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Review

Neural regulation of sex-pheromone glands in Lepidoptera

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ABSTRACT Substantial progress has been made toward understanding the neuroendocrine regulation of sex-pheromone glands in Lepidoptera, but several recent studies have revealed that direct contact of the pheromone gland with blood-borne factors is not necessary to induce pheromone biosynthesis and release in some species. The nervous system provides an alternate route of activation. Evidence from several species indicates that the pheromone gland is innervated and regulated by neural activity. Electrical stimulation of efferent axons arising from the terminal abdominal ganglion results in a significant increase in pheromone production, and neural stimulation furthermore evokes the rapid release of pheromone into the surrounding air. In some heliothine moths, the biogenic monoamine octopamine stimulates pheromone production, and octopamine has also been isolated from pheromone gland tissue. Moreover, the critical period for maximal octopamine action mirrors the time when peak levels of octopamine are present in the gland. These findings suggest that octopamine is involved in the regulation of pheromone biosynthesis and/or release, but its actions depend on additional factors associated with age and photoperiod. The combined evidence using anatomical, electrophysiological, and biochemical methods indicates that the pheromone gland is innervated and regulated by neurons that arise in the terminal abdominal ganglion. Indirect evidence suggests that at least some of this innervation is octopaminergic. In these respects, the pheromone gland in Lepidoptera exhibits characteristics of other neuroeffector systems in insects.

KEY WORDS: innervation; neural regulation; octopamine; pheromone biosynthesis; pheromone release; terminal abdominal ganglion

Introduction

Over the past decade, there has been a renewed interest in understanding the mechanisms regulating the periodic production and release of female sex pheromones in many lepidopteran insects, and several hypotheses are now being examined to help explain this regulation in different species (Fig. 1). One of the major advances in this area is the discovery that neuropeptides play a pivotal role in the control of the pheromone gland in many species of moths. To date, more than a dozen peptides that stimulate pheromone biosynthesis have been isolated and sequenced, and a number of studies suggest that they exert a direct action on the pheromone gland through the hemolymph (see Raina, 1993; Jacquin *et al.*, 1994 and references therein). A direct role in the regulation of lepidopteran pheromone glands, however, has yet to be established for any of these peptides for several reasons:

First and foremost, we still lack crucial information regarding the localization and characterization of peptide receptors associated with any tissues involved in the pheromone biosynthetic pathway, including the pheromone gland itself.

Secondly, many of these peptides have multiple functions, and, in addition to their pheromonotropic action, mediate processes that are unrelated to pheromone production (Nachman *et al.*, 1993). A number of recent studies have revealed that peptides that were first characterized as pheromonotropic, such as the pheromone biosynthesis activating neuropeptides (PBANs) from the moths *Helicoverpa zea* and *Bombyx mori* (see Raina, 1993; Kelly *et al.*, 1994 and references therein), act also on other diverse targets (Fig. 1). These peptides are collectively referred to as FXPRLamides because they all share a common C-terminal pentapeptide sequence (Nachman *et al.*, 1993). Some of the FXPRLamides, for example, can induce cuticular melanization in larvae (Matsumoto *et al.*, 1990), while also having a myotropic action on oviduct and hindgut in adults (Fonagy *et al.*, 1992a; Kuniyoshi *et al.*, 1992). Likewise, peptides that were first characterized as having myotropic properties have now been shown to be pheromonotropic in moths (Fonagy *et al.*, 1992a; Kuniyoshi *et al.*, 1992), and in some cases, more potent than the PBANs themselves (Abernathy *et al.*, 1995). Among other functionally diverse peptides is the diapause hormone from *Bombyx mori*, which induces egg diapause when injected into pupae, but has pheromonotropic properties in adult moths (Abernathy *et al.*, 1995 and references therein).

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Of particular interest is the recent finding that several locustatachykinins – molecules that share no sequence homology to the PBAN – pyrokinin family of peptides – are also pheromonotropic in moths (Fonagy *et al.*, 1992b). All of these findings suggest that members of these functionally-diverse families of peptides have many sites of action in the insect (Nässel, 1993; Homberg, 1994), and that they may also serve different functions at different stages in metamorphic development (Tublitz *et al.*, 1991).

Thirdly, the widespread distribution of pheromonotropic peptides as revealed by immunocytochemical methods suggests that such peptides are mobilized from both central (neural) and peripheral release sites (Rafaeli *et al.*, 1991; Kingan *et al.*, 1992; Davis *et al.*, 1995).

