*Pharmacology Biochemistry & Behavior*, Vol. 38, pp. 909–912. © Pergamon Press plc, 1991. Printed in the U.S.A. 0091-3057/91 $3.00 + .00

BRIEF COMMUNICATION

# An Opioid Pancreatic Peptide Produces Ileal Muscle Inhibition and Naloxone-Reversible Analgesia

CHARLES D. KIMBALL,* MUZAFFAR IQBAL,† JEN-TZAW HUANG‡ AND DWIGHT SUTTON*

**Virginia Mason Research Center, 1000 Seneca Street, Seattle, WA 98101*
*†Calzyme Laboratories, Inc., 3443 Miguelito Ct., San Luis Obispo, CA 93401*
*‡JBC, Inc., 1378 Oakridge Ct., Thousand Oaks, CA 91362*

Received 13 August 1990

KIMBALL, C. D., M. IQBAL, J.-T. HUANG AND D. SUTTON. *An opioid pancreatic peptide produces ileal muscle inhibition and naloxone-reversible analgesia.* PHARMACOL BIOCHEM BEHAV **38**(4) 909–912, 1991.—The opioid activity of immunoreactive beta-endorphin-like peptide extracted from pork pancreas duplicates the effects of morphine and synthetic beta-endorphin when measured by inhibition of isolated guinea pig ileal muscle response to electro-stimulation in vitro and by morphine-like analgesia following intravenous injection in the mouse. These responses are reversed by the opiate antagonist naloxone, indicating that a potent opioid mu receptor binding ligand is present in pancreatic extract. These findings imply a pancreatic source of plasma immunoreactive beta-endorphin that may explain a number of physiological and behavioral effects generally attributed to hypophyseal beta-endorphin alone.

Pancreas Endorphin Opiates Analgesic agents

BETA-endorphin-like peptide was reported in extracts of human pancreas by RIA using monoclonal antibody to synthetic human beta-endorphin (2). This peptide was localized within islet of Langerhans $D_2$ cells of rat, guinea pig and man by immunohistochemistry (22). Immunoreactive beta-endorphin-like peptide which displaced $^{125}I$ naloxone from rat brain opiate receptors in RRA was extracted from pork pancreas and purified by HPLC in our laboratory. Cerebroventricular injection of minute amounts of this peptide produced tail-flick analgesia in mice (8).

We now report evidence of naloxone-reversible opioid activity of HPLC purified extracts from kilogram amounts of pork pancreas that yielded sufficient material to demonstrate biological characteristics when measured by the in vitro guinea pig ileal muscle twitch inhibition test (13) and the mouse tail-flick test (3) following intravenous injections.

## METHOD

Beta-endorphin immunoreactive peptide (opioid pancreatic peptide or OPP) was extracted from six kilograms of frozen pork pancreas divided into two lots. The lots were ground, stirred in 3 volumes of −24°C acetone and centrifuged. The defatted residues (approximately 450 g from each lot) were stirred overnight in 10 liters of 0.1 M HCl at 4°C and centrifuged at 11,000 × g for 20 minutes at 0°C. The two supernatants were combined.

The acid extract (12.2 liters) was adjusted to pH 7.0 at room temperature with 1.0 M Tris base and heated to 70°C for 20 minutes (to inactivate proteolytic enzymes) followed by cooling to 4°C and recentrifugation. The 11.3 liters of supernatant was concentrated by hollow fiber filtration to approximately 2 liters (excluding material less than 1000 mol.wt.), and freeze-dried to yield 160 g of powder.

The freeze-dried powder was chromatographed in 0.01 M sodium acetate buffer (pH 4.5) on a G-25 Sephadex column. The fractions were monitored for beta-endorphin reactivity by the New England Nuclear Company RIA Kit and for protein by the Lowry method (14). Reactive fractions were combined, freeze-dried, dissolved in distilled water and applied to a Sephadex G-50 column which had been equilibrated with sodium acetate buffer (pH 4.5) and standardized with bovine serum albumin and cytochrome C. The immunoreactive fractions eluting at the cytochrome C position were again assayed for beta-endorphin reactivity and protein, and then were freeze dried. This G-50 residue was dissolved in 0.05 M ammonium phosphate (pH 4.5), centrifuged and applied to Perkin Elmer automated HPLC. Aliquots of 1 ml were inserted on a 2.35 × 25 cm C-18 column to a 3–60% linear gradient of acetonitrile against ammonium phosphate. This separated ten sequential homogeneous peaks during 45 minutes at a flow rate of 10 ml/minute. The peak eluates were collected from the HPLC, pooled, desalted on a G-25 Sephadex column in 1.2 M

