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# A Monoclonal Antibody to the Insect Prothoracicotropic Hormone

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**The prothoracicotropic hormone (PTTH) is an insect cerebral peptide that stimulates the prothoracic glands to produce the steroid hormone ecdysone thus initiating molting and metamorphosis. "Big" PTTH, one of several molecular forms of the neurohormone, was isolated from brains of the tobacco hornworm *Manduca sexta,* and fractionated by high-pressure liquid chromatography (HPLC) for use in antibody production. A murine polyclonal antiserum and a monoclonal antibody (MAb) have been generated using this highly purified preparation of big PTTH. Antisera and hybridoma supernatants were screened with an indirect, brain whole-mount immunocytological assay, and antibody specificity was confirmed by immunocytological, ELISA, and functional criteria. In brain whole-mount preparations, the MAb (A2H5) and antiserum specifically immunostained the lateral protocerebral neurosecretory cells (L-NSC III), the prothoracicotropes, which produce PTTH. This immunostaining was blocked by preadsorbing the antibodies with big PTTH. Analysis of the elution of HPLC-fractionated big PTTH with an *in vitro* bioassay for the neurohormone and an ELISA employing the A2H5 MAb resulted in peaks of activity that were superimposable. Finally, the antiserum and A2H5 MAb inhibited big PTTH activation of the prothoracic glands to synthesize ecdysone in the *in vitro* bioassay for the neurohormone. With these specific antibodies, the organization of the PTTH neuroendocrine axis has been defined. It is now evident that both of the peptidergic neurons that comprise the L-NSC III are prothoracicotropes, and that the corpora allata are the neurohemal organs for the release of big PTTH into the hemolymph. This study indicates that these specific antibodies will be useful in investigations of numerous aspects of the biology of this cerebral neuroendocrine axis.**

Peptidergic neurons in the CNSs of both vertebrates and invertebrates play integral roles in the regulation of development, homeostasis, and reproduction (Scharrer, 1977). These neurons produce molecular effectors, including neurohormones, neurotransmitters, and neuromodulators; conceivably, a single peptide product could serve all of these functions (Hökfelt et al., 1980; Krieger, 1983).

Vertebrate neuroendocrine axes are organizationally complex. By comparison, invertebrate neuroendocrine axes, and particularly those of some insects, are structurally simple, consisting of a limited number of easily identifiable neurosecretory cells (NSC) (Steel, 1978; Agui et al., 1979; Berlind and Maddrell, 1979; Taghert and Truman, 1982; Tublitz and Truman, 1985; Copenhaver and Truman, 1986a). Thus, these invertebrate neuroendocrine axes offer model systems amenable to the study of the fundamental properties of peptidergic neuron function.

In the lepidopterous insect *Manduca sexta,* the tobacco hornworm, the major cerebral neurosecretory cell groups, their axonal pathways, and their dendritic fields have been well characterized by dye-filling techniques (Nijhout, 1975; Buys and Gibbs, 1981; Carrow et al., 1984; Copenhaver and Truman, 1986b). The neurohormone products of 2 of these neurosecretory cell groups have been determined. A group located dorsolaterally in each hemisphere of the protocerebrum, the L-NSC III, produces prothoracicotropic hormone (PTTH) (Agui et al., 1979), the peptide that initiates postembryonic development by stimulating the prothoracic glands to produce ecdysone (see Bollenbacher and Granger, 1985). Five cells of a second lateral group, L-NSC Ia, produce eclosion hormone, which drives ecdysial behaviors (Copenhaver and Truman, 1986a). While the cerebral neurosecretory cell groups of *Manduca* have been morphologically characterized, and our knowledge of the physiology of their functions is increasing, little is known about the biochemistry and molecular biology of the neuropeptides they produce. The limited progress in these areas has been largely due to the fact that only trace quantities of neurohormones are produced by the few neurosecretory cells in any particular group. Consequently, purification has been difficult and few insect neuropeptides had been sequenced (Starratt and Brown, 1975; Stone et al., 1976) until very recently (Cook et al., 1986; Hayes and Keeley, 1986; Holman et al., 1986; Nagasawa et al., 1986; Kono et al., 1987; Marti et al., 1987).

Efforts to purify PTTH have spanned more than 20 years, but only in the past few years has substantial progress been made. These studies have focused principally on the silkworm, *Bombyx mori,* and *Manduca sexta* (see Bollenbacher and Granger, 1985). In these lepidopterans, PTTH appears to exist in multiple molecular forms that fall into 2 groups, according to apparent molecular weights (Bollenbacher and Gilbert, 1981; Gilbert et al., 1981; Ishizaki et al., 1983; Bollenbacher et al., 1984; Nagasawa et al., 1984; Kataoka et al., 1987). In *Manduca,* these peptides have been termed "big" (~29 kDa) and "small" (~7 kDa) PTTH, with each size group consisting of additional variants (Bollenbacher et al., 1984). In *Bombyx,* the 2 classes

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are 4.4 kDa and ~22 kDa, although the 4.4 kDa variants are not active in *Bombyx* (Nagasawa et al., 1986). In contrast, big and small PTTHs from *Manduca* are active both *in vivo* and in the *in vitro* prothoracic gland bioassay. However, prothoracic glands from different developmental stages exhibit differential sensitivities to these PTTHs. Pupal glands are substantially more sensitive to big PTTH, and larval glands are equally sensitive to both peptides, suggesting that the PTTHs might function at different times during development (Bollenbacher et al., 1984).

In *Manduca,* PTTH is known to be produced by at least one of the L-NSC III group (Agui et al., 1979). The axons of these monopolar neurons project to the contralateral cerebral lobe and exit the brain posteriorly via a retrocerebral nerve, the fused nervi corporis cardiaci I and II. This nerve innervates the glands of the retrocerebral complex, the corpus cardiacum plus corpus allatum. These are the established neurohemal organs for the cerebral peptidergic neurons. The L-NSC III axons traverse the corpora cardiaca and reach the corpora allata via the nervus corporis allati (Nijhout, 1975; Carrow et al., 1984; Copenhaver and Truman, 1986b). The axons terminate in this gland, forming a neurohemal organ for the release of PTTH (Agui et al., 1980; Carrow et al., 1984).

This laboratory has recently isolated several micrograms of highly purified big PTTH for the generation of antibodies to this peptide. Hybridoma technology, in combination with a brain whole-mount immunocytological assay to screen for antibody production, enabled the generation of big PTTH-specific antibodies. Both polyclonal murine antiserum and a monoclonal antibody (MAb) have been characterized by immunocytological, ELISA, and functional criteria that establish that they are specific for the big PTTH of *Manduca.*

## Materials and Methods

*Animals.* Larvae of the tobacco hornworm *Manduca sexta* were reared on an artificial diet (Bell and Joachim, 1976) under a nondiapausing photoperiod (LD 16:8) at 26°C and 60% relative humidity. Last instar larvae and pupae were staged at the time of ecdysis, which was designated as day 0.

BALB/c mice, 6–8 weeks old, were obtained from Charles River Laboratories (Raleigh, NC) and maintained under standard vivarium conditions.

*Immunogen purification.* PTTHs were purified from day 1 pupal brains of *Manduca* by homogenization (Polytron; Brinkmann Instruments, Westbury, NY) in 0.5 M acetic acid, followed by centrifugation at 10,000 × *g* for 15 min. The pellet was reextracted twice with acetic acid. The combined supernatants were heated at 100°C for 2 min and then centrifuged at 10,000 × *g* for 15 min. This supernatant was lyophilized, followed by extraction in 0.1 M acetic acid, as described above. The combined supernatants were ultrafiltered (YM-10; Amicon, Danvers, MA) to separate big PTTH (retentate) from small PTTH (eluate). The retentate containing big PTTH was lyophilized and resuspended in distilled water.

