

COMPARISON OF Ca^{2+} CURRENTS OF PEPTIDERGIC NEURONS DEVELOPING DIFFERING MORPHOLOGY WITH TIME IN CULTURE

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Accepted 27 November 1996

Summary

The whole-cell patch-clamp technique was used to examine Ca^{2+} currents (I_{Ca}) in mature neurons cultured in defined medium and derived from the principal neurosecretory system of decapod crustaceans, the X-organ–sinus gland. After 1 day in culture, X-organ neurons of the crab *Cardisoma carnifex* showed vigorous outgrowth characterized either by the production of broad lamellipodia (veils) or, from smaller somata, a branching morphology. The neurons developing veils (veilers) had a large I_{Ca} (approximately 650 pA) and I_{Ca} current density (approximately $5 \mu\text{A cm}^{-2}$) while other types of neuron had little or no I_{Ca} . This distinction between the two types was still present after 5–6 days in culture. However, morphologies observed after additional outgrowth, when correlated with the I_{Ca} responses, allowed four groups to be distinguished: (1) veilers and (2) branching veilers, which developed from veilers and had a similar I_{Ca} density (approximately $3 \mu\text{A cm}^{-2}$); and, developing from the 1 day branchers, (3) spiny branchers or (4) small cells (I_{Ca} density

approximately $0.8 \mu\text{A cm}^{-2}$). Immunoreactivity indicative of the presence of crustacean hyperglycemic hormone was found in all veilers and branching veilers tested, while molt-inhibiting hormone reactivity, when observed, was seen in cells having a robust I_{Ca} density ($\geq 1.2 \mu\text{A cm}^{-2}$). Normalized average current–voltage curves for each morphological group were examined for changes with increasing time in culture. The curves were consistent with the I_{Ca} being produced by a population of high-voltage-activated Ca^{2+} channels whose properties are biophysically indistinguishable and unaffected by time in culture. The averaged peak current did not change, despite an increase in neuronal surface area as outgrowth proceeded, and this resulted in a reduction of I_{Ca} density. This indicated that net addition of Ca^{2+} channels did not match the addition of new membrane under our culturing conditions.

Key words: neurosecretion, neurohormones, Ca^{2+} channel, patch-clamp, immunoblot, regeneration, crab, *Cardisoma carnifex*.

Introduction

Isolating neurons in primary culture allows their individual membrane ionic currents to be studied under experimentally well-defined conditions. However, the generalization of observations to the undisturbed, *in situ* condition requires knowledge of whether and how membrane currents are affected by increasing time in culture. The cultured cells of the mature crab peptide–neurosecretory system, known as the X-organ–sinus gland, are particularly suited for examining the characteristics of voltage-dependent ionic currents in relation to time in culture because: (1) when these monopolar neurons are isolated with an axonal stub, they show immediate, vigorous outgrowth in a simple medium *without the addition of any conditioning or growth factors*, and (2) distinctive forms of regenerative outgrowth are produced by different neuronal types (Cooke *et al.* 1989; Grau and Cooke, 1992). One type (designated veilers) are large cells ($>30 \mu\text{m}$ in diameter) initially extending a broad lamellipodium or veil from the axonal stub. This type contains crustacean hyperglycemic

hormone (CHH, Keller *et al.* 1995). Other types extend smaller growth cones and branch extensively; they include cells smaller than $30 \mu\text{m}$ in diameter.

Previous patch-clamping studies of these peptidergic neurons have been limited to observations made during the first 2 days in culture. Different outgrowth morphologies were associated with distinct differences in the density of voltage-operated Na^{+} and Ca^{2+} channels (Meyers *et al.* 1992). The veilers showed regenerative responses, usually overshooting action potentials, under current-clamp conditions (Meyers, 1993) and net inward currents under voltage-clamp conditions (Meyers *et al.* 1992). In contrast, cells with other morphologies rarely had a detectable net inward current. Using conditions appropriate for the isolation of ionic currents, the veilers were found to have significant inward currents attributable to Na^{+} and Ca^{2+} , as well as outward currents corresponding to the delayed rectifier K^{+} current (I_{K}) and early outward K^{+} current (I_{A}). Inward currents were difficult to detect in branching neurons, while the current densities of

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their outward currents were similar to those of the veilers. The observations on I_{Ca} thus far are consistent with the hypothesis that the Ca^{2+} current is controlled by a single type of high-voltage-activated Ca^{2+} channel that has proved resistant to generally available toxins and drugs (Richmond *et al.* 1995). The properties of the I_{Ca} studied in peptide secretory terminals of X-organ cells isolated from the sinus gland are indistinguishable from those of the neuronal somata (Richmond *et al.* 1996).