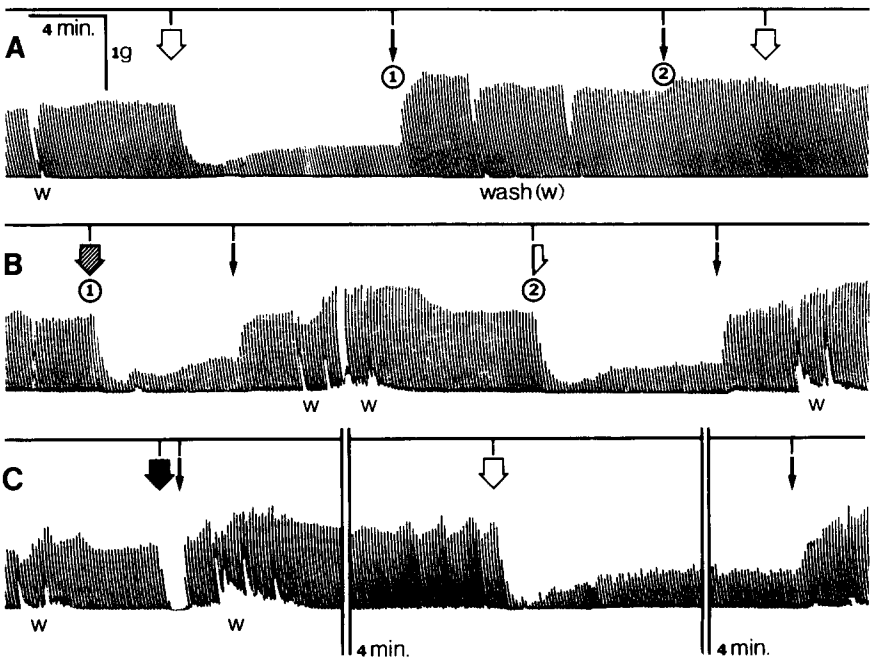


FIG. 1. Ileal muscle response to opioid pancreatic peptide. (A) Opioid pancreatic peptide (OPP) 20 μg (open arrow) added to 10 ml oxygenated Tyrode solution tissue bath inhibited the ileal muscle response to pulsed 10-second electro-stimuli (50 V, 0.5 ms). Inhibition reversed (1) and blocked (2) by addition of naloxone 0.2 μg (dart). (B) Ileal muscle response, (1) to synthetic beta endorphin 20 μg (shaded arrow) and, (2) to OPP 10 μg (½ open arrow) added to tissue bath; inhibition reversed by naloxone 0.2 μg. (C) Effect of morphine HCl 0.2 μg (solid arrow) followed by OPP 20 μg and reversed by naloxone 0.2 μg.

acetic acid, freeze-dried, dissolved in 1 ml deionized water and finally freeze-dried to approximately 700 μg of powder. Repetition of this extraction process yielded approximately 800 μg of powder.

*Receptor Specificity*

The opiate receptor binding effect on guinea pig ileum myenteric plexus was used to compare the effect of the pancreatic extract with morphine and synthetic beta-endorphin. Pieces of ileal muscle (2.0 cm), taken 15 cm proximal to the caecum of a freshly decapitated guinea pig, were suspended on a force transducer (Grass FT03) and maintained at 1 g tension in 10 ml of 32°C oxygenated Tyrode's solution. Coaxial electrodes were positioned to provide an electrical stimulation field across the muscle using rectangular constant voltage pulses (50 V; 0.5 ms) presented at 10-second intervals throughout the testing procedure. Testing consisted of adding prescribed amounts of each compound to the tissue bath while recording continuously the effects on twitch amplitude. Each test substance was followed at variable delays by introduction of naloxone to the tissue bath. New trials were preceded by multiple washouts with Tyrode's solution.

