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NEUROPEPTIDE PROCTOLIN ASSOCIATED WITH AN IDENTIFIED SKELETAL MOTONEURON[1]

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Abstract

Our objective was to find large and identifiable peptide-containing neurons and their postsynaptic targets. For this, a whole mount immunohistochemical method was used to locate cell bodies of neurons in the cockroach central nervous system immunoreactive to antibodies raised against the pentapeptide proctolin (H-Arg-Tyr-Leu-Pro-Thr-OH).

The morphology and projections of the immunoreactive neurons were investigated by combining intracellular dye injection and electrophysiological and immunochemical procedures. The presence of proctolin in specific immunoreactive neurons was check by high pressure liquid chromatography combined with bioassay applied to extracts made from individually identified and isolated cell bodies.

Using these approaches, we have identified a large proctolin-containing motoneuron which innervates muscles in a proximal segment of the cockroach leg. This study establishes a well characterized cellular preparation in which the physiological actions and functions of a peptide-containing neuron can be studied *in vivo*.

Proctolin was described initially as a myotropic peptide and proposed as a candidate neurotransmitter in the hindgut or proctodeum of the American cockroach *Periplaneta americana* (Brown, 1967, 1975; Brown and Starratt, 1975). Later, it was extracted, purified, and sequenced from 125 kg of whole cockroach (Starratt and Brown, 1975) and shown to have the following structure: H-Arg-Tyr-Leu-Pro-Thr-OH. The presence of proctolin in the proctodeal nerves and the similarity of contractions of the hindgut produced either by nerve stimulation or application of proctolin (Brown, 1975) support both its neural origin and its transmitter status. More recent evidence has supported both conclusions and, in addition, suggests that proctolin's role is not confined to the cockroach hindgut (see below). Proctolin, or closely related peptides, may have widespread distribution and diverse physiological actions in the invertebrates.

The first direct evidence that proctolin is associated with neurons was its localization to the lateral white neuron of the cockroach abdominal ganglia (O'Shea and Adams, 1981). This neuron projects to the cardiac nerve and does not innervate the hindgut musculature. The probability of nonvisceral roles for proctolin also is supported by the presence of proctolin-like immunoreactivity (PLI) throughout the cockroach central nervous system (CNS) (Bishop et al., 1981). The distribution, determined by radioimmunoassay (RIA), correlates well with the distribution and number of neurons shown to be proctolin immunoreactive by histochemical techniques (Bishop and O'Shea, 1982). These immunological studies suggest the presence of a morphologically diverse and perhaps functionally diverse class of proctolin-containing neurons in the cockroach CNS. Nothing is yet known about the specific physiological roles of neurally localized proctolin. Proctolin is, however, known to be bioactive at low (nanomolar) concentrations on a wide variety of insect neuronal and muscular preparations (May et al., 1979; Miller, 1979; Walker et al., 1980). Moreover, proctolin probably is not confined to the insects and may be present in vertebrates. Recently, Sullivan (1979) showed that a peptide released from crab pericardial organ is chromatographically indistinguishable from proctolin; we have purified proctolin from crayfish nervous system (M. O'Shea and C. A. Bishop, unpublished data); and in an immunochemical study, PLI has been shown to be widely distributed in the lobster nervous system (Schwartz et

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al., 1981). Proctolin also is known to be active in crustaceans (Schwartz et al., 1980; Benson et al., 1981; Sullivan et al., 1981). In a recent report, proctolin was shown to cause contractions of the ileum in rats and mice at a threshold of 10^{-8} M (Penzlin et al., 1981). This, combined with growing evidence that neuropeptides well known in vertebrates occur in insects and other invertebrates (O'Shea, 1982) and the reported presence of an invertebrate neuropeptide (molluscan FMRF-amide) in mammals (Dockray et al., 1981; Weber et al., 1981), suggests the possibility that proctolin may be present in vertebrates.

Proctolin or similar peptides may be present in higher animals and a study of its action on the cellular level may be greatly facilitated by finding large, identifiable proctolin-containing neurons and their postsynaptic targets in invertebrate organisms. As a prelude to a physiological study, therefore, we have examined the nervous system of the cockroach and attempted to characterize experimentally accessible proctolin neurons and their postsynaptic targets.

Here, we report on the combination of immunohistochemical, biochemical, and intracellular microelectrode techniques which show that a large and uniquely identifiable neuron in the cockroach CNS contains proctolin. In addition, we reveal its morphology and show that it is an identified motoneuron which innervates a well characterized skeletal muscle. This system may provide a convenient *in vivo* preparation in which to study the physiological action of a specific peptide-containing neuron.

Materials and Methods

Specimens of the American cockroach (*Periplaneta americana*) were obtained from Carolina Biological Supply, Burlington, NC. Specimens of locusts (*Schistocerca nitens*), used for bioassay, were obtained from our laboratory culture. Authentic proctolin for immunization was obtained from Sigma. Tritiated proctolin ([^{3}H-Tyr^2]proctolin) was prepared by catalytic reduction (New England Nuclear) of an I_2-Tyr^2 analog of proctolin synthesized by Professor Emil T. Kaiser and William F. DeGrado of the Department of Chemistry, University of Chicago.

Immunohistochemistry and intracellular dye injection. Twenty female New Zealand rabbits were immunized subcutaneously with conjugates of proctolin with bovine serum albumin emulsified with Freund's adjuvant. Serum titer, sensitivity, and specificity were checked by radioimmunoassay using proctolin to displace serum-bound radioiodinated ^{125}I-proctolin. Details of the methods and characteristics of the sera raised have been described elsewhere (Bishop et al., 1981). Immunoreactivity to proctolin antiserum (antiserum No. 9 of Bishop et al., 1981) was visualized by the peroxidase-antiperoxidase technique (Sternberger, 1979) in unsectioned whole ganglia and peripheral nerves. Details of these methods are provided elsewhere (Bishop and O'Shea, 1982). All cell staining in cockroach using proctolin antiserum No. 9 is prevented by preincubation with 0.5 mg/ml of authentic (Bishop et al., 1981).

