Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 9250–9253, September 1995
Agricultural Sciences

A nonpeptide agonist of the invertebrate receptor for SchistoFLRFamide (PDVDHVFLRFamide), a member of a subfamily of insect FMRFamide-related peptides

ANGELA B. LANGE*, IAN ORCHARD*, ZHIXANG WANG*, AND RONALD J. NACHMAN†‡

*Department of Zoology, University of Toronto, Toronto, ON Canada, M5S 1A1; and †Veterinary Entomology Research Unit, Food Animal Protection Research Laboratory, 2881 F&B Road, U.S. Department of Agriculture, College Station, TX 77845

Communicated by Edward F. Knipling, U.S. Department of Agriculture, Beltsville, MD, July 10, 1995

ABSTRACT **We describe a nonpeptide mimetic analog of an invertebrate peptide receptor. Benzethonium chloride (Bztc) is an agonist of the SchistoFLRFamide (PDVDHVFLRFamide) receptors found on locust oviducts. Bztc competitively displaces [^{125}I-labeled Y^1]SchistoFLRFamide binding to both high- and low-affinity receptors of membrane preparations. Bztc mimics the physiological effects of SchistoFLRFamide on locust oviduct, by inhibiting myogenic and induced contractions in a dose-dependent manner. Bztc is therefore recognized by the binding and activation regions of the SchistoFLRFamide receptors. This discovery provides a unique opportunity within insects to finally target a peptide receptor for the development of future pest management strategies.**

SchistoFLRFamide (PDVDHVFLRFamide) is a member of a subfamily of insect FMRFamide-related peptides that share the sequence X^{1}DVX^{4}HX^{6}FLRFamide (where X^1 is pQ, P, or T; X^4 is D or V; and X^6 is V or S) and have been identified in such diverse insect species as the cockroach *Leucophaea maderae*, locusts *Locusta migratoria* and *Schistocerca gregaria*, fleshfly *Neobelleria bullata*, fruitfly *Drosophila melanogaster*, and the tobacco hornworm *Manduca sexta* (1, 2). Members of this subfamily have actions on insect muscle, being potent inhibitors (myosuppressins) of cardiac and visceral muscle, in addition to having effects on skeletal muscle, longitudinal flight muscles, and salivary glands (2, 3). SchistoFLRFamide (4), isolated from locusts, is associated with their oviducts, where it inhibits spontaneous contractions and contractions induced by high-potassium saline, neural stimulation, and the pentapeptide proctolin (RYPLT) (5). Leucomyosuppressin or LMS (pQDVDHVFLRFamide), isolated from the cockroach *Leucophaea maderae*, is inhibitory on cockroach hindgut, oviduct, and heart spontaneous contractions and also inhibits evoked transmitter release at the neuromuscular junction of the mealworm *Tenebrio molitor* (6, 7). Structure–activity studies using truncated forms of both SchistoFLRFamide and LMS have revealed that the active core for SchistoFLRFamide lies in the sequence HVFLRFamide (which has similar potency to the parent compound) (1), whereas the active core for LMS lies in the sequence VFLRFamide, although DHVFLRFamide is markedly more potent (3).

Recently, it was observed that the nonpeptide benzethonium chloride (Bztc) shares several chemical features with the sequence VFLRFamide (8). As can be seen in Fig. 1, Bztc has zones with branched-chain and basic character and two separate zones with a phenyl ring. It was conjectured that two or more of these structural zones of Bztc might bind with those portions of the LMS receptor that interact with side chains of Leu or Val, Arg, and two Phe residues of the C-terminal pentapeptide and, thus, may be a ligand for the LMS receptor.