Consistent with observations from a broad range of preparations, a critical role for voltage-operated Ca^{2+} channels in the control of secretion has been demonstrated for the X-organ-sinus gland system (Stuenkel and Cooke, 1988), including the control of CHH secretion (Keller *et al.* 1994). In this paper, we examine the biophysical characteristics of Ca^{2+} currents (I_{Ca}) in neurons dissociated from the crab X-organ that have developed distinctive morphologies after 5–6 days in culture. Our objectives were (1) to determine whether additional Ca^{2+} channels are incorporated into the membrane as it increases in area during outgrowth, as indicated by an unchanged or increased current density; (2) to show whether, with outgrowth, any changes occur in the biophysical characteristics of I_{Ca} which would be indicative of changes in channel properties, and (3) to determine whether additional Ca^{2+} channel types, or alterations in the proportions of a mixture of channel types, occur. An abstract describing some of these observations has been published (Meyers and Cooke, 1992).

Materials and methods

Culture techniques

The procedures used to dissociate and culture X-organ neurons from the semi-terrestrial tropical crab *Cardisoma carnifex* Herbst have been described in detail elsewhere (Cooke *et al.* 1989; Meyers *et al.* 1992). Briefly, the X-organ with less than 1 mm of the axon tract was removed from the eyestalk of adult male crabs and agitated in the dark for 1.5 h in a Ca^{2+}/Mg^{2+} -free saline containing 0.1% trypsin (Gibco). A large volume of Ca^{2+}/Mg^{2+} -free saline was then added to retard enzymatic activity, and the cells were dissociated by gentle trituration in a 60 μ l drop of culture medium on 35 mm Primaria dishes (Falcon 3801). The dishes were carefully flooded after allowing 1–2 h for the cells to adhere to the substratum. The culture medium consisted of Leibowitz L-15 (Gibco) diluted 1:1 with double-strength crab saline to which D-glucose (120 mmol l⁻¹, Fisher), L-glutamine (2 mmol l⁻¹, Sigma) and gentamicin (50 mg ml⁻¹, Gibco) were added. Cultures were maintained in humidified incubators (Billups-Rothenberg) in the dark at 22–24 °C.

Electrophysiological techniques

Voltage-clamp recordings were made from isolated somata using tight-seal whole-cell recording techniques. Whole-cell voltage-clamp recordings were made with an Axopatch-1C patch-clamp amplifier (Axon Instruments) equipped with a CV-4 headstage. Voltage-clamp data were filtered at –3 dB at 1 kHz and stored on the fixed disk of an 80286-based personal

computer using commercial data acquisition hardware (TL-1, Axon Instruments) and software (pCLAMP, Axon Instruments). Off-line analysis was performed using Clampan, the analysis package provided with pCLAMP.

Estimates of membrane capacitance were made from the average of six transients generated by 20 mV hyperpolarizing commands from a holding potential (V_h) of –40 mV. After subtraction of the baseline offset (i.e. the difference between zero current and holding current), the averaged transient was integrated over the full length of the command hyperpolarization (6–20 ms). An estimate of the resistive component of the transient was then subtracted from the integral to yield an estimate of membrane capacitance. The resistive component was estimated by averaging the last five data points of the current response (i.e. the average steady-state value of the commanded current) and multiplying this by the integration period.

Pipettes were made using 1.5 mm o.d. thin-walled capillary tubing (World Precision Instruments, TW150F-4 or PG61150-4, or Drummond Scientific, 1-00-7500) and a vertical pipette puller (David Kopf Instruments, model 700C). Pipettes were coated with Sylgard (Dow Corning, 184 elastomer kit) and fire-polished (Narishige, model MF-83) just before use. The intracellular and extracellular solutions used for the various experiments were as follows (in mmol l⁻¹; chemicals from Fisher, F, Sigma, S, or Baker, B). For measuring net current: intracellular, 150 KCl (B), 150 potassium gluconate (S), 10 NaCl (F), 5 Mg-ATP (S), 5 EGTA (S) and 50 Hepes (F), pH adjusted to 7.4 with KOH (B); extracellular, 440 NaCl (F), 11 KCl, 24 MgCl₂ (S), 13.3 CaCl₂ (F), 26 Na₂SO₄ (F), 10 Hepes and 120 D-glucose, pH adjusted to 7.4 with NaOH (B). For measuring Ca^{2+} current: intracellular, 300 N-methyl-D-glucamine (NMG)-methane sulphonate (MeSO₃) or NMG-Cl (S), 10 NaCl, 5 Mg-ATP, 5 cesium 1,2-bis(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid (BAPTA, Molecular Probes), 50 Hepes and 10 tetraethylammonium (TEA) bromide (S), pH adjusted to 7.4 with CsOH (Alfa); extracellular, 440 NaCl (sometimes partially or completely replaced with NMG-MeSO₃), 24 MgCl₂, 13.3 CaCl₂, 10 Hepes, 20 TEA-Cl (Aldrich), 3 4-aminopyridine (4-AP, S), 120 D-glucose and 0.5–1.0 μ mol l⁻¹ tetrodotoxin (TTX, Calbiochem), pH adjusted to 7.4 with NaOH or CsOH. The osmolarity was adjusted with sucrose (F) to 1095–1100 mosmol l⁻¹ for intracellular solutions and 1100 mosmol l⁻¹ for extracellular solutions. All solutions were prepared on the day of the experiment. Solutions containing TTX and TEA-Br were prepared from concentrated aqueous stocks, whereas the remaining solutions were prepared from the crystalline compound. The resistance of cesium- and NMG-Cl-containing pipettes was 1–2.5 M Ω , NMG-MeSO₃-containing pipettes had resistances in the range 2–5 M Ω and K⁺-containing pipettes had resistances of \leq 1 M Ω .

