"event" was an episode in the history of Earth from approximately 1000 to 2000 Ma of widespread intrusion of rocks composed nearly exclusively of plagioclase feldspar, or "anorthosites." Additional evidence for this postulated change in mantle source may also be provided by its close correlation in time with the minimum age of "secondary mantle isochrons" (*22*).


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# Does the Binding of Cyclosporine to Calmodulin Result in Immunosuppression?


STEPHEN J. LEGRUE, RODNEY TURNER, NORMAN WEISBRODT, JOHN R. DEDMAN



**The cyclosporines are a family of cyclic endecapeptides that cause a profound suppression of primary immune stimulation both in vitro and in vivo. Recently, the regulatory protein calmodulin (CaM) has been implicated as a target for cyclosporin A (CsA) binding. This study utilized two less-active isomers of CsA to evaluate the specificity and biological significance of CaM binding. The three cyclosporines exhibited equivalent in vitro binding to CaM, regardless of immunosuppressive activity. Furthermore, CaM-dependent enzyme systems were inhibited equally by active and inactive cyclosporines, but only at concentrations 100 times those necessary to block lymphocyte activation. Thus the exquisite immunosuppressive stereospecificity displayed by cyclosporine isomers is not reflected in the binding to and inhibition of CaM, suggesting that inhibition of CaM-dependent processes is not sufficient to explain the immunosuppressive activity of CsA.**


SYNTHETIC AND NATURALLY OCCURring isomers of the cyclosporines exhibit a wide range of biological activity, from the strongly immunosuppressive cyclosporin A (CsA), to the weakly suppressive [$Val^2$]cyclosporine (CsD), to the immunologically inactive stereoisomer [(D)-*N*-methyl-$Val^{11}$]cyclosporine (CsH). Amino acid substitutions at or adjacent to the unique *N*-methyl-4-butenyl-4-methyl-threonine (MeBmt) at position 1 appear to have the greatest effect upon drug uptake and immunosuppressive activity (*1, 2*).

Specific defects in helper T (thymus-derived) lymphocytes treated in vitro with CsA include a blockade in transcriptional activity of at least two lymphokine genes: γ-interferon and interleukin-2 (*3, 4*). The hydrophobic CsA molecule does not interact with specific cell surface receptors but rather partitions through the plasma membrane, where it may interact with cytoplasmic target sites (*2*). Two candidates for CsA-binding proteins are calmodulin (CaM) (*5*) and an unrelated protein termed cyclophilin (*6*). Inhibition of CaM-dependent cascades during lymphocyte activation would be consistent with the central regulatory role of CaM in $Ca^{2+}$-mediated cellular processes (*7*).

The binding of $Ca^{2+}$ ions by CaM results in the formation of hydrophobic domains through which CaM can bind to and regulate the activity of target proteins (*7, 8*). Lipid-soluble compounds of both synthetic and natural origin can antagonize the activity of CaM by binding to and blocking the exposed hydrophobic domain (*8*). Thus, CaM antagonists such as *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7) (Sigma), trifluoperazine (TFP) (Sigma), and chlorpromazine also block the lymphoproliferative response to antigens, mitogenic lectins, or calcium ionophores without inhibiting the response to exogenous interleukin-2 (*5, 8, 9*). Analysis of the structure-function relations of synthetic CaM antagonists suggests that inhibitory activity is related to the hydrophobic index of the compound and stereospecific interactions of the ligand with its binding site (*8*). The availability of structural and stereoisomers of cyclosporine has allowed us to examine the structure-function relations between CaM binding and immunosuppressive activity.

The cyclosporines under investigation contain single amino acid replacements in the clinically useful isomer CsA (*3*). Substitution of valine for α-aminobutyric acid at position 2 yields CsD, while CsH has an L to D stereoisomerization of methylvaline at position 11. The immunosuppressive activities of the three cyclosporine isomers were profoundly affected by the single amino acid substitutions, as demonstrated by their relative abilities to block lymphoproliferation of murine spleen cells in response to the mitogenic lectin concanavalin A (Con A) (Fig. 1). The dissociation constant (binding affinity) ($K_d$) for CsA "binding" to lymphocytes and the dose of CsA yielding a 50% suppression of mitogen-induced lymphoprolifera-


