C. O. Dires, *Tech. Bull. Maine Agri. Exp. Stn.* **26**, 1 (1967); J. Dorr and E. C. Martin, *Q. Bull. Mich. Agri. Exp. Stn.* **48**, 437 (1966); R. J. Reader, *Can. J. Bot.* **55**, 2279 (1977); D. Lomond and D. J. Larson, *Can. Entomol.* **115**, 1647 (1983); J. B. Free, *Insect Pollination of Crops* (Academic Press, New York, 1970).
6. K. L. Kelly, "ISCC-NBS color name charts illustrated with centroid colors," *Suppl. Natl. Bur. Stand. Circ. 553* (1965).
7. M. Käpylä, *Ann. Bot. Fenn.* **15**, 85 (1978).
8. For information regarding techniques, see A. A. Blaker, *Field Photography* (Freeman, San Francisco, 1976); R. J. Hill, *Brittonia* **29**, 382 (1977); R. E. Silberglied, *Funct. Photogr.* **11** (No. 2), 20 (1976); *ibid.*, p. 30; R. Scogin, D. A. Young, C. E. Jones Jr., *Bull. Torrey Bot. Club* **104**, 155 (1977); F. H. Utech and S. Kawano, *Bot. Mag. Tokyo* **88**, 9 (1975).
9. R. E. Silberglied, *Annu. Rev. Ecol. Syst.* **10**, 373 (1979); M. W. Frohlich, *Science* **194**, 839 (1976).
10. K. Faegri and L. van der Pijl, *The Principles of Pollination Ecology* (Pergamon, New York, 1979); W. Wickler, *Mimicry in Plants and Animals* (McGraw-Hill, New York, 1968).
11. We also casually observed pollinators visiting wilted leaves of the following species infected by the respective fungi: *Monilinia amelanchieris* on *Amelanchier canadensis*, *M. johnsonii* on *Craetegus viridis*, *M. megalospora* on *Vaccinium uliginosum*, *M. oxycocci* on *V. macrocarpon*, and *M. polycodi* on *V. stamineum*.
12. We thank H. M. Fales for assistance with sugar analysis and N. Chaney for help with scanning-electron microscopy. Diptera and Lepidoptera were identified by R. J. Gagné, D. Ferguson, and F. C. Thompson. The cooperation of M. Hunt during several years is appreciated. E. M. Barrows, P. O. Batra, and R. W. Lichtwardt read the manuscript.

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Intracellular Stimulation of an Identified Neuron Evokes Cardioacceleratory Peptide Release

Abstract. *The central nervous system of the tobacco hawkmoth,* Manduca sexta, *is known to contain two cardioacceleratory peptides (CAP's), both of which function in vivo as cardioregulatory neurohormones. Intracellular electrical stimulation of a single abdominal ganglion neuron evokes the release of CAP-like bioactivity. This stimulation-evoked bioactivity is destroyed by prior treatment with protease. The possibility that intracellular stimulation of a CAP-containing neuron synaptically activated additional spiking neurons is eliminated.*

Neuropeptides in the central nervous system (CNS) are capable of acting as neurotransmitters (*1–3*) and as neurohormones (*4–6*). It is often easier to define a role for a neuropeptide if that neuropeptide can be unequivocally associated with an identifiable neuron or neurons. There are several physiological, anatomical, and pharmacological criteria that must be met before a neuropeptide can be established as a neurochemical mediator at the cellular level (*7*). Most of these criteria are similar to those for the rigorous identification of conventional neurotransmitters (*8*). One crucial criterion frequently overlooked is the demonstration that the neuropeptide is released when the putative peptidergic neuron is individually depolarized above threshold. Although peptide release from the CNS has been shown in several preparations by treatment with K^+-rich saline (*9–11*) or by electrical stimulation of peripheral nerve roots (*12–14*), it has been difficult to demonstrate peptide release resulting from the activity of single cells regardless of whether the neuropeptide is acting as a neurotransmitter or as a neurohormone. We show here that intracellular electrical stimulation of a single, identified neuron is sufficient to elicit the release of neuropeptide activity from its terminal endings.