For combined intracellular dye marking and immunohistochemistry, cell bodies of neurons in the isolated third thoracic ganglion were impaled with Lucifer Yellow-filled microelectrodes (4% Lucifer Yellow in 1 M lithium chloride; see Stewart, 1978). The dye was introduced into the cell by passing hyperpolarizing current pulses (500 msec duration, 1 to 5 nA) at 1 Hz for about 20 min. Dye-filled cells were visualized (Zeiss epifluorescence microscope) in the whole unsectioned ganglion before and after fixation in 4% paraformaldehyde and processing for whole mount immunohistochemistry. In some preparations, immunohistochemistry was omitted and the fixed ganglion was dehydrated and cleared in methyl benzoate for inspection of the dye-filled neuron.

Cell isolation and extraction. Living third thoracic ganglia were removed and secured dorsal face up in a Sylgard-coated Petri dish under physiological saline (140 mM NaCl, 5 mM KCl, 5 mM $CaCl_2$, 1 mM $MgCl_2$, 4 mM $NaHCO_3$, 5 mM TES (2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}ethanesulfonic acid), 5 mM trehalose, pH 7.2). Under dark-field illumination, the cell bodies of some neurons can be seen. Neurons with particularly granular cytoplasm appear opaque or white when illuminated in this way. Individual neuronal cell bodies were isolated by removing the ganglionic sheath and then dissecting single cell bodies free of the ganglion. Single cell bodies then were cleaned of adhering debris from other cells, sucked into a glass capillary (80 μm in diameter), and then transferred to a small test tube containing 50 μl of 2 N acetic acid. The glass capillary was inspected to be sure that the transfer was made and then the same volume of saline which was transferred with the cell (about 1 μl) from the same capillary was transferred to another 50-μl volume of 2 N acetic acid. The same individually identified neurons in different specimens was pooled in the same extraction tube. Extraction tubes were sonicated and frozen and thawed after each cell was added. Tubes were stored at −70°C. After centrifugation, the supernatant solution was transferred to another tube and evaporated under reduced pressure at 60°C. The dried samples then were redissolved in either isotonic physiological saline (physiological saline plus 100 mM sucrose) for bioassay or chromatography solvents for high pressure liquid chromatography (HPLC) purification (see below). Further details concerning the identity of the cells involved in these extractions will be provided under "Results."

Assay procedures. Extracts of individually identified neurons were chromatographed on a 10-μm μBondapak C_{18} reverse phase column (Alltech). A volatile liquid phase (50 mM ammonium acetate, 15% acetonitrile, pH 4.5) was pumped at 1.5 ml · min^{-1}, 1800 psi and 0.3-min fractions were collected. Elution time of proctolin in this system was determined by chromatography of tritium-labeled proctolin ([^{3}H-Tyr^2]proctolin). Use of the volatile solvent system prevented interference by salts of the bioassay used to monitor proctolin-like bioactivity (PLB) in dried and saline-suspended HPLC fractions. Chromatographed fractions were bioassayed after evaporating the liquid phase under reduced pressure and redissolving each dried fraction in isotonic physiological saline.

A sensitive and convenient proctolin bioassay has been developed from a specialized bundle of muscle fibers which form part of the main extensor muscle (the extensor tibialis) of the locust (*Schistocerca nitens*) metathoracic leg. These fibers produce a spontaneous myogenic

rhythm of contraction and relaxation (Hoyle and O'Shea, 1974). The frequency of this rhythm is known to be altered by neurotransmitters (Evans and O'Shea, 1978; Piek and Mantel, 1977; O'Shea and Adams, 1981). Octopamine, for example, decreases its frequency (threshold, 10^{-6} M) and serotonin (threshold, 10^{-6} M) and proctolin (threshold, $\sim 10^{-10}$ M) increase both the rhythm frequency and muscle tone.

A metathoracic leg of immature adult female specimens of *S. nitens* was removed and dissected to expose the myogenic fibers in the proximal region of the femur. The muscle was bathed in 10 μl of isotonic physiological saline and 1-μl aliquots of test solutions (standards and extracts) were applied to the muscle. The small movements of the tibia produced by the myogenic fibers were monitored with a photoelectric movement detector. After each 1-μl sample application and response, the muscle was washed with about 5 ml of isotonic saline prior to the next application. For quantification, the interval between contractions prior to adding a test sample was divided by the minimal interval reached during the response. Therefore, increased frequency is represented by a ratio of greater than 1. The threshold sensitivity of different assays ranges from about 10^{-10} to 5×10^{-9} M proctolin in the applied aliquot. Since the muscle is bathed in about 10 μl, the real sensitivity is about 10 times this (i.e., 10^{-11} to 5×10^{-10} M). One preparation can be used to test as many as 100 1-μl samples. Samples must be diluted to within about an order of magnitude of the threshold because rapid sustained contraction occurs at about 10^{-8} M applied proctolin.

The assay readily allows identification of subfemtomole amounts of proctolin in a 1-μl volume.

Electrophysiological technique. Intracellular recordings were made from the cell body using 3 M potassium acetate-filled microelectrodes (50 to 80 megohms). Current could be passed through the microelectrode while recording by the use of an active bridge. An extracellular suction electrode served to monitor spiking activity in peripheral nerve roots of the ganglion and also was used for nerve stimulation. Conventional recording and display techniques were employed.

