides for compounds that are an unexpectedly efficient means of stimulating insulin production in vitro and in vivo that will be useful for the treatment of diabetes mellitus, as well as specific inhibitors therof.

-continued

(1) GENERAL INFORMATION:

(i i i) NUMBER OF SEQUENCES: 7

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: peptide

(i x) FEATURE:
(A) NAME/KEY: Peptide
(B) LOCATION: 1..39
(D) OTHER INFORMATION: /label=Exendin-3

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
His Ser Asp Gly Thr Phe Thr Ser Asp Leu Ser Lys Gln Met Glu Glu
1               5                  10                  15

Glu Ala Val Arg Leu Phe Ile Glu Trp Leu Lys Asn Gly Gly Pro Ser
            20                  25                  30

Ser Gly Ala Pro Pro Pro Ser
        35
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: peptide

(i x) FEATURE:
(A) NAME/KEY: Peptide
(B) LOCATION: 1..39
(D) OTHER INFORMATION: /label=Exendin-4

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
His Gly Glu Gly Thr Phe Thr Ser Asp Leu Ser Lys Gln Met Glu Glu
1               5                  10                  15

Glu Ala Val Arg Leu Phe Ile Glu Trp Leu Lys Asn Gly Gly Pro Ser
            20                  25                  30

Ser Gly Ala Pro Pro Pro Ser
        35
```

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: peptide

(i x) FEATURE:
(A) NAME/KEY: Peptide
(B) LOCATION: 1..31
(D) OTHER INFORMATION: /label=Exendin-1-31
/ note="Exendin-4(1-31)"

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
His Gly Glu Gly Thr Phe Thr Ser Asp Leu Ser Lys Gln Met Glu Glu
1               5                  10                  15

Glu Ala Val Arg Leu Phe Ile Glu Trp Leu Lys Asn Gly Gly Pro
            20                  25                  30
```

-continued

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: peptide

(i x) FEATURE:
(A) NAME/KEY: Peptide
(B) LOCATION: 1..31
(D) OTHER INFORMATION: /label=Y31-Exendin4
/ note="Y-31-Exendin-4(1-31)"

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
His Gly Glu Gly Thr Phe Thr Ser Asp Leu Ser Lys Gln Met Glu Glu
1               5                  10                  15

Glu Ala Val Arg Leu Phe Ile Glu Trp Leu Lys Asn Gly Gly Tyr
            20                  25                  30
```

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: peptide

(i x) FEATURE:
(A) NAME/KEY: Peptide
(B) LOCATION: 1..31
(D) OTHER INFORMATION: /label=Exendin-9-39
/ note="Exendin-4(9-39)"

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
Asp Leu Ser Lys Gln Met Glu Glu Glu Ala Val Arg Leu Phe Ile Glu
1               5                  10                  15

Trp Leu Lys Asn Gly Gly Pro Ser Ser Gly Ala Pro Pro Pro Ser
            20                  25                  30
```

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: peptide

(i x) FEATURE:
(A) NAME/KEY: Peptide
(B) LOCATION: 1..30
(D) OTHER INFORMATION: /label=GLP-1-7-36
/ note="GLP-1(7-36) fragment"

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```
His Ala Glu Gly Thr Phe Thr Ser Asp Val Ser Ser Tyr Leu Glu Gly
1               5                  10                  15

Gln Ala Ala Lys Glu Phe Ile Ala Trp Leu Val Lys Gly Arg
            20                  25                  30
```

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 amino acids
(B) TYPE: amino acid

-continued

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:
(A) NAME/KEY: Peptide
(B) LOCATION: 1..29
(D) OTHER INFORMATION: /label=Glucagon

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

```
His Ser Gln Gly Thr Phe Thr Ser Asp Tyr Ser Lys Tyr Leu Asp Ser
1               5                  10                  15