Capacity compensation was adjusted to the maximum that could be achieved while maintaining a smooth decay for any remaining transient. This provided the best conditions for effective transient subtraction, which was performed on-line using the P/N protocol provided by pCLAMP or off-line by

subtracting a suitably scaled average of six hyperpolarizing responses to a 20 or 30 mV command. Capacity transients were rarely fully compensated. Series-resistance compensation ($\leq 60\%$) was always employed for the recording of inward currents. Recordings made with K^+ -containing pipettes benefited less from compensation, presumably because of the low resistance of the pipettes. For currents of 2.5 nA or below evoked by depolarizations from -40 mV, series-resistance compensation of 60% or less had little effect on the peak current.

Tests for statistical significance were performed on averaged observations ($N \geq 6$) using Welsh's unpaired two-tailed *t*-test, which does not assume equal variance of the groups compared. Comparison of normalized $I_{Ca}(V)$ curves, averaged for cells of similar morphology and age, utilized repeated-measures analysis of variance (ANOVA) over the eight voltage commands used. It examined whether there was a significant difference between the combined means of two cell types and also whether there were significant differences between cell types at any of the command voltages.

Immunoblots

Thirty-seven neurons from which patch-clamp recordings had been obtained were tested for immunoreactivity with either anti-CHH antiserum or anti-MIH antiserum on nitrocellulose membranes ('dot-blotting'). These antisera, made in rabbits against purified peptides from *Carcinus maenas*, were kindly supplied by R. Keller and are described in Dirksen *et al.* (1988). Their reactivity with the *Cardisoma* hormones is discussed in Keller *et al.* (1994, 1995). After recording from a neuron, its contents were sucked into an unfilled microelectrode and stored frozen until the dot-blot was to be made. Nitrocellulose transfer and immobilization membrane, 0.45 μ m pore size (Schleicher & Schuell, Keene, NH), was wetted in distilled water and dried. Samples, usually 2–8 μ l,

were applied so as to minimize spreading (approximately 2 μ l per application); a similar volume of saline from the culture dish was also applied as a control. Positive controls were included on each filter and consisted of 2 μ l applications of sinus gland extract (one sinus gland, approximately 2 mg wet mass, homogenized in 200 μ l of distilled water) at dilutions of 1:1, 1:10 and 1:100. For CHH, 1, 10 and 25 ng of the purified *Carcinus maenas* peptide were applied as standards. Negative controls included application of 2 μ l each of the blocking solution, the Tween-Tris-buffered saline (TTBS, 154 mmol⁻¹ NaCl, 100 mmol⁻¹ Tris, pH 7.5, 1 ml⁻¹ Tween-20), distilled water and saline from the culture dish. Reagents and procedures for immunostaining were those from the Vectastain Elite ABC Kit for rabbit IgG (Vector Laboratories, Inc., Burlingame, CA), which uses a preformed avidin and biotinylated horseradish peroxidase complex (the 'ABC' reagent). Non-specific binding to the blotted membrane was blocked by a 20 min application of goat serum in TTBS, and the membrane was then incubated overnight with the primary antiserum diluted in TTBS (anti-CHH, 1:5000; anti-MIH, 1:1000) on a rocker at room temperature. The ABC reagent was then applied, and the precipitate was visualized with a diaminobenzidine-peroxide-nickel reaction.

Results

General comparison between cells after 1 day and 5–6 days in culture

The observations reported here were made on 75 neurons dissociated from the X-organ neurosecretory cell group and held in defined culture for 5–6 days. An additional 14 neurons were cultured together with the 5–6 day neurons but were examined after 1 day in culture. These two groups were compared with our previous observations on similarly cultured neurons held for 1–2 days in culture (Meyers *et al.* 1992;

Table 1. Activity of neurons isolated from the X-organ of *Cardisoma carnifex* under current-clamp conditions and net current under voltage-clamp conditions

	Action potentials		Oscillations		N	Net inward current	N	Reference
	Spontaneous	Evoked	Spontaneous	Evoked				
Veilers								
1–2 days	4*	6	2	0	9	4	4	1
Somata†	1	4	0	3	7	ND		2
Lamellipodia‡	1	10	0	0	12	ND		2
5–6 days	0	4	0	0	5	6	6	3
Others								
1–2 days	0	0	6	0	9	1	5	1
5–6 days	0	1	0	0	4	3	9	3

*Two of these exhibited burst firing.

†Somata recorded immediately after severing the principal neurite.

‡Lamellipodia recorded after separation from somata

References: 1, Meyers *et al.* (1992); 2, Meyers (1993); 3, this study.

ND, not determined.

Meyers, 1993). Membrane potential responses of cells cultured for 1–2 days and 5–6 days under current-clamp conditions and net currents in physiological salines under voltage (patch)-clamp conditions are briefly considered before describing the characteristics of the pharmacologically and ionically isolated I_{Ca} under patch-clamp conditions.

