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Leucosulfakinin, a Sulfated Insect Neuropeptide with Homology to Gastrin and Cholecystokinin

RONALD J. NACHMAN,* G. MARK HOLMAN, WILLIAM F. HADDON, NICHOLAS LING

A sulfated, myotropic neuropeptide termed leucosulfakinin (Glu-Gln-Phe-Glu-Asp-Tyr(SO_3H)-Gly-His-Met-Arg-Phe-NH_2) was isolated from head extracts of the cockroach *Leucophaea maderae*. The peptide exhibits sequence homology with the hormonally active portion of the vertebrate hormones human gastrin II and cholecystokinin, suggesting that these peptides are evolutionarily related. Six of the 11 amino acid residues (55 percent) are identical to those in gastrin II. In addition, the intestinal myotropic action of leucosulfakinin is analogous to that of gastrin.

INSECTS UTILIZE VERTEBRATE-LIKE neuropeptides for a variety of regulatory functions. Partial sequence analyses of the amino termini of several neuropeptides that have prothoracicotropic hormone (PTTH) activity reveal homology with the human insulin A chain. Prothoracicotropic hormones, which have been isolated from silkworm (*Bombyx mori*) head extracts, mediate molting hormone synthesis and metamorphosis. Melanization and reddish coloration hormone from the same silkworm species has been partially sequenced at the amino terminus to reveal homology with the carboxyl terminal region of insulin-like growth factor II (*1*). Homology has also been reported between the amino terminus of the vertebrate peptide glucagon and the cardioacceleratory-hypertrehalosemic peptide CC-2, the latter isolated from the corpora cardiaca of the cockroach *Periplaneta americana* (*2*). In addition, material that reacts with antisera to at least nine vertebrate neuropeptides, including gastrin and cholecystokinin (CCK), has been noted in insect tissues (*3*). The presence of gastrin or CCK-like immunoreactivity in tissues of the tobacco hornworm moth *Manduca*, silkworm *Bombyx*, drone fly *Eristalis*, cockroach *Periplaneta*, and in neurons of the central nervous system (CNS) and the neuroendocrine system of the blowfly *Calliphora* has been demonstrated by fluorescence immunocytochemistry. Antisera specific for the carboxyl terminus and amino terminus of both gastrin and CCK peptides have been used in these studies. The antisera directed against the carboxyl terminus of gastrin or CCK stained a number of neurons in the brain, thoracic ganglion, and corpora cardiaca, whereas the antisera directed against the amino terminus did not cross-react with any cells of young blowflies (*4*).

We report here the isolation, characterization, and synthesis of a sulfated myotropic neuropeptide from head extracts of the Madeira cockroach *Leucophaea maderae* that exhibits sequence homology with the carboxyl terminus of the human brain-gut hormones gastrin II and CCK. The sequence homology and intestinal myotropic activity analogous to that of gastrin suggest that molecular evolution of the gastrin-CCK peptides did not begin in vertebrates, but rather began earlier in time. Unlike other known neuropeptides from invertebrates, this neuropeptide, designated leucosulfakinin (LSK), is sulfated. LSK has also been identified in extracts of *L. maderae* corpora cardiaca, which are the major neurohumoral organs of insects and are analogous to the vertebrate hypothalamus-hypophyseal system.

We isolated LSK from methanol–water–acetic acid (90:9:1) extracts of 3000 *L. maderae* heads by a four-step high-performance liquid chromatography (HPLC) purification procedure with Waters μBondapak phenyl, Rainin Microsorb C1, Techsphere 3 C18, and Waters I-125 Protein-Pac columns (*5*). Activity was detected by observing the myotropic effect of various fractions, that is, changes in the frequency or amplitude of spontaneous contractions of the cockroach proctodeum (hindgut) (*6*). Initial separation of the extracts on a μBondapak phenyl column yielded five fractions of active material. Four of these fractions were also found in brain–corpora cardiaca extracts of *L. maderae* (*6*). One of those four, eluting at 62 to 64 minutes, was purified on a Microsorb C1 column and an active fraction eluting at 43 to 45 minutes was collected (*7*). The active fraction was further purified on a Techsphere 3 C18 column and a single active peak at 51 minutes was isolated. Final purification was effected on a Waters I-125 Protein-Pac column and 1.3 μg of pure peptide (eluting at 55.3 minutes) was obtained (Fig. 1). Amino acid analysis of the pure peptide revealed the molar ratio composition as Arg(1), Asx(1), Glx(3), Gly(1), His(1), Met(1), Phe(2), and Tyr(1). Microsequence analysis (*8*) of the peptide yielded the primary structure Glu-Gln-Phe-Glu-Asp-Tyr-Gly-His-Met-Arg-Phe, which accounts for all the amino acids. However, two synthetic replicas of the established sequence—one with a carboxyl terminal amide and the other with a carboxyl terminal carboxylic acid (*9*)—behaved differently from the active substance on HPLC analysis and were also inactive in the hindgut bioassay up to a concentration of $2 \times 10^{-6} M$.