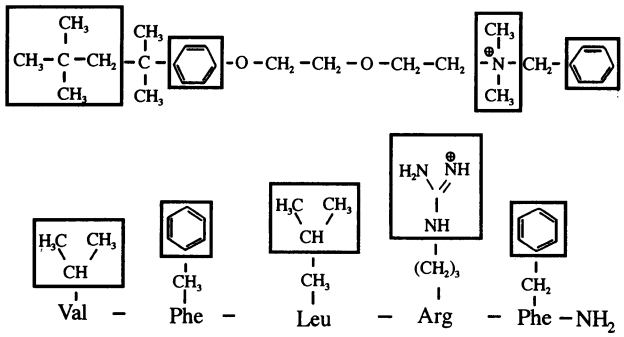

FIG. 1. Comparison of structural features of the nonpeptide Bztc (*Upper*) and the myosuppressin C-terminal fragment HVFLRFamide (*Lower*).

Interestingly, it was found that Bztc mimicked the physiological effect of LMS on both cockroach hindgut and mealworm neuromuscular junction (7, 8). Thus, Bztc reversibly inhibited spontaneous contractions of hindgut at a threshold of 6×10^{-8} M, essentially equivalent in potency to the myosuppressin C-terminal pentapeptide core. Bztc reversibly suppressed evoked excitatory postsynaptic potentials at mealworm neuromuscular junctions, with 50% inhibition induced by 10^{-4} M. As with LMS, both the suppression of mealworm neuromuscular junction excitatory postsynaptic potentials and inhibition of cockroach hindgut contraction evoked by Bztc were blocked by nordihydroguiaretic acid, an inhibitor of lipoxygenase (8). However, it could not be definitively established whether the effects of Bztc were attributable to interaction with the LMS receptor or via another mechanism.

Locust oviducts have proven a useful preparation for studying the physiological actions of SchistoFLRFamide, and recently an *in vitro* binding assay was developed to demonstrate and characterize putative receptors for SchistoFLRFamide associated with this muscle (9). This assay utilized [^{125}I-labeled Y^1]SchistoFLRFamide (Y^{1}DVDHVFLRFamide) as ligand, and the binding assay revealed the presence of two receptors, a high-affinity receptor (K_d of 9.5×10^{-10} M) and a low-affinity receptor (K_d of 1.9×10^{-7} M). Binding to both receptors was saturable, specific, reversible, and competitively inhibited by [Y^1]SchistoFLRFamide, SchistoFLRFamide, and a closely related locust peptide, ADVGHVFLRFamide. More recently, we have shown (1, 10) that the essential core for binding, which is comparable to the parent compound, lies in the sequence VFLRFamide as opposed to the active core for physiological activity, which is HVFLRFamide.

In the present study we have used the locust oviduct bioassay and binding assay to examine more closely the possibility that Bztc may be a ligand of the myosuppressin receptors.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

‡To whom reprint requests should be addressed.

EXPERIMENTAL PROCEDURES

Insects and Chemicals. Mature adult female locusts, obtained from a colony of *Locusta migratoria*, were used for all experiments. The locusts were reared at 30°C under crowded conditions and kept on a 12-h light/12-h dark regime. The locusts were fed on freshly grown wheat supplemented with bran. $Na^{125}I$ was obtained from Amersham. SchistoFLRFamide and proctolin were obtained from Peninsula Laboratories. All other chemicals were obtained from Sigma.

Preparation of [^{125}I-Labeled Y]DVDHVFLRFamide and Oviduct Plasma Membranes. [Y^1]SchistoFLRFamide (YDVDHVFLRFamide) was radioiodinated by a typical chloramine-T method as described (9). The plasma membranes of locust oviducts were prepared as described (9). Briefly, the oviducts from 28-day-old adult female locusts were dissected out and homogenized by using a Potter-type glass homogenizer in ice-cold extraction buffer (150 mM NaCl/4 mM $CaCl_2$/2 mM $MgCl_2$/10 mM KCl/4 mM $NaHCO_3$/5 mM Hepes, pH 7.2/1 mM phenylmethylsulfonyl fluoride/1 mM 2-mercaptoethanol; 4 ml of buffer per g of tissue). The homogenate was centrifuged at 800 × *g* for 10 min at 4°C to remove cell debris, and the resulting supernatant was centrifuged at 30,000 × *g* for 1 h to collect plasma membranes. The pellet was washed with the same buffer. Finally, the pellet was resuspended in the extraction buffer and adjusted to a concentration of 10 μg of protein per μl. Total protein was determined by the Bio-Rad assay with γ-globulin as the protein standard.