S. J. LeGrue, Department of Immunology, The University of Texas M. D. Anderson Hospital and Tumor Institute (Box 178), Houston, TX 77030.
R. Turner, N. Weisbrodt, J. R. Dedman, Departments of Physiology and Cell Biology, The University of Texas Medical School, Houston, TX 77025.

tion ($SD_{50}$) have both been reported to be in the range of 50 to 100 n*M* (*2, 4, 5*). The $SD_{50}$ for CsD was three- to fivefold higher than for CsA, while CsH was unable to inhibit lymphocyte activation at concentrations up to 30 μ*M*. For comparison, we also examined the in vitro immunosuppressive activities of two synthetic CaM antagonists (Fig. 1): W-7 and TFP had $SD_{50}$ values of 5 μ*M* and 2 μ*M*, respectively.

The binding of agonists and antagonists to dansylated proteins can lead to conformational changes that are manifested by change in the fluorescence intensity and are sometimes accompanied by a moderate shift in the emission spectrum toward shorter wavelengths (*5, 10*). We used this property to examine the binding of the cyclosporine isomers and of known CaM antagonists to dansyl-CaM. All three cyclosporines induced a similar $Ca^{2+}$-dependent increase in dansyl-CaM fluorescence intensity, regardless of the biological activity of the isomer (Fig. 2). Concentrations of CsA, CsD, and CsH ranging from $3 \times 10^{-7}M$ to $3 \times 10^{-5}M$ yielded reproducible shifts, with a maximum effect achieved at $1 \times 10^{-5}M$. The half-maximal response at $10^{-6}M$ was tenfold above the $SD_{50}$ for CsA. Addition of $0.3 \times 10^{-6}M$ to $1 \times 10^{-6}M$ melittin, a 26–amino acid CaM antagonist from bee venom, induced very large increases in the fluorescence intensity of dansyl-CaM. However, W-7 yielded only a slight shift in the emission spectrum, even at the highest concentrations tested (Fig. 2). TFP could not be analyzed in this assay because of its spectral properties. Addition of 2 m*M* EGTA to the reaction mixtures in all cases returned the fluorescence profiles to the ground state, demonstrating the requirement of $Ca^{2+}$ for binding.

Because the hydrophobic domain on CaM is the binding site for both calmodulin acceptor proteins (CAP's) and antagonists, CaM also associates with other hydrophobes such as phenyl-Sepharose in a $Ca^{2+}$-dependent fashion. CsA, CsH, and TFP had comparable effects on the binding of $^{125}I$-labeled CaM to phenyl-Sepharose (Fig. 3). At micromolar concentrations, where TFP is biologically active, it increased the relative hydrophobicity of CaM and promoted the association of labeled CaM with phenyl-Sepharose. The cyclosporines showed a comparable, but weaker, promotion of binding (Fig. 3). These data demonstrate that TFP and the cyclosporines increased the hydrophobic index of activated CaM, consistent with their binding at or near the hydrophobic domain.

The equivalent binding to CaM by the three cyclosporines without apparent stereospecificity or correlation with biological activity led to an investigation of the functional significance of the CSA-CaM association. The equivalent binding of CsA and CsH to CaM suggested that the drugs should be equipotent in the inhibition of CaM-dependent assays: the binding of CaM to its isolated CAP's and the activation of cyclic nucleotide phosphodiesterase. CAP's were isolated by affinity chromatography by means of immobilized CaM in the presence of 100 μ*M* $Ca^{2+}$ and eluted with 1 m*M* EGTA. The binding of $^{125}I$-labeled CaM to affinity-purified CAP's immobilized on nitrocellulose paper was abrogated by the CaM antagonist melittin at micromolar concentrations (Fig. 3). Conversely, binding was not blocked by CsA at concentrations up to $10^{-5}M$, 100-fold above the $SD_{50}$ for suppression of lymphoproliferation. Although the $Ca^{2+}$-dependent activation of