Our realization that LSK exhibited sequence homology with human gastrin and CCK (Fig. 2) provided an important clue to

R. J. Nachman and W. F. Haddon, Western Regional Research Center, U.S. Department of Agriculture, 800 Buchanan Street, Berkeley, CA 94710.
G. M. Holman, Veterinary Toxicology and Entomology Laboratory, U.S. Department of Agriculture, F and B Road, College Station, TX 77841.
N. Ling, Laboratories for Neuroendocrinology, The Salk Institute, La Jolla, CA 92037.

*To whom correspondence should be addressed. Some of this work was done when R.J.N. was a visiting scientist in the Laboratories for Neuroendocrinology, The Salk Institute, La Jolla, CA 92037.

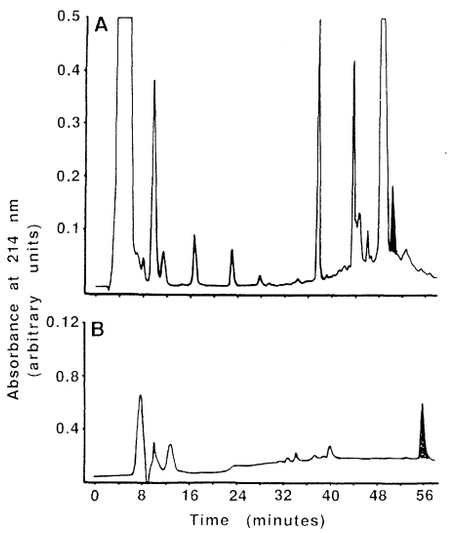

Fig. 1. Separation of myotropic activity by HPLC. (A) Fractionation on Techsphere 3 C18 of area eluting 43 to 45 minutes from Microsorb C1. Peak eluting at 51 minutes contained myotropic activity (*5*). 0.5 absorbance units at full scale. (B) Fractionation on Waters I-125 Protein column of active peak collected in (A). Myotropic activity eluted in a peak at 55.3 minutes (*5*). 0.2 absorbance units full scale.

its structure. Residues Glu^1, Glu^4, Tyr^6, Gly^7, Met^9, and Phe^{11} of the LSK sequence match positions 7, 10, 12, 13, 15, and 17 of human gastrin I (the nonsulfated form of gastrin). Thus, 6 of the 11 (55 percent) amino acid residues of LSK are identical with those of human gastrin, the highest percentage reported between insect and vertebrate neuropeptides. These residues are retained in minigastrin-14, minigastrin-13, and minigastrin-6, naturally occurring truncated forms of gastrin (*10*), and are located in the active portion of the hormone. Furthermore, LSK shares 4 of 6 (66 percent) residues with minigastrin-6. Sequence homology is potentially greater, as the sequence differences Gln^2, Asp^5 of LSK and Glu^8, Ala^{11} of gastrin I could arise from single-base substitution in the respective codons (Fig. 2).