We have studied the cardioacceleratory peptide (CAP) system in the tobacco hawkmoth, *Manduca sexta*. Earlier investigations (*15–17*) have shown that two cardioactive neuropeptides, known as cardioacceleratory peptide 1 (CAP_1) and cardioacceleratory peptide 2 (CAP_2), are present in the pharate adult ventral nerve cord (VNC). The two CAP's are coreleased into the hemolymph from the segmentally repeated transverse nerves (Fig. 1B) immediately after adult emergence, and they act to increase heart rate significantly and to facilitate inflation of

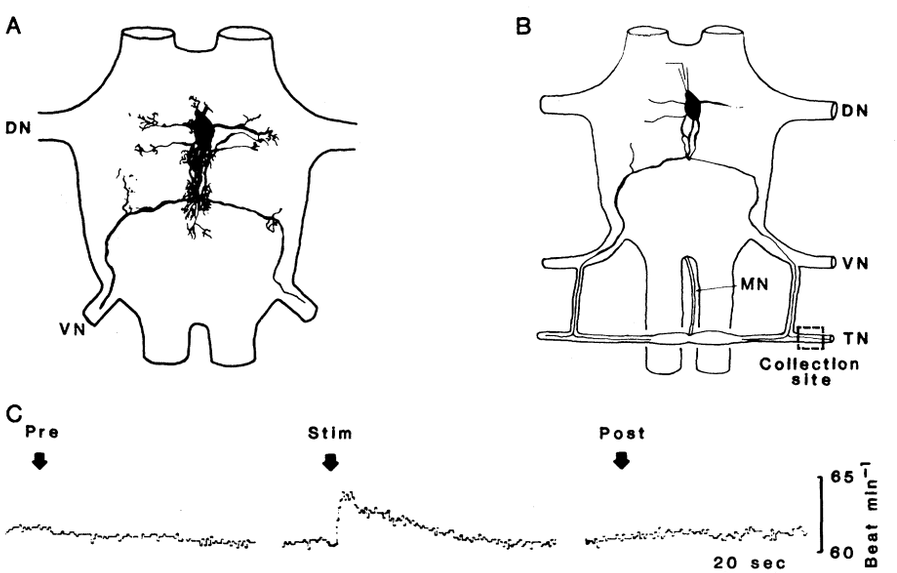

Fig. 1. Stimulation of a new MB neuron causes the release of CAP-like bioactivity. (A) Camera lucida drawing of a new MB neuron in a pharate adult abdominal ganglion. We stained the cell by passing positive current through an intracellular micropipette filled with 48 percent hexamine cobaltous chloride. In the preparation a modification of Timm's silver intensification procedure was used (*23*). The bifurcating axon exits the ganglion via both ventral nerves. The cell terminates bilaterally in neurosecretory endings along the length of the transverse nerve (*24*). (B) Diagrammatic representation of the experimental protocol. We impaled a soma of a new medial bilateral (MB) cell, using standard glass microelectrodes, and depolarized it by passing d-c current pulses for up to 15 minutes at a frequency of not greater than 0.5 Hz. Although not visible in situ, each new MB cell was identified unequivocally on the basis of its cell body position in the ganglion, the trajectory of its axons, and the characteristic electrical properties of its soma. As is typical of insect neurosecretory cells (*24, 25*), the somata of the new MB neurons were electrically excitable, capable of supporting overshooting action potentials with durations of approximately 50 msec. Thus, as a group these neurons were uniquely recognizable during recording sessions. As it proved impossible to maintain somatic activity with dye-filled microelectrodes, we were unable to distinguish between the two anteriormost pairs of new MB cells. We collected CAP activity by erecting a Vaseline well (volume ~0.1 ml) around the transverse nerve at a point distal to the transverse nerve–ventral nerve anastomosis. The contents of the well were collected at various times, frozen on Dry Ice, and stored at −20°C, usually for less than 24 hours, until bioassayed for CAP activity. Abbreviations: TN, transverse nerve; DN, dorsal nerve; VN, ventral nerve; MN, median nerve. (C) Cardioacceleratory activity of samples collected during intracellular stimulation of a new MB cell. Each sample was sequentially bioassayed on the same in vitro *Manduca* heart as described (*16, 17, 21*). For these experiments, the variability in the basal heart rate was ≤1 percent. Arrows denote application of samples. The heart rate increased after application of the Stim sample. A standard lepidopteran saline (*16*) was used in all experiments.