Current clamp

Previous reports of spontaneous and evoked activity under current-clamp conditions on X-organ cells that had been in

culture for 1–2 days are compared in Table 1 with observations from this study on cells maintained in culture for 5–6 days. It will be seen that spontaneous action potentials and impulses in response to depolarizing current injection were observed in most of the veiling neurons of both age groups. One of four branching neurons maintained for 5–6 days in culture responded to depolarizing current injection with an overshooting action potential; the others showed responses to depolarizing current having an active component, but were not able to produce regenerative potentials, even as a rebound from

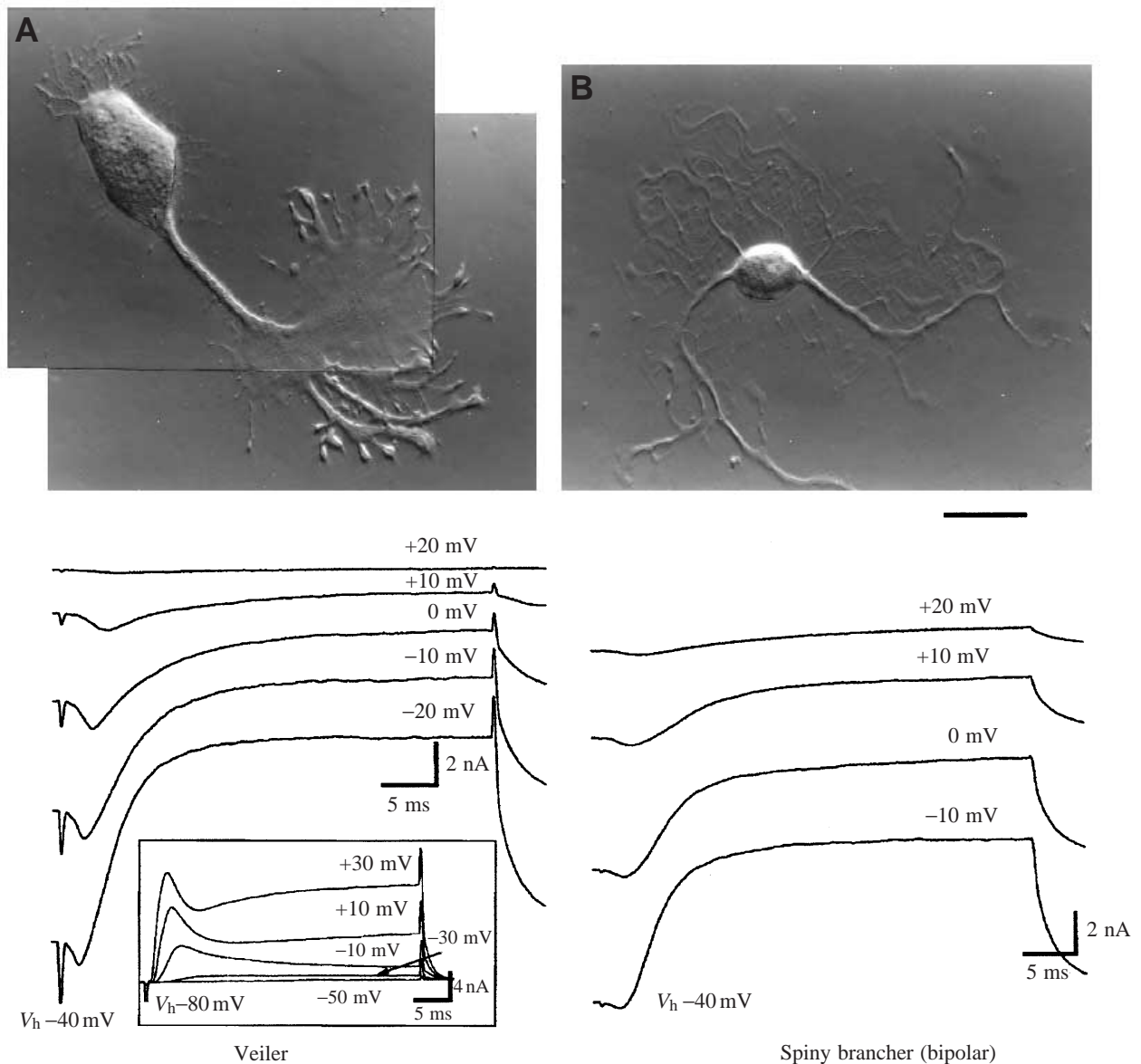


Fig. 1. Currents recorded from crab peptidergic neurons after 5–6 days in defined culture under voltage-clamp conditions in physiological salines. A photomicrograph (Hoffman modulation contrast) taken just prior to the recording is shown above the current traces obtained during a voltage command to the values indicated above the traces from the holding potential (V_h), which was -40 mV unless otherwise indicated. (A) Recordings (not leak- and capacitance-subtracted) from a typical veiling neuron (veiler), where the initial net inward current is followed by an outward current, with the currents nearly cancelling at +20 mV (the baseline has been shifted for each trace to clarify presentation). Inset: currents from a V_h of -80 mV show an initial rapidly activating and inactivating outward current (I_A) that obscures inward currents. (B) Net current recorded from a spiny brancher with a bipolar outgrowth morphology (capacitance- and leak-subtracted in this and subsequent records shown); note the small net inward current. Scale bar, 50 μ m.

hyperpolarization. Six small branching neurons maintained for 1–2 days in culture showed spontaneous, small-amplitude (<10 mV) oscillations but no regenerative responses. Thus, other than the lack of spontaneous firing by veiling neurons, there was no clear difference between the electrical behavior under current-clamp conditions of the neurons cultured for 5–6 days and that of those cultured for 1–2 days.