Results

On the dorsal surface, laterally and posteriorly in the third or metathoracic thoracic ganglion of *Periplaneta americana*, there is a single, large (60-μm-diameter), bilaterally symmetric pair of proctolin-immunoreactive cell bodies (Fig. 1). These cells are seen consistently in the same location in different preparations. Immunoreactive cells that may be homologous also are seen laterally on the dorsal surface of the first and second (pro- and mesothoracic) thoracic ganglia (see Fig. 5 of Bishop and O'Shea, 1982). The possibility that the metathoracic immunoreactive cells represent the same identified neuron in different individual animals is suggested by their bilateral symmetry and the constancy of their location and appearance from animal to animal. That these cells contain proctolin is indicated by the immunochemical staining and by the fact that all staining is blocked by incubation of antiserum with proctolin.

The results presented here address the following questions. Are the stained immunoreactive cells in Figure 1*A* individually identifiable neurons? Do they contain proctolin? What are their likely postsynaptic sites of action?

Anatomical identification. When the living metathoracic ganglion is illuminated against a dark background, a large and opaque cell can be seen in the location of the immunoreactive cell bodies. A cell with a similar appearance appears in this location consistently in different individuals.

Under visual control, a Lucifer Yellow-filled microelectrode can readily be placed in this cell and its anatomy can be revealed by dye injection and fluorescence microscopy. Figure 1*C* and Figure 2 illustrate the central anatomy of this cell in the third thoracic ganglion. Although there are small differences between individuals, the following is a summary of the features shared by five preparations. The cell body (about 60 μm in diameter) gives rise to a single ventrally and anteriorly directed neurite which branches close to the soma. Branches are directed anteriorly and medially and end near the midline near a group of large, dorsal cell bodies. These cells are the octopaminergic dorsal unpaired median (or DUM) neurons (Evans and O'Shea, 1978; O'Shea and Evans, 1979; Dymond and Evans, 1979). The primary neurite loops anteriorly, laterally, and then posteriorly. This loop forms an expanded (~20-μm) region from which conspicuous ipsilateral processes arise which pass anteriorally, dorsally, and medially to end near the dorsal midline. Another major process arises from the expanded region and passes anteriorally along the lateral edge of the ganglion. In the ganglion at the point of exit of ipsilateral nerve 5 (the major nerve that supplies the ipsilateral hindleg), the expanded region narrows abruptly and forms an axon-like process in the nerve.

To summarize, the cell body identified visually has the form typical of an insect monopolar neuron which projects an axon peripherally. Insect sensory neurons typically have peripherally located cell bodies and this cell, therefore, is probably a motoneuron. Moreover, the constancy of the anatomical features suggest that it is a uniquely identifiable motoneuron with specific and constant targets.

Does this pair of cells, characterized anatomically, correspond to the pair of immunoreactive cells illustrated in Figure 1*A*? The results of experiments in which a ganglion containing a dye-injected cell was processed for whole mount immunohistochemistry confirm the correspondence (Fig. 1, *B* to *D*). The immunochemical processing reduces the dye fluorescence (Fig. 1, *B* and *D*), particularly around the periphery of the cell body, and the presence of dye interferes with the immunoreactivity. It is clear, however, that the injected cell corresponds both to the neuron described above and to the proctolin-immunoreactive neuron on the uninjected side in Figure 1*D*. On the injected or doubly labeled side, there are no additional immunoreactive cells of appropriate size to correspond to those on the uninjected side.

Identity of soma content. Taken together, the above evidence suggests the hypothesis that the neuron identified with a peripheral process in nerve 5 (Fig. 2) contains proctolin or a proctolin-like peptide. This idea was tested by bioassaying crude and HPLC-purified extracts made from the isolated cell body of the immunoreactive neuron