It has become clear within the last decade that certain neuropeptides are sufficient to stimulate pheromone biosynthesis *in vivo* and *in vitro*, and the supporting literature has been reviewed extensively (Altstein *et al.*, 1993; Rafaeli *et al.*, 1993; Raina, 1993; Raina and Menn, 1993 and earlier references therein). It is also apparent, however, that other mechanisms are also sufficient to induce pheromone production in some species of moths (Hollander and Yin, 1982, 1985; Tang *et al.*, 1987, 1989; Teal *et al.*, 1989; Thyagaraja and Raina, 1994). There is a growing body of evidence, for example, that the pheromone glands in several species of Lepidoptera are innervated and regulated by the central nervous system. It is this particular aspect of pheromone regulation in Lepidoptera that we review here.

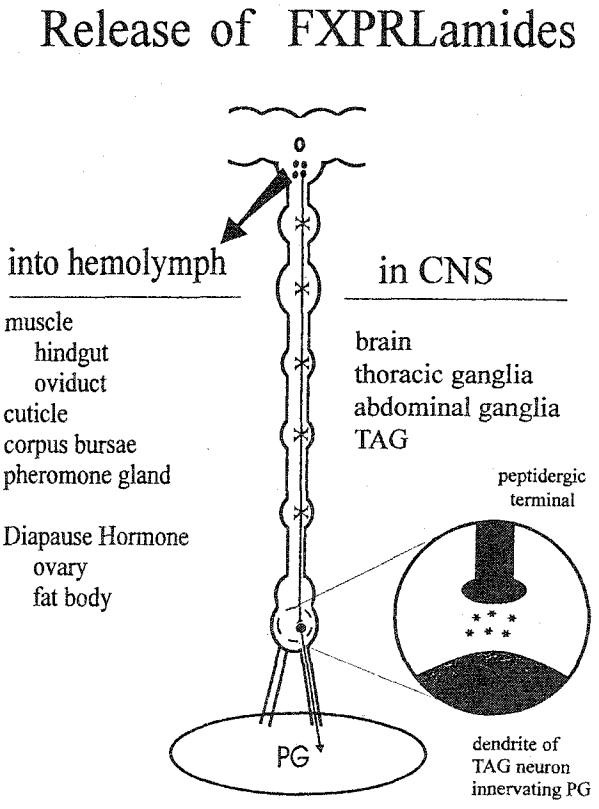

Fig. 1. Diagram summarizing the proposed sites of action for FXPRLamides in Lepidoptera. *Peptide-immunoreactive cells arising in the subesophageal ganglion display what appear to be both peripheral and central release sites. Peptides may be released into the blood from the corpora cardiaca and other neurohemal sites (left side of diagram), or they may be carried by descending neurons and released within the CNS where they would affect central nervous targets (right side). Descending fibers extend varicose processes into every ganglion they pass through, including the fused terminal abdominal ganglion (TAG) where they terminate. These varicose processes resemble nerve terminals and therefore possible release sites for peptides in the neuropil of the TAG. Inset at lower right depicts an enlargement of a putative peptidergic release site in the TAG. PG, pheromone gland.*

Evidence for innervation

Anatomy

The neural connections between the terminal abdominal ganglion and the pheromone gland were first discovered in the adult female corn earworm moth *Helicoverpa zea* (Christensen *et al.*, 1991), but innervation has since been observed at the ultrastructural level in the tobacco budworm moth *Heliothis virescens* (Christensen *et al.*, 1991), and the tobacco hornworm moth *Manduca sexta* (T. Christensen, C. Cuzzocrea and J. G. Hildebrand, unpublished observations). Nerves arising from the terminal ganglion in *H. zea* were filled with cobalt–lysine or biocytin, and the staining revealed the innervation of the pheromone gland as finely branching nerve fibers studded with varicosities (Fig. 2; Christensen *et al.*, 1991). These "blebbed" elements are not associated with muscle, nor are they associated with peripheral cell bodies, which are typical of sensory neurons that are sometimes observed in these preparations. Electron microscopy revealed that these nerve fibers penetrated the basement membrane of the gland, were enwrapped by glia or Schwann-like cells, and contained dense-core vesicles typical of neurosecretory nerve endings (Fig. 2). Similar profiles were observed in the pheromone gland of *Heliothis virescens* females (Christensen *et al.*, 1991; R. A. Steinbrecht, unpublished observations).

