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Peptidergic neurons of the crab, *Cardisoma carnifex*, in defined culture maintain characteristic morphologies under a variety of conditions

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Summary. Peptidergic neurons dissociated from the neurosecretory cell group, the X-organ, of adult crabs (*Cardisoma carnifex*) show immediate outgrowth on unconditioned plastic dishes in defined medium. Most of the neurons can be categorized as small cells, branchers or veilers. A fourth type, "superlarge," found occasionally, has a soma diameter greater than 40 μm and multipolar outgrowth. We report here the effects on morphology that follow alterations of the standard defined culturing conditions. The three common types of neurons are present when cells are grown in crab saline or saline with L-glutamine and glucose (saline medium). Changes of pH between 7.0 to 7.9 have no effect. Osmolarity changes cause transient varicosities in small cells. In some veilers, pits rapidly appear in the veil and then disappear within 35 min. In cultures at 26° C instead of 22° C, veilers extend processes from the initial veil in a pattern similar to branchers, and the processes of adjacent veilers sometimes form appositions. Culturing in higher $[K^+]_o$medium ($[K^+]_o = 15$–110 mM; standard = 11 mM) has no long-term effect, but growth is arrested by $[K^+]_o$ greater than 30 mM. Cultures were also grown in media in which $[Ca^{2+}]_o$ ranged from 0.1 μM to 26 mM (standard = 13 mM). Outgrowth occurred from all neuronal types in all $[Ca^{2+}]_o$ tested. Thus, the expression of different outgrowth morphologies occurs under a wide variety of culturing conditions.

Key words: Cultured neurons – Potassium elevation – Calcium alteration – Defined culture – Neurosecretion, peptidergic – Growth cones – Neuronal regeneration – *Cardisoma carnifex* (Crustacea)

Outgrowth of neurons in low-density cultures generally requires the presence of tissue-derived conditioning factors or serum. This greatly complicates the elucidation of mechanisms regulating morphology. The culture of peptidergic neurons from the crustacean X-organ (Cooke et al. 1989) offers unusually promising material for such studies because, in these cultures, immediate regenerative outgrowth of at least four distinct types of neurons occurs without conditioning of the dishes or the addition of factors or serum to the medium. Correlation of the neuronal outgrowth pattern of some of these neuron types with hormonal immunoreactivity (Cooke et al. 1989) and with electrophysiological characteristics (Cooke et al. 1989; Meyers et al. 1992) indicates that the different morphological types may represent distinct phenotypes.

The presence of neurons having different morphologies in these cultures reflects the heterogeneity of the cluster of neurosecretory cells from which they are isolated. We have used the X-organ – sinus gland system of the eyestalk of the crab (*Cardisoma carnifex*). This system is analogous, physiologically and structurally, to the peptidergic component of the vertebrate hypothalamo-neurohypophyseal system. The peptide hormones secreted by this neuroendocrine system govern complex physiological processes, such as molting, hemolymph glucose levels and color change (for a review, see Cooke and Sullivan 1982).

Reports of the successful culture of crustacean neurons have only recently appeared (for a review, see Thomas et al. 1987). In addition to the X-organ neurons, stomatogastric neurons from lobster have now been cultured without conditioning factors (Graf and Cooke 1990), and from crayfish with the addition of fetal bovine serum (Krenz et al. 1990).

In this paper, we have extended our initial description (Cooke et al. 1989) of the outgrowth and general morphology of the several types of X-organ neurons seen under a standard culturing regime. We have attempted to probe the mechanisms regulating this outgrowth and morphology by manipulating the culturing conditions. We have found that the morphological distinctions among our heterogeneous population of neurons are clearly preserved under a very wide range of altered, but defined culturing conditions.

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Materials and methods

Animals and dissection

Adult, male crabs (*Cardisoma carnifex*; 300 g) were obtained from Christmas Island, Republic of Kiribati. They were kept in outdoor cages supplied with clay flower pot shelters and provided with both fresh and sea water. The animals were fed rat pellets (Purina, Richmond, Ind., USA) and occasionally *Pandanus* nuts, carrots, lettuce and broccoli stalks. The cages had a northeasterly exposure and received up to two hours of direct morning sun. Animals were generally used within a month, but individuals have been held under our conditions for over a year. We have never observed a crab to molt.

Eyestalks were removed, rinsed with 70% ethanol, then opened and exposed to 3 changes of sterile crab saline (*crab saline*: 440 mM NaCl, 11.3 mM KCl, 13.3 mM $CaCl_2$, 26 mM $MgCl_2$, 23 mM Na_2SO_4, 10 mM HEPES, pH 7.4 with NaOH, 1050 mOsm; after Cole, 1940) that contained antibiotics (100 units/ml penicillin, 0.1 mg/ml streptomycin, 0.25 µg/ml fungizone; Gibco, Grand Island, N.Y., USA). Upon removing the exoskeleton, and muscle and connective tissue overlying the optic ganglia, differences in the consistency of the tissue were observable from time to time. These differences account for the greatest part of the uncontrolled variability in the culturing results and must be attributed to the history of the animals previous to their captivity. Preparations having firm, translucent tissue in which the X-organ cell group, often distinctly blue-white, was compact and slightly protruding from the surface of the medulla terminalis consistently yielded good cultures; if the tissue proved sticky and opaque, the dissection was discontinued. The X-organ with approximately 2 mm of the sinus gland tract was dissected free of surrounding tissue (see Fig. 1 A) in the antibiotic saline, placed in nominally Ca-free and Mg-free saline with 0.1% trypsin (Gibco), and agitated on a Dubnoff shaker (Precision Scientific, Chicago, Ill., USA) in the dark for 90 min at 22° C. Enzymatic activity was arrested by agitating the tissue in a large volume of the Ca-free and Mg-free saline.

Cell isolation and standard culture conditions

The *standard medium* consisted of Liebowitz L-15 (Gibco, as powder) reconstituted to 100% and mixed with an equal volume of crab saline of 1.75× normal concentration that was buffered with 20 mM HEPES (pH 7.6–7.8). The final solution also contained 0.1 mg/ml gentamicin, 120 mM D-glucose, and 2 mM L-glutamine, which were added immediately before use. Total osmolarity was 1100 mOsm. Determinations of the osmolarity of hemolymph (after stirring to remove clot-forming material) for 5 crabs held for over two weeks in our cages averaged 1060 mOsm.

One dissected enzyme-treated X-organ was transferred to a culture dish (Primaria, Falcon 3801, Bectin-Dickinson, Lincoln Park, N.J., USA) containing a 50-µl drop of medium. Approximately 1/4 of the cells (~150) were disaggregated in the drop. The X-organ was transferred to another dish, more cells disaggregated, and the procedure continued so that the cells of one X-organ were distributed among ~4 dishes. To disaggregate the cells, the X-organ was held by the sinus gland nerve tract with a fine pair of forceps and agitated with a direct stream of medium delivered from a P20 Pipetman (Gilson, Villier-le-Bel, France). After completion of the dissociation, the dishes were left undisturbed for an hour or more to permit the cells to adhere to the dish. Then the volume of the culture medium was increased to 2.0 ml. By contrast with neurons of the stomatogastric ganglion (Krenz et al. 1990; Graf and Cooke 1990), X-organ (and other CNS) neurons are not closely enveloped in accessory cells (glia) that present problems for adhesion. Cultures were held in moist chambers (Billups-Rothenberg, Del Mar, Calif., USA) in the dark at 22–24° C. The culture medium was not changed, except as noted. Cells were examined on an inverted microscope (Diaphot, Nikon, Torrence, Calif., USA) equipped with phase optics (40–400x). Photomicrographs (Nikon FE, Technical Pan film, Kodak, Rochester, N.Y., USA) of cells were taken at regular intervals after plating.

Altered culturing solutions

Cells were cultured in crab saline as described above. Cells were also cultured in *saline medium*, viz. crab saline to which 120 mM D-glucose, 0.1 mg/ml gentamicin, and 2 mM L-glutamine were added, osmolarity, 1140 mOsm. *Hypo-osmotic medium* consisted of standard medium mixed 1:1 with crab saline, and had a final osmolarity of 1050 mOsm (as compared with 1100 mOsm for standard medium, or of approximately 1200 mOsm measured on samples of medium removed from 3-day-old cultures). *K-enriched media* containing raised concentrations of KCl were prepared by corresponding reductions in the NaCl concentration. In *calcium-deleted saline* and *calcium-deleted saline medium*, $CaCl_2$ was omitted (replaced by NaCl) from crab saline medium, which was otherwise as above. Because the concentration of $CaCl_2$ in L-15 medium is 1.0 mM, it was not possible to make Ca-deleted, but otherwise standard medium. Using calcium-deleted crab saline at a 1.75x concentration mixed 1:1 with L-15 medium reduced the value of $[Ca^{2+}]_o$ (external Ca^{2+} concentration) to approximately 0.5 mM. The addition of 1.0 mM EGTA (Sigma, St. Louis, Mont., USA) to this solution resulted in a calculated $[Ca^{2+}]_o$ of 0.1–0.3 µM. For all other Ca concentrations the amount of $CaCl_2$ was varied by adjustment of NaCl in the 1.75x crab saline component of the standard medium. Osmotic pressure was measured using a µOsmette osmometer (Precision Systems, Sudbury, Mass., USA) or Wescor Osmometer (Logan, Utah, USA).

Viability

Seven X-organs were prepared for culture and distributed among 20 dishes (Primaria, Falcon 3801). On day 1 and day 2 in culture, cells showing outgrowth equal to or greater than one soma diameter were designated as growing; cells with outgrowth of less than one soma diameter were designated as surviving. The remaining cells that showed signs of non-viability (blebs, granulation, vacuolization) were counted. All counts were made at 400x using the perimeter of the photo mask on a Nikon Diaphot inverted microscope as a reference for each field.

Microscopy

X-organs were processed and embedded as for transmission electron microscopy, serially sectioned at 0.5 µm, and stained with 1% paraphenylenediamine. The tissue used was an approximation of the mass used for tissue culture (Fig. 1 A). Total cell counts were made at the level of each cell nucleus from photomicrographs of every third section. Cultures that were to be examined under the scanning electron microscope (SEM, Cambridge S-150, Cambridge, UK) were grown on poly-L-lysine (1 mg/ml, Sigma)-coated circular glass coverslips (12 mm) that were attached to the bottom of plastic culture dishes in which a hole had been bored. The seal between the coverslip and culture dish was made using melted Cenco Softseal Tackiwax (Central Scientific, Chicago, Ill., USA). Cells were plated on the coverslip portion of the dish and when ready for examination, the coverslip was prized away from the dish with a razor blade, immersed in fixative and processed for SEM using conventional methods (Postek et al. 1980).