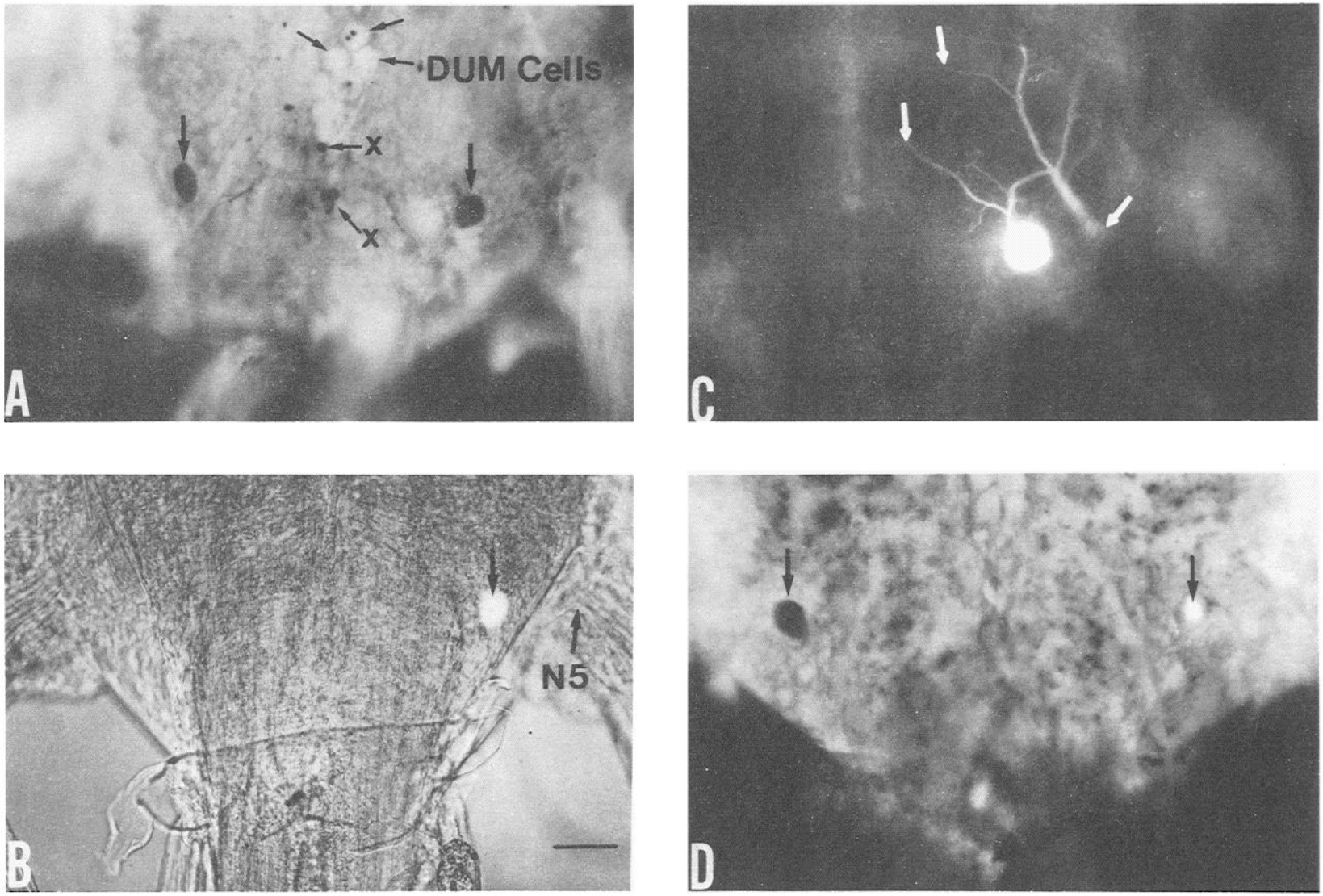

Figure 1. Whole mount proctolin immunoreactivity on the dorsal surface of the third thoracic ganglion. *A*, A pair of large (60-μm-diameter) bilaterally symmetrical immunoreactive cell bodies is indicated by the *arrows* on the lateral margins of the ganglion. Several small immunoreactive cell bodies on the dorsal midline are indicated also (×). The large medial nonimmunoreactive cell bodies are the somata of dorsal unpaired medial neurons (*DUM cells*). *B* to *D*, in a living ganglion, a cell in the region of the large immunoreactive cell shown in *A* was injected with Lucifer Yellow dye. This ganglion is shown in whole mount epi-illuminated with blue light to excite the dye fluorescence in *B* and *C*. In *B*, the ganglion also was exposed with transmitted white light in order to show the outline of the ganglion and the origin of nerve 5 (*N5*). The *arrows* in *C* indicate processes which project to the midline and an axon-like process projecting to nerve 5. The same preparation is illustrated in *D* after processing for whole mount immunohistochemistry. The bilateral (uninjected) homolog of the cell revealed in *C* is clearly proctolin immunoreactive. The injected cell (*right arrow*) shows the presence of fluorescent dye in the soma and a rind of immunoreactivity. See the text for further interpretation. *Scale bar*, 100 μm.

and comparing the results to the effects of authentic proctolin. Figure 3 shows the response of the myogenic oscillator bioassay to an unpurified acid extract made from the visually identified cell body. Control extracts made from unidentified cells in the vicinity of the immunoreactive neuron, but not including it, do not show PLB in the myogenic oscillator bioassay. This assay is capable of detecting PLB in an aliquot of the extract containing less than the content of 1 cell (about 0.1 cell). In general, however, extracts were prepared by pooling up to 30 individually identified, isolated, and cleaned cell bodies (2 cells per animal). This was to provide sufficient material to detect PLB in fractions of chromatographically purified extract (see below).

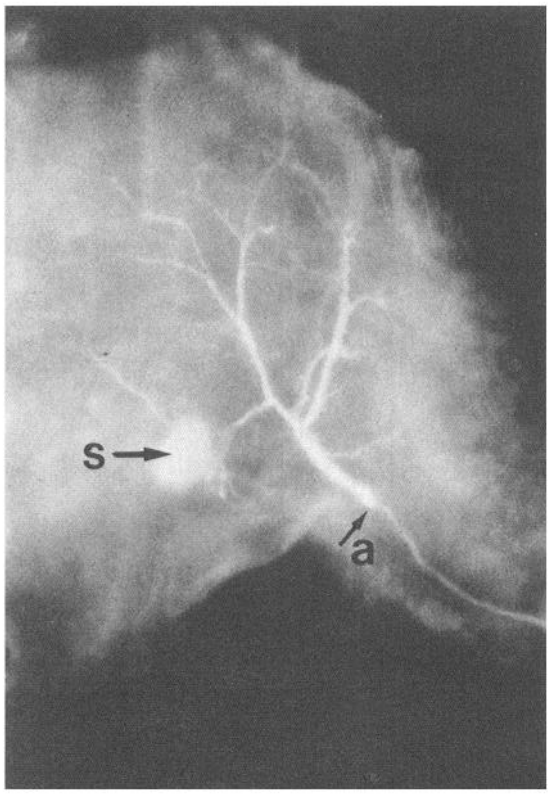

Figure 2. Whole mount of the third thoracic ganglion showing a Lucifer Yellow-injected neuron. This cell is homologous to the lateral proctolin-immunoreactive cell shown in Figure 1. Compare the details of branching with Figure 1*C*. This preparation was dye filled long enough to reveal a process leaving the ganglion in nerve 5. Note the abrupt narrowing (*a*) which occurs at the exit of the process from the ganglion. *s*, Soma. The scale is the same as in Figure 1.