Physiology

What role(s) might this innervation play in the regulation of pheromone biosynthesis and release? The pheromone glands of female heliothine moths contain

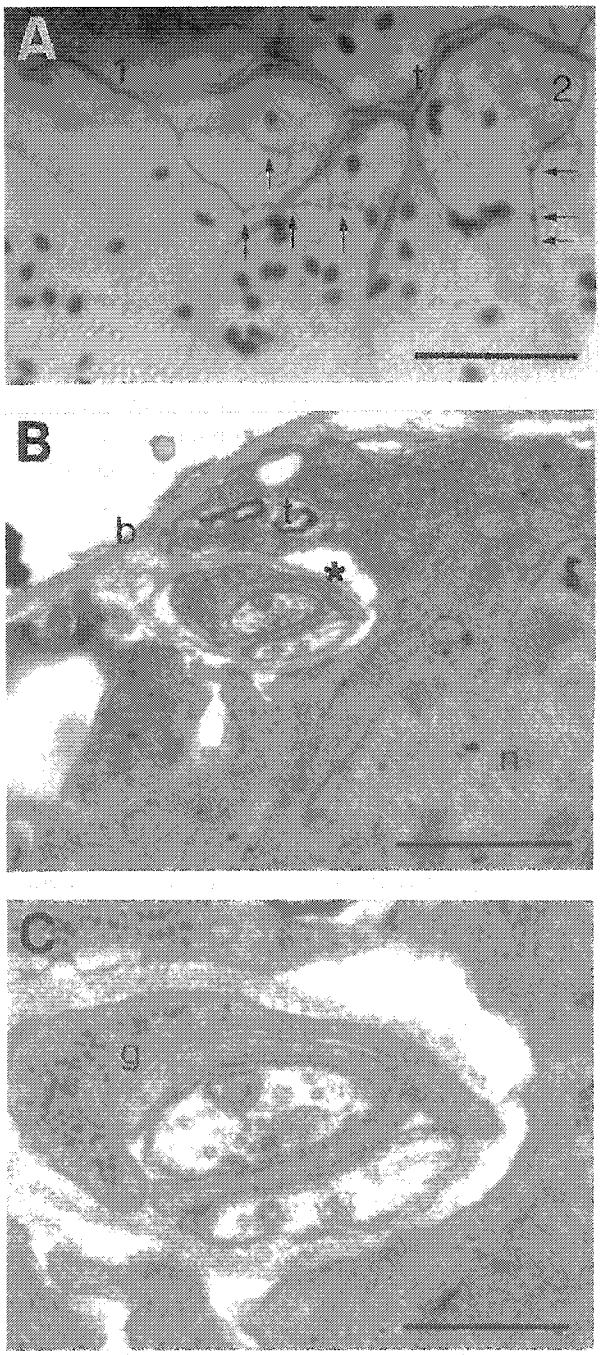

Fig. 2. Anatomical evidence for innervation of the pheromone gland through the terminal nerves in *Helicoverpa zea* females. *A. Anterograde impregnation of axons in the terminal nerves using the tracer biocytin. Two terminal-like processes branch over the surface of the gland, which lines the ventral epicuticle between abdominal segments 8 and 9. The branching processes, labeled 1 and 2, are studded with varicosities (arrows) near their distal ends. t, tracheole. B. Electron micrograph of a pheromone gland cell showing innervation that penetrates the basement membrane (b) of the gland. n, nucleus. C. Enlargement of the area indicated by the asterisk in B. One of the neural processes contains numerous vesicles and is enwrapped by a glial cell (g). Scale bars: A, 100 μm; B, 800 nm; C, 300 nm.*

little pheromone during the photophase (quantified by gas–liquid chromatography following solvent rinses of pheromone glands), but electrical stimulation of the connectives anterior to the terminal ganglion or the nerves posterior to the terminal ganglion resulted in a significant increase in the amount of pheromone in the gland (Christensen *et al.*, 1991). Stimulation for as little as 5 min. evoked pheromone production, but stimulation for longer periods (up to 1 h) did not result in greater production. One possible explanation is that over the longer periods of stimulation, the pheromone being produced was released into the air, and therefore unavailable for analysis. To test this hypothesis, a male electroantennogram bioassay was used to monitor the pheromone being released as an immediate consequence of the electrical stimulation. Using this method with *M. sexta*, it was found that electrical stimulation of the ventral nerve cord leads to the release of pheromone from the gland, and that this release occurs on a sub-second time scale (Christensen *et al.*, 1994). The electrically-evoked response increased gradually as the number of stimulus pulses increased, but the response decreased and leveled off with stimulus trains of more than about 10 pulses. The pheromone gland therefore seems to resemble other typical neuroeffector systems such as muscle, in that an increase in the number of stimuli yields a gradual increase in the magnitude of the effector response, and excessive stimulation leads to response depression. The response, furthermore, is completely eliminated if the terminal nerves are severed (Christensen *et al.*, 1994), indicating the necessity of this particular neural connection in mediating the response.