Comparison of LSK with naturally occurring, active CCK-8 (*11*) (Fig. 2) reveals a homology between Asp^5-Tyr^6 of the LSK sequence and the corresponding amino acid pair at positions 1 and 2 of CCK-8. The residues Glu^4 and Asp^5 in LSK and Asp^1 and $Tyr^2 \cdot SO_3H$ in CCK-8 are related by their acidic character and by the fact that their codons could differ by only a single nucleotide (Fig. 2). In addition, the tyrosine residues of CCK and another form of gastrin, gastrin II, are present as sulfate esters, which suggested the presence of a sulfated tyrosine in native LSK. The sulfate group in LSK could have been readily removed under the acidic conditions of amino acid analysis and sequencing. The presence of a sulfate ester on tyrosine in LSK, a modification that is present in only a small number of peptides (*12*), further emphasizes the resemblance to gastrin II. The sequence homology and analogous biological activity exhibited by LSK and gastrin II suggests that the probability that their structural convergence is coincidental is remote.

The sulfate group was incorporated on the synthetic peptide-amide by treatment with concentrated sulfuric acid at −5°C for 30 minutes (*13*). Purification by reverse-phase HPLC on a Vydac C18 column (product eluted at 83 to 86 minutes) and by a μBondapak phenyl column (*5*) yielded a peak at 62.9 minutes, corresponding to the retention time of LSK. The synthetic peptide caused an increase in the spontaneous contractions of the cockroach hindgut at a threshold concentration of $2.2 \times 10^{-10} \pm 1.1 \times 10^{-10} M$ (SD, $n = 5$), a value virtually identical to that recorded for the natural product ($1.9 \times 10^{-10} \pm 0.6 \times 10^{-10} M$ (SD, $n = 5$) (Fig. 3). The sulfated peptide had a retention time of 50.8 minutes on a Techsphere 3 C18 column, again similar to the value obtained for the natural product (51.0 minutes). Amino acid analysis confirmed that the amino acid residues and molar ratios were identical to those of LSK. Fast atom bombardment mass spectra of synthetic LSK showed molecular ions at 1537.6 (MH^+) and 1457.6 ($MH^+ - SO_3H$), a result consistent with a single sulfate group in the structure (*14*). The identification of tyrosine sulfate in the $Ba(OH)_2$ hydrolysates of both synthetic and natural LSK pinpointed the location of the sulfate (*13*). The complete structure of LSK was therefore established as Glu-Gln-Phe-Glu-Asp-Tyr(SO_3H)-Gly-His-Met-Arg-Phe-NH_2. The content of LSK was approximately 0.5 ng per head (0.00034 nmol per head) in the insect. The presence of the neuropeptide in the neurosecretory corpora cardiaca of the brain, its myotropic activity at the distant hindgut, and homology with vertebrate hormones suggests that LSK may have a hormonal function in *L. madeira*.

Fig. 3. Response of the hindgut preparation of *Leucophaea maderae* (*6*) to leucosulfakinin (LSK). Concentration, $6 \times 10^{-10} M$ (three times threshold dose); time base, 1 minute; vertical calibration, 2-mm tissue movement. The point of LSK application is indicated by the arrow.

The sulfated terminal carboxylic-acid analog of LSK, prepared by the above procedure for LSK, proved inactive even up to a concentration of $10^{-6} M$. Synthetic CCK-8 (Peninsula Laboratories), gastrin I, and gastrin II were also found to be inactive up to $10^{-6} M$. This was expected because of the disparity between the basic His^8 and Arg^{10} residues of the carboxyl terminus of LSK and the analogous nonpolar Trp and acidic Asp residues present in the same relative positions of gastrin and CCK (Fig. 2). This disparity is consistent with the evolutionary distance between their respective hosts and suggests that LSK and gastrin have dissimilar conformations. Furthermore, gastrin-CCK immunoreactive substances from the crab *Cancer magister* and sea hare *Aplysia californica* are inactive in mammalian bioassays, consistent with the presence of sequence differences in the carboxyl terminal region of invertebrate CCK-gastrins that affect biological activity more than immunoreactivity (*15*).