Receptor Binding Assay. Binding assays were carried out as described (9). Briefly, 100 μg of membrane preparation was incubated with either 1 nM or 30 nM radioiodinated [Y^1]SchistoFLRFamide (10^5 or 3 × 10^6 cpm, respectively) in 1.5-ml polypropylene microcentrifuge tubes in the presence of various concentrations of unlabeled peptides in a final volume of 100 μl of binding buffer (50 mM Hepes/10 mM $CaCl_2$/10 mM $MgCl_2$/50 mM NaCl/0.5% bovine serum albumin/1 mM phenylmethylsulfonyl fluoride/1 mM 2-mercaptoethanol) at room temperature. After a 60-min incubation, ice-cold binding buffer (0.5 ml) was added, and the tubes were centrifuged at 16,000 × *g* for 30 min. The pellet was washed once with 0.5 ml of ice-cold binding buffer. The tip of the microcentrifuge tube was cut just above the pellet and its radioactivity was measured in a γ counter. Binding affinities were studied by competitive displacement. The affinity (K_i) was established from the concentration of competitor giving half-maximal inhibition of receptor binding (IC_{50}). The curves were fitted with a two-site competition model. The equation used was $B = B_{maxh} \times [IC_{50h}/(L + IC_{50h})] + B_{maxl} \times [IC_{50l}/(L + IC_{50l})]$, where B is the binding of radioligand, L is the concentration of the competitor, B_{maxh} is the binding to the high-affinity receptor with no competitor, B_{maxl} is the binding to the low-affinity receptor with no competitor, IC_{50h} is the concentration of competitor causing half-maximal competition for high-affinity receptor, and IC_{50l} is the concentration of competitor causing half-maximal competition for low-affinity receptor. The affinity (K_i) of ligand for the receptor was estimated from the IC_{50} value by the Cheng–Prusoff relation: $K_i = IC_{50}/(1 + D/K_d)$, where D is the concentration of the radioligand and K_d is the dissociation constant. Based on the dissociation constants (K_d) and maximum binding capacities (B_{max}) from Wang *et al.* (9) and estimated from a two-site model, 72% of the initial binding at a concentration of 1 nM [^{125}I-labeled Y^1]DVDHVFLRFamide can be attributed to the high-affinity receptor, whereas 84% of the initial binding at a concentration of 30 nM can be attributed to the low-affinity receptor. Therefore, we used a competition curve with the concentration of [^{125}I-labeled Y^1]DVDHVFLRFamide at 1 nM to estimate the IC_{50} and K_i values for the high-affinity receptor and a competition curve with the concentration of [^{125}I-labeled Y^1]DVDHVFLRFamide at 30 nM to estimate the IC_{50} and K_i for the low-affinity receptor. Curve fitting was done by using the SIGMAPLOT curve fitter program (Jandel, Corte Madera, CA).

Physiology and Bioassay. Oviducts were dissected from day 10 adult females under physiological saline (150 mM NaCl/4 mM $CaCl_2$/2 mM $MgCl_2$/10 mM KCl/4 mM $NaHCO_3$/5 mM Hepes, pH 7.2/90 mM sucrose/10 mM trehalose) and set-up for measuring isotonic contractions as described (5). The application of 20 μl of 10^{-9} M proctolin directly to the locust oviducts results in a sustained contraction that is reversible upon washing. Both Bztc and SchistoFLRFamide were applied along with this dose of proctolin. The effects of Bztc and SchistoFLRFamide were quantified by measuring the amplitude of the proctolin-induced contraction in their presence relative to proctolin alone (counted as 100%). Oviducts were also set up for measuring neurally evoked contractions as described (5). The oviducal nerves were stimulated at 30 Hz for 2 sec every 10 sec, with each burst of stimulation resulting in a sustained contraction. The amplitude of neurally evoked contractions in saline was taken as 100% and the effect of SchistoFLRFamide or Bztc was quantified as a reduction of this contraction.