There is a striking qualitative similarity between the bioassay responses to authentic proctolin and to the cell extract (Fig. 3). Both, for example, cause an increase in the basal muscle tone and the frequency of the myogenic rhythm. The bioassay response to the cell extract, proctolin, and other compounds which may alter the myogenic oscillator can be compared in a semiquantitative way by dividing the interval between contractions prior to application of a test sample by intervals measured during the response. This ratio, therefore, is greater than 1 for an increase in frequency. Ratios greater than 1 are produced by nanomolar concentrations of proctolin and by serotonin at much higher concentrations (see Fig. 4). The assay is insensitive to micromolar concentrations of the following neuropeptides: enkephalin, neurotensin, somatostatin, and vasoactive intestinal peptide. Figure 4 shows changes in the ratio during responses to proctolin, the cell extract, and serotonin. Serotonin is a putative transmitter in insects and therefore may be present in the extract. The response to the cell extract or proctolin, however, is unlike the effect of serotonin in its duration and sensitivity to washing (Fig. 4). This suggests that the biological activity in the extract is not due to the presence of serotonin. A comparison of the extract and proctolin responses, however, shows considerable similarities in the onset, magnitude, decay, and recovery after washing with saline (Fig. 4). In addition to these general features, the biological responsiveness to the cell extract is reduced by extract dilution in a way which parallels changes in the responsiveness to different concentrations of authentic proctolin (Fig. 5). These results show that an unpurified extract of the proctolin-immunoreactive neuron contains a bioactive factor which is not serotonin and which is not distinguished from proctolin by a specific proctolin bioassay. By comparison of the responses to authentic proctolin and the cell extract, the bioassay data suggest that about 4×10^{-2} pmol of proctolin may be present in each cell body.

Additional evidence for the structural identity of PLB in the immunoreactive cell is provided by HPLC purification. Figure 6 shows co-elution of PLB and [^{3}H]proctolin in reverse phase liquid chromatography. The single peak of PLB recovered from the cell extract purification cannot be distinguished from authentic proctolin. It is

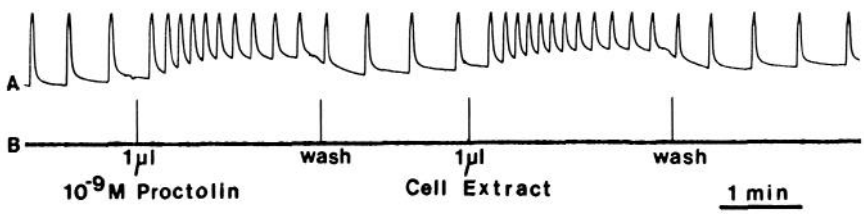

Figure 3. Proctolin bioassay. *Trace A* shows a continuous record of the rhythmic myogenic contractions of the locust extensor tibia muscle. Proctolin and proctolin-immunoreactive cell extract applied in 1-µl aliquots (trace B) to this muscle produce qualitatively similar responses (i.e., a slow tonic contracture and an increase in the frequency of the rhythmic contractions). In this example, the 1 µl of the cell extract contained the acid-soluble content of 0.3 cells. Note the rapid return to basal frequency after washing with physiological saline.

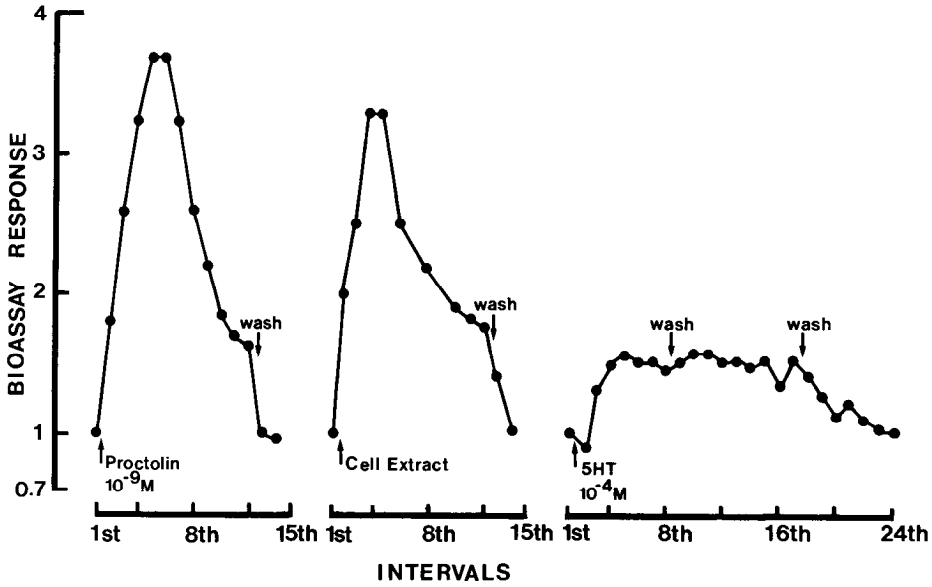

Figure 4. Comparison of the bioassay response to proctolin, proctolin-immunoreactive cell extract, and serotonin (*5HT*). Note the quantitative similarity of the responses to proctolin and the cell extract. Serotonin also increases the rhythm frequency, but the response is prolonged and more resistant to washing the preparation with physiological saline.

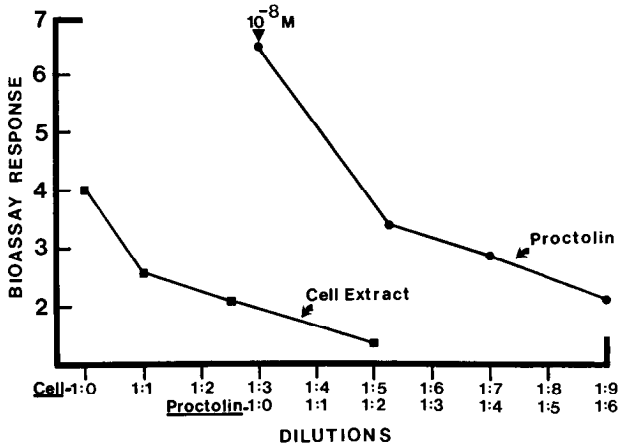

Figure 5. Parallel responsiveness of the bioassay to dilutions of a proctolin standard solution and an extract of the proctolin-immunoreactive cell body.

likely therefore that both the proctolin-like immunoreactivity and PLB in the cell soma are due to the presence of proctolin.

