ABSTRACT

The survival and outgrowth of neurons in culture has usually required conditioning factors. We now report that crustacean neurons, taken from the peptidergic neurosecretory system of the eyestalk of crabs (Cardisoma carnifex) and lobsters (Panulirus marginates), show immediate outgrowth, sustained for a week or more, in defined medium as simple as physiological saline with glucose and glutamine. The neurons show peptide hormone immunoreactivity that is prominent at growth cones, exhibit differences in form correlated with their immunoreactivity, release peptides to the medium, and have voltage-dependent currents, including a well-sustained Ca current. Cd blocks secretion, growth, and the Ca current. Peptidergic secretory neurons may be able to utilize existing membrane from their store of granules and already active synthetic, transport, and secretory mechanisms for immediate outgrowth.

The mechanisms governing the outgrowth of neuronal processes and the mature form of neurons remain largely unknown. Isolation of neurons in culture may provide the possibility of undertaking experimental manipulations under controlled conditions. However, although outgrowth in low-density cultures has now been demonstrated for vertebrate (1–5), annelid (6, 7), and molluscan (8–11) preparations, the need for addition of undefined factors has frustrated rigorous studies of the control of outgrowth aforesaid. We report that crustacean neuroendocrine cells show immediate, vigorous outgrowth on a variety of substrates in defined medium as simple as physiological saline and glucose. We propose that this capability for immediate outgrowth is made possible by the utilization of already active synthetic, transport, and secretory mechanisms for growth. Different forms of outgrowth are consistently obtained from the heterogeneous group of neurons cultured, and reactivity with antisera raised against peptide hormones suggests correlations with the biosynthetic capabilities of the neurons. These neurons in culture, with their differences in form, thus provide a promising defined starting point for testing hypotheses about mechanisms governing the control of growth and form in regenerating neurons. We are unaware of any report of crustacean neurons in culture (12). Abstracts describing some of our work have appeared (13–15).

The neurons cultured in these studies form the major neuroendocrine system of crustaceans, the X-organ–sinus gland system of the eyestalk (ref. 16 and, for review, see ref. 17). For the tropical land crab Cardisoma carnifex used for most of the work to be discussed, there have been studies of the morphology of nerve terminals (18), electrophysiological characterization of somata, axons, and terminals (19–22), studies of secretory capabilities (22, 23), and characterization of the hormonal peptides present (24) and their biosynthesis (25). These provide a background against which the cultured cells can be evaluated.

The X-organ of crabs is a discrete cluster of about 200 iridescent-white neuronal somata (26). These neurons send their axons to terminate as clusters of large (up to 30 μm) dilatations in a neurohemal organ (the "sinus gland"). Two size-classes of neurons are present and are associated with different hormonal peptides on the basis of reactivity to antisera as observed in histological studies: small neurons (soma 15–25 μm in diameter) with red-pigment-concentrating hormone (27), and large neurons (30–70 μm) with several peptides and hormonal activities (28), including crustacean hyperglycemic hormone (29), the most prevalent peptide hormonal activity, and molt-inhibiting hormone (30). The cells showing outgrowth in culture reflect these size classes and the immunoreactivity shown by the X-organ cells in situ. The observations suggest that the form of outgrowth may be correlated with the biochemical competence of the neuron.

MATERIALS AND METHODS

Animals and Dissection. Cardisoma carnifex were obtained from Christmas Island, Republic of Kiribati; Panulirus marginates were bought in local fish markets. Dissections were performed in a horizontal-flow culture hood (Baker). Eye-stalks were removed, rinsed with 70% (vol/vol) ethanol, then opened and exposed to three changes of sterile crab saline (440 mM NaCl/11.3 mM KCl/13.3 mM CaCl2/26 mM MgCl2/23 mM Na2SO4/10 mM Hepes, pH 7.4 with NaOH) with antibiotics (penicillin at 100 units/ml, streptomycin at 0.1 mg/ml, and fungizone at 0.25 μg/ml). The X-organ with a 2-mm of the sinus gland tract was dissected in antibiotic saline, placed in nominally Ca- and Mg-free saline with 0.1% trypsin (Sigma, type IV, or GIBCO) for 90 min (24°C), then rinsed thoroughly with saline. Substitution of collagenase (Sigma, type IV), protease (Sigma, type IX), or collagenase-dispase (Boehringer Mannheim) for trypsin gave similar results.

Cell Isolation and Culture Conditions. The routine culture medium consisted of Liebowitz L-15 (GIBCO, as powder) reconstituted and mixed with an equal volume of crab saline of 1.75× normal concentration buffered with 20 mM Hepes (pH 7.6–7.8) and containing gentamycin at 0.1 mg/ml, 120 mM NaCl, and 2 mM glucose, added immediately before use. Total osmolality was 1.1. The dissected X organs were transferred to a 50-μl drop of medium, the cells were separated by agitation with a stream of medium and transferred to a culture dish, usually Primaria (Falcon 3801), as for all examples shown. Cultures were held in moist chambers (Billups Rothanber) in the dark at 22–24°C.