Estimation of outgrowth

Estimates of cell growth in culture were made from measurements of cells in photomicrographs. The cell outline was digitized using

the cursor and digitizing pad of a Zidas Digitizer (Version 1.77, Carl Zeiss, Thornton, N.Y., USA), which calculated an approximately scaled estimate of the area encompassed by the digitized outline (i.e., approximately half the actual membrane surface-area). Cell growth was calculated as the percent increase in the area of the cell outline at day 1 and day 2 to the area when plated.

Results

Outgrowth morphology under standard culturing conditions

Four morphological types of neurons. The majority of cells in cultures of dissociated crab X-organ neurons can be assigned to one of four categories on the basis of their size and pattern of outgrowth during the first few days in culture. We suggest that these differences reflect inherent differences in phenotype of the neurons, because neither the dishes nor the medium are conditioned in any way. Fig. 1A shows micrographs of a section through an intact cluster of peptidergic secretory neurons, the X-organs, from which the neurons to be cultured are dissociated. Figure 1B illustrates the plating density typical of our cultures, and shows that, except for a few incompletely dissociated groups of small cells (see below), the cells are so widely separated that conditioning as a result of factors released from adjacent cells is unlikely. In a series of 20 plates in which all cells were counted, the average number of cells per dish was 156 ± 24 (SE). It should be noted that most of the neurons were plated with a length of neurite. Isolated axons produce a lamellipodium during the first day in culture, but growth then ceases (Fig. 1D). When cells are plated without an axonal stub, their morphology is generally stellate, and their phenotypic relationship to other neuronal types is uncertain (Fig. 1E).

An exception is the type of cell shown in Fig. 1C. Neurons of this size (soma diameter >40 μm) were not observed at the time of our previous report (Cooke et al. 1989). Dubbed "superlarge cells", they have not been plated with a surviving neurite, but unlike other neurons, they consistently regenerate one or more major processes. Cells of this type reach a stable morphology within 8 days and do not show withdrawal for over 12 days in culture. This type may represent cells of similar size that are seen in sections at the external surface of the X-organ. Perhaps because of this location and their large soma size, they rarely survive the dissociation and plating procedure. This cell type does not show immunoreactivity to antisera made to crustacean hyperglycemic hormone (CHH, Dircksen et al. 1988), or to antisera to molt-inhibiting hormone (MIH, Dircksen et al. 1988). Reactivity to the other antisera against X-organ peptides (Cooke et al. 1989) has not been tested.

The outgrowth patterns for three neurons representative of the categories into which most of the cultured neurons can be grouped are shown in Fig. 2. These are as follows.

(1) "Small cells" (Fig. 2A): these neurons have somata of less than 25 μm diameter, which are usually spherical with a prominent, centrally placed nucleus. They often fail to be completely dissociated and are plated as clumps of 3–20 cells. They possibly represent cell groups at the periphery of the X-organ (e.g., left of the left arrow, Fig. 1A). We have not done further staining and thus have not been able to confirm our earlier association of this type with red pigment-concentrating hormone (RPCH)-like immunoreactivity (Cooke et al. 1989), but in our hands very few neurons in X-organ sections show reactivity to the antiserum made against the N-terminus of insect adipokinetic hormone (Code: AKH (1–4) 433, Schooneveld et al. 1987) that is reactive with RPCH (Mangerich et al. 1986). When plated with a surviving neurite, the small cells produce an array of fine (<1 μm wide) processes from the axonal stump, one or more of which continues outgrowth. This type often forms a stable morphology by the second day. After day 4, the cells become increasingly withdrawn.

(2) "Veilers" (Fig. 2B): these neurons have soma diameters of 25–50 μm that, when plated with an axonal stub, produce one or more broad lamellipodia or veils. The additional veils occur at bends in the initial neurite (see Fig. 3A). These neurons show more extensive outgrowth during the first day in culture than any other type; within 18 h, the area of the lamellipodium may exceed the surface area of the soma. Filamentous processes extend from the soma and from points along the neurite where it appears to adhere to the substrate. Within 2–3 days the veil may double or triple its area. Certain regions along the margin of the veil begin to appear engorged (rounded up and phase-bright), and with additional time in culture, some of these grow out to become thick processes that may reach 20–50 μm in length. The margins of the veil between the filopodia and processes begin to withdraw after day 4. Usually, no further outgrowth is seen after 6 days in culture, even if the medium is changed. Withdrawal is evident in cultures held longer than 10 days. As previously reported (Cooke et al. 1989), cells of this type consistently show immunoreactivity to CHH antisera, and to anti-sinus gland homogenate serum and anti-peptide D (Newcomb 1987) serum; they are not reactive to MIH anti-sera or to AKH antiserum. The relative abundance and size range of these neurons is consistent with their homology with neurons showing CHH immunoreactivity in the X-organ of *Carcinus maenas* (Dircksen et al. 1988).

(3) "Branchers" (Fig. 2C): these are neurons having distinctly ovoid somata (minor axis 25–40 μm), that when plated with a neurite consistently show outgrowth in a monopolar, extended branching form occurring by the extension of filopodia from a few compact growth cones. The arbor reaches distances of over 100 μm within 4 days. Withdrawal of some processes and extension of others continues for up to 7 days in culture, after which time, withdrawal predominates. Some neurons of this type, including cells having soma diameters at both the larger and smaller ends of the observed size range, show immunoreactivity to anti-MIH sera. Branchers have also shown immunoreactivity to anti-sinus gland homogenate serum and to anti-D serum. A few neurons of this

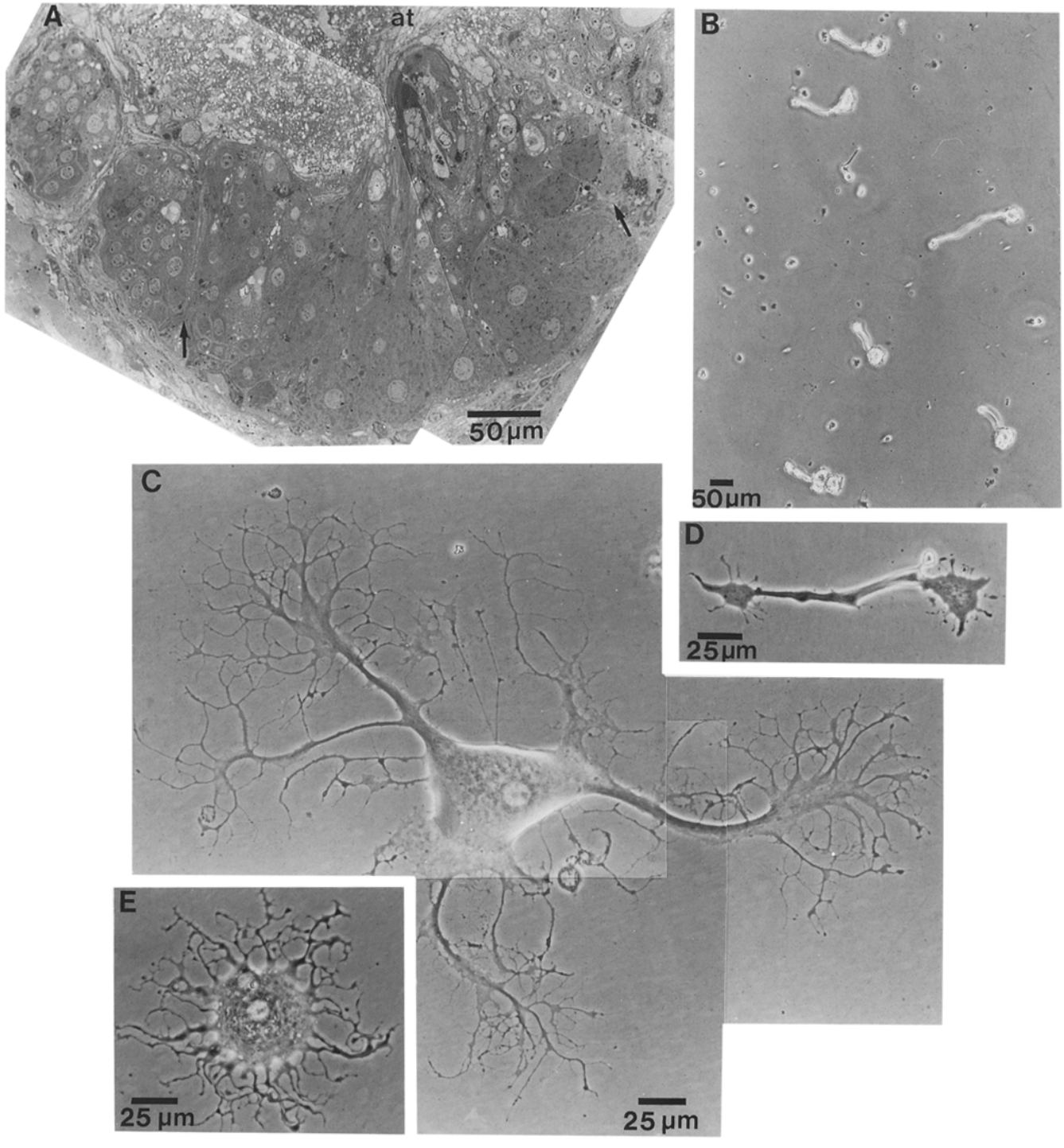

Fig. 1. **A** The X-organ, a group of peptidergic neurosecretory cell somata, from which the dissociated cultures were made. Photomicrographs (montage) of a 0.5 µm plastic-embedded section stained with 1% paraphenylenediamine. Axons of the cells between the *arrows* could be traced to the axon tract (*at*) through the central neuropil of the medulla terminalis. It is unclear whether the clusters of small cells peripheral to the *arrows* contribute axons to the axon tract, but they may be included in the cultures. **B** Culture immediately after plating to show sparse plating density. The majority of neurons are plated with a surviving neurite (many are out of focus because they are not adhering to the plate). A number of the cells have a soma diameter (~40 µm) corresponding to that of the larger cells with nuclei shown in **A**. **C** A "superlarge" cell on day 12. Two major branching neurites were produced from the soma, which did not possess a neurite when plated. **D** Outgrowth from a neurite plated without a soma; day 2. A growth cone formed at each end, but no further growth occurred. **E** Typical outgrowth from a soma plated without a neurite, d 7. A halo of thin branches but no major neurite was produced. Standard, fully defined culturing conditions (unconditioned Primaria dishes, standard medium, 22° C) were used unless otherwise noted