Neurochemistry

To begin to identify neuroeffector candidates that could be involved in regulation or modulation of pheromone gland activity, several biogenic amines that have diverse functions in insects were tested. Of these substances, only the monoamine octopamine had significant effects on pheromone production (Christensen *et al.*, 1991, 1992). Octopamine was injected into intact *H. virescens* females (Christensen *et al.*, 1991) or applied to isolated abdomens in the absence of the terminal ganglion (Christensen *et al.*, 1992). In the latter experiments, it was shown that the effects of octopamine were not always present, but strongly depended on photoperiod. Abdomens treated up to the 13th hour of a 14 h photophase did not respond to octopamine, whereas abdomens treated during the period beginning 1 h prior to scotophase and ending 1 h into scotophase produced significant amounts of pheromone. This critical period for octopamine action furthermore coincides with the

time when peak levels of this amine are present in the pheromone gland tissue (Christensen *et al.*, 1992).

Our results with *H. zea* females also suggest that age-related changes can affect dramatically the actions of octopamine on the pheromone glands in these short-lived insects. Octopamine, when injected into females during the second day of adult life, stimulated significant increases in pheromone production, whereas the same treatments did not have an effect on animals in their third or fourth photophase following emergence (Christensen *et al.*, 1992). In this regard, another study has reported that octopamine has no pheromonotropic effect in older *H. zea* females (Jurenka *et al.*, 1991). In order to test for an age-dependent effect in *H. virescens*, we repeated the abdomen incubations using animals during their first, second, or third day (24 h period) after emergence. As shown in Fig. 3, octopamine induced significant amounts of pheromone production in day-2 females, but not in day-1 or day-3 females. These results were essentially the same as those previously reported, but with one major difference. The pheromone levels obtained from both the control and the treated day-2 animals were substantially greater than the corresponding levels in earlier trials (Christensen *et al.*, 1992). This result illustrates two important principles that have been encountered repeatedly with respect to the operation of the pheromone gland: not only is there considerable variability from trial to trial in the amount of extractable pheromone induced by various treatments, but there is also significant variability in the basal levels of pheromone in the gland, even in the same species. The endogenous and/or environmental factors that influence this variability are unknown and difficult to control, making the results of the various pharmacological treatments in different populations of insects difficult to interpret.