Adherence and outgrowth were obtained on the following substrates: (i) uncoated plastic dishes, including Falcon 3801 and 3001 dishes, Permanox (Lux 5223) dishes, Corning 2500 dishes, and Tekmat Plastek dishes; (ii) Falcon 3001 dishes

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coated with one of the following attachment factors, poly(t-Lysine) (Sigma) at 1 mg/ml (31), laminin (Bethesda Research Laboratories) at 10 mg/ml, fibronectin (Cappel Laboratories–Organon Teknika) at 0.1 mg/ml (32); and (iii) glass coated with poly(t-Lysine). Cells did not show outgrowth on uncoated glass or on plastic coated with Cell-Tak (Biopolymers) at 2.5–10 μl per dish or gelatin (GIBCO) at 1 mg/ml. For immunofluorescence, a hole was drilled in a plastic dish and a poly(t-Lysine)-coated cover glass was attached.

Staining with Antisera. Cultures were fixed in 1% paraformaldehyde in crab saline. Cells were permeabilized with 0.1% Triton X-100 (33) and exposed to 3% (wt/vol) powdered nonfat milk (Carnation) to prevent nonspecific binding and incubated with antisera for 24 hr at 4°C and with goat anti-rabbit IgG conjugated with fluorescein (Sigma) or Texas Red (Cappel Laboratories) for 30 min. The following antisera, all from rabbit, were used: (i) anti-sinus gland homogenate conjugated to bovine serum albumin; (ii) anti-peptide D (24) conjugated to bovine serum albumin; (iii) anti-insect adipokinetic hormone, code 433, kindly supplied by H. Schooneveld, (Agricultural University, Wageningen, The Netherlands) (34, 35); (iv) anti-crustacean hyperglycemic hormone (28); and (v) anti-molt-inhibiting hormone (28). The latter two were kindly provided by R. Keller (Free University of Berlin). In no case did all cells exposed to antisera show staining. Preimmune serum and antiserum preabsorbed with sinus gland extract were used as controls and were negative.

Electron Microscopy. Cultures were fixed in 4% (wt/vol) glutaraldehyde buffered at pH 7.4 with 0.1 M Sorenson's phosphate buffer (sodium salts) and postfixed with 1% OsO4, dehydrated with ethanol, and embedded in LX 112 (Ladd Research Industries, Burlington, VT) in the culture dish.

Electrophysiology. Voltage-clamp measurements were made using the whole-cell form of the patch-clamp technique, using an Axopatch-1C (Axon Instruments) patch-clamp amplifier with a CV3 0.1 headstage (50-MΩ feedback resistor). The culture medium was replaced by crab saline; the pipette contained 300 mM potassium aspartate, 300 mM mannitol, 2 mM ATP, 2 mM MgCl2, 2 mM EGTA, 0.3 mM CaCl2, 1 mM streptomycin, and 100 mM Heps (buffered to pH 7.4 with KOH). For the study of Ca current with minimal contamination by outward current, solutions were K+-free. Extracellular solutions contained 44 mM NaCl, 24 mM MgCl2, 13.3 mM CaCl2, 10 mM Heps, 10 mM D-glucose, and 0.0002 mM tetrodotoxin (pH 7.5, adjusted with NaOH). Intracellular solutions contained 300 mM N-methylglucamine methanesulfonate, 5 mM Mg-ATP, 5 mM 1,2-bis(2-aminophenoxy)ethane-N,N',N"N'-tetraacetic acid (tetracesium salt, BAPTA), 50 mM Heps, 2 mM streptomycin, and 10 mM tetraethylammonium chloride. Toxicity was maintained at 1100 mosM by addition of sucrose. Patch pipettes were pulled from WPI capillary tubing (TW150F-4), fire polished, and coated with beeswax. Pipette-to-bath resistances were 6 MΩ. Seal resistances were >1 GΩ.

RESULTS

Conditions and Time-Course of Neuronal Outgrowth in Culture. As rapidly as the culture can be put under the microscope after completion of dissociation procedures, cells that have adhered are found to have produced a corona of fine processes around the soma. Outgrowth from the axonal stump, if present and adhering, is seen at the same time. Within 16 hr, the form to be taken by each neuron is well defined. Growth continues for ~7 days in our routine defined medium, after which processes show various degrees of retraction. Cells survive for at least 3 weeks. Assuming that the land crab X-organ has 200 neurons, ~40% survive and exhibit outgrowth. We conclude that neither outgrowth nor form is dependent on enzymatic treatment of the neurons, because we have obtained outgrowth of the same types after dissociations performed without enzyme treatment, as well as after treatment with a number of different enzymatic preparations. However, the survival rate, particularly of larger neurons, is improved by enzymatic treatment that causes the cells to dissociate more easily, a result that we attribute to reduction of the trauma experienced by the neurons. Application of the same culturing procedures to the X-organ of spiny lobsters, a phylogenetically well-separated group, has given cultures showing comparable neuronal survival and forms of outgrowth. Thus we believe that our observations have general applicability and are not the reflection of some unique quality of the land crab principally used for this work.