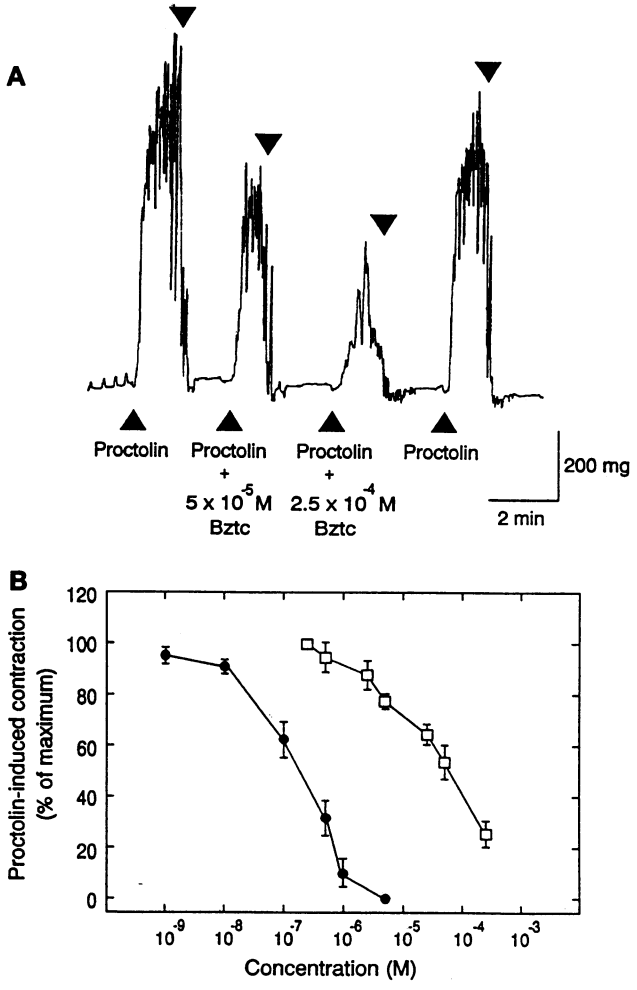

FIG. 2. Effects of Bztc on proctolin-induced contractions of locust oviduct. (*A*) The contraction induced by 10^{-9} M proctolin is reduced in the presence of Bztc. The effect of Bztc is reversible. Upward arrowheads denote application of chemicals; downward arrowheads denote wash off. (*B*) The effect of Bztc (□) upon proctolin-induced contractions (10^{-9} M) is dose-dependent. For comparative purposes, the effect of SchistoFLRFamide (●) upon proctolin-induced contractions is also illustrated. Values are mean ± SEM of four experiments.