*Analgesia*

Sixteen Swiss-Webster mice (26–30 g) were utilized. Each animal was tested individually. The tail-flick latency response to a high intensity light beam focused on an ink-blackened spot located at midlength along the tail was recorded electronically on a custom-built tail-flick latency timing device that automatically terminated heat stimulation after ten seconds to avoid tissue injury. Five control trials at 2-minute intervals provided baseline latency data. Following the fifth control trial, nine animals were given tail vein injections of approximately 75 μg of the purified pancreatic extract dissolved in sterile water (0.1 cc). Four additional animals received 0.1 cc sterile water containing no peptide. All animals in each group then underwent five subsequent test trials, which recurred on a two-minute intertrial interval schedule. OPP recipients were then given 160 μg naloxone IV ten minutes after receiving OPP, and five additional tests of tail flick were recorded. Three animals were given 150 μg morphine IV for comparison with effects of OPP.

## RESULTS

*Experiment 1. Ileal Myenteric Plexus Response*

Profound suppression of ileum contraction occurred following application of OPP [Fig. 1A, B(2)], synthetic beta-endorphin (Fig. 1B), and morphine (Fig. 1C). All three substances yielded similar response patterns. At similar dose levels of OPP and synthetic beta-endorphin (20 μg), twitch magnitudes were reduced to approximately 12–15% of control values within 40–60 seconds. OPP at a reduced dosage (10 μg) exerted an effect similar to 20 μg synthetic beta-endorphin (Fig. 1B). The initial twitch reduction was maintained for 40–80 seconds, followed by a slight recovery to approximately 25–30% of control with no further change. Naloxone (0.2 μg) in each case resulted in recovery of baseline twitch performance within 30–90 seconds. Further, naloxone administered prior to OPP blocked the opioid response (Fig. 1A; dart 2). These data were subsequently reproduced on a second muscle strip preparation.

*Experiment II. Analgesic Effect: Tail-Flick Response*

The mean response latency for the control trials was 3.85 s.

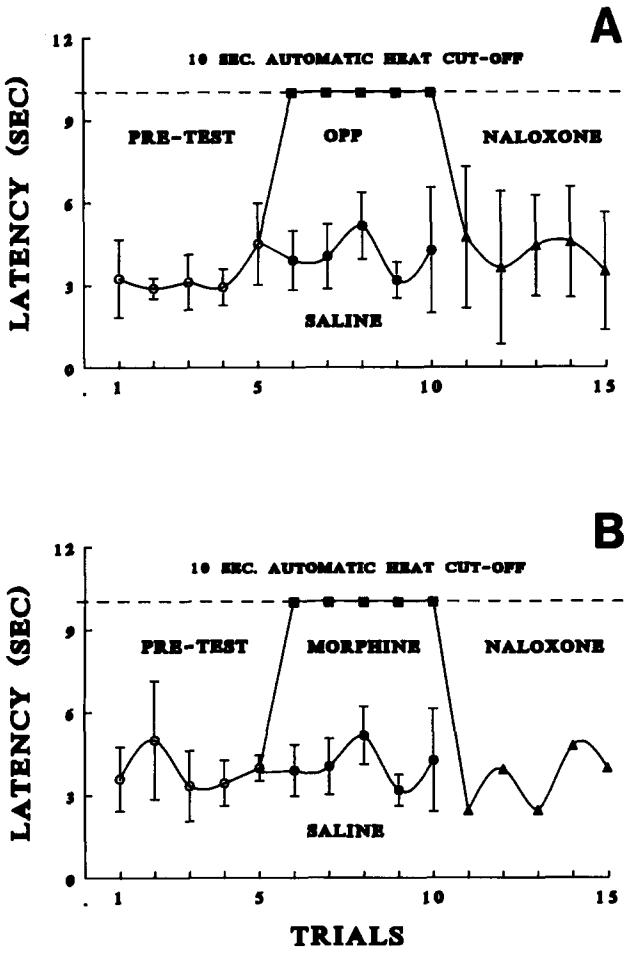


FIG. 2. Tail-flick response to opioid pancreatic peptide and morphine. (A) Open circles: Preinjection tail-flick response times of Swiss-Webster mice (22–25 g) to 5 exposures to radiant heat at 2 minute intervals, N = 16. Solid circles: Tail-flick latency of mice injected in tail vein with 0.1 ml normal saline, N = 6. Squares: Tail-flick latency of mice given opioid pancreatic peptide (OPP, approx.) 75 μg in tail vein exceeds automatic 10-second heat cut off, N = 10. Triangles: Tail-flick latency of mice given IV naloxone 160 μg 5–30 minutes subsequent to OPP injections, N = 10. (B) Open circles: Mean of 5 preinjection tail-flick latency trials, N = 7. Solid circles: Tail-flick latency following IV N. saline 0.1 ml, N = 4. Squares: Tail-flick latency after IV morphine HCl 150 μg exceeds automatic 10-second heat cutoff, N = 3. Triangles: Mean tail-flick response to naloxone 160 μg tail vein injection, N = 3.