The big PTTH sample was precipitated sequentially with cold acetone (25, 55, and 80%), followed by centrifugation at 10,000 × *g* for 10 min after each precipitation. The 55% pellet, containing big PTTH, was resuspended in distilled water and subjected to reverse-phase high-pressure liquid chromatography (HPLC) on a preparative C-18 column (7.8 mm × 30 cm; LKB Instruments, Gaithersburg, MD) with a solvent system of acetonitrile–water [10–100% linear gradient over 90 min, 0.1% trifluoroacetic acid (TFA)] at a flow rate of 0.5 ml $min^{-1}$. Fractions containing big PTTH were pooled, vacuum-centrifuged (Speed Vac; Savant Instruments, Hicksville, NY), and resuspended for reverse-phase HPLC on an analytical C-4 column (4.6 mm × 25 cm; Vydac, Hesperia, CA) with a solvent system of acetonitrile–water (20–40% linear gradient over 60 min, 0.1% TFA) at a flow rate of 0.5 ml $min^{-1}$.

The initial immunogen was fractionated further on a diphenyl reverse-phase HPLC column (4.6 mm × 25 cm; Vydac) with a solvent system of acetonitrile–water [30–50% linear gradient over 120 min, 0.2% heptafluorobutyric acid (HFBA)] at a flow rate of 0.5 ml $min^{-1}$. This HPLC separation was subsequently omitted in the preparation of immunogens for booster injections because it did not significantly increase the purity of big PTTH. At each step, PTTH activity was assessed by the *in vitro* prothoracic gland assay (see below) and expressed in units; 1 unit is the PTTH activity in a day 1 pupal brain (Bollenbacher et al., 1984).

*Immunization protocol.* Highly purified big PTTH was vacuum-centrifuged to dryness and dissolved in 0.25 ml PBS (0.01 M, 0.15 M NaCl, pH 7.2), followed by the addition of 0.25 ml of 20% Maalox in PBS. The aluminum hydroxide in the Maalox forms an alum precipitate with the antigen (Oi and Herzenberg, 1980). A BALB/c mouse was immunized intraperitoneally with 65 units of big PTTH (15 μg total protein). Boosts of PTTH were given 1, 6, and 7 months after the initial immunization (~20 units per boost, ~4 μg total protein). The last boost was given in PBS without Maalox 3 d prior to the fusion.

Blood was collected from the retrobulbar sinus of the mouse after each immunization, and the resulting serum (~0.1 ml/bleed) was tested for an immune response using the brain whole-mount immunocytological assay (see below).

*Hybridoma production.* Hybridomas were produced using standard procedures (Köhler and Milstein, 1975; Galfrè et al., 1977) with minor modifications. The myeloma cell line used for the fusions was the nonsecretor P3-X63-Ag8.653 (Kearney et al., 1979). Cells were grown in Dulbecco's modified Eagle medium (DMEM) (Hazleton, Denver, PA), supplemented with 10–20% fetal calf serum (Gibco, Grand Island, NY), 2 mM glutamine, 1 mM sodium pyruvate, and 0.1 mM nonessential amino acids (Sigma, St. Louis, MO). Spleen cells ($10^8$) from the responding BALB/c mouse were fused in polyethylene glycol 1000 (Sigma) with myeloma cells ($10^8$) from a log phase culture (Pontecorvo, 1975; Gefter et al., 1977). The cells were diluted in the supplemented DMEM, which also contained 10% NCTC-109 (Gibco), 0.002 units of insulin (Sigma), and hypoxanthine–aminopterin–thymidine (HAT) (Hazleton), for selection of hybrids (Littlefield, 1964), and were then aliquoted (0.1 ml) into six 96-well tissue culture plates (Cambridge, MA, 3595; Costar). Additional HAT medium (0.1 ml) was added after 24 hr and at 1 week the medium was changed. The cultures were incubated under 10% $CO_2$ at 37°C until cell growth was observed. Supernatants from wells with hybridoma growth were screened with the whole-mount immunocytological assay, and positive hybridoma cells were cloned by limiting dilution (Oi and Herzenberg, 1980; Galfrè and Milstein, 1981) to yield monoclonal cell lines.

*Brain whole-mount immunocytology.* Antisera and hybridoma supernatants were screened for antibody production with an indirect immunofluorescent cytochemical assay (Coons, 1958) that employed *Manduca* brain whole-mount preparations. Brains were dissected in Grace's medium (Gibco), fixed in aqueous Bouin's for 2 hr at room temperature, and rinsed overnight in PBS (0.05 M, pH 7.4) at 4°C. Following removal of the neurolemma, brains were permeabilized in PBS containing 2% Triton X-100 at 4°C. They were then incubated overnight in either antiserum (2% in PBS) or undiluted MAb culture medium at 4°C, and then rinsed in PBS (3 washes) for 1 hr, followed by a 2 hr incubation in secondary antibody (1:20 dilution in PBS) at room temperature. Various affinity-purified fluorescein- or rhodamine-conjugated goat anti-mouse IgG antibodies were used with equal success (e.g., Cooper Biomedical, Malvern, PA; Jackson Laboratories, West Grove, PA). The brains were rinsed 3 times in PBS for a total of 2 hr, followed by a final rinse in distilled water. Specimens were dehydrated with ethanol (70, 95, and 100%, 15 min each) and then mounted in methyl salicylate. Fluorescence was viewed with a Nikon epifluorescence microscope. This protocol enabled an individual to screen 200 clones/d.

*PTTH block of cytological immunoreactivity.* To determine whether the immunoreactivity in brains could be blocked with PTTH, they were incubated with antiserum or MAb culture medium that had been preadsorbed with either big PTTH or control protein. Antiserum and MAb culture medium were titrated for their cytological immunoreactivity to determine the threshold dilutions of each that would insure detection of any blocking with PTTH or control protein. Antiserum at a dilution of 1:2000 was incubated with either C-4 HPLC-purified PTTH (25 units, 4.5 μg total protein) or a control protein mixture of ovalbumin and myoglobin (4.5 μg) in a 0.1 ml volume overnight at 4°C. Likewise, MAb culture medium at a 1:600 dilution (40 ng $ml^{-1}$ IgG) was incubated with big PTTH (30 units, 5.4 μg total protein) or control protein (5.4 μg). Samples of antiserum and MAb culture medium with no protein addition were also diluted and incubated. After the overnight preincu-

bation, day 1 pupal brains (3) and day 3 last instar larval brains with attached retrocerebral complexes (3) were incubated in each of the solutions for ~24 hr at 4°C and then prepared for immunocytology.

*Enzyme-linked immunosorbent assay (ELISA).* An amplified enzyme-linked immunosorbent assay (ELISA) (Engvall and Perlman, 1971) was performed in 96-well microtiter plates (Nunc, Roskilde, Denmark). Big PTTH and other antigens being tested were diluted in carbonate buffer (0.1 M, pH 9.6), and aliquots of each (0.1 ml) were added to wells, followed by a 3 hr incubation at 37°C. Wells were then washed (3 times) with PBS (0.01 M, pH 7.2) containing 0.1% Tween-20. MAb supernatants were diluted in PBS, aliquots were added to each well (0.09 ml) and incubated overnight at 4°C. For competition experiments, which assessed the effects of increasing doses of big PTTH, small PTTH, or other proteins on the MAb binding to wells coated with big PTTH, the MAb supernatant was preincubated with titrations of each of the proteins before being added to the wells. After 3 washes with PBS, an affinity-purified rabbit anti-mouse Ig antibody (Jackson) (22 ng $ml^{-1}$ in PBS) was added to each well (0.1 ml) and incubated for 1 hr at room temperature. Wells were then washed 3 times with PBS, followed by the addition of an alkaline phosphatase-conjugated goat anti-rabbit IgG antibody (1:1000 in PBS) (Sigma) to each well (0.1 ml). After a 1 hr incubation, followed by 3 washes in PBS, freshly prepared substrate was added to each well (0.09 ml). The substrate solution consisted of *p*-nitrophenyl phosphate disodium (0.6 mg $ml^{-1}$) (Sigma) dissolved in diethanolamine buffer (1 M, 0.5 mM $MgCl_2$, pH 9.8). The enzyme reaction was carried out at room temperature, and the resulting color reaction was spectrophotometrically quantified with a micro-ELISA reader (Dynatech, Alexandria, VA).