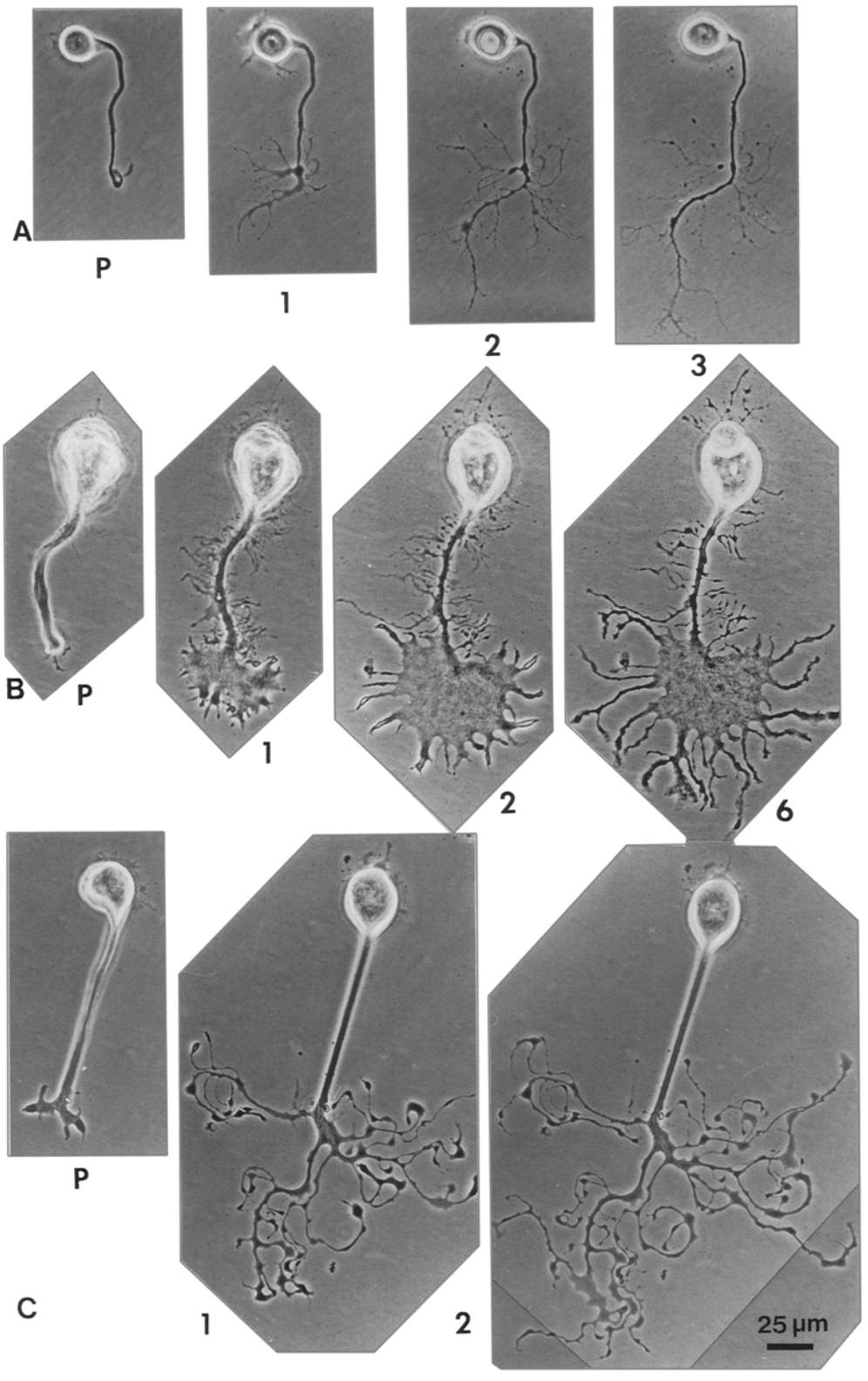

Fig. 2A–C. Outgrowth pattern distinguishing three categories of neurons in X-organ cultures. **A** "Small cell": small (<25 µm) round soma with prominent central nucleus and a fine branched outgrowth from the surviving neurite. **B** "Veiler": soma 25–45 µm, here 25 µm, a large lamellipodium is produced from the surviving neurite. At day 6, withdrawal of the veil between the filopodia is evident. This cell type shows immunoreactivity to crustacean hyperglycemic hormone (CHH) antisera. **C** "Brancher": outgrowth produces a number of complex branched processes. *P* Plating; *numerals* days in culture

type have shown reactivity to the anti-AKH serum, suggesting that they contain RPCH.

Survival. Survival and outgrowth were evaluated in one series of 20 culture dishes prepared by the dissociation of neurons from seven X-organs. Some 3122 neurons were counted, of which 1497 (45%) showed outgrowth of at least a soma diameter by 24 h in standard medium. In order to estimate the number of neurons present in the seven X-organs, an intact X-organ, dissected as for culturing, was fixed, serially sectioned (at 0.5–1 µm), and the neurons counted at the level of the nucleus for each cell (see Fig. 1A). There were 538 cells whose axons were clearly directed to the sinus gland tract. An uncounted

number of small neurons that occurred in clusters on the periphery of the major X-organ mass, and whose axons could not be followed, may have doubled the total cell number. It is likely, on the basis of their size and tendency to plate as clumps of cells, that some of the "small cells," as categorized above, are derived from these peripheral clusters. Their possible homology with the group of distal small putative RPCH neurons described by Mangerich et al. (1986) remains to be determined. It is possible that some may not represent secretory neurons. The larger neurons are from the group clearly contributing to the sinus gland tract. Counting all neurons (including small neurons) in the cultures, and calculating survival by comparison with the neurons counted in the X-organ, we have obtained a figure of 89% surviving dissociation. It seems probable that this number is inflated by the inclusion in the starting material of uncounted numbers of cells from the peripheral small neuron clusters.

The outgrowth and morphology of neurons observable when the dissociation was made without use of enzymes was the same as under standard culturing conditions, but the number surviving (particularly of the larger neurons) was reduced, presumably as a result of increased trauma during dissociation; superlarge cells were not observed without enzymatically-aided dissociation. We conclude that the use of enzymes for the dissociation does not alter the outgrowth morphology.

Effects of altered culture conditions on morphology

Table 1 presents a summary of alterations in culturing conditions tested, the number of cultures and approximate numbers of neurons for which photographic documentation was obtained, and the most prominent changes observed (or lack of change). In all cases, many additional cells were surveyed to confirm that the photographed neurons were representative examples. It may be noted that neurons showing initial veiling outgrowth were affected by altered conditions while other neuronal types showed little change. Further details and comments are given below.

Outgrowth in simple media. We wished to explore the minimal requirements of the medium that would support outgrowth. We found that during the first 2 days, neurons plated on untreated plastic dishes in crab saline show forms of outgrowth similar to those in standard medium (Fig. 3A), and the overall survival of neurons was comparable. However, after 2 days, growth ceased, growth cones became blunt and rounded, and processes began to retract.

In saline medium (crab saline with glucose, L-glutamine and gentamicin as in the standard medium), the period of growth was 5 days, compared with up to 7 days in standard medium. The extent of outgrowth was generally reduced. Withdrawal followed cessation

Table 1. Effects of altered culturing conditions on outgrowth of neurons

Treatment	Cultures	Cells photographed	Effect	Figure
Crab saline	5	17	Normal outgrowth, all neuronal types, early withdrawal (day 2 vs day 7)	3A
Crab saline with glucose, L-glutamine and gentamicin	14	42	Normal outgrowth, all neuronal types, early withdrawl (day 5)	3B
Plated on 1 mg/ml poly-L-lysine-coated substrate	8	15/16	Veiling neurons form "terminals" at day 1, then veils	4
	4	12	Branching neurons normal	
Plated in restricted volume	14	64	Anticipated veilers form "terminals" at day 1, no further growth	
	14	25	Branching neurons normal	
Cultured at 26° C (vs 22° C)	22	95	Veiling neurons become branched after day 3	5
	19	46	Occasionally formed contacts Branching neurons, no difference	6
Change of pH, 7.0–7.9	15	53	Normal outgrowth of all neuronal types	
Medium hypo-osmotic	16	24/35	Pits form in veils	7A–C
	8	15/16	Beading and varicosities in small cell processes	
	11	23	Branching neurons, no effects seen	
Transfer on day 1 to $[K^+]_o$ enriched medium (2x-10x normal)	31	116	Transient effects as in hypo-osmotic medium, arrested outgrowth	
Transfer on day 1 to $10 \times [K^+]$, ~ 0 $[Ca]_o$	10	47	Change in cytoplasmic appearance	7D, E
$[Ca^{2+}]_o$ varied from nominally 0 to 26 mM, from plating	24	85	Normal outgrowth, all types; broad optimum at 5 mM for veilers	8, 9
$[Ca^+]_o$ varied from $\sim$0–26 mM, day 1	20	97	Normal outgrowth, all types	

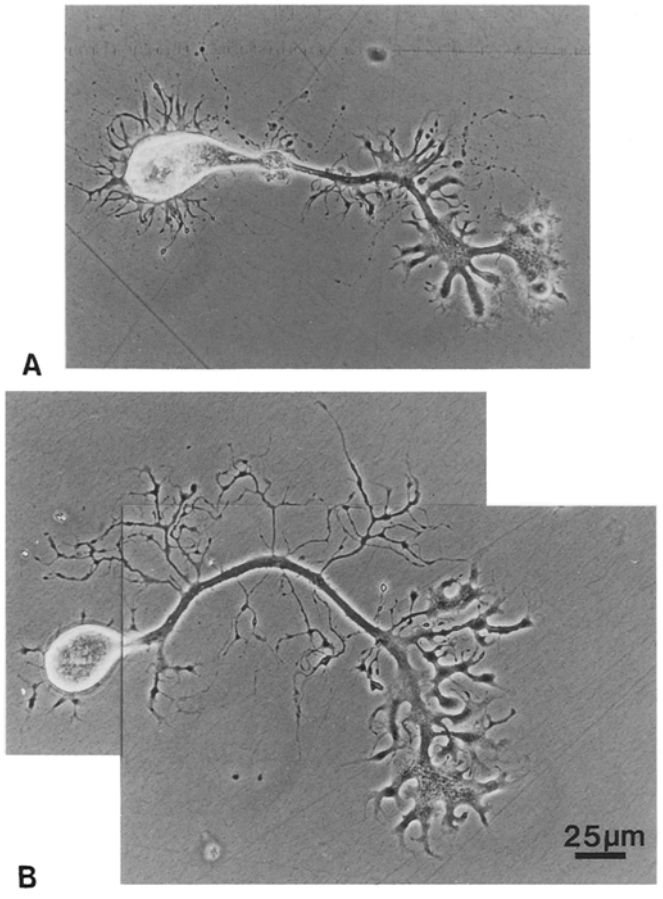

Fig. 3A, B. Outgrowth in simple media. **A** Neuron in crab saline; day 3. Withdrawal from the lamellipodium has begun. **B** Neuron in saline medium (crab saline + glucose + L-glutamine), day 4

of outgrowth without a period of 2–3 days of stable morphology. The example shown in Fig. 3B is among the most developed branchers observed in saline medium.