All nine animals receiving IV OPP (approximately 75 μg) exhibited no tail-flick response to heat stimulation at the first postinjection test. The record reflects latencies in excess of 10 s to this and each of four subsequent exposures to the test stimulus (Fig. 2A). No overt tail flick was detected from any animal injected with OPP within the ten seconds maximum exposure time.

Upon completion of the five consecutive test trials following OPP injection, the mice were injected with 0.16 μg naloxone (again into the tail vein), and the tail-flick latency trials were continued, resulting in prompt restoration of normal response to the heat stimulus (mean latency: 5.40 s). Four animals subject to control injection (normal saline) exhibited no change from baseline response values (Fig. 2A). Tail-flick testing with three mice that received 150 μg morphine IV yielded typical response suppression which was reversed by administration of naloxone (Fig. 2B).

## DISCUSSION

These data demonstrate that OPP 1) binds opiate receptors, 2) competes for binding sites with naloxone, 3) paralyzes ileal muscle, and 4) produces analgesia following intravenous injection. The report (1) of naloxone-reversible analgesic effects of ingested sucrose on infant rats subjected to hot plate stimulation and separation distress also may be related to OPP hormone released with sucrose-evoked insulin. The recent report (16) of immunoreactive beta-endorphin-like peptide with similar Sephadex G-25 elution profile extracted from eel pancreas acetone powder suggests some ubiquity and conservation of OPP in species. The elution profiles of RIA reactivity in pancreatic extracts on Sephadex G-50 columns standardized with synthetic human beta-endorphin and bacitracin indicated by previously published (8) and unreported data that the immunoreactive eluates are larger than synthetic beta-endorphin. On the Sephadex G-50 column standardized by bovine serum albumin and cytochrome C, the immunoreactive OPP herein reported appeared to be about 14,000 daltons in comparison with 3,450 daltons for human beta-endorphin, indicating these are different peptides. The G-25 elution profile of placental beta-endorphin-like peptide (9) also showed a molecular size significantly larger than synthetic human beta-endorphin.

The naloxone reversible analgesic effects of intravenous OPP in the mouse tail-flick test for analgesia is congruent with similar findings reported (20) by Tseng et al. The binding site appears to be the naloxone and morphine sensitive central receptors, although spinal and somatic sites cannot be ruled out.

The beta-endorphin antiserum in the RIA kit from the New England Nuclear Laboratory, used throughout our studies, does not react significantly with Met- or Leu-enkephalin or alpha-endorphin, but cross reacts approximately 50% with beta-lipotropin, indicating recognition of the c-terminus of the OPP beta-endorphin-like peptide.

In addition to the manifest analgesic and ileal muscle data, the conjecture that pancreatic islet $D_2$ cells reported by Watkins, Bruni and Yen (22) may be a source of plasma immunoreactive beta-endorphin can be supported by other reports linking it with islet cell function (4), insulin and glucagon secretion (18), obesity (type-2) diabetes (6), the influence of synthetic beta-endorphin, opiates and naloxone on appetite satiety and feeding behavior (15), the analgesic effects of sucrose ingestion on rat pups (1), and the common use of sucrose sugar tits to comfort human infants under circumcision, reinforcement of breast feeding (5), reward conditioned behavior (11,19), place preference and drug addiction (7, 10, 23).

Further investigation of this pancreatic opiate receptor ligand is necessary and in progress to determine the molecular structure and its possible clinical potential in obesity, diabetes, and drug-seeking behavior.