Isotyping and quantification of the MAbs by ELISA were essentially as described previously, except that wells were coated with goat anti-mouse immunoglobulins recognizing IgG, IgM, and IgA antibodies (Cooper), and incubated overnight at 4°C. After washing in PBS, known titers of mouse antibody standards and titrations of test antibody were added to the wells and incubated at 37°C for 1.5 hr. The plates were again washed and alkaline phosphatase-conjugated goat anti-mouse antibodies specific for the heavy chain isotypes $IgG_1$, $IgG_{2a}$, $IgG_{2b}$, $IgG_3$, IgM, IgA, and for kappa and lambda light chains were incubated at room temperature for 2 hr. Substrate was added as described above.

In vitro *prothoracic gland bioassay for PTTH.* Big PTTH activity in HPLC column fractions was assessed and quantified with the *in vitro* prothoracic gland assay previously described (Bollenbacher et al., 1983, 1984). One gland of each pair was incubated for 2 hr in a 0.025 ml standing drop of Grace's medium containing an aliquot from a column fraction, and the other gland in 0.025 ml of medium only. Ecdysone synthesized by the glands was quantified by radioimmunoassay (macro-RIA) (Bollenbacher et al., 1983). The labeled ligand for the RIA was $^3$H-ecdysone (New England Nuclear, Boston, MA) adjusted to 4 Ci $mmol^{-1}$, and the unlabeled ligand was ecdysone (a gift from Dr. D. H. S. Horn, CSIRO, Melbourne, Victoria, Australia). The amount of ecdysone synthesized was computed using a log–logit computer program. Activation by big PTTH was expressed as a ratio: the amount of ecdysone synthesized by the PTTH-stimulated gland divided by that synthesized by the contralateral control gland (Bollenbacher et al., 1979).

*Antibody inhibition of PTTH activity* in vitro. The *in vitro* prothoracic gland assay for PTTH was used to confirm antibody specificity for big PTTH. To demonstrate this, dose–responses of prothoracic gland activation were generated in the presence of 1% control preimmune serum, test antiserum, control hybridoma culture medium, and MAb culture medium. Each dose of big PTTH (in 0.099 ml of Grace's medium) was preadsorbed with the particular serum or medium (0.001 ml) overnight at 4°C. For these experiments, the pH of the Grace's medium was adjusted from 6.2 to 6.8 to enhance antibody binding. For the assay of big PTTH activity, experimental glands were incubated in Grace's medium containing the PTTH preadsorbed with 1% antibody. Control glands of each pair were incubated in Grace's medium alone, because it was determined that neither 1% control serum nor 1% control hybridoma culture medium affected basal ecdysone synthesis or PTTH-stimulated ecdysone synthesis. For each dose of PTTH, activation ratios were computed and used to assess dose-dependent activation (Bollenbacher et al., 1979, 1984). In this way, dose–responses of gland activation by PTTH in control serum and control hybridoma medium could be compared with dose–responses obtained with antiserum and MAb culture medium. Inhibition of PTTH activity by an antibody was denoted by an increase in the $ED_{50}$, indicated by a shift to the right for the dose–response curve. Although the maximum activation ratio was somewhat variable, owing to the nature of the bioassay, the $ED_{50}$ value was consistent. To minimize variability, control and experimental dose–responses were always run concurrently.

## Results

### *Immunocytological screen for antibody production*

On the basis of previous findings that PTTH in the *Manduca* brain was localized in the L-NSC III (Agui et al., 1979), an immunocytological brain whole-mount assay was developed to screen for antibodies specific for the prothoracicotropes (Flanagan et al., 1988). Pupal brains, which contain the highest level of PTTH activity (O'Brien et al., 1986), were used initially to screen serum for antibody production. Preimmune serum from the mouse yielded no immunoreactivity, but after an initial immunization, immunostaining was observed in the prothoracicotropes, as well as in other neurosecretory cells. After each boost, immunoreactivity became progressively stronger in the L-NSC III, but decreased in the other cells. After the final boost, the serum yielded immunoreactivity specific for the L-NSC III at a 1:2000 dilution (Fig. 1*A*). Since the serum was specific, had a high antibody titer, and yielded good immunostaining of the somata, axons, and dendritic processes of the prothoracicotropes, it appeared that the mouse had responded to the immunogen and was producing antibodies against big PTTH.

This mouse was then used for hybridoma production, and hybridomas grew in 33% of the wells. Of the 200 clones screened with the immunocytological assay for antibody production to big PTTH, a single clone demonstrated strong immunostaining of the prothoracicotropes. The clone's immunocytological specificity was similar to that of the serum; however, the signal was stronger, with virtually no background immunofluorescence (Fig. 2*A*). Clones producing antibodies specific for other neurosecretory cell groups were also identified—for example, for the large medial neurosecretory cells (group $IIa_2$; Copenhaver and Truman, 1986b). All the positive primary clones were subcloned by limiting dilution, and rescreened and then classified for antibody isotype using the ELISA. The medial NSC-specific clone, designated A1C11, was subsequently used as a control for this study.

The L-NSC III-specific clone, designated A2H5, originally contained both IgM and $IgG_1$ antibodies, as determined by the ELISA. After subcloning, the L-NSC III-specific immunoreactivity was shown to be a product of the $IgG_1$ antibody. This $IgG_1$-producing hybridoma was subcloned again to insure that it was monoclonal. The $IgG_1$ antibody produced by the A2H5 clone was quantified with an ELISA and was ~24 $\mu g\ ml^{-1}$ culture medium.

### *Blocking of L-NSC III-specific immunoreactivity by big PTTH*

If the L-NSC III-specific antiserum and A2H5 MAb immunoreactivities reflected antibody binding to big PTTH, then preadsorption of the antibodies with excess big PTTH before their incubation with *Manduca* brains should abolish the immunostaining. For this study, antiserum and A2H5 MAb culture medium, at appropriate dilutions, were incubated either (1) alone, (2) with purified big PTTH, or (3) with control protein at the same concentration as the PTTH. Strong immunostaining of the L-NSC III was observed in pupal brains incubated with antiserum alone (Fig. 1*A*) or with antiserum preincubated with control protein (Fig. 1*B*). The L-NSC III somata, axons, and dendritic fibers were clearly visible in both, indicating that con-