Net current under voltage-clamp conditions

Observations of net current under whole-cell patch-clamp conditions using ‘intracellular-like’ solutions in the pipette and crab saline in the bath and depolarizations of –30 mV or more showed a pattern of net inward current (Table 1) followed by outward current for all of the six veilers examined after 5–6 days in culture, but for only three of the nine branchers (all with large somata) examined (see Fig. 1A,B). As seen in Fig. 1A (inset), if a holding potential (*V_h*) of –80 mV instead of –40 mV was used, a robust early transient outward current (*I_A*) dominated the initial 10 ms of the response, obscuring the inward current. Branching neurons also have an *I_A* when depolarized from a holding potential of –80 mV (not shown). These results do not differ from similar observations made on neurons maintained for 1–2 days in culture where four of four veiling neurons examined showed a net inward current (from a *V_h* of –40 mV), but only one of five branching neurons examined did so (Meyers *et al.* 1992).

Ca²⁺ current

On the basis of our earlier experiments, comparisons of veilers and cells of other morphology for each age group were made with regard to the average size of the cells, as evaluated either by the recorded total membrane capacitance (*C_m*) or the soma diameter, the peak amplitude of *I_{Ca}* and the current density (i.e. peak *I_{Ca}*/*C_m*). All of these measurements were significantly larger for veilers than for cells of other morphology, and these differences were as prominent in cells cultured for 5–6 days as for cells cultured for 1–2 days (*P*<0.0001, see wide bars, Fig. 2A,B,C). Thus, the veiling neurons consistently showed inward current in the hundreds of picoamps range, while other neurons showed significantly smaller (*P*≤0.009) or no inward currents. In comparing the values for cells cultured for 1–2 days and 5–6 days, there was a significant (*P*=0.0016 for *C_m*) increase in the average size (capacitance) of veiling cells but not in their peak *I_{Ca}* (*P*=0.73) and, consequently, a significant decrease in their average *I_{Ca}* density (*P*=0.0005). In cells showing all other morphologies, none of the measurements changed significantly with time in culture (*P*>0.09).

Correlation of I_{Ca} characteristics with morphology

While the differences between veiling neurons and other neurons were unambiguous, measurements of size and *I_{Ca}* in non-veiling neurons showed a wide variation. Since such neurons developed distinctive morphological characteristics if maintained in culture for 5–6 days, we examined whether the variation between cells could be correlated with morphological

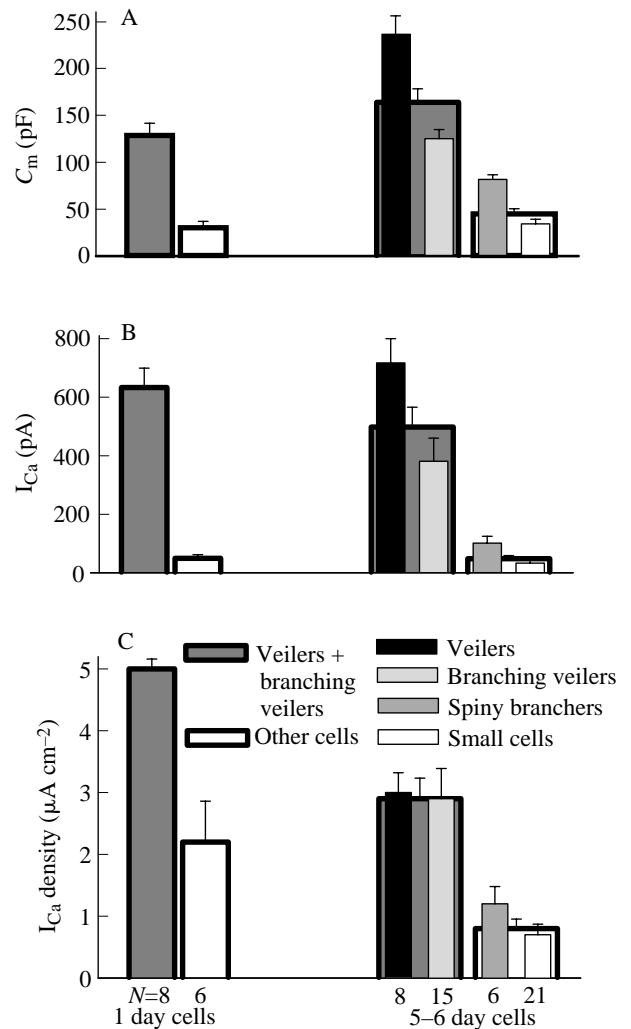


Fig. 2. Correlation of capacitance (*C_m*), peak Ca²⁺ current (*I_{Ca}*) and *I_{Ca}* density with age and morphology. Wide bars surrounded by thick lines compare the mean value (with standard error) for veilers and branching veilers (dark gray) with the mean value for neurons of other morphology (white). Thinner bars show means for 5–6 day neurons (cell numbers are given below the graph) in each morphological category. See text for statistical comparisons.