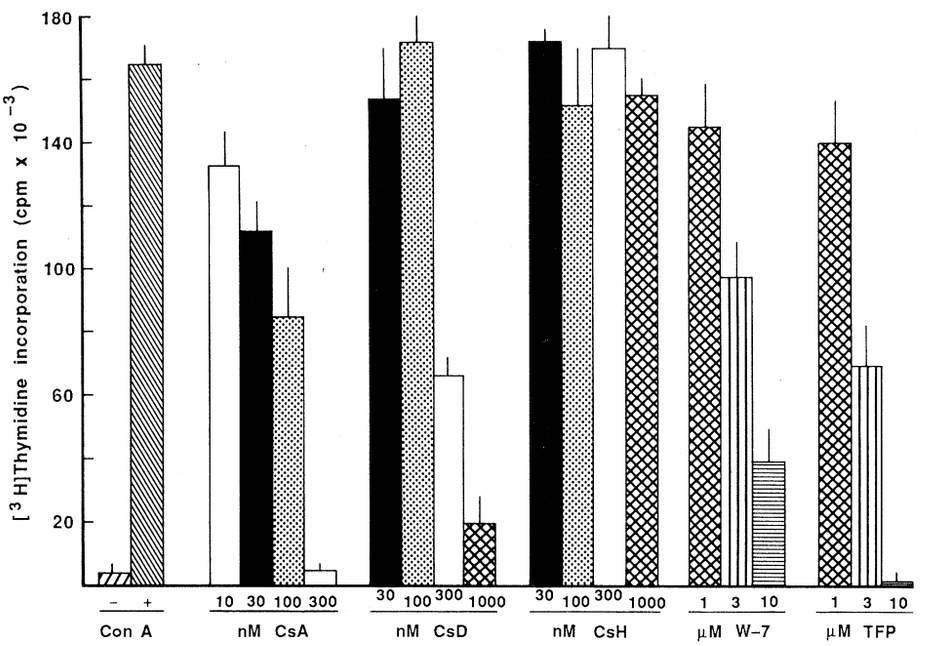


Fig. 1. Immunosuppressive activities of the cyclosporines (CsA, CsD, and CsH), W-7, and TFP. Splenic lymphocytes were obtained from pathogen-free C57BL/6 mice (Charles River Breeding Laboratories, Kingston, NY) by buoyant density centrifugation on Lympholyte-M (Accurate Chemical, Westbury, NY). The proliferative response of splenocytes to the mitogenic lectin Con A was assessed after 3 days of in vitro culture by the addition of 0.5 μCi [$^3H$]thymidine (ICN, Irvine, CA). Values are the mean ± SE of triplicate assays. The compounds were dissolved in ethanol to yield $10^{-2}M$ stock solutions (*2*). Prior to use, stocks were diluted tenfold with ethanol before being added to complete medium or buffer at the appropriate concentrations.

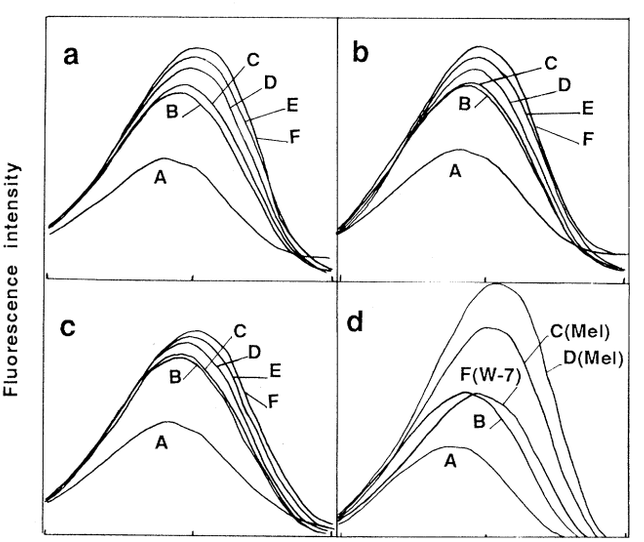


Fig. 2. Dansyl-calmodulin binding assay. The binding of cyclosporine to dansyl-CaM was monitored as an increase in fluorescence intensity (excitation at 345 nm, emission maximum at 495 nm). A cuvette containing 54 μg (1 μ*M*) of dansyl-CaM (Sigma) in 50 μ*M* MOPS [2-(*N*-morpholino)ethanesulfonic acid], 200 m*M* KCl, and 1 m*M* $CaCl_2$ was supplemented with increasing concentrations of (a) CsA, (b) CsD, (c) CsH, (d) W-7 or melittin (Mel) and the emission spectrum was obtained. The $Ca^{2+}$ requirement for binding was demonstrated by the return of the emission spectrum to the ground state after the addition of 2 m*M* EGTA. Lines: A, 2 m*M* EGTA; B, with 1 m*M* $CaCl_2$; C, $3 \times 10^{-7}M$ ligand; D, $1 \times 10^{-6}M$ ligand; E, $3 \times 10^{-6}M$ ligand; and F, $1 \times 10^{-5}M$ ligand.