These observations support the conclusion that outgrowth from these peptidergic neurons does not require external chemical signals. We consider it unlikely that the neurons provide their own conditioning factor(s) for the following reasons: (1) single neurons that were plated in the normal volume of 2 ml showed immediate and normal outgrowth; (2) neurons grew normally when the culture dish was flushed with fresh medium within 1 h of plating and daily thereafter. We cannot, however, rule out the possible continuing influence of factor(s) to which the neurons were exposed prior to their dissociation.

Effect of substrate and plating density. Our general observation is that if dissociated X-organ neurons adhere to the substrate, outgrowth follows immediately. All types of untreated plastic culture dishes supported outgrowth, whereas glass required treatment with poly-L-lysine ($<$ 0.1 mg/ml for normal morphology, see below). Outgrowth did not occur or was limited on substrates treated with gelatine (Gibco) or Cell-Tak (Bio Polymers, Farmington, Conn., USA). Outgrowth occurred on laminin (Gibco) and fibronectin (Cappel-Organon Teknika, Westchester, Pa., USA), but was not enhanced; moreover, the respective morphologies were not altered on these materials (Cooke et al. 1989).

A characteristic change in morphology observed under two quite different conditions deserves comment. On glass that was treated with poly-L-lysine (Sigma) at 1.0 mg/ml for 24 h, rinsed with saline followed by distilled water, and aged for 1 week, adhesion and outgrowth occurred, and eventually the usual types of morphology were seen. However, lamellipodia, characteristic of veilers, were not obvious until the second day, whereas during the first 18 h, rounded protrusions appeared at the end of the axonal stub (Fig. 4). The veil, when it appeared, pushed out from under the "terminal blebs," and the blebs continued to increase in number and volume. Under the SEM, these had a striking resemblance to terminals of the in situ gland (see Fig. 2B in Stuenkel and Cooke 1988). Where the interior was exposed, the

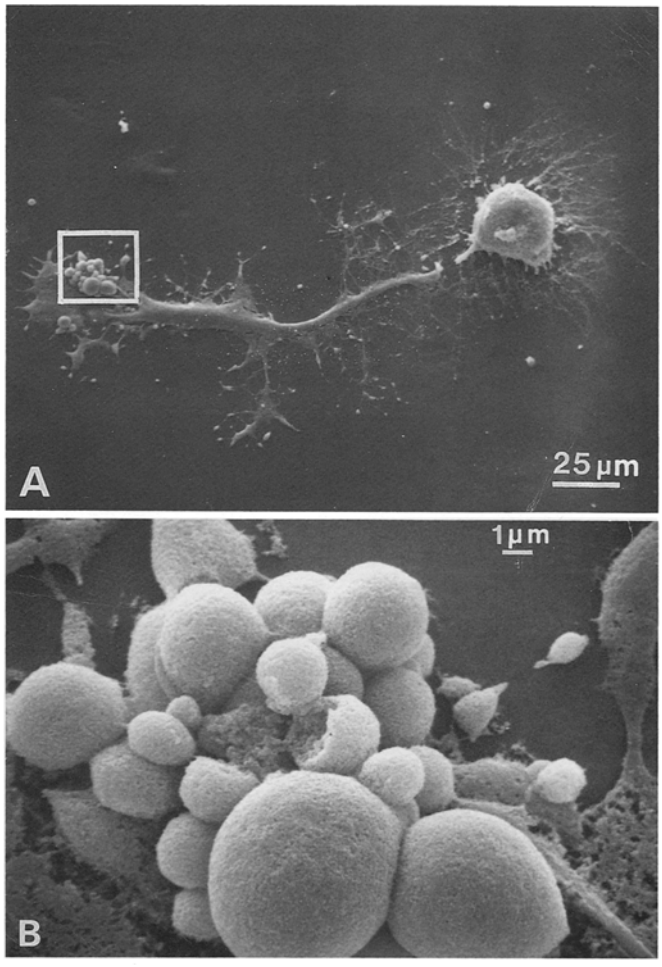

Fig. 4A, B. Formation of "terminals" in culture. Cells cultured on poly-L-lysine (1 mg/ml)-coated glass, and fixed for SEM on day 7. Rounded protrusions were apparent by day 1. With a change of medium, an underlying lamellipodium was seen on day 2, but did not enlarge, whereas "terminals" increased in number and volume until the culture was fixed. **B** Higher-resolution micrograph of *boxed area* shown in **A**. "Terminals" resemble those of the in situ sinus gland. Note the presumed neurosecretory granules in broken terminal

blebs appeared to contain secretory granules, as in the sinus gland (Fig. 4, bottom). Outgrowth of branching neurons appeared not to be affected by this or other conditions under which terminals were formed by veilers.

A second condition under which the formation of "terminals" was consistently observed was during efforts to culture the neurons at greater density. Thus, for example, blebs were observed when approximately 160 X-organ neurons were plated within a 24 mm diameter circle formed by a wax ring in a small volume (< 0.5 ml, compared with 2 ml). The same occurred when culturing in multiwell plates (Primaria).

Effects of temperature. A series of cultures were prepared by standard methods, but were held at 26° C rather than the standard 22° C. Veilers in cultures at 26° C continued to enlarge the area of the lamellipodium during the first 3 days (Figs. 5A–C, 6A). At this time, a major difference relative to cultures at the cooler temperature began to appear: veilers developed a markedly more branched morphology by extension and enlargement of a few of the filopodia, and withdrawal of the thin portions of the veil (Figs. 5D, 6B).

An observation unique to the cultures held at the higher temperature was that veilers undergoing directed outgrowth, as described above, seemed to extend a process preferentially toward a nearby, originally veiling neuron, as judged by the bending of the neurite toward the neighbor (Fig. 6A, B). In four cases, the neurites formed such close contacts that they could not be resolved as separate processes under phase optics. Immunostaining with anti-CHH sera confirmed the CHH reactivity of both neurons, as anticipated from their earlier veiling morphology.

Apposition of neuronal processes was never observed in cultures held at the cooler temperature. In contrast, outgrowing processes held their trajectories even when in close proximity to neighbors, or showed preferential outgrowth away from neighboring neurons (Fig. 6C–E).

The pattern of outgrowth for the neuronal types other than veilers was not clearly different in the cultures held at the higher temperature.

Altered pH and medium osmolarity. Neurons were cultured in medium that had been buffered to different values of pH over the range 7.0 to 7.9. We did not observe any consistent differences in the time-course or morphologies of outgrowth.

Exposure to hypo-osmotic medium produced almost immediate morphological changes in veilers and small cells, but no obvious effects in branchers. The medium, at 1050 mOsm, was 50 mOsm hypo-osmotic to the standard medium; however, samples of medium removed from cultures after 3 days measured 1194 mOsm. The effect on veilers consisted of a beading or thickening at growth cones and the edge of veils. In addition, a majority (24 out of 35 photographed) of the veilers also developed marked pits in the "palm" of the veil (Fig. 7A–C). These appeared within 1 min, increased in number (from 2–20), and widened to diameters of up

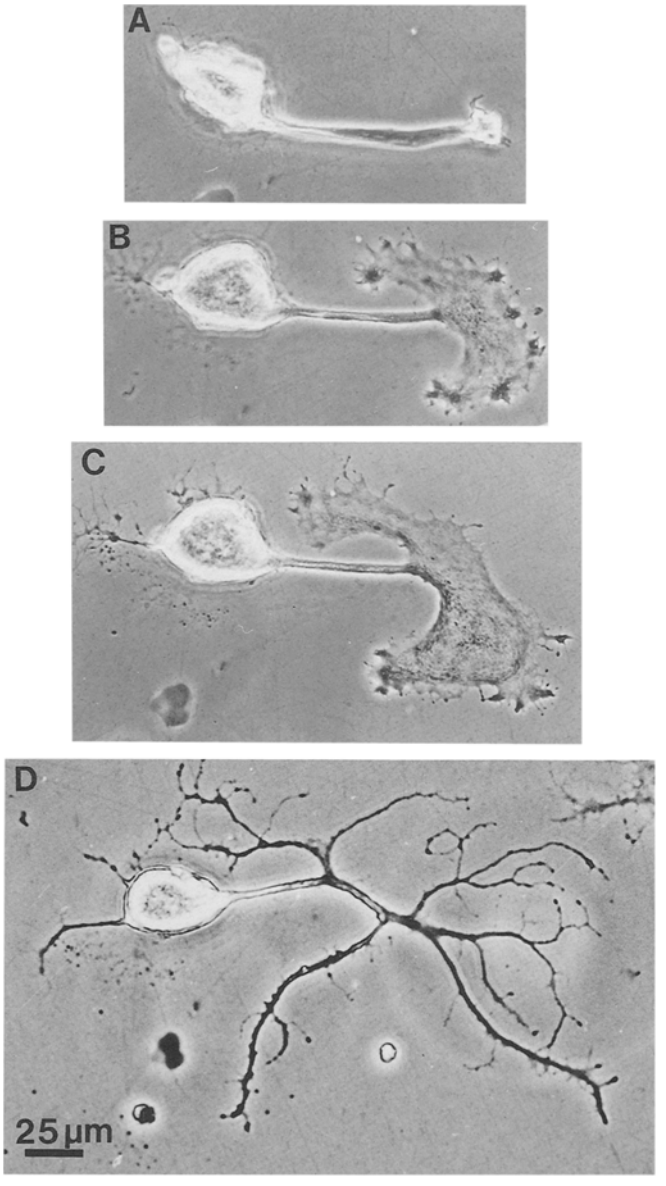

Fig. 5A–D. Branching outgrowth of a veiler cultured at a higher temperature (26° C). **A** 10 min after plating. **B** A lamellipodium has been produced by day 1. **C** Lamellipodium has enlarged by day 2. **D** Extension of processes and withdrawal of the veil has resulted in a branching morphology by day 6

to 5 µm during the first 10 min, and then disappeared within the next 15–25 min, in the continued presence of the hypo-osmotic medium. In additional experiments, similar changes, including the appearance of pits, occurred when crab saline (1050 mOsm) replaced the standard medium (1100 mOsm). We observed the reappearance of pits at similar locations upon return of the cultures to standard medium. The pits gradually diminished in number and diameter and disappeared within 30 min.