characteristics and, furthermore, whether differences in the characteristics of *I_{Ca}* were correlated with differences in morphology. We have divided the 5–6 day cells of ‘other morphology’ into three subgroups, each of which show significant differences in size and peak *I_{Ca}*. The groups will be referred to as (1) branching veilers, (2) spiny branchers, and (3) small cells.

Morphological subdivisions of 5–6 day neurons

Some branching veilers were seen to develop from cells that in the first 2 days in culture had the broad growth cones associated with veilers, and it seems likely that all branching veilers derive from cells which earlier had a veiler morphology. These cells as a group had a mean surface membrane area (as indicated by capacitance, Fig. 2A) and peak

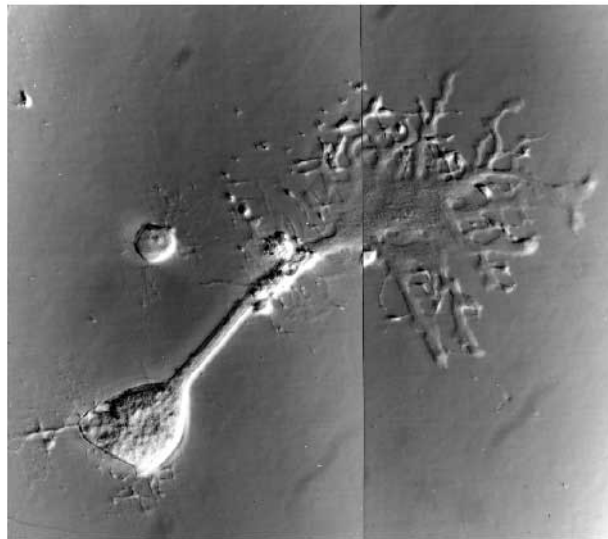


Fig. 3. Inward (Ca^{2+}) current recorded under whole-cell patch-clamp conditions from a veiling neuron (veiler). Cells in this and subsequent figures were cultured for 5–6 days in defined medium. The $I(V)$ curve plotted from recordings such as those shown (inset) represents measurements from the neuron pictured. Note that activation occurred at potentials more positive than -20 mV; peak inward current (approximately 700 pA) was observed at $+10$ mV and did not show reversal at $+60$ mV. The contents of this cell were reactive to crustacean hyperglycemic hormone (CHH) antiserum when immunoblotted. Scale bar, 50 μm .

inward current (Fig. 2B) that were significantly ($P=0.0005$ for C_m , $P=0.009$ for I_{Ca}) lower than the mean values for these characteristics in veilers in culture for 5–6 days. The mean current density (Fig. 2C) of branching veilers, however, was indistinguishable from that of veilers ($P=0.86$). Examples showing the morphology of cells categorized as veilers are given in Figs 1A and 3, while the appearance of cells identified as branching veilers is illustrated in Fig. 4A,B.

The processes from spiny branchers formed an extensive arborization of variable appearance, as illustrated in Figs 1B and 5. They differed from the branching veilers both in appearance and in the mean size of the cells as recorded from measurements of C_m (Fig. 2A) or measurements of soma