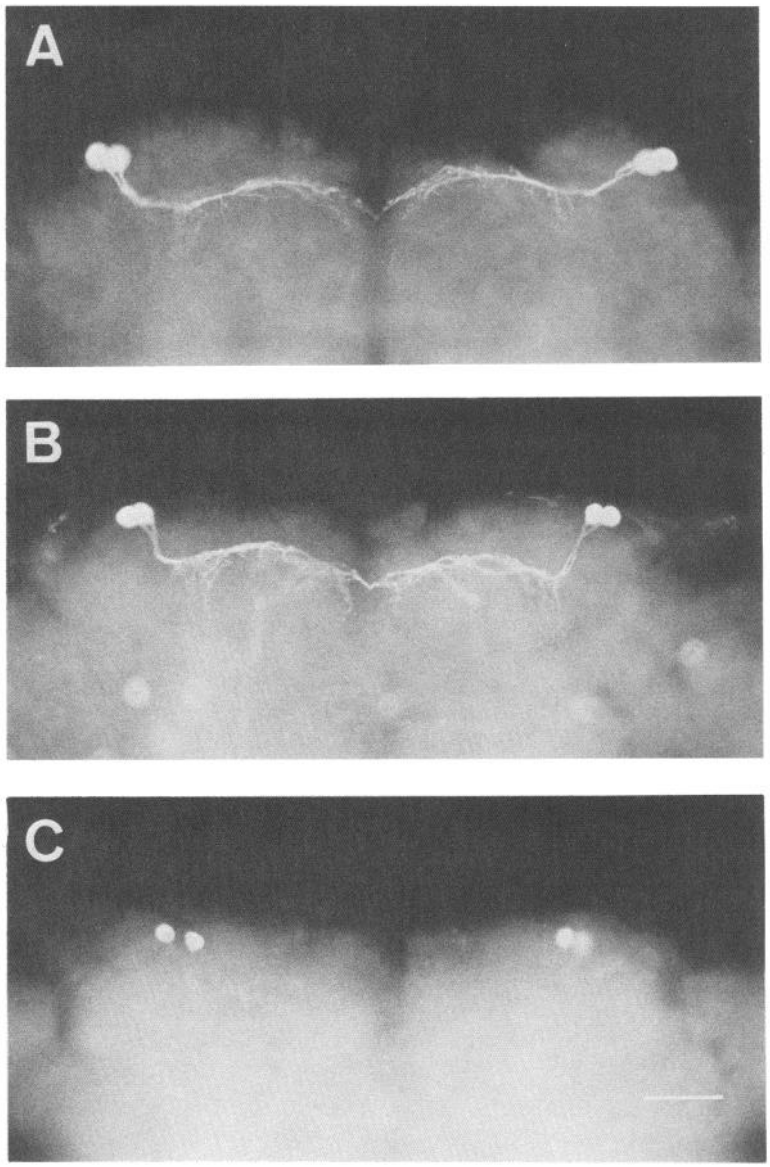


*Figure 1.* Photomicrographs of the neurosecretory cell specificity of the murine antiserum in day 1 pupal brain whole-mounts of *Manduca sexta.* The immunofluorescent staining is specific for the L-NSC III, the prothoracicotropes (*A*). The L-NSC III immunostaining is not blocked by preincubating the antiserum with control protein (*B*), but is partially blocked by preincubation with big PTTH (*C*). Scale bar, 100 $\mu$m.

trol protein did not affect the specific staining. However, preincubation of the antiserum with big PTTH resulted in a substantial decrease in immunoreactivity (Fig. 1*C*). The axons and dendritic processes of the L-NSC III were not visible and the somata were only faintly immunoreactive. Big PTTH thus partially blocked the antiserum's L-NSC III-specific immunostaining. The inability to block the immunoreactivity completely was probably due to the limited amount of big PTTH available for preadsorption and to the range of avidities of antibodies present in the serum. However, the presence of antibodies specific for other components of the L-NSC III cannot be ruled out as a possible reason for this faint immunostaining.

Using the A2H5 MAb, pupal brains (Fig. 2*A*) and day 3 last larval instar corpora allata (Fig. 2*B*) were specifically immunostained. This immunostaining was confined to the L-NSC III somata, their axons, dendritic fibers, and terminal endings in the corpora allata. Preincubation of the A2H5 MAb with control protein did not affect the L-NSC III-specific immunoreactivity in either the brain (Fig. 2*C*) or corpora allata (Fig. 2*D*). However, the immunoreactivity of the A2H5 MAb was blocked completely following preadsorption with big PTTH. There was no immunostaining in the brain, nerves, or corpora allata (Fig. 2, *E, F*). The arrows in Figure 2 denote the immunoreactivity in the axons in the nervus corporis allati, which connects the corpora cardiaca to the corpora allata (Fig. 2, *B, D*), and the complete loss of this immunoreactivity after preadsorption with big PTTH (Fig. 2*F*). Thus, the A2H5 MAb and antiserum cytological immunoreactivity appeared to reflect the presence of antibodies directed against big PTTH.

### *HPLC/ELISA assessment of antibody specificity*

An amplified ELISA was developed for use in conjunction with HPLC of big PTTH to characterize the A2H5 MAb antigen specificity. Antiserum was not used in this study because it generally resulted in high backgrounds in the ELISA. First, the specificity of the assay was defined by testing the A2H5 MAb for immunobinding to big PTTH, *Manduca* small PTTH (Bollenbacher et al., 1984), bovine insulin, ovalbumin, and myoglobin. The A1C11 medial NSC-specific MAb was used as a control antibody in this study. The A2H5 MAb was specific for

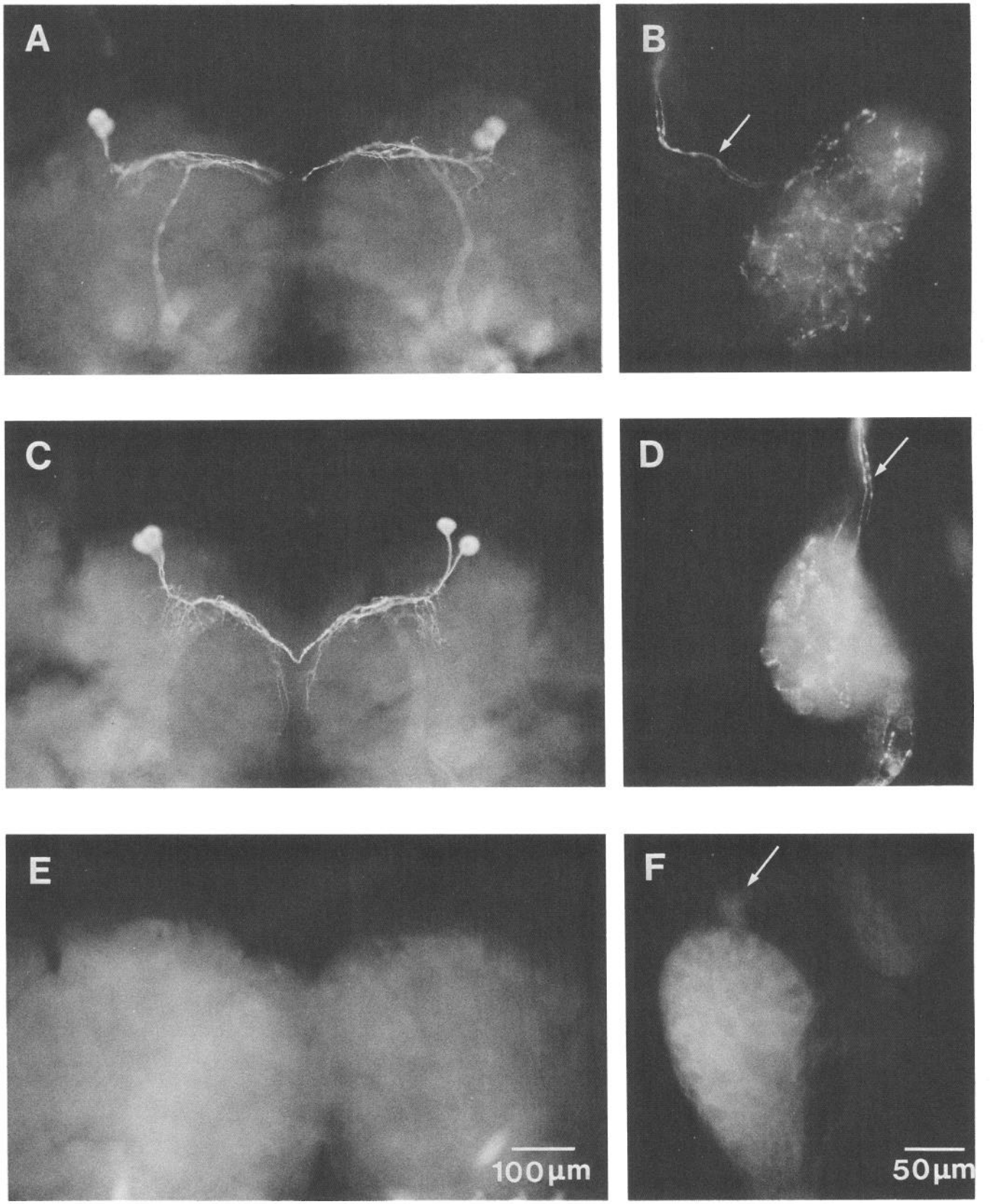


*Figure 2.* Photomicrographs of the neurosecretory cell specificity of the A2H5 MAb in whole-mounts of day 1 pupal brains and day 3 last larval instar corpora allata of *Manduca*. The immunofluorescent staining is specific for the L-NSC III (*A*) and their terminals in the corpora allata (*B*). The L-NSC III immunostaining in the brain (*C*) and corpora allata (*D*) is not blocked by preincubating the A2H5 MAb with control protein, but is completely blocked in the brain (*E*) and corpora allata (*F*) by preincubation with big PTTH. *Arrows* denote the L-NSC III axons in the nervus corporis allati.

