Two-Dimensional Gel Electrophoresis of Neurosecretory Polypeptides in Crustacean Eyestalk

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Summary. A knowledge of the precise location of neurosecretory cell bodies is a prerequisite for studying the synthesis and subsequent processing of neurosecretory polypeptides stored in axon terminals comprising the sinus gland of the crustacean eyestalk. Structural data establish that the X organ in the medulla terminalis ganglion (mtXo) of the crayfish eyestalk represents 90–95% of the cell bodies actively synthesizing neurosecretory vesicles stored in the neurohemal sinus gland (Fig. 4). These cell bodies transport rather than accumulate neurosecretory vesicles as judged by light and electron microscopy suggesting that neurohormone precursors, but not subsequently stored products, might be found there. Two-dimensional electrophoresis of sinus gland and mtXo homogenates support this hypothesis. In crayfish, lobster and blue crab, stained two-dimensional gels display a number of sinus gland-specific polypeptides whose high concentrations and low molecular weights are consistent with stored neurosecretory material (Table 1). These neuropeptides are not detected in mtXo homogenates or in non-neurosecretory neural tissue with Coomassie Blue staining. By decreasing the porosity of the second dimension, the two-dimensional gel technique has proven useful in determining the molecular weights of a variety of neurosecretory polypeptides stored in the sinus gland. The crayfish and lobster store several polypeptides of ca. 7,000 Dalton. The blue crab stores two 7,000, two 13,000 and three 20,000 Dalton sinus gland polypeptides detected in stained gels.

Following a 4 h incubation in $^3$H-labelled amino acids, predominantly labelled 19,000–21,000 Dalton polypeptides are detected in crayfish mtXo homogenates by 2-D gel autoradiography (Fig. 12). Concomitantly, three labelled polypeptides (4,000–10,000 Dalton) appear in the sinus gland (Fig. 13), suggesting that they are cleaved from 19,000–21,000 Dalton molecules. This study is the first to examine neurosecretory precursors and their putative cleavage products in the Crustacea.

Introduction

The X organ on the medulla terminalis ganglion of the decapod eyestalk consists of neurosecretory cell bodies whose axons terminate in the neurohemal sinus gland. These cell bodies are a synthetic site of neurosecretory material stored in the crayfish sinus gland as observed with light microscopy (Durand, 1956). Single cell cobalt iontophoresis and electron microscopy have shown that the dendrites of these cells receive synaptic input from the central nervous system within neuropil of the medulla terminalis ganglion (Andrew and Saleuddin, 1978). Recent cobalt backfilling of fibres entering the crayfish sinus gland has revealed, in addition to the medulla terminalis X organ (mtXo), sources of other sinus gland axons (Andrew et al., 1978; Jaros, 1978). These fibres are primarily from the brain and are devoid of neurosecretory vesicles in the crayfish (Andrew et al., 1978).

This study determines the proportion of sinus gland axons contributed by the mtXo. Then, homogenates of both mtXo and sinus gland are examined with respect to synthesis and storage of neurosecretory material. These tissues are analyzed with two-dimensional polyacrylamide gel electrophoresis (O'Farrell, 1975), a powerful separation technique that can resolve hundreds of proteins from microgram amounts of crude biological material. Separation is based on two parameters. Isoelectric focusing of proteins in one dimension is followed by migration
Materials and Methods

Animals

Male or female crayfish (Orconectes virilis), lobster (Homarus americanus) and blue crab (Callinectes sapidus) were held at 14–18 °C under a 12L:12D photoperiod. Young Orconectes (3.0 g) or adults (25–40 g) were in intermolt or early premolt. Homarus (300–450 g) and Callinectes (100–200 g) were in intermolt. Eyestalks ablated from crayfish were dissected under saline (van Harreveld, 1936); those from Homarus and Callinectes, under artificial sea water.