Small cells exposed to the hypo-osmotic conditions showed a beading of the major processes; varicosities were obvious within 3 min, were maintained during the exposure to hypo-osmotic medium, but disappeared rapidly (within 10 min) upon return to standard medium.

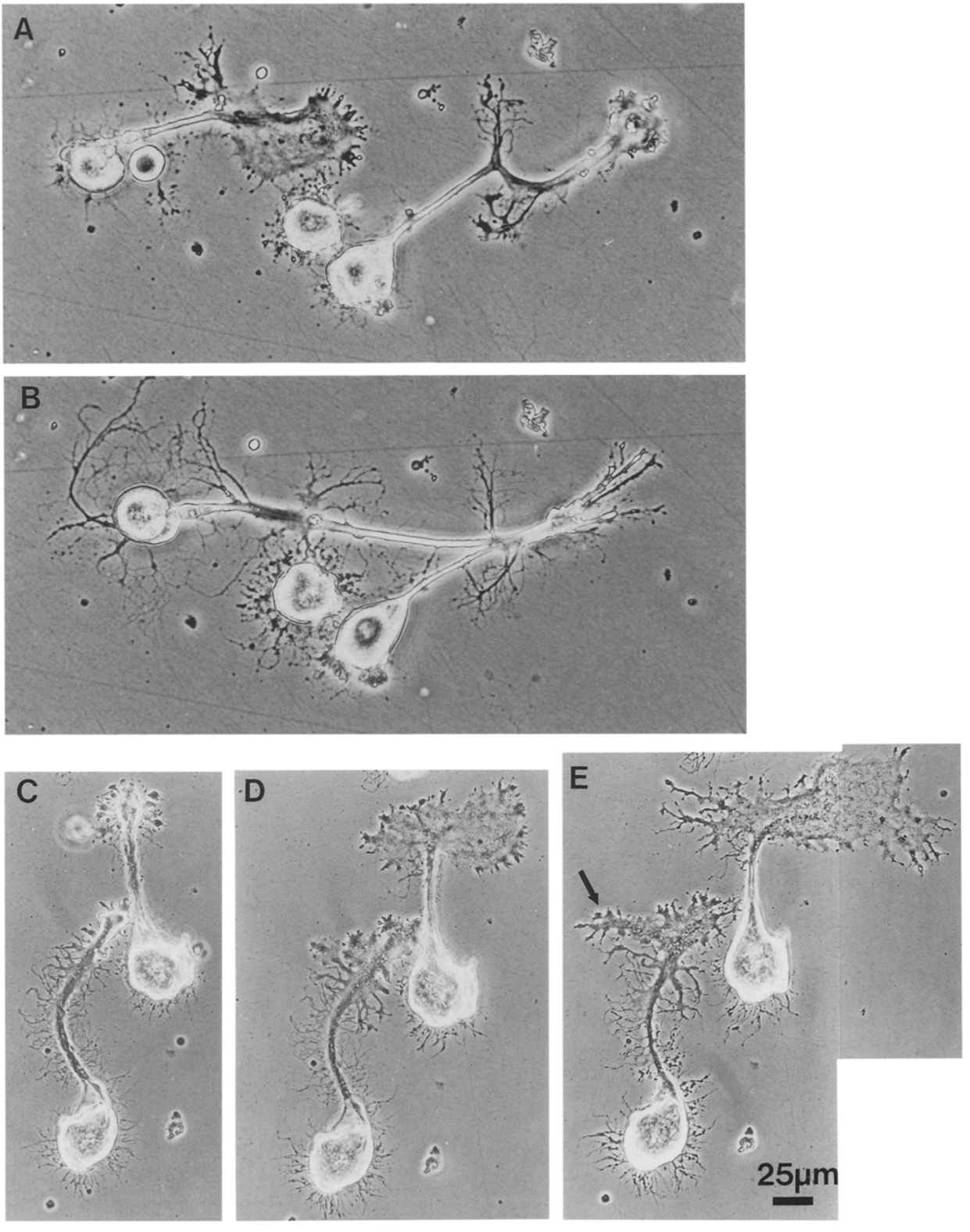

Fig. 6. A, B Formation of appositions between veilers at 26° C. **A** Two large cells plated with neurites have produced veils (2 neurons without neurites are also present); day 2. **B** At day 6, veils have been withdrawn, and the processes of the cells are in close apposition. **C, D** Lack of apposition between veilers at 22° C. **C** Veiling outgrowth has begun 2 h after plating. **D** At day 2, the veil of the left neuron is adjacent to the neurite of the other. **E** At day 6, the outgrowth from the veil of the *left* neuron has grown predominantly away from the adjacent neuron (*arrow*), as has that of the *right* neuron

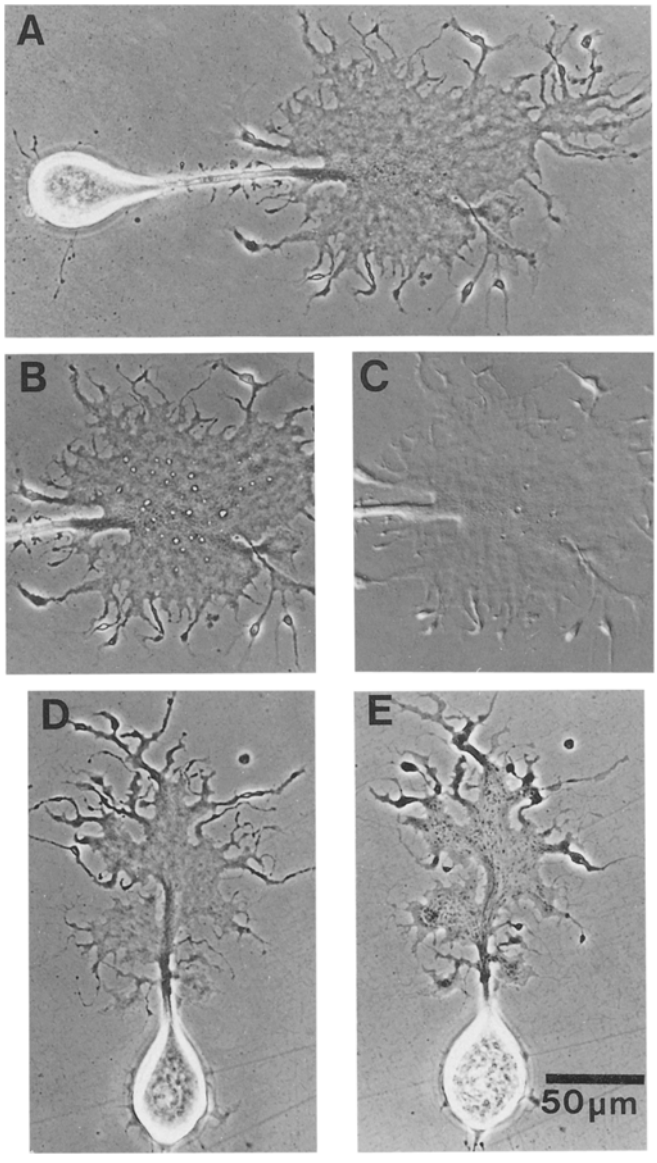

Fig. 7. A–C Immediate response to change in osmolarity. **A** Veiler in culture just prior to change; day 12. **B, C** 5 min and 20 min after a change to medium that was hypo-osmotic to the standard medium. Apparent openings in the central domain of the veil begin to appear within 1 min. There are fewer by 20 min, and appear as pits in the Hoffman-modulation contrast image. Pits disappear by 30 min. They reappear transiently on a return to standard medium. **D, E** Effect on a veiling neuron of $10 \times [K^+]_o$-nominally 0 $[Ca^{2+}]_o$. **D** Neuron before change to altered medium on day 1; **E** after 48 h in 110 mM K−Ca-deleted saline medium. Note the dark reticulated lines in the cytoplasm

Altered medium K concentration. A series of cultures were established in standard medium and were then moved on day 1 to K-enriched media (20, 25, 30, 40 and 110 mM K, standard = 11 mM). In veilers, there was an immediate (within 2–30 min) beading or rounding up of a few of the growth cones and portions of the edge of the veil. Varicosities formed on some small cells. The similarity of this response to that observed following exposure to osmotically changed conditions as just described suggests that, although these K-enriched media were nominally isosmotic, they were not isotonic, as might be expected if membrane permeability to K^+ is greater than that to Na^+. The morphological changes had disappeared in most cells by the next day. Cells were followed for up to 6 days (except in 110 mM K). Outgrowth continued in the elevated $[K]_o$ up to 30 mM but was sparse, consisting of blunt extension of preexisting growth cones or of long, thin processes. At 40 mM and 110 mM, growth was arrested, although cells appeared normal after 6 days in the case of 40 mM, and 2 days (the longest period tested) in 110 mM K. One set of cultures was changed back to standard medium after being held in 110 mM K-enriched medium for about 18 h. The cells in these cultures resumed normal outgrowth. Some of the veiling neurons exposed to a 40–60 min pulse of 110 mM K during the first 2 days appeared to withdraw material from the veil 1–2 days earlier than is usually seen. Neurons of the other morphological types appeared to be unaffected by the changes in $[K^+]_o$.