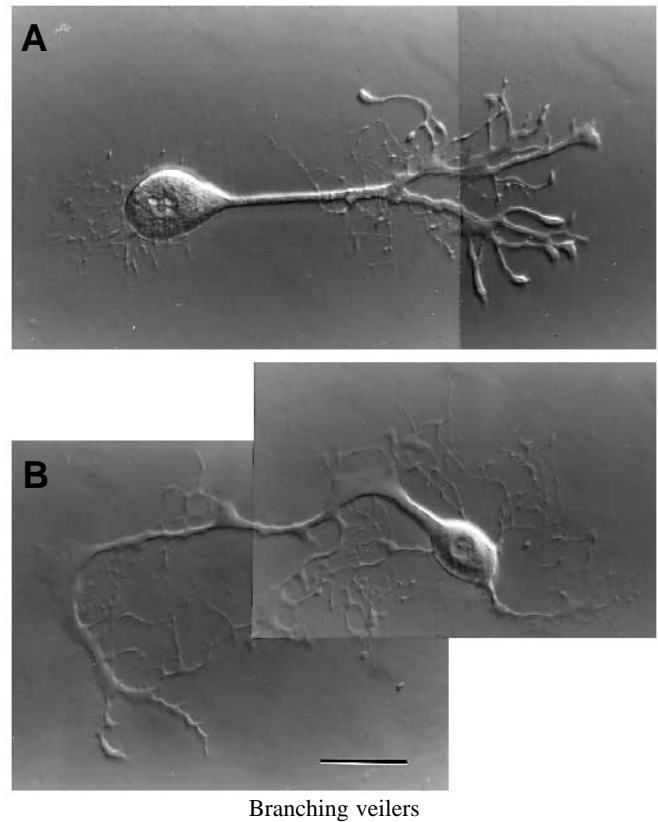


Fig. 4. Morphology of branching veilers. (A) This neuron had a peak I_{Ca} of 1.1 nA, which was among the largest observed, and had an I_{Ca} density of 6.5 $\mu\text{A cm}^{-2}$. (B) An example of a cell that had one of the lowest peak I_{Ca} values (174 pA) and I_{Ca} densities (1.2 $\mu\text{A cm}^{-2}$) in the group. The contents of this cell were reactive to molt-inhibiting hormone (MIH) antiserum. Scale bar, 50 μm .

diameter (33 ± 1 μm , mean \pm S.E.M., $N=6$, compared with a soma diameter of 42.5 ± 1.4 μm , $N=15$, for the branching veilers). Correlated with this difference, the spiny branchers had a significantly smaller mean peak inward current ($P=0.0038$) and lower inward current density ($P=0.007$) than the branching veilers.

The regenerative outgrowth of small cells (see Fig. 6) was similar to that of many of the spiny branchers (Fig. 5), but they were classified as a separate group on the basis of their smaller soma diameter (<28 μm , 23 ± 1 μm , mean \pm S.E.M., $N=21$). Examination of the 27 cells whose branching morphology placed them in the spiny brancher or small cell categories showed that there was a gap in the distribution of soma diameters between 28 μm and 33 μm , with only one cell falling between these values. Furthermore, a division on the basis of soma diameter placed 10 of the 11 cells from which no inward current was detected in the small cell category. Small cells have a mean capacitance that is significantly lower than that of all the other cell types (34.4 ± 4.9 pF, $N=21$, $P<0.001$) and a lower mean peak inward current ($P=0.035$) compared with spiny branchers. However, they do not differ significantly from the spiny branchers in their small I_{Ca} density ($P=0.175$, Fig. 2).

Thus, on the basis of both morphological similarities and

comparable I_{Ca} densities, we believe that veilers and branching veilers represent the more differentiated forms developed after 5–6 days in culture by the neurons that are identified as veilers within the first 2 days in culture. Similarly, the spiny branchers and small cells represent later subdivisions of cells that are grouped together as ‘other cells’ or ‘branchers’ in the 1- to 2-day-old cultures described in this and previous reports. In Fig. 2, the mean measurements of cell characteristics are shown, grouping veilers with branching veilers and spiny branchers with small cells (wide, heavily outlined bars), as well as the averaged measurements of the subdivided groups identified in this report (thin bars). Except for their size (capacitance or diameter), none of the subgroup measurements differs significantly from the combined means of that group. Among the combined groups (wide bars), only the current density shows a significant ($P < 0.0001$) difference with time in culture.

Immunoreactivity of neurons of different morphology

Neurons that had been in culture for 5–6 days were aspirated into a micropipette after electrical recording and then tested by immunoblotting for reactivity with anti-CHH or anti-MIH antisera (see Materials and methods). Previous observations

have shown that a high proportion of veiling neurons contain large amounts of CHH (Keller *et al.* 1995). The 13 cells tested for CHH immunoreactivity confirmed these observations: all the veiling neurons ($N=4$) and the branching veilers tested ($N=2$) were reactive, while only one of the two spiny branchers and one of the four small cells tested were immunopositive for CHH. Five of the 18 cells examined for MIH reactivity were positive, including the one veiling neuron tested, three of the five branching veilers and the one spiny brancher tested, while none of ten small cells was MIH-positive. Six cells were tested for MIH reactivity after 1 day in culture: reactive cells included one of three veilers, the one branching veiler and one of two small cells. It is noteworthy that the spiny brancher and the small cell showing MIH immunoreactivity had exceptionally large inward currents compared with those of other neurons of their group.

Comparison of $I_{Ca}(V)$ curves for neurons of different morphology

We examined $I_{Ca}(V)$ curves for clamp commands from a V_h of -40 mV at 10 mV increments to $+50$ mV for the neurons of each of the morphological types and ages. In order to compare the form of the curves for the neurons grouped according to the morphological types discussed above, independently of their very different current amplitudes, the inward current response observed for each neuron was normalized for each voltage command (V_c) with respect to the peak inward current of that particular neuron. Cells showing a conspicuously non-exponential onset of current, which could result from inadequate space clamping, or which showed evidence of a significant residual outward current at large depolarized potentials, were excluded from the analysis. This left 34 neurons that had been maintained for 5–6 days in culture, and 14 cells from the same set of cultures that had been kept for 1 day in culture. The normalized currents in response to each voltage-clamp step were averaged for each of the groups discussed above ($N \geq 6$) and are presented together with