Arg Arg Ala Gln Asp Phe Val Gln Trp Leu Met Asn Thr
            20                  25
```

What is claimed is:

1. A polypeptide having the amino acid sequence: HGEGTFTSDL SKQMEEEAVR LFIEWLKNGG P[SEQ ID No:3].

2. A polypeptide having the amino acid sequence: HGEGTFTSDL SKQMEEEAVR LFIEWLKNGG Y[SEQ ID No:4].

3. A pharmaceutical composition which comprises an effective insulinotropic amount of a substantially pure polypeptide, synthetic or purified from natural sources, having the amino acid sequence of claim 1 in a suitable carrier, which will stimulate the secretion of insulin in vivo.

4. A pharmaceutical composition which comprises an effective insulinotropic amount of a substantially pure polypeptide, synthetic or purified form natural sources, having the amino acid sequence of claim 2 in a suitable carrier, which will stimulate the secretion of insulin in vivo.

5. A method of stimulating insulin release in a mammal comprising administering an effective insulinotropic amount of a substantially pure polypeptide, synthetic or purified from natural sources, having the amino acid sequence:

HSDGTFITSDL SKQMEEEAVR LFIEWLKNGG PSSGAPPPS (SEQ ID NO: 1),

wherein the resulting insulinotropic effect is greater than that attainable by administration of GLP-1.

6. A method of stimulating insulin release in a mammal comprising administering an effective insulinotropic amount of a substantially pure polypeptide, synthetic or purified from natural sources, having the amino acid sequence:

HGEGTFTSDL SKQMEEEAVR LFIEWLKNGG PSSGAPPPS (SEQ ID NO: 2),

wherein the resulting insulinotropic effect is greater than that attainable by administration of GLP-1.

7. A method of inhibiting insulin release in a mammal comprising administering an effective amount of a substantially pure polypeptide, synthetic or purified from natural sources, having the amino acid sequence:

DL SKQMEEEAVR LFIEWLKNGG PSSGAPPPS (SEQ ID NO: 5).

* * * * *

EXHIBIT C

Maintenance Fee Statement receipts received for U.S. Patent Number 5,424,286 for years four and eight

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If the statement of small entity status is defective the reason will be indicated below in the "Small Entity" status column. THE STATEMENT OF SMALL ENTITY STATUS WILL BE ENTERED UPON RECEIPT OF ACCEPTABLE CORRECTION.

PATENT NUMBER	FEE AMT	SUR CHARGE	U.S. APPLICATION NUMBER	PATENT ISSUE DATE	APPL. FILING DATE	PAYMENT YEAR	SMALL ENTITY?	STAT	ATTY DKT NUMBER
5,424,286	**$525.00**	**$0.00**	**08/066,480**	**06/13/95**	**05/24/93**	**04**	**YES**	**PAID**	**93084**

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MAINTENANCE FEE STATEMENT

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PATENT NUMBER	FEE AMT	SUR CHARGE	U.S. APPLICATION NUMBER	PATENT ISSUE DATE	APPL. FILING DATE	PAYMENT YEAR	SMALL ENTITY?	STAT	ATTY DKT NUMBER
5,424,286	**$1,010.00**	**$0.00**	**08/066,480**	**06/13/95**	**05/24/93**	**08**	**YES**	**PAID**	**93084**

Direct any questions about this notice to:
Mail Stop M Correspondence
Director of the U.S. Patent and Trademark Office
P.O. Box 1450
Alexandria, VA 22313-1450

EXHIBIT D

FDA Fax

TELEFAX

TO: Sidney Gilman, PhD
Ref. NDA 57,725

FAX: 619-625-0737

PHONE:

FROM: Jean Webb

Food and Drug Administration
Division of Metabolic and Endocrine Drug Products
5600 Fishers Lane, HFD-510
Rockville, Maryland 20857-1706

FAX: (301)443-9282

PHONE: (301)827-6430

DATE: 2/10/99

PAGES: 02 (Inclusive)

Food and Drug Administration
Division of Metabolic and Endocrine Drug Products
5600 Fishers Lane-HFD-510
Rockville, Maryland 20857-1706

Amylin Pharmaceuticals, Inc.
9373 Towne Center Drive
San Diego, CA 92121

Attention: Sidney Gilman, Ph.D.

Fax: 619-625-0737

Ref: IND 57,725 (Exendin-4) original submission dated January 12, 1999.

We have completed our review of the clinical section of your IND submission, and your study may continue. However, given the number of hypotensive episodes that occurred in previous studies, we recommend that you include cardiac and blood pressure monitoring during your study. Please address this and any subsequent information in writing to your IND file.

This information was faxed to Dr. Gilman on February 10, 1999.

CLEARED FOR FAXING

2/9/99
Jena Weber, CSO

2/10/99
Saul Malozowski, M.D.

EXHIBIT D

FDA Fax

TELEFAX

TO: Sidney Gilman, PhD
RE: NDA 57,725

FAX: 619-625-0737

PHONE:

FROM: Jean Webb

Food and Drug Administration
Division of Metabolic and Endocrine Drug Products
5600 Fishers Lane, HFD-510
Rockville, Maryland 20857-1706

FAX: (301)443-9282

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9373 Towne Center Drive
San Diego, CA 92121

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Fax: 619-625-0737

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Jena Weber, CSO

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Saul Malozowski, M.D.