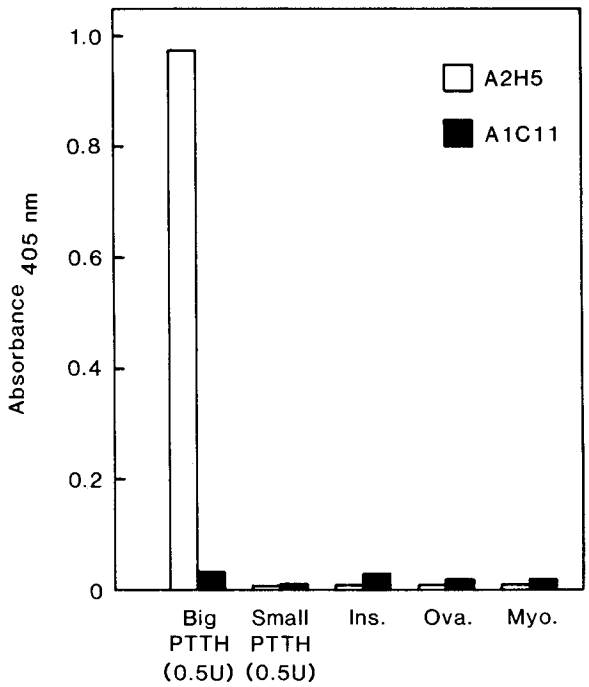


*Figure 3.* Specificity of the A2H5 MAb for big PTTH in the ELISA. Big PTTH (0.5 units, 0.1 μg of protein), small PTTH (0.5 units, 1 μg of protein), bovine insulin (0.1 μg), ovalbumin (0.1 μg), and myoglobin (0.1 μg) were each assayed in duplicate; the absorbance is the average of the 2. Small PTTH was only partially purified; hence the higher protein concentration. The medial cell-specific A1C11 MAb was used as a control antibody in the ELISA. Small PTTH was also tested at 0.1 μg of protein (not shown) and the results were the same.

big PTTH (Fig. 3), exhibiting no cross-reactivity with the other proteins, and the A1C11 MAb did not cross-react with big PTTH or any of the other proteins. Thus, using the A2H5 MAb in the ELISA was suitable for a chromatographic characterization of the antigen specificity of the antibody.

A reverse-phase C-18 HPLC-fractionated preparation of big PTTH was separated on HPLC using an analytical reverse-phase C-4 column. The elution of big PTTH was assessed by both ELISA and the *in vitro* prothoracic gland bioassay (Fig. 4). In both assays, activity was detected in fractions 59–64. The peak of activity with the ELISA (absorbance, 1.045) occurred at fraction 62; this corresponded precisely with the fraction representing the peak of biological activity (activation ratio, 6.3). The protein elution profile for this separation (Fig. 4, inset) revealed a sharp peak of absorbance at this same fraction.

Together, these ELISA and bioassay results provided additional evidence that the A2H5 MAb is directed against big PTTH in its native, biologically active configuration. In addition, the precise agreement between the ELISA and bioassay data indicated that the A2H5 MAb could be used in the ELISA to monitor the purification of big PTTH.

The impure state of the small PTTH used in the ELISA to determine the specificity of the A2H5 MAb may have precluded binding to the microtiter wells, and consequently detection of any cross-reactivity. To better assess cross-reactivity with small PTTH, a competition ELISA was employed. This approach is less dependent on the purity of the hormone. The A2H5 MAb, preincubated with increasing doses of either big PTTH, small PTTH, or control protein, was added to microtiter wells previously coated with 0.5 units of big PTTH. Unbound big PTTH, from 0.3 to 5.0 units, caused a sharp decrease in the ELISA

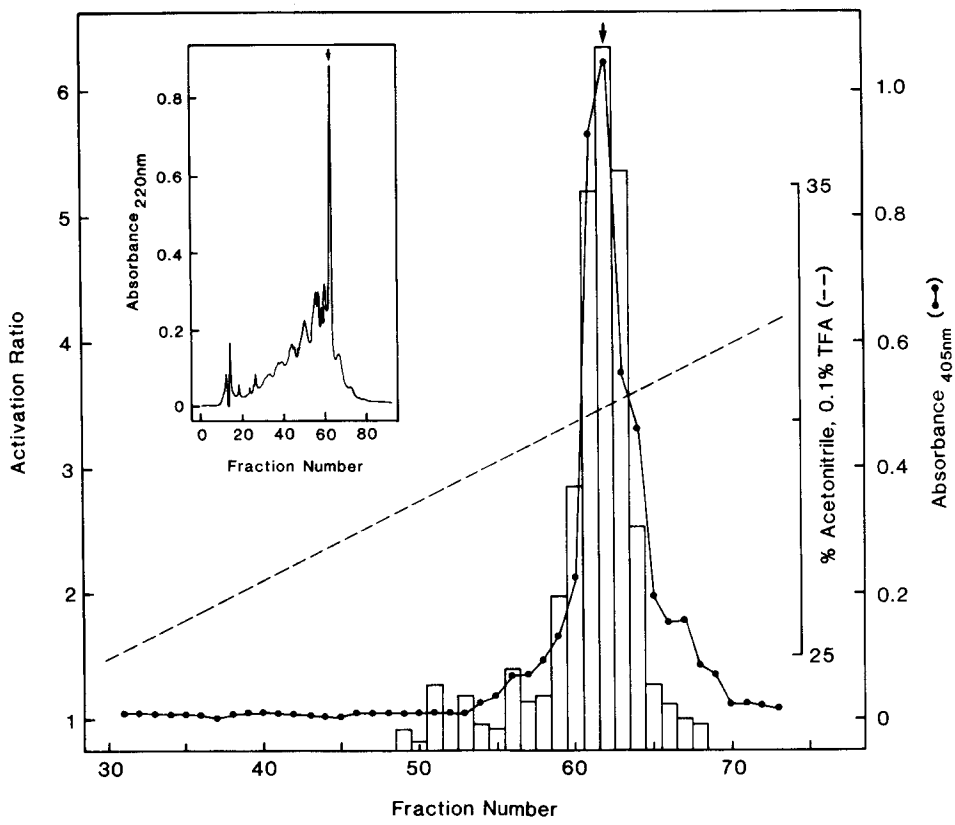


*Figure 4.* Elution profile of big PTTH from a C-4 reverse-phase HPLC fractionation assessed by ELISA (●) and the *in vitro* prothoracic gland bioassay (*bars*). *Dotted line*, the acetonitrile–water gradient employed; *inset*, the protein elution profile for the separation.