RESULTS AND DISCUSSION

Initially, we assessed the physiological effects of Bztc on locust oviduct. Fig. 2 shows that Bztc can mimic the effects of SchistoFLRFamide in being able to reversibly inhibit proctolin-induced contractions of locust oviduct. The dose–response curve for Bztc shows a shift to the right with the threshold for Bztc being higher than for SchistoFLRFamide (5×10^{-7} M vs. 10^{-9} M, respectively) and the concentration causing 50% inhibition of the proctolin-induced contraction also higher for Bztc (6×10^{-5} M vs. 2×10^{-7} M). Similarly, Bztc reversibly inhibits neurally evoked contractions of locust oviduct (Fig. 3), but once again threshold is higher than for SchistoFLRFamide and the concentration for 50% inhibition is higher (3×10^{-5} M for Bztc vs. 10^{-8} M for SchistoFLRFamide). Bztc is also able to inhibit spontaneous contractions of locust oviduct, as it does in cockroach hindgut, and lower basal tonus (Fig. 3*A*). Thus, with regard to biological activity, the nonpeptide Bztc is able to mimic the peptide SchistoFLRFamide on locust oviduct. By using binding studies that give a direct assessment of an interaction on receptors, we found that Bztc competitively displaced [^{125}I-labeled Y^1]SchistoFLRFamide from both high- and low-affinity receptors (Fig. 4). Binding to both high- and low-affinity receptors is competitively displaced by [Y^1]SchistoFLRFamide with K_i values of 6.9×10^{-10} M and 5.0×10^{-7} M, respectively. Bztc also competitively displaces the radioligand, although as with biological activity, higher doses are required and the displacement curve is shifted to the right. The K_i for Bztc is 6.3×10^{-8} M for the high-affinity site and 1.5×10^{-4} M for the low-affinity site. For comparative purposes, the displacement curve for Bztc is similar to that of FLRFamide, which has a K_i of 5.1×10^{-8} M for the high-affinity site and a K_i of 8.5×10^{-6} M for the low-affinity site (10).

The data provide clear evidence from both biological activity and displacement from binding sites that the nonpeptide Bztc is recognized by SchistoFLRFamide receptors, and, thus, Bztc is a nonpeptide ligand for a peptide receptor in invertebrates. In the vertebrates, especially mammals, nonpeptide ligands have been described for the cholecystokinin system (11), substance P (12), and vasopressin (13).

The discovery of nonpeptide ligands is an important lead. Substances that mimic or block the action of endogenous peptides are useful for defining the physiological functions of these peptides. However, they also have considerable potential for insect pest management. Peptides control a vast array of physiological activities in insects. For instance, this subfamily of FMRFamides, the myosuppressins, controls muscular activity associated with locomotion, reproduction, and digestion, and disruption of these activities would have damaging con-