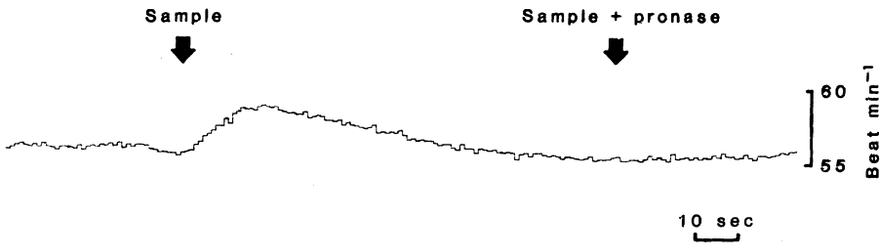

Fig. 2. The effect of protease treatment on the cardioacceleratory activity collected after stimulation of a new MB neuron. A new MB cell was impaled and stimulated as described in Fig. 1. The sample obtained after somatic stimulation was divided into two equal portions and frozen at −20°C for 12 hours. After thawing, one portion was treated with Pronase (0.5 mg/ml) dissolved in saline. An equivalent amount of saline was added to the control sample. Both samples were incubated at 30°C for 1 hour and then were boiled for 5 minutes to inactivate the protease. Both samples were then immediately bioassayed for cardioacceleratory activity on the in vitro *Manduca* heart.

the adult wings (*16–18*). Preliminary evidence (*19*) suggests that the CAP-containing neurons are restricted to the new medial bilateral (MB) neurons, three pairs of neurons that arise during adult development and have somata lying along the midline of each abdominal ganglion (*20*). These cells have a distinctive morphology (Fig. 1, A and B) and are the only cells in this portion of the ganglion that project bilaterally to the posterior transverse nerves, the primary neurohemal release site for the insect VNC.

After impaling a new MB soma and obtaining a stable intracellular recording, we stimulated the cell body at 0.5 Hz with suprathreshold current pulses for 15 minutes. Three samples were obtained from the collection site on the transverse nerve (Fig. 1B) during each experiment: one prior to stimulation (Pre), one immediately after intracellular stimulation (Stim), and a third after the preparation was washed for 60 seconds (Post). Each sample was sequentially bioassayed for cardioacceleratory activity on the in vitro *Manduca* heart, whose variability in basal rate was ≤1 percent (*16, 17, 21*). Only the Stim sample exhibited any cardioacceleratory activity (Fig. 1C). Bioassay of the Stim sample produced a 4 percent increase in heart rate, equivalent to the release of 0.02 CAP unit. In contrast, the Pre and Post samples exhibited no demonstrable cardioacceleratory activity. Using the same protocol, we found that CAP-like bioactivity was released in five out of six preparations. Expressed in terms of the percentage increase in heart rate, the mean ± SE (n = 6) values for the Pre, Stim, and Post samples were 0.50 ± 0.34, 4.00 ± 0.63, and 0.20 ± 0.06, respectively.