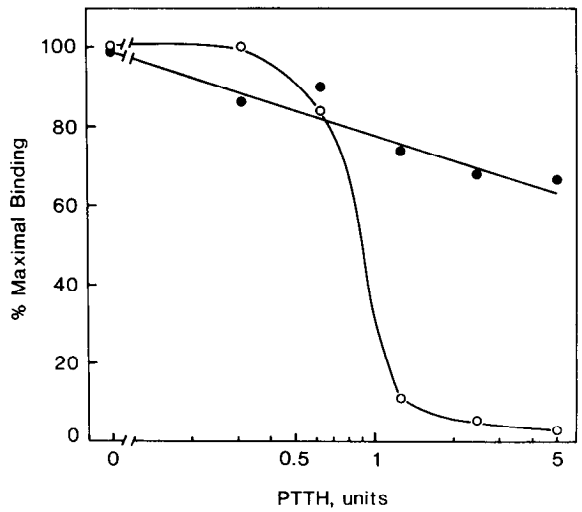


*Figure 5.* Assessment of the cross-reactivity of small PTTH with the A2H5 MAb by a competition ELISA. The A2H5 MAb was preincubated with increasing units of big PTTH (○) and small PTTH (●) before testing in the ELISA. Each datum point represents the average of 2 absorbance determinations expressed as the percentage of maximal binding (absorbance resulting from the A2H5 MAb incubated alone).

response, from 100% of maximal binding to 3% (Fig. 5). Equivalent doses of small PTTH resulted in a gradual decrease in the ELISA response, from 100% to 67% of maximal binding (Fig. 5). Since the small PTTH extract has approximately 10 times more protein than the equivalent units of big PTTH, increasing doses of a control protein (myoglobin) at the same protein concentration as the small PTTH extract were also assessed in the competition ELISA. Myoglobin at the highest dose (9 $\mu$g well$^{-1}$) decreased the A2H5 MAb ELISA response to 86% of maximal binding. It appears that high levels of control protein slightly inhibit the A2H5 MAb binding to big PTTH adsorbed to microtiter wells. The results suggest that if the A2H5 MAb does recognize small PTTH, the epitope is not identical to that of big PTTH and cross-reactivity is minimal.

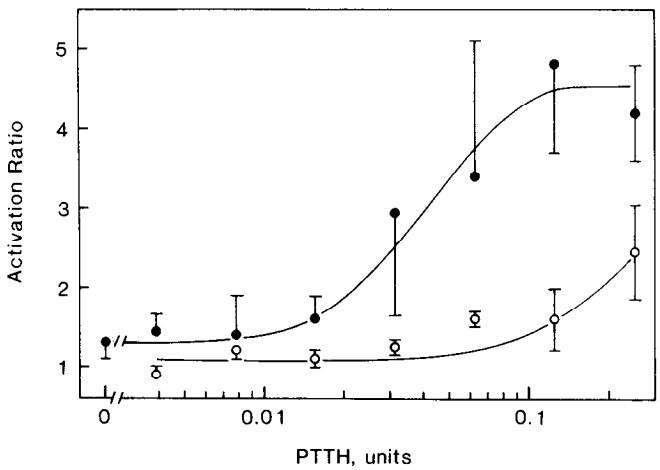


*Figure 6.* Effect of the murine antiserum on the dose–response of day 0 pupal prothoracic gland activation by big PTTH. The control dose–response was generated with big PTTH preincubated with preimmune serum (●), and the experimental with big PTTH preincubated with antiserum (○). Each datum point represents the mean ± SEM of 3 determinations.

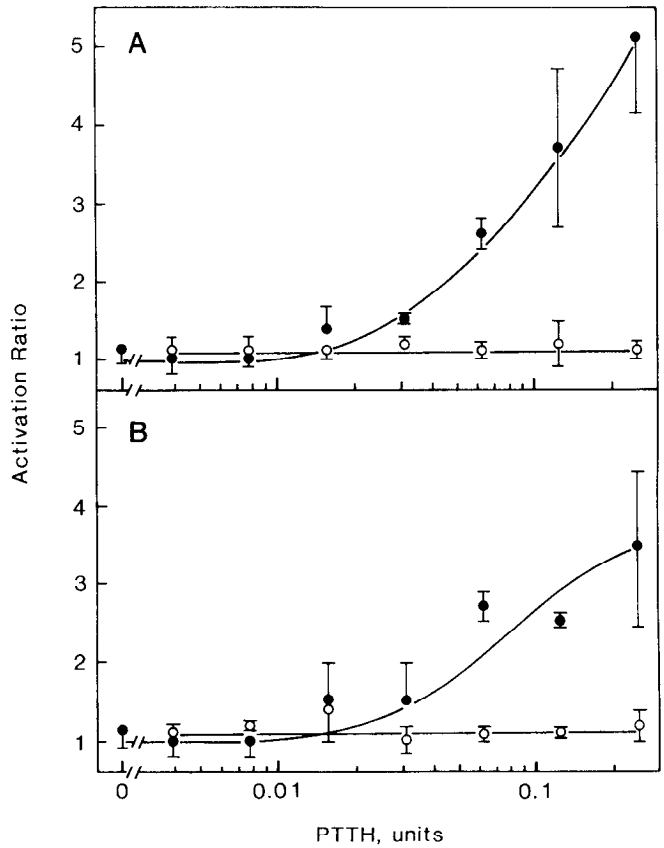


*Figure 7.* Effect of the A2H5 MAb on the dose–response of day 0 pupal prothoracic gland activation by big PTTH. *A*, Dose–responses of activation obtained with big PTTH preincubated with control hybridoma medium (●) and A2H5 MAb medium (○). *B*, Dose–responses of activation obtained with big PTTH preincubated with A1C11 MAb medium (●) and A2H5 MAb medium (○). Each datum point represents the mean ± SEM of 3 determinations.

*Antibody inhibition of PTTH activity* in vitro

While the immunocytological and ELISA/HPLC studies provided convincing evidence that the antiserum and A2H5 MAb were directed against big PTTH, conclusive proof required the demonstration of antibody inhibition of the neurohormone's biological activity. This was shown by assessing the effect of the antibodies on big PTTH's dose-dependent activation of the prothoracic glands. This approach was possible since 1% control preimmune serum or control hybridoma culture medium did not affect basal or PTTH-stimulated ecdysone synthesis by the prothoracic glands (results not shown). The dose–response of prothoracic gland activation by big PTTH preincubated with preimmune serum (Fig. 6) demonstrated the typical stimulation of the glands (Bollenbacher et al., 1983, 1984). Gland activity increased from a basal activation ratio of 1 at 0.02 units of big PTTH to a maximum activation ratio of 4.5 at 0.1 units. A concurrent dose–response analysis of big PTTH preincubated with antiserum (Fig. 6) revealed a significant decrease in gland stimulation. Here, a dose of big PTTH as high as 0.1 units did not elevate gland activity above a basal level, and at 0.25 units, the activation ratio was only 2.5. Quantification ($ED_{50}$ analysis) of PTTH activity from these dose–response curves (Bollenbacher et al., 1979, 1984) revealed that the antiserum had inhibited ~85% of the big PTTH activity.