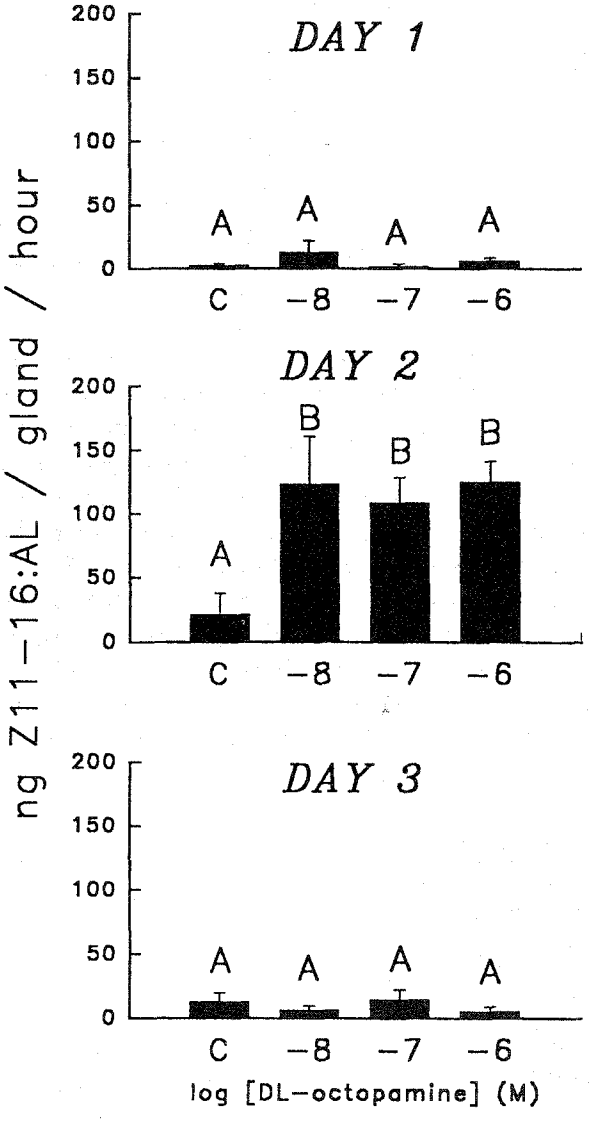

Fig. 3. Age-dependent effect of DL-octopamine on isolated abdomens in *H. virescens* females. *Each bar represents mean ± SEM of (Z)-11-hexadecenal (Z11–16:AL – the major component of the sex pheromone) produced in 1 h by treatment with either 10^{-8}, 10^{-7}, or 10^{-6} M octopamine in physiological saline (Christensen et al., 1992); C = saline control. Means capped by the same letter are not significantly different ($P < 0.05$); Duncan's multiple range test; n = 5–8 glands per treatment.*

Thus, while the combined physiological and biochemical evidence suggests a role for octopamine in the regulation of the pheromone gland, its exact role and even its site of action as a neurotransmitter or neuromodulator is still unclear (Rafaeli and Gileadi, 1995; Ramaswamy *et al.*, 1995). It is of interest to note that the potential role of octopamine in regulating the release of adipokinetic hormones from the insect corpora cardiaca is also controversial (Passier *et al.*, 1995 and references therein). Interestingly, there is significant variation in the amount of hormone released from these glands in response to different experimental treatments, much like the variation in pheromone titers observed in a given population of female moths. Understanding the nature of these variations, and the possible regulatory and modulatory roles of biogenic amines and peptides, is an important challenge in furthering our understanding of stimulus–secretion coupling mechanisms in these glands.

Integrating two views of pheromone regulation

The regulation of pheromone biosynthesis and release in insects has become a vigorous area of research, requiring the combined efforts of biochemists, behaviorists, anatomists, electrophysiologists and molecular

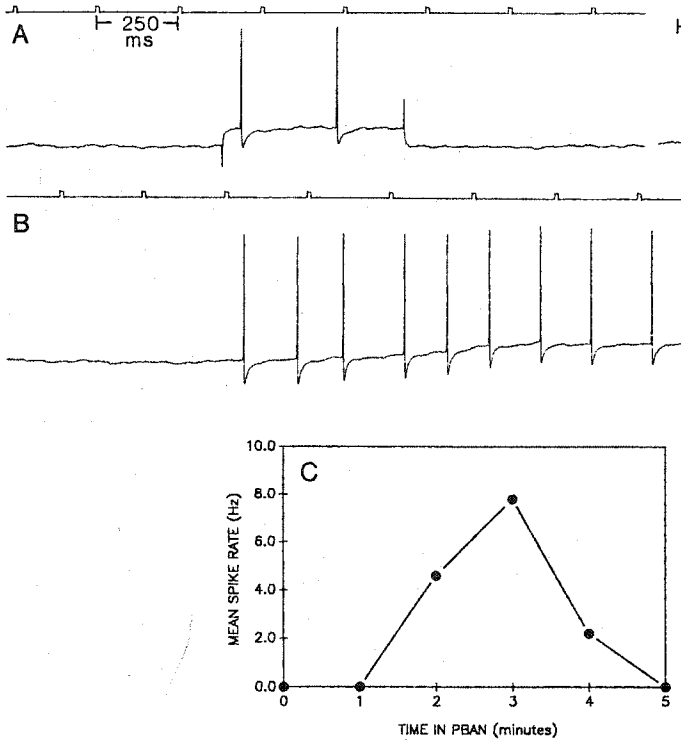

Fig. 4A–C. Some neurons in the terminal abdominal ganglion are activated by bath application of PBAN. *A. Before application of a PBAN first identified in* Helicoverpa zea *(Hez PBAN), this neuron produced a spike only if depolarizing current was injected through the microelectrode into the soma. Inset at right shows spike on an expanded time scale. Note relatively broad spike width and deep undershoot, characteristic of neurosecretory neurons. B. Two min. after applying 5 pmol Hez-PBAN to the 0.5 ml well surrounding the terminal ganglion, the cell depolarized gradually by 5–10 mV and produced spikes. C. Time course of mean activity in four impaled neurons in as many animals.*