Although the *Manduca* heart bioassay has been shown to be quite sensitive to low concentrations of either CAP (*16, 17*), it also responds in a qualitatively similar fashion to several other neuroactive substances, particularly serotonin and octopamine (*16, 17, 21*). To demonstrate that this stimulation-evoked cardioacceleratory activity was due to the presence of a peptide, we impaled an MB neuron and stimulated it as described above. The sample taken immediately after stimulation was divided into two portions, one of which was treated with Pronase (Sigma), a nonspecific protease that does not interfere with the in vitro *Manduca* heart bioassay (*16, 22*). After incubation and heating, each sample was bioassayed for CAP bioactivity. The control sample (Fig. 2) produced a demonstrable elevation in heartbeat frequency, whereas the protease-treated sample (Fig. 2) was devoid of CAP activity. These data demonstrate that the cardioactivity exhibited after stimulation was proteinaceous and, on the basis of the site of its release and its action on the in vitro heart, was likely to be CAP_1 or CAP_2, or both.

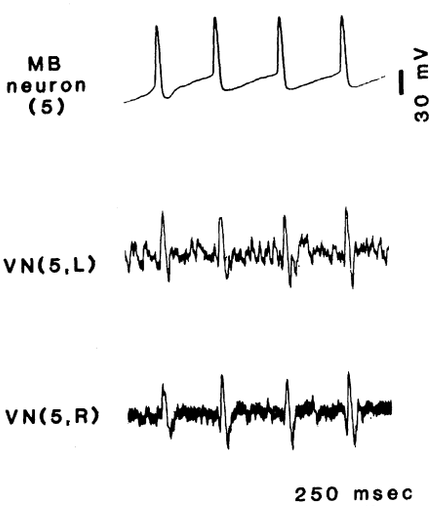

Fig. 3. Extracellular activity recorded from the right (R) and left (L) ventral nerves during depolarization of a new MB cell. (Top trace) Intracellular recording from a new MB neuron located in the fifth abdominal ganglion (A5) of a pharate adult. (Bottom traces) Extracellular recordings from the paired ventral nerves of the same ganglion. Glass-tipped suction electrodes were placed on the cut ends of each ventral nerve in ganglion A5 close to where the nerve exits the ganglion. Action potentials in the MB neuron are associated with a single extracellular spike in each root.

It was important to establish that intracellular stimulation of the MB neuron did not synaptically activate other neurons that projected to the transverse nerve. An MB cell was therefore depolarized above threshold, and the activity leaving the ganglion was monitored by extracellular electrodes placed bilaterally on the ventral roots. As we predicted from its anatomy (Fig. 1A), depolarization of an MB neuron revealed a single extracellular unit in each of the ventral roots that was phase-locked to the intracellular activity (Fig. 3). Distinguished by its very long duration (40 to 50 msec), this extracellular unit was similar in length to the MB intracellular action potential and much longer than the duration of a standard insect axon spike (1 to 2 msec). The failure to find other units activated coincident with MB cell stimulation suggests that CAP release was due solely to the depolarization of the MB terminals and not to synaptic activation of other transverse nerve-projecting neurons, all of which fire action potentials (*9, 24*). This experiment, however, does not rule out the slim possibility that the stimulated neuron depolarized a follower cell, which released CAP activity without firing action potentials.

This evidence demonstrates that intracellular stimulation of a new MB soma causes release from its terminals of a protease-sensitive, cardioactive factor that is pharmacologically identical to the CAP's. This result supports our contention that peptide activity can be elicited from the excitation of a single, identified, peptidergic neuron.

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References and Notes

1. L. Y. Jan and Y. N. Jan, *Frontiers Neuroendocrinol.* **7**, 211 (1982).
2. S. Konishi, A. Tsunoo, M. Otsuka, *Nature (London)* **294**, 80 (1981).
3. T. Takahashi *et al.*, *Brain Res.* **73**, 59 (1974).
4. R. Guillemin, *Science* **202**, 390 (1978).
5. J. W. Truman, in *Insect Biology in the Future*, M. L. Locke and D. S. Smith, Eds. (Academic Press, New York, 1980), p. 384–401.
6. I. Kupfermann, *J. Neurophysiol.* **33**, 877 (1970).
7. J. L. Barker, in *Peptides in Neurobiology*, H. Gainer, Ed. (Plenum, New York, 1977), pp. 295–343.
8. R. Werman, *Comp. Biochem. Physiol.* **18**, 745 (1966).
9. P. H. Taghert and J. W. Truman, *J. Exp. Biol.* **98**, 373 (1982).
10. M. E. Adams and M. O'Shea, *Science* **221**, 286 (1983).
11. S. H. P. Maddrell and J. Gee, *J. Exp. Biol.* **61**, 155 (1974).
12. S. B. Kater, *Science* **160**, 765 (1968).