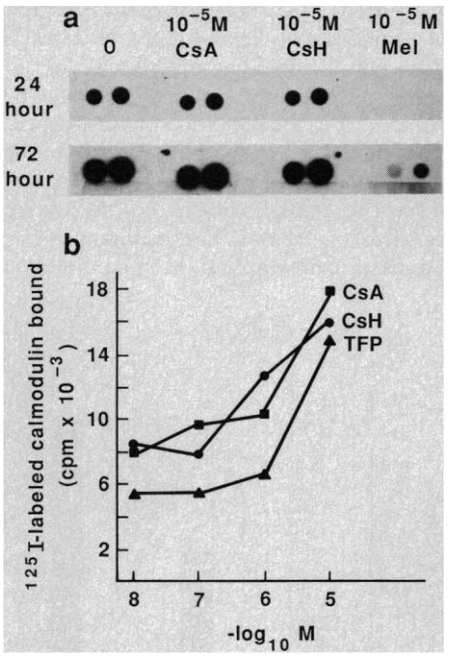


Fig. 3. $^{125}$I-labeled calmodulin binding assays. (a) The binding of $^{125}$I-labeled CaM to its isolated acceptor proteins was measured with a dot-blot radiobinding assay. Aliquots containing 5 μg (left) or 10 μg (right) of CAP's protein in buffer were immobilized on nitrocellulose paper, and unreacted sites blocked with 1% bovine serum albumin. Radiolabeled CaM (10 ng; 6 μCi/μg) was preincubated with $10^{-5}M$ antagonist in $Ca^{2+}$-containing buffer for 30 minutes and then applied to the immobilized CAP's. After a 2-hour incubation, the blots were washed, dried, and autoradiographed for the times indicated. (b) Aliquots containing 10 ng of $^{125}$I-labeled CaM (6 μCi/μg) were incubated with the indicated concentrations of CsA, CsH, or TFP for 10 minutes in the presence of 1 m$M$ $CaCl_2$. Phenyl-Sepharose (Pharmacia, Uppsala, Sweden) was pretreated with 1% bovine serum albumin to reduce nonspecific binding. The reaction mixtures were added to 50 μl of phenyl-Sepharose in a microcentrifuge tube, and the incubation was continued for an additional 10 minutes. The resin was washed three times, and the amount of bound radioactivity was determined. The net counts per minute of $^{125}$I-labeled CaM bound was obtained by subtracting the counts bound in the absence of $Ca^{2+}$. The three drugs had no effect on binding in the absence of $Ca^{2+}$.

3′,5′ cyclic nucleotide phosphodiesterase (PDE) has been reported to be inhibited by CsA (*5*), we did not observe a significant inhibition of PDE activity at CsA concentrations below $3 \times 10^{-5}M$, whereas TFP and melittin significantly blocked PDE activity in the 10 μ$M$ concentration range (*11*).

The inability of CsA to inhibit either the $Ca^{2+}$-dependent binding of CaM to immobilized CAP's or the activation of PDE, despite the apparent low-affinity binding of CsA and CsH to purified CaM, led us to investigate the effects of CsA on contraction of an isolated smooth muscle tissue. In smooth muscle, the $Ca^{2+}$ mobilized during excitation activates CaM that binds to and activates myosin light chain kinase, resulting in light chain phosphorylation and muscle contraction (*12*). Stimulation of smooth muscle isolated from the opossum esophagus by either carbachol or electrical pulses was not attenuated by CsA or CsH concentrations up to $10^{-5}M$ (Fig. 4). Compound W-7 caused a slight reduction in tension development. In contrast, TFP significantly inhibited contractions at concentrations of $10^{-6}M$ and $10^{-5}M$, analogous to its effects on other CaM-activated systems.