No outgrowth is observed unless the cell adheres to the substrate. We now routinely use Primaria dishes, but have obtained adhesion and outgrowth of the same types on every type of untreated plastic tested, as well as on polylysine-treated glass.

A number of lines of evidence suggest that the neurons do not require any conditioning factors for their outgrowth. The sparsity of cells relative to the volume of medium makes self-conditioning unlikely. Cells exhibit outgrowth at normal rates and of normal form under the following circumstances: when the culture dish is flushed with fresh medium within an hour of plating the cells and changed daily thereafter; when individual neurons are grown in isolation; and when cells are plated in crab saline and glucose without any other additions. In saline, growth slows after 2 days and ceases soon after. If glucose and glutamine are included growth is sustained for up to a week. This indicates that the standard medium includes no unidentified critical component(s), and that the antibiotic routinely included is not influencing neuronal development.

Numerous processes like those from the soma also grow from the neurite at areas of adhesion to the substrate. Growth ceases in 7–10 days, and retraction of the veil is seen after 14 days.

Cells of <30 μm consistently show a different, branching form of outgrowth (Fig. 1 C and D). No processes are extended from the soma; they remain monopolar, with the major outgrowth occurring from the remaining axon at compact growth cones. Neurites reach ~150 μm within 7 days, after which growth slows or stops.

A few of the larger cells plated from an X-organ also show outgrowth of a branching form (Fig. 1C). Unlike the smaller cells, processes are also frequently extended from the soma and proximal neurite.

Cells plated without an axonal stump, or whose axonal stump does not adhere, fail to develop a predominant neurite and instead augment the initial halo of fine processes (Fig. 1E).

Reactivity of Cultured Neurons to Antisera. The selective-ness of reactivity to different antisera confirms that neurons of the several biosynthetic types present in the X-organ–sinus gland system survive in culture and retain their distinct capabilities. Cultures having neurons with outgrowth of the various forms described above were exposed to the five antisera reactive to peptides of the X-organ–sinus gland system that were available to us. In each case, some, but not all, cells showed reactivity. Certain correlations of cell size and form of outgrowth with antisera reactivity have been consistently observed. Thus, for example, antiserum to insect adipokinetic hormone N terminus, known to react with red-pigment-concentrating hormone (27, 34, 35), shows re-
activity with a few of the small cells with branching morphology in a given culture (Fig. 2C), but never with large cells. Antiserum raised against crustacean hyperglycemic hormone (28) shows reactivity with cells having large veils (Fig. 2E), as does antiserum raised against peptide D (24) (Fig. 2B), while antiserum raised against molt-inhibiting hormone (28) is reactive with large cells of branching morphology (Fig. 2D). The antiserum raised against crude sinus gland extract reacts with some of the large cells, but not with small cells in a given culture (Fig. 2A). Immunoreactivity is particularly intense in lamellipodia and filopodia.

**Electron Microscopy.** Electron micrographs of large cells show an abundance of microtubules and accumulations of secretory granules in branching processes (Fig. 1F). Granules are aligned with bundles of microtubules, suggestive of ongoing rapid transport. Somata (data not shown) show extensive arrays of rough endoplasmic reticulum, indicating that intense synthetic activity is ongoing, as is also seen in sections of large X-organ somata in situ (unpublished observations). Small cells in situ have fewer granules in their somata and proximal neurite and less extensive endoplasmic reticulum, which we suggest may have a bearing on their different form of outgrowth.

**Secretion.** If outgrowth involves incorporation of granule membrane by exocytosis, we would expect the release of neuropeptides into the medium by growing neurons. Medium samples gave positive red-pigment-concentrating hormone bioassays (36), even when medium had been changed at 2-day intervals. Secretory responsiveness of the cells was indicated by increased amounts of neuropeptides present in samples taken after a 40-min exposure to elevated concentrations of K⁺ (100 mM) compared to amounts present after 2 days in normal medium.