The structure of LSK, with an Asp and two Glu residues preceding the Tyr sulfate moiety, substantiates a hypothesis developed from studies of other sulfated peptides—a sequence composed of acidic residues preceding tyrosine is a recognition site for tyrosylprotein sulfotransferase (*12*). Like LSK, an Asp along with other acidic residues precedes the Tyr sulfate residue in CCK, the related frog peptide caerulein, and

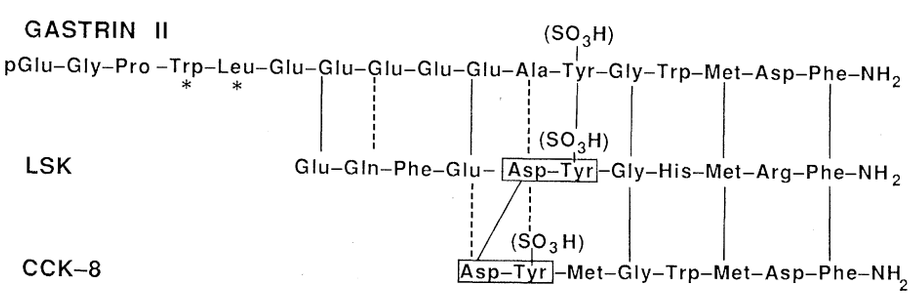

Fig. 2. Sequence homology between leucosulfakinin (LSK) and human gastrin II and cholecystokinin-8 (CCK-8); * denotes the amino termini of minigastrin-14 and minigastrin-13, active naturally occurring, truncated forms of gastrin. Solid lines indicate direct homology and dashed lines correspond to residues in which the nucleotide codons could differ by a single nucleotide.

the sulfated fibrinopeptides. The Tyr sulfate residue in gastrin II and in hirudin is also preceded by several acidic amino acids.

The biological actions of CCK in mammalian systems include the stimulation of pancreatic enzyme secretion, gall bladder contraction, and possible CNS involvement in the mechanism of satiety (*13, 16*). Nonsulfated CCK's are 250 to 350 times less effective in stimulating amylase secretion and gall bladder contraction than the sulfated peptide (*13, 17*), demonstrating the importance of the sulfate moiety. Although the primary gastrointestinal function of gastrin in mammals is to induce gastric secretion, it also stimulates smooth muscle contraction, increases blood circulation and water secretion in the stomach and intestine, and stimulates pancreatic secretion. The sulfate ester is not required in gastrin to elicit gastric acid secretion (*18, 19*). Contraction of visceral muscle promoted by LSK in the cockroach hindgut and its attendant stimulation of hemolymph (blood) circulation (*20*) are analogous to gastrin-induced motility and increased blood circulation in mammalian intestines. It is unknown whether LSK is also involved in the secretion of digestive enzymes and regulation of water balance or satiety in the cockroach, other insects, or even other invertebrates.