Identification of postsynaptic targets. The morphology of the identified neuron (Figs. 1 and 2), in particular, the presence of a dye-filled process in nerve 5, suggests a motor function. Support for this is provided by electrophysiological and further morphological evidence. For example, intracellular recording from the soma of the immunoreactive neuron and extracellular recording and stimulation from the stump of nerve 5 show that the cell body is connected electrophysiologically to the nerve (Fig. 7). In most preparations, spontaneous trains of small (4- to 8-mV) depolarizing potentials are seen in the soma which correspond 1:1 with action potentials recorded extracellularly from the cut end of nerve 5. These soma potentials cannot be distinguished from soma potentials activated by antidromic stimulation of the axon (Fig. 7). It is likely therefore that, in common with other identified insect motoneurons, the soma membrane is electrically inexcitable and that the depolarizing potential is the electrotonic potential derived from a remote site of spike initiation.

The cell bodies of insect motoneurons are located almost entirely on the ventral side of the central ganglia. The dorsally placed immunoreactive neuron is therefore an exception. In fact, a single large dorsal motoneuron with a cell body in the same location as the immunoreactive cell has been identified in the cockroach (Pearson and Fourtner, 1974). This neuron is known as the Ds or slow coxal depressor motoneuron (Pearson and Iles,

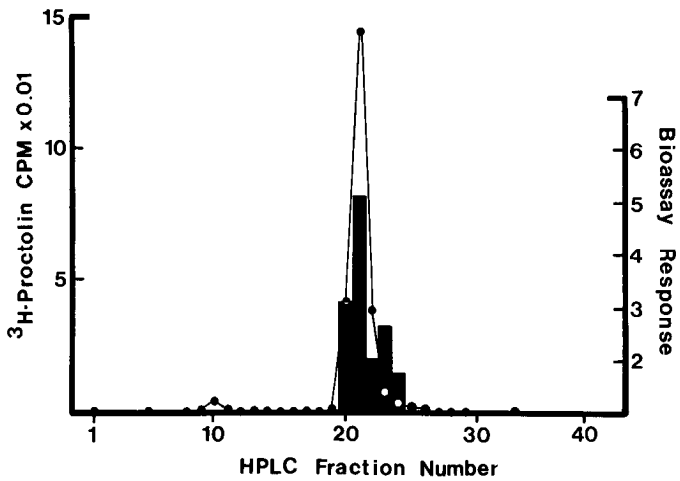

Figure 6. Co-elution of [^{3}H]proctolin (●——●) and proctolin-like bioactivity (*solid bars*) in reverse phase high pressure liquid chromatography.

1971). The Ds axon is one of five large axons (>5 μm) contained in the first branch of nerve 5 (branch 5rl). In this branch, the Ds axon is about 15 to 20 μm in diameter and is the second largest. The Ds axon branches to innervate four subdivisions of a coxal depressor muscle (177d, 177d′, 177e, and 177e′; see Pearson and Iles, 1971).

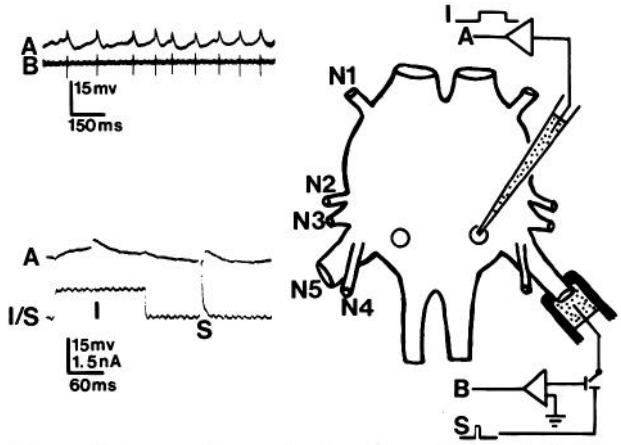

Figure 7. Axon-spike projection from the proctolin-immunoreactive neuron to nerve 5. The *diagram on the right* represents the third thoracic ganglion and shows an intracellular microelectrode (*A*) in the neuron's soma and an extracellular suction electrode (*B*) on the cut stump of nerve 5 (*N5*). Current (*I*) can be passed across the soma membrane while recording and a stimulus (*S*) can be delivered to nerve 5. On the *left*, the *upper recordings* show spontaneous depolarizing potentials (*A*) in the soma which correlate 1:1 with extracellular spikes (*B*). The *lower recordings* show the response of the soma (*A*) to depolarizing current (*I*) and antidromic stimulation (*S*) of nerve 5. See the text for interpretation.

That the proctolin-immunoreactive neuron identified here and the Ds motoneuron are indeed the same cell is supported by the following evidence. First, the central anatomy of the immunoreactive cell corresponds to the published descriptions and drawings of the Ds neuron (Pearson and Fourtner, 1974). The size and position of the cell body, the shape of central arborizations, and the axon projection to nerve 5 are all characteristic of Ds. Second, prolonged intracellular injection of Lucifer Yellow into the cell soma reveals the morphology and branching of the immunoreactive cell's axon in the periphery. Figure 8 shows that this branching is consistent with that of the axon of Ds. For example, the filled axon leaves nerve 5 in branch 5rl and then continues to divide into the branches which project to subdivisions of the coxal depressor muscle 177. Finally retrograde or backfilling of the branch 5rl with cobaltous chloride reveals only one cell body (Ds) on the dorsal-lateral posterior surface of the metathoracic ganglion. The cell that we have identified chemically and morphologically is therefore the Ds motoneuron.