13. J. M. Lundberg *et al.*, *Neuroscience* **4**, 1539 (1979).
14. I. Kupfermann and E. R. Kandel, *J. Neurophysiol.* **33**, 865 (1970).
15. N. J. Tublitz and J. W. Truman, *Soc. Neurosci. Abstr.* **7**, 253 (1981).
16. ———, *J. Exp. Biol.*, in press.
17. N. J. Tublitz, thesis, University of Washington, Seattle (1983).
18. N. J. Tublitz and J. W. Truman, *J. Exp. Biol.*, in press.
19. ———, *Soc. Neurosci. Abstr.* **9**, 19 (1983).
20. H. M. Taylor and J. W. Truman, *J. Exp. Biol.* **58**, 821 (1974).
21. The in vitro *Manduca* heart bioassay consisted of the abdominal portion of the pharate adult heart, which was isolated and placed in a 250-μl superfusion chamber and attached to a Bionix F-200 isotonic displacement transducer. The amplified signal was fed through a window discriminator and a digital-to-analog frequency monitor, which allowed for the measurement of instantaneous heart rate. An open perfusion system was used with the open point suspended 5 cm above the superfusion chamber. During each bioassay, saline flowed from the open point directly to the superfusion chamber. Samples (100 μl) were pulse-applied at the open point with a 100-μl gas-tight Hamilton syringe. Samples were applied only when the variability in the heart was ≤1 percent for a 15-minute period. In addition to the CAP's, octopamine and serotonin increase heart rate as measured on the in vitro *Manduca* heart bioassay; these biogenic amines can be separated from the CAP's by gel filtration or high-performance liquid chromatography, or both.
22. In these experiments, crude homogenates or partially purified CAP_1 or CAP_2 were incubated in the presence of Pronase which had been previously inactivated by boiling. The results show that inactivated Pronase neither interferes with the basal rate of the in vitro heart nor curtails the ability of the heart bioassay to respond to CAP_1 or CAP_2 (*16*).
23. J. P. Bacon and J. S. Altman, *Brain Res.* **138**, 359 (1977).
24. P. H. Taghert, thesis, University of Washington, Seattle (1981).
25. S. Miyazaki, *J. Comp. Physiol.* **140**, 43 (1980).
26. We thank J. Weeks for technical assistance and advice during the course of these experiments. We are grateful to S. H. P. Maddrell, P. K. Schofield, and J. E. Treherne for critical reviews of this manuscript. Supported by a National Science Foundation predoctoral fellowship and an National Institutes of Health traineeship to N.J.T. and by grants to J.W.T. from NIH, NSF, and the McKnight Foundation.
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Durability of the Accretion Disk of Millisecond Pulsars

Abstract. *Pulsars with pulsation periods in the millisecond range are thought to be neutron stars that have acquired an extraordinarily short spin period through the accretion of stellar material spiraling down onto the neutron star from a nearby companion. Nearly all the angular momentum and most of the mass of the companion star is transferred to the neutron star. During this process, wherein the neutron star consumes its companion, it is required that a disk of stellar material be formed around the neutron star. In conventional models it is supposed that the disk is somehow lost when the accretion phase is finished, so that only the rapidly spinning neutron star remains. However, it is possible that, after the accretion phase, a residual disk remains in stable orbit around the neutron star. The end result of such an accretion process is an object that looks much like a miniature (about 100 kilometers), heavy version of Saturn: a central object (the neutron star) surrounded by a durable disk.*

Spin-up of pulsars in a binary accretion scenario has been proposed by a number of investigators (*1*). We have argued that neutron stars formed in supernova events would be surrounded by a disk of material from the parent star (*2, 3*). Most but not all of the stellar material from the supernova is either exploded to form a surrounding nebula or imploded to form a neutron star. We estimate that approximately 10^{-4} solar mass is left in orbit around the neutron star to form a disk. Dynamo currents between the remnant disk and the magnetized neutron star drive the observed pulsar emissions (*3–5*).