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5. Operating conditions for columns on Waters ALC-100 HPLC system (columns i-v): (i) Waters μ-Bondapak phenyl, 4.6 mm by 30 cm (Waters Associates, Milford, MA). Solvent A, 0.1% trifluoroacetic acid (TFA) in water; Solvent B, 25% acetonitrile in 0.1% aqueous TFA. Conditions: 100% A for 8 minutes, then linear gradient to 100% B over 1 hour; flow rate, 1.5 ml per minute; detector, 2.0 absorption units at full scale (AUFS) at 214 nm. (ii) Rainin Microsorb C1, 4.6 mm by 25 cm (Rainin Instrument Co., Woburn, MA). Solvents and conditions: same as μBondapak phenyl except final condition was 50% B. (iii) Techsphere 3 C18, 4.6 mm by 15 cm (Phenomenex, Rancho Palos Verdes, CA). Solvents A and B: same as μBondapak phenyl. Conditions: 50% A for 8 minutes, then linear gradient to 100% B over 40 minutes; flow rate, 1.0 ml per minute; detector, 0.5 AUFS at 214 nm. (iv) Waters I-125 Protein-Pac, 7.8 mm by 30 cm. Solvent A, 95% acetonitrile made to 0.01% TFA. Solvent B, 75% acetonitrile made to 0.01% TFA. Conditions: 100% A for 8 minutes, then linear gradient to 100% B over 40 minutes; flow rate, 1.5 ml per minute; detector, 0.2 AUFS at 214 nm. (v) Waters Novapak C18. Solvent A, 95% 1 m*M* sodium acetate, *p*H 5.25; 5% acetonitrile, Solvent B, acetonitrile and water (3:2). Conditions: 100% A, then manufacturer's program 5 initiated upon injection to 80% B over 24 minutes; flow rate, 1 ml per minute; detector, 0.05 AUFS at 269 nm. (vi) Vydac C18 (Phenomenex), 1 cm by 25 cm, on Beckman 332 HPLC system. Solvent A, 0.1*N* ammonium acetate, *p*H 6.5. Solvent B, 80% acetonitrile, 20% 0.1*N* ammonium acetate, *p*H 6.5. Conditions: 12 to 20% B over 90 minutes, then 21 to 80% B over 2 minutes and held at 80% B for 10 minutes; flow rate, 2 ml per minute; detector, 1.0 AUFS at 280 nm.
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8. Amino acid sequence was determined by Edman degradation with an Applied Biosystems model 470A gas-phase sequencer. Phenylthiohydantoin derivatives of amino acids obtained at each cycle of the Edman degradation were determined by reversed-phase HPLC on Waters Novapak C18 (*5*).
9. Solid phase synthesis of the peptide amide and peptide acid utilized *p*-methylbenzhydrylamine and Merrifield resins, respectively, on a Beckman 990 peptide synthesizer. Derivatized amino acids were purchased from Protein Research Foundation, Osaka, Japan. Amino acid side chain groups were protected as follows: His and Arg, tosyl; Tyr, 2,6-dichlorobenzyl ether; Asp, cyclohexyl ester; Glu, benzyl ester. The protected peptide-resins were treated with mixture of 1.5 ml of anisole, 0.25 ml of methylethyl sulfide, and 10 ml of hydrogen fluoride per gram at 0°C for 1 hour to cleave the peptide from the resin anchor and free it of side-chain protecting groups (*7*).
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Neurons Containing NADPH-Diaphorase Are Selectively Resistant to Quinolinate Toxicity

JAE-YOUNG KOH, STEPHEN PETERS, DENNIS W. CHOI*

Exposure of cultures of cortical cells from mouse to either of the endogenous excitatory neurotoxins quinolinate or glutamate resulted in widespread neuronal destruction; but only in the cultures exposed to quinolinate, an *N*-methyl-D-aspartate agonist, was there a striking preservation of the subpopulation of neurons containing the enzyme nicotinamide adenine dinucleotide phosphate diaphorase (NADPH-d). Further investigation revealed that neurons containing NADPH-d were also resistant to the toxicity of *N*-methyl-D-aspartate itself but were selectively vulnerable to the toxicity of either kainate or quisqualate. Thus, neurons containing NADPH-d may have an unusual distribution of receptors for excitatory amino acids, with a relative lack of *N*-methyl-D-aspartate receptors and a relative preponderance of kainate or quisqualate receptors. Since selective sparing of neurons containing NADPH-d is a hallmark of Huntington's disease, the results support the hypothesis that the disease may be caused by excess exposure to quinolinate or some other endogenous *N*-methyl-D-aspartate agonist.

A SMALL SUBPOPULATION OF NEURONS in the mammalian central nervous system (CNS) contains high activities of the enzyme nicotinamide adenine dinucleotide phosphate diaphorase (NADPH-d), the presence of which can be detected histochemically by the enzyme-mediated reduction of a tetrazolium dye to a visible reaction product (*1*). These NADPH-d–containing [NADPH-d(+)] neurons are scattered in "solitary" fashion throughout the neocortex, striatum, and other brain regions (*1, 2*), as well as in the retina (*3*). Neither the functional significance of the NADPH-d enzyme, nor the physiologic role of the NADPH-d(+) neurons is known, although in both the cortex and striatum the enzyme is colocalized with either somatostatin or avian pancreatic polypeptide–neuropeptide Y (or both) immunoreactivity (*4*).

Recently, this distinct class of neurons has been found to be selectively spared in the striatum of patients with Huntington's disease (HD) (*5*), in contrast to the general loss of intrinsic striatal neurons in HD. Biochemical markers for striatal γ-aminobutyric acid (GABA)–containing, cholinergic, and some peptidergic neurons (*6*) are all decreased in HD, but somatostatin levels are actually increased (*7*), probably reflecting

Department of Neurology, Stanford University, Stanford, CA 94305.

*To whom correspondence should be sent.