Our evidence suggests that the soma of the Ds motoneuron contains the peptide proctolin. Therefore, proctolin may be the transmitter of this cell and may be transported in the Ds axon from the soma to the coxal depressor muscles. It should be mentioned here that the presumptive excitatory transmitter at the insect neuromuscular junction is L-glutamate (see "Discussion") and that proctolin immunoreactivity is *not* associated with the majority of the skeletal motoneurons (Bishop and O'Shea, 1982). The possibility, therefore, that proctolin may be present in an axon innervating the coxal depressor muscles would suggest that a small proportion of

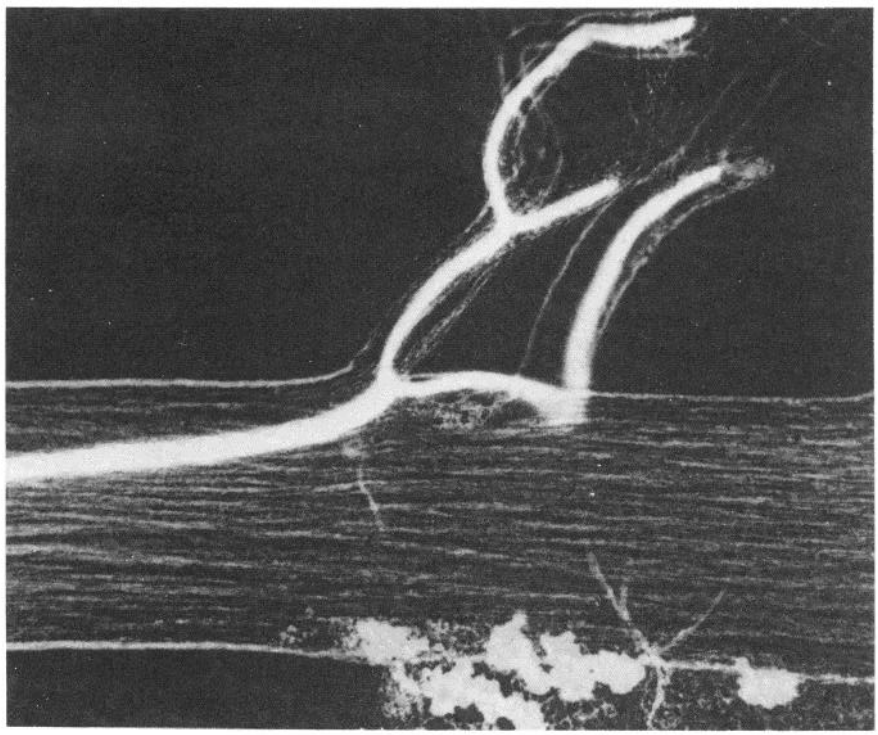

Figure 8. Peripheral projections of a single Lucifer Yellow-filled axon in nerve 5. This axon was filled from the soma of the immunoreactive neuron in the metathoracic ganglion. The axon leaves nerve 5 in the two primary branches of the nerve which innervate the coxal depressor muscles.

insect motoneurons are peptidergic (see "Discussion"). In order to check for the presence of a proctolin-containing axon in the nerve innervating the coxal depressor muscles, we have applied our immunochemical whole mount methods to the branch of nerve 5 (nerve 5rl) which contains the large Ds axon and the other motor axons of the coxal depressors. Figure 9*A* shows the presence in this nerve of a large (~20-μm-diameter) proctolin-immunoreactive axon. We think that this is the Ds motor axon and, therefore, that the Ds motoneuron projects with a proctolin-containing axon to the coxal depressor muscles. Moreover, whole mount immunocytochemistry applied to the coxal depressor muscles shows the presence of immunoreactive motor nerve fibers (Fig. 9*B*). It appears from this figure that the muscle fibers are innervated multiterminally, which is typical of the motor innervation pattern of insect muscle (Usherwood, 1980). The significance of this in relation to the possible transmitter status of proctolin at the neuromuscular junction is discussed below.

Discussion

We have characterized a bilaterally symmetric individual motoneuron in the metathoracic ganglion of *Periplaneta americana*. The cell body is immunoreactive to rabbit serum raised against the neuropeptide proctolin (H-Arg-Tyr-Leu-Pro-Thr-OH). Extracts of the cell body contain a factor with biological activity indistinguishable from that of proctolin and which also co-migrates with proctolin in reverse phase liquid chromatography. Combined intracellular dye injection, immunohistochemistry, and electrophysiology show that the neuron is the slow excitatory motoneuron of the coxal depressor muscles (the Ds motoneuron) of the ipsilateral metathoracic leg. Immunohistochemical evidence indicates that proctolin