biologists to unravel its secrets. Over the years, different arguments have been put forth in support of the "neuroendocrine" or "neural" hypotheses to explain pheromone regulation, but it remains unclear exactly how these mechanisms contribute to pheromone production in any species. From the results of our own work and those of others, it is now clear that more than one mechanism can govern the operation of pheromone glands, even in the same species. These mechanisms, moreover, are not mutually exclusive. One plausible explanation for the lack of agreement over a regulatory mechanism is that the two mechanisms may, in fact, be interdependent and their respective actions, therefore, difficult to separate. There is now considerable evidence for both central and peripheral sites of action for various peptidergic neurons in insects (Nässel, 1993), and this applies also to the pheromonotropic peptides discussed earlier. The patterns of staining revealed by PBAN immunocytochemistry as well as by competitive ELISA bioassays seem to indicate that there are sites of release not only into the blood, but also within the CNS itself (Fig. 1; Rafaeli *et al.*, 1991; Kingan *et al.*, 1992; Davis *et al.*, 1995). This idea is supported by the recent finding

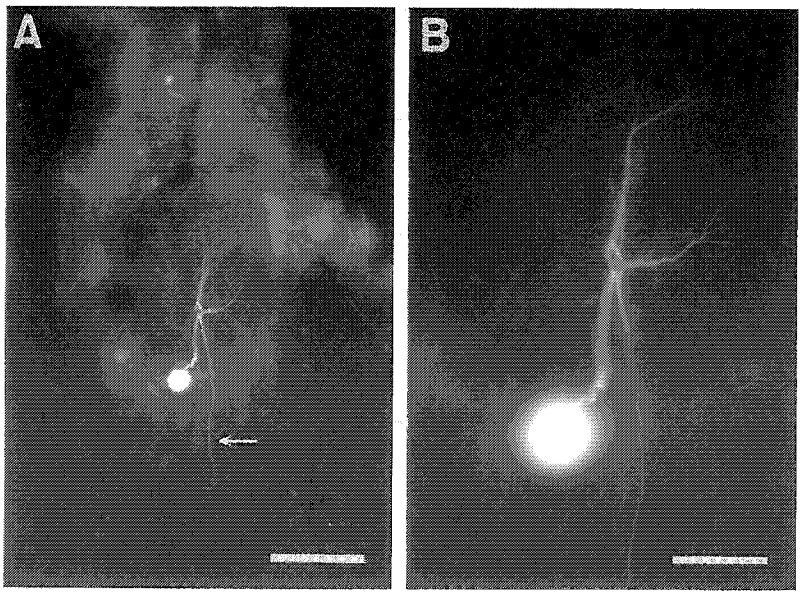

Fig. 5A–B. *A. Fluorescence micrograph of a neuron in the terminal ganglion that responded to bath application of Hez-PBAN and was then stained with Lucifer Yellow CH. The morphology is similar to some posterior midline cells described in an earlier report by Thorn and Truman (1989). Note the single axon exiting the terminal nerve posteriorly (arrow). B. Detail of neuritic branches, most of which appear to be ipsilateral to the soma. Scale bars: A, 100 μm; B, 25 μm.*

that some neurons in the terminal abdominal ganglion are depolarized and activated by bath application of PBAN (Fig. 4). The morphology of these PBAN-receptive neurons has been revealed in a few cases by intracellular staining, and they each displayed a unilateral axon that exited the terminal nerve (Fig. 5). Unfortunately, we have been unable up to this point to trace this axon the several mm distance to its termination point, but these experiments are still in progress.

In summary, pheromone biosynthesis in Lepidoptera is not an all or nothing process, and many factors influence its regulation. Recent evidence supports the idea that some physiological processes in insects are controlled through multiple mechanisms (Hewes and Truman, 1991; Sakurai *et al.*, 1991), and we believe that the mechanisms of pheromone biosynthesis and release are no exception (Christensen *et al.*, 1992; Rafaeli and Gileadi, 1995). Normal activation of the pheromone gland may involve both neural and humoral mechanisms, or alternatively, the two regulatory mechanisms may be differentially activated at different times during the short lifespan of these insects or due to changing environmental conditions. Many questions still remain, and great care must be taken in interpreting results before the definitive experiments are in hand.

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