For millisecond pulsars (if they are formed by accretion events), a durable disk evolves from the accretion disk after the secondary object stops feeding the disk. Once the forced accretion stops, the portion of the disk inside the corotation distance continues to move inward and falls onto the neutron star. However, the portion outside the corotation distance moves away from the neutron star at a relatively slow rate because of the torque applied to it by dynamo currents between the disk and the star (*3*).

The viscous dissipation in the shrinking inner disk suggests that the disk would accrete entirely in a short time. However, some of the spin-down energy is pumped back into the disk and acts instead to eject it. We can calculate a critical viscosity ν_{crit} that dissipates energy in the disk faster than power is supplied by the central object as follows. The time derivative of the inward mass rate, $\dot{M}$, is $3\pi\nu\Sigma$ (*6*), where Σ is the surface mass density of the disk (in grams per cubic centimeter). By equating the spin-down output $I\Omega\dot{\Omega}$ (where I is the moment of inertia and Ω and $\dot{\Omega}$ are the spin rate and its time derivative, respectively) to the increased binding produced by a mass rate $G\dot{M}M/R_c$ [where R_c is the corotation distance, $(GM/\Omega^2)^{1/3}$, and G is the gravitation constant], we find ν_{crit} equal to $R_c I\Omega\dot{\Omega}/3\pi G\Sigma$. For a millisecond pulsar, Ω is about 4000 sec^{-1}, $\dot{\Omega}/\Omega$ is about 1.3×10^{-19}, Σ is about 10^{12} g/cm^2, and R_c is about 3×10^6 cm. With I approximately 10^{45} g cm^2 as the moment of inertia of a typical neutron star, this gives a ν_{crit} of about 5 cm^2/sec. A value for ν of approximately 1 to 100 cm^2/sec has been calculated for matter at densities typical of white dwarfs (that is, essentially the disk) (*7*). The electron degenerate disks proposed here differ greatly from the nondegenerate disks proposed for pulsating x-ray sources, which are thought to have a viscosity 10^{14} times larger than that calculated by Flowers and Itoh (*7*).

The remnant of the companion star that provided the material for the disk and for spinning up the neutron star may be reasonably expected to form a degenerate dwarf (*8*). It has been argued that the companion star would spiral inward because of gravitational radiation and would eventually become tidally disrupted to add material to the disk (*8*). This scenario reinforces our proposal that a remnant disk is formed in the spin-up of the neutron star.

The only other suggestion for removing a disk is that such a disk could not survive if the companion star at a distance of only a few solar radii were to become a supernova (*9*). There are two mechanisms by which a disk might be dissipated in such a supernova event: (i) the gravitational impulse caused by the sudden passage of the ejected mass might shake the disk loose from the neutron star, and (ii) the direct impact of supernova ejecta might sweep the disk away.

The first mechanism cannot disrupt the disk. The neutron star and the disk are so close to each other that they receive almost identical gravitational impulses. The second mechanism, in which it is proposed that direct impact of supernova debris can blow away the disk of a neutron star, is also ineffective. To evaluate this possibility, we must make some assumptions that are dependent on our model. The disk is so stable that, even in the most unfavorable case, it can survive a nearby supernova explosion.

For example, we will suppose that an extremely energetic event propels the outer envelope of the exploding star outward with a velocity V of 10^4 km/sec and a peak mass density ρ of the order of 1 g/cm^3. If the star undergoes a vigorous phase of mass loss just before detonation, smaller densities could be appropriate. In the most unfavorable case, the disk would be oriented face-on to the blast and not shielded by the neutron star