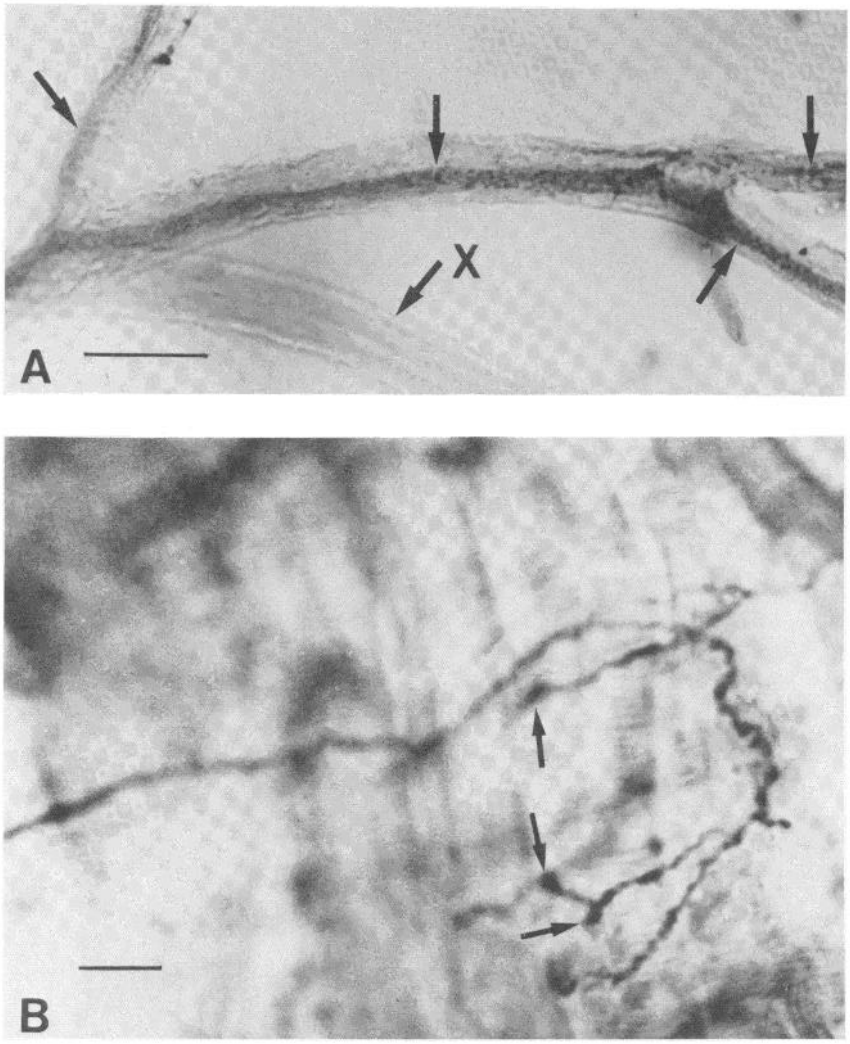

Figure 9. Whole mount preparations showing proctolin-immunoreactive axon and motor terminals associated with the innervation of the coxal depressor muscles. In *A*, the *arrows* point to branches of a single immunoreactive axon in coxal depressor nerves. One of these nerves (×) does not contain a branch of the axon. *Scale bar*, 100 μm. In *B*, a single proctolin-immunoreactive motor axon is shown entering the picture from the *left*. This axon gives rise to branches, varicosities, and terminals which are closely associated with muscle fibers of the coxal depressor muscle (177). The *arrows* indicate immunoreactive varicosities apparently associated with a single muscle fiber. *Scale bar* 50 μm.

is present in the axon of the Ds neuron which innervates the coxal depressor muscles.

These observations strongly implicate proctolin as a peptide neurotransmitter in insect skeletal motoneurons. Although the presence of a neuroeffector in a neuron does not necessarily indicate a transmitter role, this possibility is supported by other observations. For example, insect skeletal muscle is known to be highly sensitive to proctolin (Piek and Mantel, 1977; May et al., 1979; O'Shea and Adams, 1981). With very low concentrations (10^{-10} to 10^{-9} M) of proctolin, the autorhythmic activity of the main extensor muscle of the locust hindleg is modulated. This high sensitivity of the myogenic oscillator forms the basis for our proctolin bioassay. At higher concentrations (above 10^{-8} M), proctolin produces a more rapid contraction of the extensor muscle similar to the contraction produced by nerve stimulation. Proctolin applied to the muscle via an iontophoretic microelectrode produces depolarizing iontophoretic potentials up to 15 mV (May et al., 1979). The iontophoretic potentials can initiate an active membrane response and muscle contraction. Moreover, proctolin potentials occur only at localized sites on the extensor muscle fibers (May et al., 1979). Finally, in coxal depressor muscles, we see proctolin-immunoreactive processes and endings of motoneurons on muscle fibers (Fig. 9*B*). From this combined evidence, we infer that proctolin may have a transmitter role at the insect neuromuscular junction.

It should be emphasized here, however, that there is considerable evidence supporting the view that L-glutamate is the excitatory transmitter at the insect neuromuscular junction (see Sattelle et al., 1980, for reviews). With this in mind, several possibilities are suggested by our results. For example, proctolin may be a transmitter associated with a special class of motoneurons. We know from our immunohistochemical studies (Bishop and O'Shea, 1982) that proctolin immunoreactivity is associated with a small proportion of motoneurons. This idea that a small and specialized population of peptidergic motoneurons exists, also is supported by the fact that there appears to be only one proctolin-immunoreactive axon in the nerve supplying the coxal depressor muscles (Fig. 9*A*). This nerve contains several motor axons which, based on immunocytology, may not contain proctolin. The existence of a distinct class of proctolin-containing motoneurons does not exclude the possibility that L-glutamate functions as their neurotransmitter. Such neurons may have actions mediated by both proctolin and glutamate.

Clearly many questions remain to be resolved. There are, however, reasons to suspect that actions of the Ds neuron on coxal depressor muscles may be proctolinergic. This question will be resolved by future physiological experiments, but some pertinent studies have already been made on the physiological response of the muscles. It is known, for example, that Ds produces potentials in the coxal depressor which differ from potentials produced by a different motoneuron in the same muscle fiber (Pearson and Iles, 1971). In more recent experiments on the coxal muscles (Chesler and Fourtner, 1981), contractions evoked by the Ds neuron are shown to be followed by a residual tension requiring several minutes for complete relaxation. Whether this can be attributed to the presence and to the release of proctolin from Ds will await further experiments.

The anatomy that we describe suggests both a peripheral action at the neuromuscular junction and possibly central actions of Ds on dorsal unpaired median (DUM) neurons (see Fig. 1). This latter possibility also is supported by recent evidence that the DUM cells are highly sensitive to applied proctolin (Walker et al., 1980). The preparation that we have described may represent a highly convenient situation for studying the action of a neurally localized peptide in a cellular *in vivo* system which is unusually simple, accessible, and well characterized. Discoveries made in this system may have significant implications for general questions concerning the physiological roles of neuropeptide-containing neurons.

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