**Electrophysiology.** Exocytic secretion requires increased internal Ca²⁺ concentration that, in neurons, involves the participation of voltage-dependent Ca channels (37). Studies
FIG. 2. Immunofluorescence of crab peptidergic neurons in primary culture. The fluorescent second antibody was labeled with Texas Red, except for E, which was fluorescein isothiocyanate; the primary antisera, and days in culture of the cells were as follows. (A) Anti-sinus gland homogenate conjugated to bovine serum albumin and diluted 1:500 at 10 days. (B) Anti-peptide D conjugated to bovine serum albumin and diluted 1:1000 at 8 days. (C) Anti-AKH N terminus diluted 1:2000 (reactive with red-pigment-concentrating hormone) at 8 days. (D) Anti-molt-inhibiting hormone diluted 1:1000 at 7 days. (E) Anti-crustacean hyperglycemic hormone diluted 1:5000 at 7 days. Note brilliant staining of veils (A, B, and E), granular appearance of staining in C (processes extending to the left and right edge were dimly stained but did not reproduce).

demonstrate elevated levels of Ca$^{2+}$ in growth cones relative to other parts of the neuron (38, 39). Our proposal links these observations. Voltage-clamping of cultured veiling cells by using tight-seal patch electrodes reveals, in addition to tetraethylammonium- and 4-aminopyridine-sensitive outward currents (data not shown), an inward current that is attributable to Ca: it is unaffected by tetrodotoxin and blocked by the addition of Cd (Fig. 3), and its amplitude is markedly decreased in saline containing 10% of the normal Ca concentration. It is unchanged by changes of membrane holding potential between −40 and −60 mV, observable for steps to >−30 mV, and maximal at +10 mV. Minimum time to peak is ≈5 ms. Inward current initially decays rapidly ($\tau < 20$ ms) and then more slowly ($\tau > 50$ ms); at the end of a 160-ms pulse to +10 mV, >50% remains. Both inward and outward currents have been recorded from cells that have been in culture from 2 hr to 10 days.

Arrest of Outgrowth by Addition of a Ca-Current Blocker. Consistent with an important role of Ca currents in growth (and a link with exocytosis), we observed that addition of 0.2 mM Cd to the culture medium halts outgrowth (see ref. 39). Cd also inhibits secretion from the isolated X-organ–sinus gland system (22).

DISCUSSION

Our results support the proposal [an expansion of a more general one by Bray (40)] that membrane may be added at growth cones by exocytosis of granules synthesized and transported by the mechanisms normally involved in secretion; the re-uptake of membrane is omitted, however. The presence in the newly plated cells of abundant membrane, in the form of the neurosecretory granules, capable of being added to the surface of the cell, together with transporters
(microtubules) for moving them to axon terminations, as well as activated synthetic processes for maintaining the granule population may account for the capability of these cells for immediate outgrowth. The predetermined form of the outgrowth and its independence of external conditioning factors is emphasized by the unaltered initial outgrowth observed in plain crab saline. We have even observed formation of a typical veil at one end of isolated axons. It should be noted, however, that the form and extent of outgrowth in these cultures do show changes in response to manipulations of the culturing conditions, such as the addition of crab or bovine serum, incubation with whole skin gland, or brief exposure to saline containing elevated K⁺.

We suggest that differences in outgrowth form may reflect differences in abundance and distribution of granules and microtubules in concert with signals derived from surface membrane contact with the substrate. The role of internal Ca concentration in controlling the assembly and distribution of microtubules as well as the movement of granules along microtubules (41) suggests the possibility that the density and activity of Ca channels in or near the growth cones play crucial roles in governing the form of outgrowth. We were thus interested to find that cells of branching morphology have much smaller Ca currents than those reported above for large veiling cells, although these observations must be regarded as preliminary. The small cells, which consistently show a branching morphology, have less abundant endoplasmic reticulum and granules in the soma. Additional work correlating fine structure, electrophysiological characteristics, biochemical competence, and the form of outgrowth should provide further insight.

To our knowledge, the only other neurons reported to show immediate outgrowth without conditioning factors are also peptidergic neurosecretory cells, the Aplysia bag cells (8, 42). However, in contrast to our results, outgrowth from these cells has not been reported without the addition of amino acids and vitamins to the medium. The consistency of the time course, forms, and extent of outgrowth of the crustacean peptidergic neurons in our defined culture conditions provides a starting point for experimental manipulations to explore the control of neuronal growth and form.

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Fig. 3. Current (I)-voltage (V) relationship for Ca current measured at the end of 160-ms voltage steps before (control, ○) and after (○) exposure to Cd. Whole-cell patch-clamp recordings with K⁺-free solutions, membrane potential held at -40 mV, and stepped from -80 to +50 mV in 10-mV increments (17). The magnitude of current and form of this I-V curve are typical of those obtained from >10 cells with large veils. (Lower Inset) Examples of recordings. Superimposed current traces in response to step to +10 mV before and after exposure to Cd (0.2 mM). (Upper Inset) Photomicrograph (Hoffman modulation contrast) of the neuron taken immediately before the recordings were made. The neuron was plated 12 hr prior to making the recordings. Note the large veil.