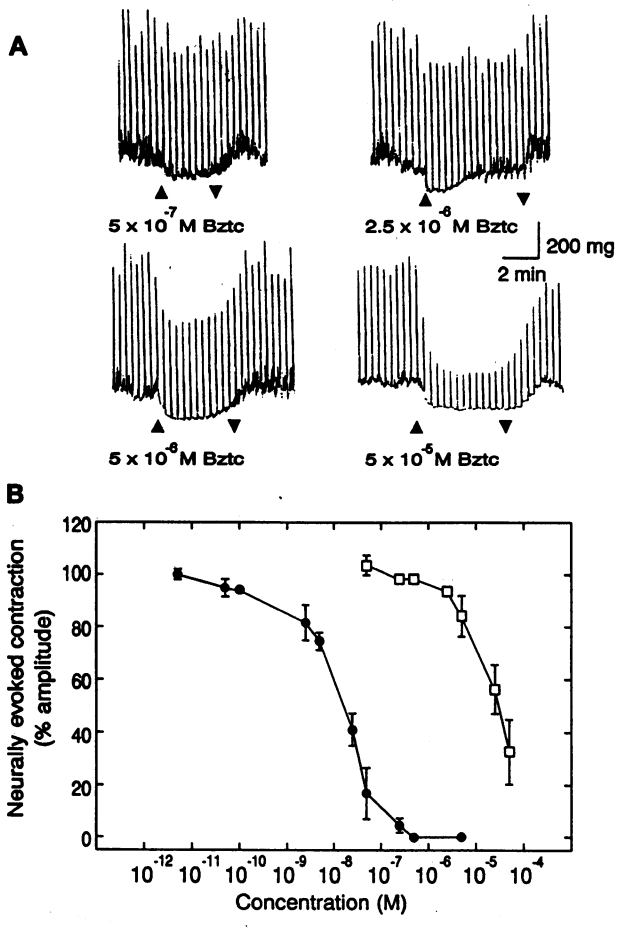

FIG. 3. Effects of Bztc upon neurally evoked contractions of locust oviduct. (*A*) Bztc reduced the amplitude of neurally evoked contractions in a reversible manner. Note also that Bztc lowers basal tonus and inhibits myogenic contractions seen in between the neurally evoked contractions. Upward arrowheads denote application of Bztc; downward arrowheads represent wash off. (*B*) The effect of Bztc (□) is dose-dependent. For comparative purposes, the effect of SchistoFLRFamide (●) upon neurally evoked contractions is also shown. The values are mean ± SEM of four experiments.

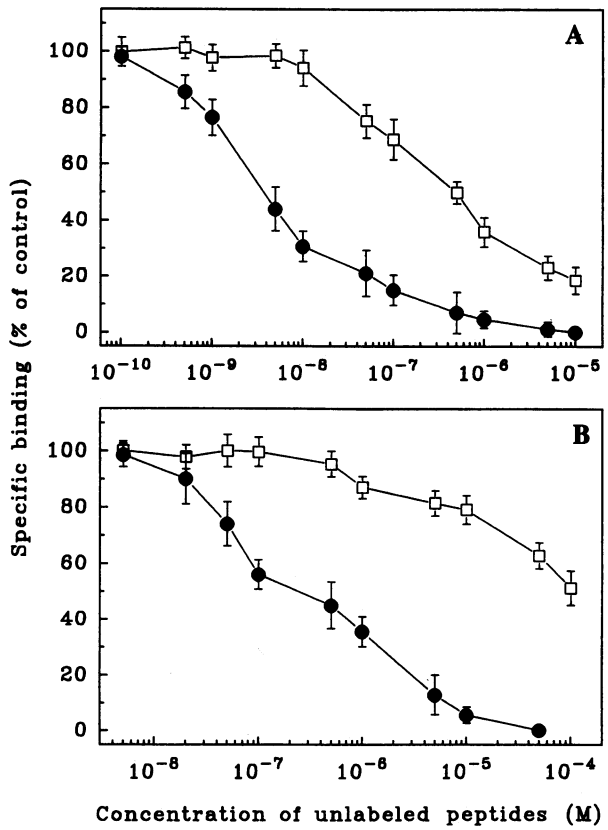

FIG. 4. Competition of specific [^{125}I-labeled Y^1]DVDHVFLRFamide binding to locust oviduct membranes by unlabeled Y^{1}DVDHVFLRFamide ([Y^1]SchistoFLRFamide) (●) and by Bztc (□). Curves were fitted with a two-site model. Each value is the mean ± SEM of four experiments run in duplicate. (*A*) Membranes were incubated with 1 nM [^{125}I-labeled Y^1]DVDHVFLRFamide. Under these conditions, the majority of initial binding is due to the high-affinity receptor, and binding was measured in the presence of increasing concentrations of [Y^1]SchistoFLRFamide or Bztc. (*B*) Membranes were incubated with 30 nM [^{125}I-labeled Y^1]DVDHVFLRFamide. Under these conditions the majority of initial binding is due to the low-affinity receptor, and binding was measured in the presence of increasing concentration of [Y^1]SchistoFLRFamide or Bztc.