Some of the cultures were studied for effects of 110 mM K in Ca-deleted saline medium. They were examined after being in the altered medium for 18 h to 2 days. None of the cell types showed further outgrowth during exposure to this medium, but neither was there evidence of cell mortality or obvious physiological stress. Cells of branching morphology showed little change, whereas veiling cells exhibited a marked change in the appearance of the cytoplasm of thick neurites and veils under phase contrast (Fig. 7D, E). This consisted of the appearance of a phase-bright reticulum and phase-dark granulation. Thin portions of the veil between filopodial extensions were withdrawn. A number of the neurons in cultures were returned to standard medium after 1 day in the altered conditions and resumed outgrowth.

Outgrowth in media having altered Ca concentrations. We examined cultures placed in medium containing different concentrations of $[Ca^{2+}]_o$ from nominally 0 to 26 mM (2x normal), and compared outgrowth in a sample of neurons. Outgrowth of all forms occurred in all of the $[Ca]_o$ tested.

Fig. 8 shows examples of outgrowth of both the branching (Fig. 8A–F) and veiling types (Fig. 8G) from an experimental series in which the neurons were allowed to settle in the dishes in the nominally Ca-free, Mg-free dissociation medium, and then flooded with Ca-deleted saline medium (see Methods) or medium having the relevant Ca^{2+} concentration to be tested. A cell cultured in saline medium with the standard Ca^{2+} concentration (13 mM) is shown in Fig. 3B. The pattern and rate of outgrowth may be compared with that in standard medium shown in Fig. 2C (some of the differences are attributable to the longer initial neurite present in the neuron of Fig. 2C).

In order to quantitate the outgrowth in altered $[Ca^{2+}]_o$, an average of 14 cells in each dish were photographed shortly after plating (selection was made for proximity to features that assisted reidentification). The cells were then rephotographed daily. Estimates of the area covered ("perimeter area") were made by digitizing

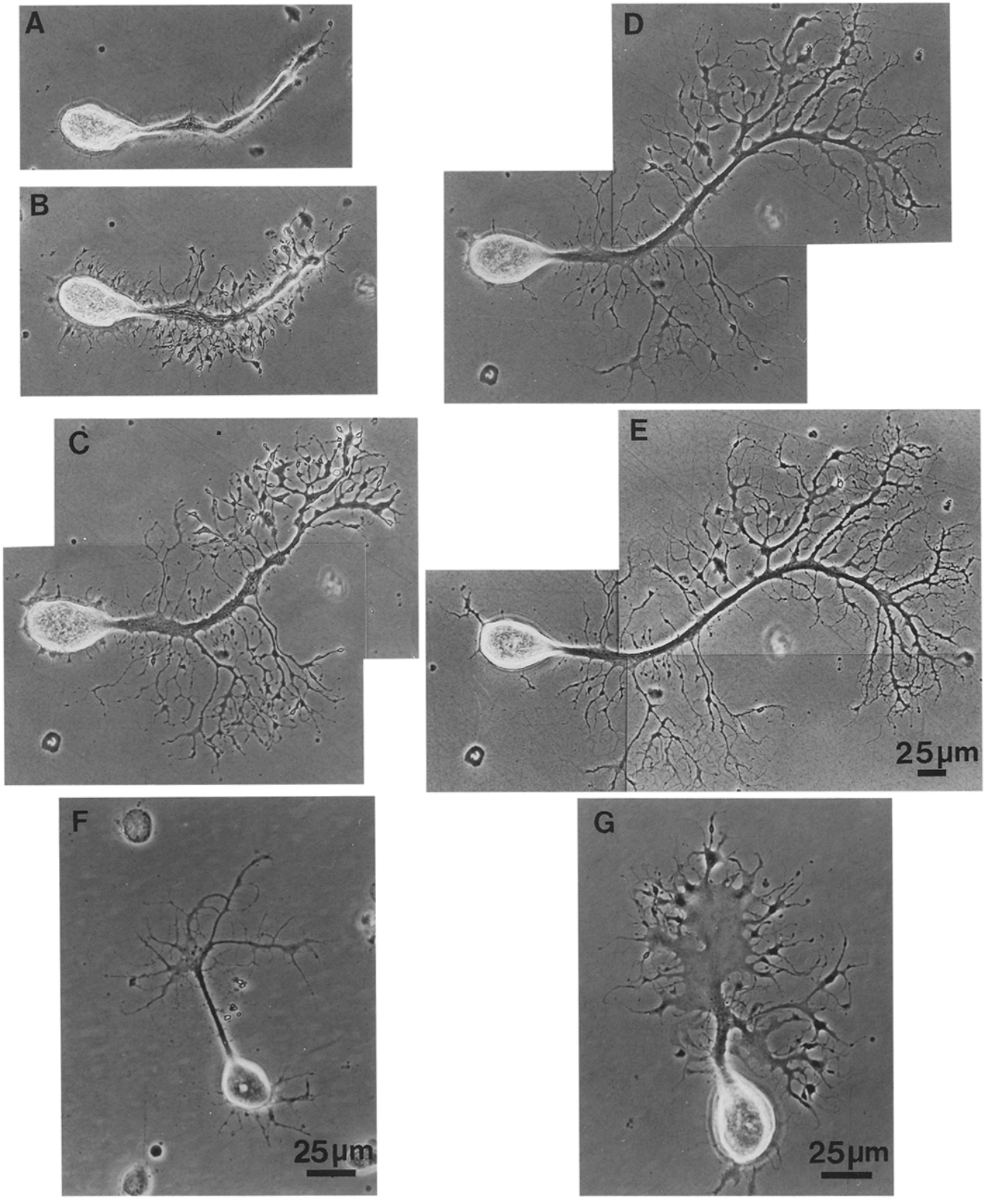

Fig. 8A–G. Outgrowth in calcium-deleted saline medium. **A–E** A large brancher shows outgrowth continuing for 7 days. **A** 30 min after plating. **B** 18 h in culture. **C** Day 2. **D** Day 4. **E** Day 7. **F** Small cell type; day 2. **G** Veiler; day 2

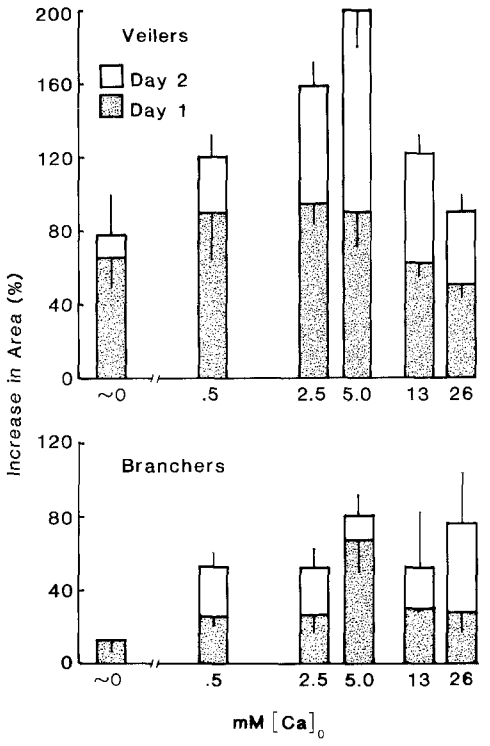

Fig. 9. Outgrowth of veilers (*top*) and branchers (*bottom*) in various $[Ca^{2+}]_o$. *Bars* at the indicated $[Ca^{2+}]_o$ (placed on a log scale) give the percent increase in cell outline area at day 1 (*dark portion*) and day 2 relative to area at plating (averages of 3–12 neurons, *lines* indicate standard error). Cultures were changed to the indicated $[Ca^{2+}]_o$ within 2 h of plating (~*0 Ca* Ca-deleted saline medium, as in Fig. 8; no observation for branchers in ~0 Ca on day 2)

the outlines of the cells from the photographs. An analysis of variance (univaried) of the perimeter areas at plating showed a normal distribution, whereas a two-way analysis of variance showed that the change in outgrowth with time in culture was highly significant ($P<$ 0.0001). However, the effect of $[Ca^{2+}]_o$ on outgrowth was not statistically significant ($P<0.08$) for either the veilers or the branchers.

Fig. 9 plots the data from this experiment normalized by calculating the percentage increase in perimeter area relative to the area of the neuron and its neurite at plating (the bars are placed on an approximately logarithmic scale for $[Ca^{2+}]_o$). The plot for veiling neurons (top) shows that there was little effect of the changed $[Ca^{2+}]_o$ on the outgrowth during the first day, most of the differences in the presence of different $[Ca^{2+}]_o$ becoming obvious during the second day in culture. The plot suggests a dose-dependence of outgrowth on $[Ca^{2+}]_o$, showing a bell-shaped curve relating the increase in area after day 2 to $[Ca^{2+}]_o$ with a maximum at 5 mM; however, the analysis of variance fails to demonstrate statistical significance ($P<0.07$). It is noteworthy that the outgrowth of veilers in the low Ca^{2+} concentration almost ceased after 24 h. It was also very limited during the second day at the highest $[Ca^{2+}]_o$.

Fig. 9, bottom, presents the normalized data for the branchers photographed in this same experimental series. The generally smaller increase in perimeter area attributable to the outgrowth of branchers relative to veilers is apparent, but there was no clear effect of the changes of $[Ca^{2+}]_o$ at days 1 or 2.

Neuronal perimeter areas were estimated for veilers in a series of cultures exposed immediately after plating to the same series of $[Ca^{2+}]_o$ in the same manner as those just discussed, but cultured at 26° C, rather than the standard 22° C (data not shown). Although differences between the groups held in different $[Ca^{2+}]_o$ were not statistically significant, the suggestion of an optimum for outgrowth at 5 mM $[Ca^{2+}]_o$ was again seen. Outgrowth on the second day in Ca-deleted saline medium was minimal. At the highest $[Ca^{2+}]_o$, withdrawal was observable after the second day.