A similar dose–response analysis of big PTTH preincubated

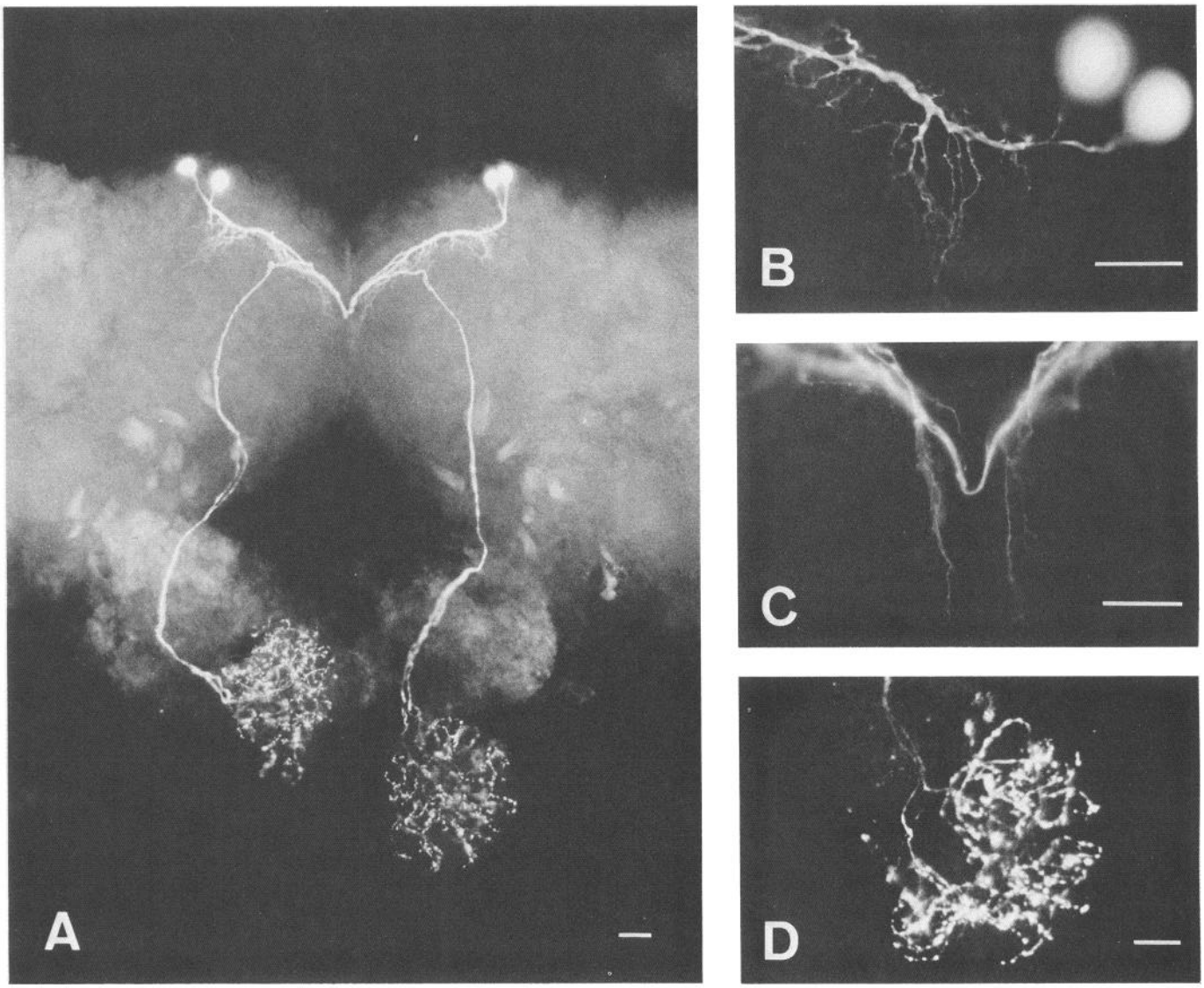


*Figure 8.* Photomicrographs of the A2H5 MAb immunofluorescent staining of the entire prothoracicotrope neuroendocrine axis in whole-mounts of day 1 pupal brain–retrocerebral complexes of *Manduca*. Immunostaining depicts the complete morphology of the big PTTH axis (*A*), the prothoracicotrope somata with proximal dendritic processes (*B*), decussation of the primary axons with descending medial dendritic processes (*C*), and the terminal arborizations in the corpora allata (*D*). Scale bars, 50 μm.

with control hybridoma medium resulted in normal activation of the prothoracic glands (Fig. 7*A*). The response increased from a basal activation ratio of 1 at 0.02 units of big PTTH to 5 at 0.25 units. A concurrent dose–response analysis of big PTTH preincubated with the A2H5 MAb resulted in no detectable activation of the glands (Fig. 7*A*), indicating that the antibody had inhibited all of the neurohormone's activity.

To insure that the apparent inhibition of big PTTH activity by the A2H5 MAb was specific, an additional dose–response was generated using PTTH preincubated with the A1C11 MAb. Activation of the glands by big PTTH was normal (Fig. 7*B*), increasing from a basal activation ratio of 1 at 0.02 units to an activation ratio of 3.5 at 0.25 units of PTTH. The concurrent dose–response analysis of big PTTH preincubated with the A2H5 MAb yielded the expected lack of gland stimulation, indicative of complete inhibition of PTTH activity (Fig. 7*B*). Therefore, the A2H5 MAb and murine serum antibodies raised against big PTTH are directed against the neurohormone. Since the antibodies inhibited big PTTH activity directly, it appears that the epitope they are binding to is near, or perhaps part of, the active site of the hormone.

*Immunocytological characterization of the prothoracicotrope*

A precise immunocytological characterization of the cellular distribution of big PTTH in the L-NSC III was now feasible, using the big PTTH A2H5 MAb. Employing the whole-mount protocol, the brain and attached retrocerebral complexes were immunostained to illustrate the complete morphology of the big PTTH axis (Fig. 8*A*). In this preparation, all of the L-NSC III were immunostained, and at a higher magnification, details of the various morphological components of these peptidergic neurons were clearly discernible (Fig. 8, *B–D*). A significant finding was that all 4 of the L-NSC III were equally immunoreactive, indicating that both cells of the L-NSC III pair are prothoracicotropes (Fig. 8*B*).

The axons of these monopolar neurons traverse medially

through the protocerebrum to the contralateral lobe (Fig. 8*C*) and then posteriorly, exiting the brain via the nervi corporis cardiaci I + II (Fig. 8*A*). They then pass through the corpora cardiaca without branching, and finally pass through the nervus corporis allati before terminating in the corpora allata. In the corpora allata, the axon terminals form numerous varicosities characteristic of a neurohemal organ, i.e., a release site for a neurohormone (Fig. 8*D*). This finding supports the idea that the corpus allatum is the release site for big PTTH synthesized by the L-NSC III (Agui et al., 1980).

Last, the immunocytological staining revealed that big PTTH is present in many dendritic fibers of the L-NSC III that project into the protocerebral neuropil (Fig. 8, *B, C*). The principal dendritic arborizations were observed in the dorsal region of the protocerebrum, near the somata (Fig. 8*B*), and in a region that extends posteriorly along the midline toward the tritocerebrum in the cerebral hemispheres (Fig. 8*C*).

## Discussion

A murine polyclonal antiserum and a MAb to the big PTTH of *Manduca sexta* have been produced. By immunocytological, chromatographic, and functional criteria, the antibodies are extremely specific. The antibodies recognize the native configuration of the neurohormone, as demonstrated by their capacity to inhibit the biological activity of big PTTH in the *in vitro* prothoracic gland assay. In this assay, it was not necessary to immunoprecipitate big PTTH to detect binding. This suggests that the A2H5 MAb and serum antibodies are binding to an epitope that is part of or near the active site of big PTTH for binding to a receptor. Thus, these antibodies to big PTTH should be very useful probes for investigating the biology of the PTTH neuroendocrine axis in *Manduca.*

A detailed immunocytological characterization of the PTTH neuroendocrine axis with the A2H5 MAb indicated that both L-NSC III were prothoracicotropes. This finding necessitates a reconsideration of the original identification of the neurosecretory cells that produce PTTH. It had been reported previously that only one of the cells of the L-NSC III pair was a prothoracicotrope (Agui et al., 1979), a conclusion based on the amount of PTTH biological activity present in individual, isolated L-NSC III somata. By contrast, the A2H5 MAb has clearly demonstrated that both of the L-NSC III contain an epitope that is part of big PTTH. The discrepancy between the results of these 2 studies may be attributed to the low amount of PTTH activity present in an individual L-NSC III soma and the sensitivity of the bioassay employed to detect this activity. In the earlier study, one soma clearly possessed a significant amount of PTTH activity, while the other possessed activity only slightly above background. The difficulty associated with manipulating individual NSC somata for activity assay may have resulted in the loss of some PTTH activity from one of the cells. Alternatively, immunocytological analysis is a direct and sensitive means of denoting the presence of big PTTH in cells; thus, it now appears that both of the L-NSC III are the prothoracicotropes.