sequences to the insect (14). However, the use of peptides *per se* as pesticides would appear impractical since their chemistry would make them susceptible to degradation under field conditions and to digestion after feeding, and their polarity would make uptake through the cuticle difficult (8, 14). Over the last few years, some progress has been made in the development of stable peptide analogues. In several of these examples, some amino acids have been modified or replaced giving the structure a degree of pseudopeptide or nonpeptide character (8, 15, 16). However, problems of degradation, transport, and excretion still remain to be resolved. Totally nonpeptide compounds overcome many of these problems and also allow leads for the synthesis of a series of related analogues and/or screening of chemical data banks for similar structures. Due to resistance to peptidase degradation, nonpeptide mimetic analogs need not be as potent as the endogenous peptides to disrupt physiological processes and/or normal behavioral patterns of insects. Thus, it is worth reiterating that the importance of neuropeptides as potential leads for pest control is not the molecule as such but the information encoded within it (14, 15). The identification of the nonpeptide Bztc that must match some of the binding and activation structures encoded within SchistoFLRFamide provides a unique opportunity within insects to finally target a peptide receptor for the development of future pest management strategies.

This work was supported by the Natural Sciences and Engineering Research Council of Canada, Insect Biotech Canada, and the U.S. Department of Agriculture.

1. Peeff, N., Orchard, I. & Lange, A. B. (1994) *Peptides* **15,** 387–392.
2. Schoofs, L., Holman, G. M., Nachman, R. J., Hayes, T. K. & DeLoof, A. (1994) in *Perspectives in Comparative Endocrinology*, eds. Davey, K. G., Peter, R. E. & Tobe, S. S. (Natl. Res. Counc. Canada, Ottawa), pp. 155–165.
3. Nachman, R. J., Holman, G. M., Hayes, T. K. & Beier, R. C. (1993) *Peptides* **14,** 665–670.
4. Robb, S., Packman, L. C. & Evans, P. D. (1989) *Biochem. Biophys. Res. Commun.* **160,** 850–856.
5. Lange, A. B., Orchard, I. & Te Brugge, V. A. (1991) *J. Comp. Physiol.* **168,** 383–391.
6. Holman, G. M., Cook, B. J. & Nachman, R. J. (1986) *Comp. Biochem. Physiol.* **85C,** 329–333.
7. Yamamoto, D., Ishikawa, S., Holman, G. M. & Nachman, R. J. (1988) *Neurosci. Lett.* **95,** 137–142.
8. Nachman, R. J., Yamamoto, D., Holman, G. M. & Beier, R. C. (1994) in *Insect Neurochemistry and Neurophysiology 1993*, eds. Borkovec, A. B. & Loeb, M. J. (CRC, Boca Raton, FL), pp. 319–322.
9. Wang, Z., Orchard, I. & Lange, A. B. (1994) *Peptides* **15,** 875–882.
10. Wang, Z., Orchard, I., Lange, A. B. & Chen, X. (1995) *Neuropeptides*, in press.
11. Evans, B. E., Bock, M. G., Rittle, K. E., DiPardo, R. M., Whitter, W. L., Veber, D. F., Anderson, P. S. & Freidinger, R. M. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 4918–4922.
12. Snider, R. M., Constatine, J. W., Lowe, J. A., III, Longo, K. P., Lebel, W. S., Woody, H. A., Drozda, S. E., Desai, M. C., Vinick, F. J., Spencer, R. W. & Hess, H.-J. (1991) *Science* **251,** 435–436.
13. Yamamura, Y., Ogawa, H., Chihara, T., Kondo, K., Onogawa, T., Nakamura, S., Mori, T., Tominaga, M. & Yabuuchi, Y. (1991) *Science* **252,** 572–574.
14. Masler, E. P., Kelly, T. J. & Menn, J. J. (1993) *Arch. Insect Biochem. Physiol.* **22,** 87–111.
15. Nachman, R. J., Holman, G. M. & Haddon, W. F. (1993) *Arch. Insect Biochem. Physiol.* **22,** 181–197.
16. Nachman, R. J., Tilley, J. W., Hayes, T. K., Holman, G. M. & Beier, R. C. (1994) in *Natural and Engineered Pest Management Agents*, eds. Hedin, P. A., Menn, J. J. & Hollingworth, R. M. (Am. Chem. Soc., Washington, DC), pp. 210–229.