In another series of experiments, neurons were held under standard conditions for the first 18 h and medium then changed to one of the series of $[Ca^{2+}]_o$ to be tested. The lowest $[Ca]_o$ tested in this experimental series was obtained in an EGTA-buffered medium that gave a Ca^{2+} concentration calculated to be 0.1–0.3 μM. An analysis of variance revealed no significant differences in the normalized outgrowth during the 24 h after changing the cultures to one of the series of different $[Ca^{2+}]_o$ (data not shown). It is noteworthy that outgrowth was not depressed in the EGTA-Ca medium.

In additional experiments, neurons were tested for their tolerance of still lower levels of $[Ca^{2+}]_o$ by placing them in Ca-deleted saline medium buffered with 0.5 mM EGTA. Neurons, whether freshly plated or showing outgrowth after one or more days in culture, disintegrated within 30 min when exposed to this medium.

In summary, these peptidergic neurons survive and grow in their recognizable forms under a wide range of $[Ca^{2+}]_o$.

Discussion

In this report, we have shown that regenerative outgrowth of mature crustacean peptidergic neurons having several distinct morphologies occurs in unconditioned dishes and simple defined media. We have studied the effects of changes in defined culturing conditions, including substantial changes of $[K^+]_o$ and $[Ca^{2+}]_o$, on the outgrowth and morphology of this heterogeneous group of neurosecretory neurons. The somewhat arbitrarily chosen "standard" conditions prove to be those under which the most distinct differences in morphology are observed. The most remarkable finding of this study is that all of the major morphological types of neurons show great robustness in their survival and ability to continue to regenerate, and that each type remains clearly distinguishable under widely varied conditions.

Outgrowth from the isolated crab X-organ neurons in cultures is immediate and extensive. This growth of neurons in culture when plated at low density in the absence of conditioning or regulatory factors appears to be exceptional. It may be significant that another exceptional system is also peptidergic, viz., the bag cells of *Aplysia* (Forscher et al. 1987). The veiling neurons in the X-organ cultures, the neuron types reliably show-

ing immunoreactivity for CHH, often double their membrane area during the first day and triple it within 2 days, under standard conditions. Outgrowth usually ceases within a week in medium having a full complement of amino acids, vitamins, and trace elements. Growth occurs even in crab saline, but ceases within 2 days; if glucose and L-glutamine are added to the saline, growth continues for 5 days. We have recently found a way of conditioning the medium that results in not just lengthened survival but in outgrowth that continues for several weeks (S. Grau and I. Cooke, unpublished). The extremely small amount of material needed suggests that the halt in outgrowth in defined medium is not the result of a lack of necessary nutrients, but represents the attenuation of a regulatory signal received before or at the time the neurons were dissociated.

Of the three common neuronal types in the defined X-organ cultures, the lamellipodia of veiling neurons display the most obvious responses to the altered conditions. Electron micrographs show the cytoplasm to be packed with neurosecretory granules with interspersed microtubules (see Fig. 1 F in Cooke et al. 1989). The immediate appearance and later disappearance of pits in veils on exposure to osmotic changes, and the formation of terminal blebs in crowded cultures or on altered substrates suggest a minimal or dynamic cytoskeletal structure.

Although controversy exists regarding the role of elevated $[Ca^{2+}]_i$ at the growth cone (discussed below), it appears well accepted that neurite stabilization requires low $[Ca^{2+}]_i$. We suggest that differences in the outgrowth morphology between the branching and veiling neurons, and the greater sensitivity of the veilers to altered ionic conditions, may have some relationship to the presence of prominent Ca currents in veiling neurons (Meyers et al. 1992), and particularly their growth cones (Meyers and Cooke 1991). In contrast, Ca currents are undetectable in the somata of small branching neurons and small cells (Meyers et al. 1992). The paucity of Ca-channels in branchers would limit the fluctuation of $[Ca^{2+}]_i$ under altered culturing conditions.

The effect of culturing veilers at the higher temperature is the appearance of a more organized structure within the veil, accompanied by more directed outgrowth. Because transport along microtubules involves a calmodulin-activated Ca-Mg-dependent ATPase (Ochs et al. 1986), the increased temperature would be expected to result in accelerated transport of Ca-sequestering materials (Aletta and Greene 1988; Dailey and Bridgman 1989) and microtubule-associated porteins that aid in stabilizing neurite structure (Mateus 1988). Temperature will also have direct effects on the polymerization dynamics of cytoskeletal elements, such as tubulin and actin (Lankford and Letourneau 1989).

It is noteworthy that the more directed outgrowth of veilers leads to the formation of appositions only at the higher temperature, and that these appositions have been observed only between the CHH-containing type of neuron. A number of examples of the formation of specific synapses between neurons in culture are now available (O'Lague et al. 1974; Dagan and Levitan 1981; Kleinfeld et al. 1990; Nicholls et al. 1990; Syed et al. 1990).

The relative lack of a sustained effect of increased $[K^+]_o$ on our cultures must be evaluated in the light of the variety of effects that have been reported in cultures following the elevation of $[K^+]_o$. These include increased survival and outgrowth of neurons (Nishi and Berg 1981; Gallo et al. 1987; Hockberger et al. 1987), the inhibition of neurite formation (Kostenko et al. 1982; Sussdorf and Campenot 1986), or withdrawal (Campenot 1986). In our cultures, as long a period as 2 days in 110 mM (10x-normal) $[K^+]_o$ produced little effect; although outgrowth ceased, the cells survived, and some were able to resume outgrowth following their return to standard medium.

We have examined the effects of altered regimes of $[Ca^{2+}]_o$ because there are interesting but conflicting observations regarding the role of Ca^{2+} in determining the outgrowth and morphology of neurons in culture. Many studies have provided evidence for the crucial role of $[Ca^{2+}]_i$ in governing growth cone elongation, stasis or retraction, motility of filopodia and the conversion of the growth cone to a stable neurite (e.g., Kostenko et al. 1983; Connor 1986; Cohan et al. 1987; Mattson et al. 1988; Goldberg 1988; Lankford and Letourneau 1989; Silver et al. 1989). The proposal that growth cone behavior is regulated by changes of $[Ca^{2+}]_i$ from an optimal 'set point' (cell-type specific differences in Ca homeostasis being present), with $[Ca^{2+}]_i$ at advancing growth cones generally being higher than in the neurite (for reviews, see Mills and Kater 1990; Kater and Mills 1991), cannot be too widely generalized. Whereas Ca^{2+} entry has been observed in processes of some types of neurons in culture (e.g., Grinwald and Farber 1981; Anglister et al. 1982; Connor et al. 1987; Lipscombe et al. 1988), examples are also available in which outgrowth occurs (1) in the apparent absence of an obvious Ca current recordable at the growth cone (Balardetti et al. 1986); (2) in the absence of $[Ca]_o$ except at the soma (Campenot and Dracker 1989); (3) with $[Ca^{2+}]_i$ at the growth cone being no higher than that in the neurite (Ross et al. 1987, 1988; Silver et al. 1990); and (4) in the absence of any fluctuations of $[Ca^{2+}]_i$ (Tolkovsky et al. 1990). A change from outgrowth to arrest has also been reported as occurring with no change of $[Ca^{2+}]_i$ (Ivins et al. 1991). The relationship between $[Ca^{2+}]_i$ and growth cone behavior may therefore differ depending on the preparation studied.

The suggestively bell-shaped relationship between membrane addition and $[Ca^{2+}]_i$ that is observed for veilers is consistent with suggestions (Mills and Kater 1990) that outgrowth requires an optimum level of $[Ca^{2+}]_o$. The appearance of such a relationship was only seen when the neurons were cultured in altered $[Ca^{2+}]_o$ during the first day. The level of $[Ca^{2+}]_o$ affected branchers little, if at all. The modest effects of changes of $[Ca]_o$ may reflect a limited effect of these changes on $[Ca^{2+}]_i$. There is increasing evidence from Ca-indicator studies for a surprising resistance of $[Ca^{2+}]_i$ to sustained perturbation (for a review, see Perry and McNaughton 1991).

We have previously suggested (Cooke et al. 1989) that the immediate outgrowth from peptidergic neurons results from exocytotic addition of pre-existing neurosecretory granule membrane. Exocytosis has also been proposed as the means of membrane addition to veils in molluscan neurons (Goldberg 1988). As would be predicted by this hypothesis, addition of cadmium to the culture medium halts outgrowth (Cooke et al. 1989; Suarez-Isla et al. 1984). Cadmium has been shown to block K-evoked secretion from X-organ-sinus gland systems (Stuenkel 1985) and to inhibit Ca current in cultured veiling neurons (Meyers et al. 1992). Thus, we were at first surprised to find that veiling neurons could produce robust veils in Ca-deleted saline medium or medium buffered to a Ca^{2+} concentration of 0.3 μM with EGTA. However, in considering growth, the rate of spontaneous exocytosis, analogous to spontaneous miniature end-plate potentials (minepps, Fatt and Katz 1952), is possibly of more relevance than Ca-dependent evoked secretion. The rate of minepps (approximately 1/s) is insensitive to $[Ca^{2+}]_o$, even when buffered to less than 10^{-8} M with EGTA (Miledi and Thies 1971). Spontaneous release of acetylcholine from growth cones has been detected (Hume et al. 1991; Young and Poo 1991). Exocytosis of one 120 nm-diameter granule per s would provide over 7000 μm^2 of additional membrane in 48 h. This is more than is required to account for the observed outgrowth of veilers, assuming that little reuptake of membrane occurs. Thus, continued growth in low $[Ca^{2+}]_o$ is not inconsistent with our original hypothesis. Further, there is evidence that spontaneous secretion of CHH, the hormone associated with the veiling neurons, is not blocked in Ca-deleted saline (Stuenkel 1985). A more definitive test of our proposal would be to use an appropriate marker of the granule membrane and to seek evidence for its appearance at the growth cone during active outgrowth.

The importance of the work reported here, in addition to establishing that mature crustacean neurons can be routinely cultured in a simple defined medium, is that it provides a stable baseline of morphological observations on several distinct types of neurons, and that it shows that the different patterns of outgrowth are not highly dependent on the culturing conditions. This permits the exploration of other characteristics of the neurons that are correlated with their morphology, such as their electrophysiological characteristics (Meyers et al. 1992), with some confidence that artifacts of culturing are minimal. We hope to use this system to explore further the effects of experimental manipulations that may reveal mechanisms governing neuronal outgrowth and morphology.