Microscopy

For light microscopy, crayfish eyestalks were fixed in aqueous Bouin’s fluid, serially sectioned at 8 µm and stained with para-phenylendiamine polycarbohyde fuchsin (PAF). Cell bodies in the mTxo were classified as type I (50–60 µm dia.) or type 2 (25–35 µm dia.) according to the light microscopical description of Durand (1956). Fixation for electron microscopy has been outlined earlier (Andrew and Shivers, 1976).

Electrophoresis in the First Dimension

Nonequilibrium pH gradient electrophoresis (NEPHGE) was performed at 600 or 1,600 v-h using 3.5–10 pH ampholines (LKB) according to the method of O’Farrell et al. (1977) with the following modifications. The mTxo region, sinus glands and eyestalk ganglia were frozen over solid CO2, freeze dried and placed into 1.5 ml Eppendorf tubes containing 20–30 µl of lysis buffer (O’Farrell, 1975). Samples were then given a one second burst with a micro-tip sonicator at 5 °C, and centrifuged for 4 min at 12,000 x g in a Brinkman 3200 Eppendorf centrifuge. The supernatant was loaded onto the gel. The diameter of the first dimension gels was 2 mm, permitting direct placement onto the top of second dimension slabs. Other extruded first dimension gels were fixed overnight in 10% trichloroacetic acid + 7% acetic acid, stained in 0.03% Coomassie Blue-R + 40% methanol + 7% acetic acid and destained in 7% acetic acid.

Isoelectric focusing in the first dimension was carried out at 400 volts for 11 h and then 1,000 volts for one hour with pH 3.5–10 ampholines. The pH of gel sections was determined according to O’Farrell (1975). Protein concentrations were determined with the technique of Bramhall et al. (1969).

Electrophoresis in the Second Dimension

For the second dimension, buffers, stacking gels and separating gels were as described by O’Farrell (1975). The migration distance of several E. coli proteins of known molecular weight were used to estimate the size of eyestalk proteins. E. coli extracts have been run routinely in our laboratory with the identical two-dimensional gel system. The following proteins (Appendix, Fig 2 in Pedersen et al., 1978) were used as molecular weight markers: $p$ subunit of RNA polymerase, $M_r=155,000$; ribosomal protein S1, $M_r=65,000$; z subunit of RNA polymerase, $M_r=39,000$ or 36,000; elongation factor T5, $M_r=34,000$ or 31,000; ribosomal protein S6, $M_r=16,000$. Slab gels were fixed in 10% trichloroacetic acid + 50% ethanol and stained as described for the first-dimension gels. They were then dried on to chromatography paper backings under vacuum and photographed through a yellow filter with Pana-tomic-X film. With one exception (Fig. 7), slab gels were oriented with acidic proteins to the right.

To improve the resolution of small proteins, a second dimension was developed using the high-urea gel system of Swank and Munkres (1971) with the slab apparatus and buffers of O’Farrell (1975). First dimension gels were placed across the top of the slab gel consisting of 12.5% acrylamide with a bis:acrylamide ratio of 1:9 and 8 M urea (Schwarz-Mann ultra pure). The urea concentration decreases gel pore size by approximately forty percent (Swank and Munkres, 1971). Electrophoresis was for 5–8 h at 2.75 W per gel. Slabs were fixed and stained overnight in 40% methanol + 0.3% Coomassie Blue-R + 7% acetic acid. Following washing in 7% acetic acid, gels were photographed over a light box. For molecular weight determinations the following polypeptide markers were loaded on slots and their migration distances in high urea gels were plotted against log molecular weight: ovalbumin, $M_r=43,000$; carboxypeptidase A, $M_r=34,000$; myoglobin, $M_r=17,000$; ribonuclease, $M_r=13,700$; cytochrome C, $M_r=12,400$; insulin, $M_r=6,000$; glucagon, $M_r=3,500$; gramicidin-D, $M_r=2,000$; bacitracin, $M_r=1,400$; oxytocin, $M_r=1,000$. Polypeptides were from Sigma Chemical Co. except glucagon, gramicidin-D and bacitracin which were from Calbiochem.

 Autoradiography

Eyestalks from adult Orconectes virilis in intermolt were ablated and the cuticle, muscle and connective tissue removed in saline