## REFERENCES

1. Blass, E.; Fitzgerald, E.; Kehoe, P. Interactions between sucrose, pain and isolation distress. Pharmacol. Biochem. Behav. 26:483–489; 1987.
2. Bruni, J. F.; Watkins, W. B.; Yen, S. S. C. B-Endorphin in the human pancreas. J. Clin. Endocrinol. Metab. 49:649–651; 1979.
3. D'Amour, F. E.; Smith, D. L. Method for determining loss of pain sensation. J. Pharmacol. Exp. Ther. 72:74–79; 1941.
4. Feldman, M.; Kiser, R. S.; Unger, R. H.; Li, C. H. Beta endorphin

and the endocrine pancreas. N. Engl. J. Med. 308:349–353; 1983.
5. Franceschini, R.; Venturini, P. L.; Cataldi, A.; Barreca, T.; Ragni, N.; Rolandi, E. Plasma beta endorphin concentrations during suckling in lactating women. Br. J. Obstet. Gynecol. 96:711–713; 1989.
6. Guigliano, D.; Ceriello, A.; Salvatore, T.; Paolisso, G.; D'Onofrio, F.; Lefébvre, P. B-endorphin infusion restores acute insulin responses to glucose in type-2 diabetes mellitus. J. Clin. Endocrinol. Metab. 64:944–948; 1987.
7. Herz, A.; Shippenberg, T. S. Neurochemical aspects of addiction: Opioids and other drugs of abuse. In: Goldstein, A., ed. Molecular and cellular aspects of the drug addictions. New York: Springer-Verlag 1989:111–141.
8. Houck, J. C.; Chang, C. M.; Kimball, C. D. Pancreatic beta endorphin-like polypeptides. Pharmacology 23:14–23; 1981.
9. Houck, J. C.; Kimball, C. D.; Chang, C.; Pedigo, N. W.; Yamamura, H. I. Placental B-endorphin-like peptides. Science 207:78–80; 1980.
10. Kalant, H. The nature of addiction: An analysis of the problem. In: Goldstein, A., ed. Molecular and cellular aspects of the drug addictions. New York: Springer-Verlag; 1989:1–28.
11. Keverne, E. B.; Martensz, N. D.; Tuite, B. Beta endorphin concentrations in cerebrospinal fluid of monkeys are influenced by grooming relationships. Psychoneuroendocrinology 14:155–161; 1989.
12. Khawaja, X. Z.; Green, I. C.; Thorpe, J. R.; Titheradge, M. A. The occurence and receptor specificity of endogenous opioid peptides within the pancreas and liver of rat. Comparison with brain. Biochem. J. 267:233–240; 1990.
13. Kosterlitz, H. W.; Watt, A. J. Kinetic parameters of narcotic agonists and antagonists, with particular reference to N-allylnoroxymorphone (naloxone). Br. J. Pharmacol. Chemother. 33:266–276; 1968.
14. Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. Protein measurment with Folin phenol reagent. J. Biol. Chem. 193:265–275; 1951.
15. Morley, J. E.; Levine, A. S. Stress induced eating is mediated through the endogenous opiates. Science 209:1259–1261; 1980.
16. Ng, T. B. The eel pancreas contains opiate-like but not corticotrophin-like materials. Biochem. Int. 20:1161–1167; 1990.
17. North, R. A.; Tonini, M. The mechanism of action of narcotic analgesics in the guinea-pig ileum. Br. J. Pharmacol. 61:541–549; 1977.
18. Reid, R. L.; Yen, S. S. C. B-Endorphin stimulates the secretion of insulin and glucagon in humans. J. Clin. Endocrinol. Metab. 52: 592–594; 1981.
19. Stein, L.; Beluzzi, J. D. Brain endorphins: Possible mediators of pleasurable states. In: Usdin, E.; Bunney, W. E.; Kline, N. S., eds. Endorphine in mental health research. New York: Oxford Univ. Press; 1979:375–388.
20. Tseng, L-F.; Loh, H. H.; Li, C. H. B-endorphin as a potent analgesic by intravenous injection. Nature 263–239–240 1976.
21. Tung, A. K.; Cockburn, E. B-Endorphin-like immunoreactivity in extracts of the fetal bovine pancreas. Diabetes 33:235–238; 1984.
22. Watkins, W. B.; Bruni, J. F.; Yen, S. S. C. B-Endorphin and somatostatin in the pancreatic D-cell, colocalization by immunocytochemistry. J. Histochem. Cytochem. 28:1170–1174; 1980.
23. Watson, S. J.; Trujillo, K. A.; Herman, J. P.; Akil, H. Neuroanatomical and neurochemical substrates of drug-seeking behavior: Overview and future directions. In: Goldstein, A., ed. Molecular and cellular aspects of the drug addictions. New York: Springer-Verlag; 1989:29–91.