Assays used in this study demonstrate the equivalent, low-affinity binding of CsA and CsH to CaM, with half-maximal binding at about $10^{-6}M$. However, at concentrations 10-fold higher and 100-fold above the $SD_{50}$ of CsA for suppression of lymphocyte proliferation, neither CsA nor CsH was able to significantly inhibit CaM-dependent enzymatic assays. Thus, though we can confirm that CsA does bind to CaM, the absence of stereospecificity and the inability of CsA to antagonize CaM function suggest that this mechanism cannot explain the in vitro or in vivo immunosuppressive activity of CsA.

In addition to the $Ca^{2+}$-dependent CaM activation, the other major metabolic pathway triggered in T lymphocytes after antigen or mitogen stimulation is the hydrolysis of phosphatidylinositol 4,5-bisphosphate to inositol 1,4,5-trisphosphate and diacylglycerol (*13*), resulting in activation of protein kinase C (PKC). We have found no evidence that CsA can inhibit the activity of partially purified rat brain PKC at rate-limiting concentrations of phospholipid and diacylglycerol (*14*). Additional $Ca^{2+}$-dependent hydrophobic proteins, the calcimedins, have been identified in a wide variety of cell types, including lymphocytes (*7, 15*). The hydrophobic nature and drug-binding specificities of the calcimedins suggest that they may be involved in the mechanism of CsA immunosuppression. Other possible sites for CsA action subsequent to the activation of CaM and PKC include downstream signal-transducing enzyme cascades, nuclear regulatory components, or cyclophilin.

The equivalent binding of CsA and CsH to CaM, despite their profound differences in immunosuppressive activities, presents a paradox. One possible explanation is that CsA does not exert its immunosuppressive activity by antagonism of CaM function. The lack of stereospecific binding and the apparent low affinity of the cyclosporines for CaM may be used as arguments against designating CaM as the target for CsA action. Alternatively, the D to L isomerization at position 11 in CsH may result in a change in the hydrophobic index of the drug and an inability to enter the cell. Evidence in support of this model is available from CsA-binding studies, where cold CsH was not able to displace radiolabeled CsA from either lymphocytes or phospholipid vesicles (*2*). Whatever the site or sites for CsA action, the mechanism of immunosuppression will likely be stereospecific and involve hydrophobic associations at or near the unique MeBmt amino acid at position 1.

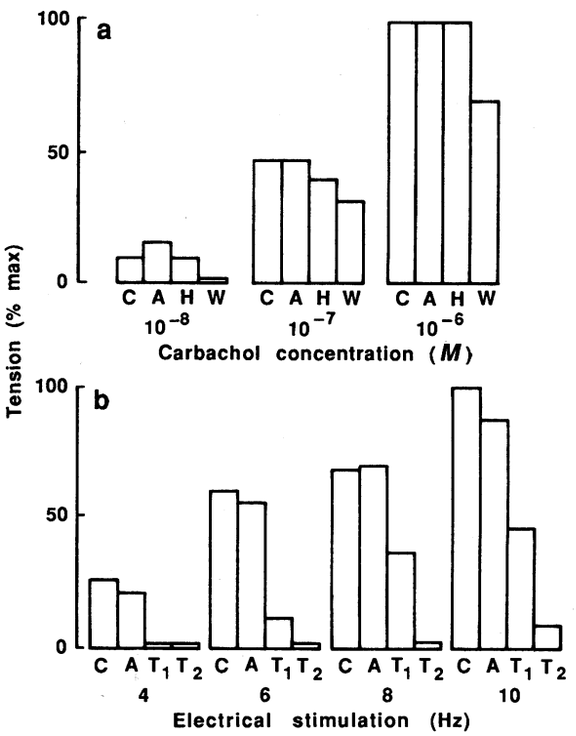


Fig. 4. Smooth muscle contraction. The isometric tensions developed by strips of esophageal muscle were recorded as described (*12*). Each strip was stimulated with either three different doses of carbachol (a) ($n$ = 1) or electrical pulses (b) (5 msec, supramaximal voltage, $n$ = 2) delivered transmurally at four different frequencies both before (C) and after the addition of $10^{-5}M$ CsA (A), $10^{-5}M$ CsH (H), $10^{-5}M$ W-7 (W), or TFP ($T_1 = 10^{-6}M$, $T_2 = 10^{-5}M$). Tension is expressed as a percentage of the maximal tension developed by each strip.

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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.