The immunocytological localization of big PTTH within the L-NSC III has also provided a more precise determination of the site of big PTTH release. Retrograde and anterograde dye-fills of the L-NSC III revealed that their axons terminate in the corpora allata (Nijhout, 1975; Carrow et al., 1984; Copenhaver and Truman, 1986b). These results, combined with the finding that the corpora allata contains far more PTTH activity than does the corpora cardiaca (Agui et al., 1980), led to the conclusion that the corpora allata were the neurohemal organs for the *Manduca* prothoracicotropes. Nevertheless, it was suggested that the corpora cardiaca were also release sites for PTTH (Carrow et al., 1981), a conclusion based on the apparent *in vitro* release of PTTH from the corpora cardiaca. The immunocytological results have revealed that the big PTTH-containing axons traverse the corpora cardiaca without branching, then terminate and arborize exclusively in the corpora allata. Thus, it appears that the corpora allata are the only neurohemal organs for big PTTH release into the hemolymph. It is possible that the PTTH activity released from the corpora cardiaca *in vitro* (Carrow et al., 1981) may represent small PTTH. To date, the NSC that synthesize this peptide and its site of release have not been established. The fact that the corpora cardiaca-associated PTTH activity was assessed with a larval prothoracic gland bioassay, which is much more sensitive to small PTTH than the pupal gland assay (Bollenbacher et al., 1984), further supports this possibility. Alternatively, the PTTH activity apparently released from the corpora cardiaca may be a nonphysiological event caused by the *in vitro* conditions that reflect PTTH leakage from the L-NSC III axons as they pass through the corpora cardiaca.

One intriguing finding of the immunocytological studies with the A2H5 MAb was the presence of big PTTH in the dendritic processes of these neurosecretory cells. It has generally been assumed that the dendritic fields of the cerebral neurosecretory cells of insects represent sites of afferent synaptic input for the control of these peptidergic neurons (Bern, 1962), but efferent output of neurosecretory material from these collaterals has also been suggested (Schooneveld, 1974). Additionally, it has been proposed that, in insects, these projections serve as a reservoir for neurosecretory material (Highnam and West, 1971). In *Manduca,* big PTTH is present in these processes in fairly high amounts, as indicated not only by strong cytological immunoreactivity, but also by measurements of PTTH activity in different regions of the protocerebrum (Agui et al., 1979). It is possible that these fibers are storage sites for the hormone, but they appear to possess terminal-like swellings containing PTTH, suggesting that the peptide may be released to act locally within the brain. A more thorough immunohistochemical analysis of these areas of the protocerebral neuropil at an ultrastructural level will be necessary to determine whether big PTTH is present in presynaptic terminals. The presence of PTTH in such terminals would support the idea that it is acting locally within the brain in a neuromodulator and/or neurotransmitter capacity. An analysis of the dendritic fibers of the different neurosecretory cell groups in *Manduca* indicates that they overlap considerably, forming a neurosecretory neuropil (Copenhaver and Truman, 1986b), and thus providing an anatomical basis for possible intercommunication (Taghert, 1981). If big PTTH terminals exist in the protocerebrum, perhaps intercommunication between neurosecretory cells can be direct via big PTTH, functioning as a neuromodulator or neurotransmitter. The evolving realization that many neurohormones act as neurotransmitters or neuromodulators in the CNS of both vertebrates and invertebrates (see Joosse, 1986) lends support to this hypothesis.

The A2H5 MAb has proven sufficiently specific for big PTTH to be used in an ELISA for the neurohormone. The PTTH–ELISA is currently being employed to monitor the purification of big PTTH, thus circumventing the use of the lengthy and comparatively tedious *in vitro* prothoracic gland assay for the neurohormone. Given the usefulness of the A2H5 MAb in the ELISA, it is anticipated that it can be employed in other assays

(e.g., RIA) suitable for quantifying the hormone. The specificity of the A2H5 MAb should also permit affinity-purification of big PTTH.

The cross-reactivity of small PTTH with the A2H5 MAb, assessed by a competition ELISA, indicated that this antibody would not be suitable for affinity-purification of small PTTH. Cross-reactivity was minimal, although the small PTTH extract caused a slight decrease in the percentage of maximal binding. It is possible that this effect is due to a related epitope on the small PTTH molecule, although the results are not conclusive. If, indeed, the A2H5 MAb is recognizing the receptor binding site of big PTTH, then perhaps weak cross-reactivity with small PTTH is a result of a lower affinity for a similar, but not identical, receptor binding site. Additional studies on this binding, e.g., antibody inhibition of the biological activity of small PTTH, will be necessary to resolve the issue of the structural relationship between big and small PTTH.

Recently, a MAb has been produced to a synthetic fragment of the cerebral insulin-like 4 kDa peptide from *Bombyx mori,* bombyxin, which has PTTH-like activity in another lepidopteran, *Samia cynthia ricini* (Suzuki, 1986; Mizoguchi et al., 1987). Although this MAb does not recognize the native peptide, it does bind to the medial NSC of the *Bombyx* brain. It is possible that this peptide is analogous to the small PTTH in *Manduca*; thus the *Manduca* small PTTH may be produced by medial neurosecretory cells. Interestingly, the initial immunization with big PTTH yielded serum that gave weak immunostaining of the medial NSC in *Manduca* brains. This staining could be due to serum antibodies binding to a peptide structurally related to big PTTH, possibly small PTTH. Alternatively, the medial NSC immunoreactivity could be a result of a contaminant in the big PTTH antigen localized in these cells. Determining the specificity of the medial NSC-specific monoclonal antibody (A1C11) derived from this mouse will be especially interesting in light of the localization of bombyxin.

It is equally likely that 22K–PTTH in *Bombyx* (Kataoka et al., 1987) may be produced by lateral cerebral neurosecretory cells. If this is the case, these MAbs could be used to investigate possible functional/structural relationships between the big and small PTTHs of these 2 lepidopterans. Additional phylogenetic studies in nonlepidopterans are being conducted currently. Preliminary immunocytological studies using the big PTTH antiserum suggest that lateral NSC in other species produce PTTH. However, this cross-reactivity has not been observed using the A2H5 MAb. The possibility that the epitope recognized by the A2H5 MAb is unique to *Manduca* is being investigated.

The regulatory mechanisms underlying the differentiation and expression of neuronal phenotypes is an important area of neurobiological research (Purves and Lichtman, 1985). The potential of the A2H5 MAb for investigating expression of a peptidergic phenotype in a small subset of neurons in a developmental context is apparent. In fact, preliminary ontogenetic studies in *Manduca* have revealed that big PTTH is detectable immunocytologically as early as at 30% of embryogenesis (A. Westbrook and W. E. Bollenbacher, unpublished observations). These results have been supported by measurements of PTTH activity in *Manduca* embryos at this stage (Dorn et al., 1987). Additionally, PTTH activity has also been found in *Bombyx* embryos (Chen et al., 1987; Fugo et al., 1987). The presence of the neurohormone in the embryonic brain suggests that it may have an endocrine function, perhaps to elicit increases in the ecdysteroid titer that may drive embryonic molting late in embryogenesis (see Hoffmann and Lagueux, 1985; Dorn et al., 1987). The big PTTH A2H5 MAb could be employed to investigate the regulation of expression of a peptidergic phenotype at the level of individual cells, as well as the functional role of a neuropeptide in embryogenesis.

In summary, a MAb and antiserum to the big PTTH of *Manduca sexta* have been generated, and they appear to have properties that should enable their use in an array of studies on the biology of the PTTH neuroendocrine axis in insects. This peptidergic system, confined to 4 large neurosecretory cells in the brain of an insect, should be very useful for investigating fundamental developmental and functional properties of peptidergic neurons.

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