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References

Aletta J, Greene LA (1988) Growth cone configuration and advance: a time-lapse study using video-enhanced differential interference contrast microscopy. J Neurosci 8:1425–1535

Anglister L, Farber IC, Shahar A, Grinvald A (1982) Localization of voltage-sensitive calcium channels along developing neurites: their possible role in regulating neurite elongation. Dev Biol 94:351–365

Belardetti F, Schacher S, Siegelbaum SA (1986) Action potentials, macroscopic and single channel currents recorded from growth cones of *Aplysia* neurones in culture. J Physiol (Lond) 374:289–313

Campenot RB (1986) Retraction and degeneration of sympathetic neurites in response to locally elevated potassium. Brain Res 399:357–363

Campenot RB, Dracker DD (1989) Growth of sympathetic nerve fibers in culture does not require extracellular calcium. Neuron 3:733–743

Cohan CS, Connor JA, Kater SB (1987) Electrically and chemically mediated increases in intracellular calcium in neuronal growth cones. J Neurosci 7:3588–3599

Cole WH (1940) The composition of fluids and sera of some marine animals and of the sea water in which they live. J Gen Physiol 23:575–584

Connor JA (1986) Digital imaging of free calcium changes and spatial gradients in growing processes of single mammalian central nervous system cells. Proc Natl Acad Sci USA 83:6179–6183

Connor JA, Tseng H-Y, Hockberger PE (1987) Depolarization- and transmitter-induced changes in intracellular Ca^{2+} of rat cerebellar granule cells in explant cultures. J Neurosci 7:1384–1400

Cooke IM, Sullivan RE (1982) Hormones and neurosecretion. In: Bliss DE (ed) The biology of Crustacea, vol 3. Academic Press, New York, pp 205–290

Cooke IM, Graf RA, Grau S, Haylett B, Meyers D, Ruben P (1989) Curstacean peptidergic neurons in culture show immediate outgrowth in simple medium. Proc Natl Acad Sci USA 86:402–406

Dagan D, Levitan IB (1981) Isolated identified *Aplysia* neurons in cell culture. J Neurosci 1:736–740

Dailey M, Bridgman P (1989) Dynamics of the endoplasmic reticulum and other membranous organelles in growth cones of cultured neurons. J Neurosci 9:1897–1909

Dircksen H, Webster SG, Keller R (1988) Immunocytochemical demonstration of the neurosecretory systems containing putative moult-inhibiting hormone and hyperglycemic hormone in the eyestalk of brachyuran crustaceans. Cell Tissue Res 251:3–12

Fatt P, Katz B (1952) Spontaneous subthreshold activity at motor nerve endings. J Physiol (Lond) 117:109–128

Forscher P, Kaczmarek LK, Buchanan J, Smith SJ (1987) Cyclic AMP induces changes in distribution and transport of organelles within growth cones of *Aplysia* bag cell neurons. J Neurosci 7:3600–3611

Gallo V, Kingsbury A, Balázs R, Jørgensen O (1987) The role of depolarization in the survival and differentiation of cerebellar granule cells in culture. J Neurosci 7:2203–2213

Goldberg D (1988) Local role of Ca^{2+} in formation of veils in growth cones. J Neurosci 8:2596–2605

Graf RA, Cooke IM (1990) Primary culture of crustacean stomatogastric ganglion neurones in a defined medium. J Exp Biol 149:521–525

Grinvald A, Farber IC (1981) Optical recording of calcium action potentials from growth cones of cultured neurons with a laser microbeam. Science 212:1164–1167

Hockberger PE, Tseng H-Y, Connor JA (1987) Immunocytochemical and electrophysiological differentiation of rat cerebellar granule cells in explant cultures. J Neurosci 7:1370–1383

Hume RI, Role LW, Fischbach GD (1991) Acetylcholine release from growth cones detected with patches of acetylcholine receptor-rich membranes. Nature 305:632–634

Ivins JK, Raper JA, Pittman RN (1991) Intracellular calcium levels do not change during contact-mediated collapse of chick DRG growth cone structure. J Neurosci 11:1597–1608

Kater SB, Mills LR (1991) Regulation of growth cone behavior by calcium. J Neurosci 11:891–899

Kleinfeld D, Raccuia-Behling F, Chiel HJ (1990) Circuits constructed from identified *Aplysia* neurons exhibit multiple patterns of persistent activity. Biophys J 57:697–715

Kostenko M, Tretjak N, Musienko V (1982) The effect of elevated potassium on the adult mollusc giant neurone survival and neurite formation in culture. Brain Res 236:183–192

Kostenko M, Musienko V, Smolikhina T (1983) Ca^{2+} and pH affect neurite formation in cultured mollusc isolated neurons. Brain Res 276:43–50

Krenz WD, Principe F del, Fischer P (1990) Crustacean nerve cells in primary culture. In: Frontiers in crustacean neurobiology. Advances in life sciences. Birkhäuser, Basel, pp 509–515

Lankford KL, Letourneau PC (1989) Evidence that calcium may control neurite outgrowth by regulating the stability of actin filaments. J Cell Biol 109:1229–1243

Lipscombe D, Madison DV, Poenie M, Reuter H, Tsien RY, Tsien RW (1988) Spatial distribution of calcium channels and cytosolic calcium transients in growth cones and cell bodies of sympathetic neurons. Proc Natl Acad Sci USA 85:2398–2402

Mangerich S, Keller R, Dircksen H (1986) Immunocytochemical identification of structures containing putative red pigment-concentrating hormone in two species of decapod crustaceans. Cell Tissue Res 245:377–386

Mateus A (1988) Microtubule-associated proteins: their potential role in determining neuronal morphology. Annu Rev Neurosci 11:29–44

Mattson MP, Taylor-Hunter A, Kater SB (1988) Neurite outgrowth in individual neurons of a neuronal population is differentially regulated by calcium and cyclic AMP. J Neurosci 8:1704–1711

Meyers DER, Cooke IM (1991) Regional distribution of ion channels in a cultured crustacean peptidergic neuron. Neurosci Abstracts 17:1335

Meyers DER, Graf RA, Cooke IM (1992) Ionic currents of morphologically distinct peptidergic neurons in defined culture. J Neurophysiol 67:1301–1315

Miledi R, Thies R (1971) Tetanic and post-tetanic rise in frequency of miniature end-plate potentials in low-calcium solutions. J Physiol (Lond) 212:245–257

Mills LR, Kater SB (1990) Neuron-specific and state-specific differences in calcium homeostasis regulate the generation and degeneration of neuronal architecture. Neuron 4:149–163

Newcomb RW (1987) Amino acid sequences of neuropeptides in the sinus gland of the land crab *Cardisoma carnifex:* a novel neuropeptide proteolysis site. J Neurochem 49:574–583

Nicholls JG, Liu Y, Payton BW, Kuffler DP (1990) The specificity of synapse formation by identified leech neurones in cultures. J Exp Biol 153:141–154

Nishi R, Berg D (1981) Effects of high K^+ concentrations on the growth and development of ciliary ganglion neurons in cell culture. Dev Biol 87:301–307

Ochs S, Gaziri J, Jersild R Jr (1986) Metabolic and ionic properties of axoplasmic transport in relation to its mechanism. In: Iqbal Z (ed) Axoplasmic transport, CRC, Boca Raton, Florida, pp 57–68

O'Lague PH, Obata K, Claude P, Furshpan EJ, Potter DD (1974) Evidence for cholinergic synapses between dissociated rat sympathetic neurons in cell culture. Proc Nat Acad Sci USA 71:3602–3606

Perry RJ, McNaughton PA (1991) Calcium regulation in neurons: transport processes. Curr Opinion Neurobiol 1:98–104

Postek MT, Howard KS, Johnson AH, McMichael KL (1980) Scanning electron microscopy – a student's handbook. Ladd Research Industries Inc, Burlington, Vermont

Ross WN, Aréchiga H, Nicholls JG (1987) Optical recording of calcium and voltage transients following impulses in cell bodies and processes of identified leech neurons in culture. J Neurosci 7:3877–3887

Ross WN, Aréchiga H, Nicholls JG (1988) Influence of substrate on the distribution of calcium channels in identified leech neurons in culture. Proc Natl Acad Sci USA 85:4075–4078

Schooneveld H, Herp F van, Minnen J van (1987) Demonstration of substances immunologically related to the identified arthropod neuropeptides AKH/RPCH in the CNS of several invertebrate species. Brain Res 406:224–232

Silver RA, Lamb AG, Bolsover SR (1989) Elevated cytosolic calcium in the growth cone inhibits neurite elongation in neuroblastoma cells: correlation of behavioral states with cytosolic calcium concentration. J Neurosci 9:4007–4020

Silver RA, Lamb AG, Bolsover SR (1990) Calcium hotspots caused by L-channel clustering promote morphological changes in neuronal growth cones. Nature 343:751–754

Stuenkel EL (1985) Simultaneous monitoring of electrical and secretory activity in peptidergic neurosecretory terminals of the crab. J Physiol (Lond) 359:163–187

Stuenkel EL, Cooke IM (1988) Electrophysiological characteristics of peptidergic nerve terminals correlated with secretion. Curr Top Neuroendocrinol 9:123–150

Suarez-Isla BA, Pelto DJ, Thompson JM, Rapoport SI (1984) Blockers of calcium permeability inhibit neurite extension and formation of neuromuscular synapses in cell culture. Dev Brain Res 14:263–270

Sussdorf WS, Campenot RB (1986) Influence of the extracellular potassium environment on neurite growth in sensory neurons, spinal cord neurons and sympathetic neurons. Dev Brain Res 25:43–52

Syed NI, Bulloch AGM, Lukowiak K (1990) In vitro reconstruction of the respiratory central pattern generator of the mollusk *Lymnaea*. Science 250:282–285

Thomas WE, Jordan FL, Townsel JG (1987) The status of the study of invertebrate neurons in tissue culture-phylum Arthopoda. Comp Biochem Physiol 87A:215–222

Tolkovsky AM, Walker AE, Murrell RD, Suidan HS (1990) Ca^{2+} transients are not required as signals for long-term neurite outgrowth from cultured sympathetic neurons. J Cell Biol 110:1295–1306

Young SH, Poo M (1991) Spontaneous release of transmitter from growth cones of embryonic neurones. Nature 305:634–637