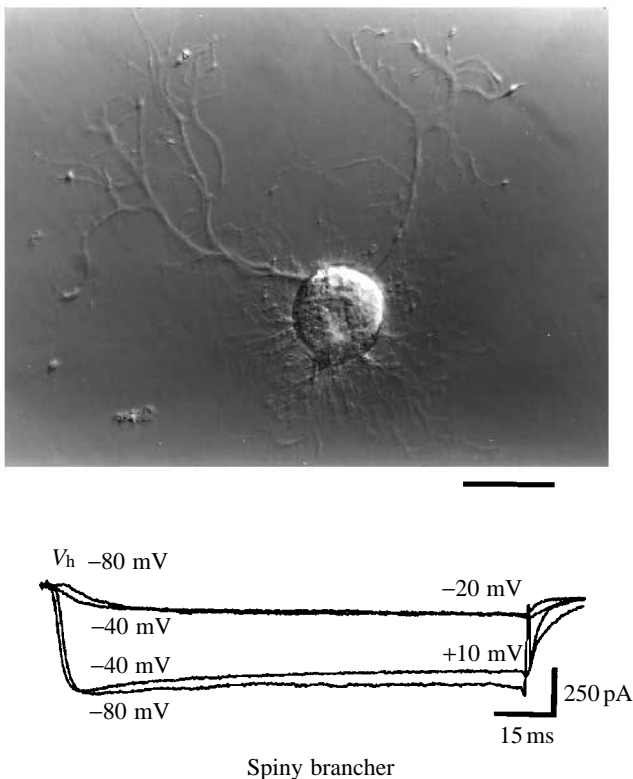
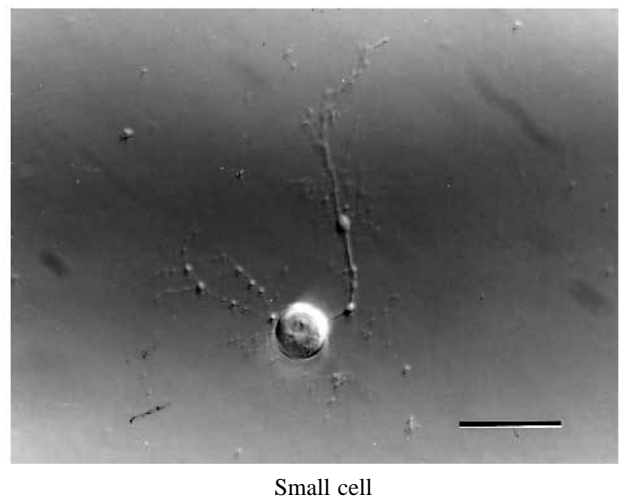


Fig. 5. The morphology and the I_{Ca} measured from different holding voltages (V_h) in a spiny brancher. Currents recorded in response to clamps to -20 mV and $+10$ mV from V_h values of -80 and -40 mV are superimposed. Note that the amplitude of the inward current differs very little, consistent with the presence of a single type of high-voltage-activated Ca^{2+} channel. The contents of this neuron were strongly immunoreactive with MIH antiserum. Scale bar, $50 \mu\text{m}$.



Small cell

Fig. 6. Morphology of a small cell. This cell had higher than average peak I_{Ca} (37 pA) and I_{Ca} density ($1.1 \mu\text{A cm}^{-2}$). The contents of this cell did not show reactivity with MIH antiserum.

standard errors in Fig. 7A,B (symbols). Fig. 7C,D compares these same currents recorded at 1–2 days and at 5–6 days in culture for cells of like morphology. The lines are fits of the averaged data for each group of neurons to a modified Boltzmann equation (see Table 2). As will be seen from Fig. 7 and Table 2, the properties of the $I(V)$ curves for all of the cell groups are very similar in that the peak current occurred at nearly the same V_c (+10 mV), the slopes are very similar and, with the exception of cells cultured for 1 day, the curves all extrapolate to a reversal potential (V_r) above +75 mV. None of the curves shows discontinuities or bulges that would be expected if populations of Ca^{2+} channels having different voltage activation characteristics were present. Table 2 gives the values of the parameters utilized for each cell group to fit its Boltzmann curve. The values for the voltage for half-activation ($V_{1/2}$) for all cells were between -10.3 and -5.9 mV, and the maximum slope ranged from 5.6 to 8.5. The V_r for cells cultured for 1 day was between +58 and 60 mV, and for cells cultured for 5–6 days, it was between +75 and 80 mV.

Analysis of variance (ANOVA) was undertaken for the four morphological types of cells found after 5–6 days in culture for responses to the eight voltage commands, and this showed no significance difference in the averages combined over all voltages ($P=0.603$), but did show significant differences when responses at individual voltages were considered ($P=0.0034$). Comparison of the groups by pairs revealed significant differences for some voltages between veilers and branching veilers ($P=0.001$), and between veilers and small cells ($P=0.001$), but not between other combinations. $V_{1/2}$ and V_r , as well as V_c for the peak current, suggest that the $I(V)$ curve of the branching veilers is shifted by approximately 4 mV in the hyperpolarizing direction relative to that of the veiling neurons. The small cells show activation beginning at a less depolarized V_c than the other types of neurons, and this is reflected in the $V_{1/2}$ for the small cell group. However, the difficulty of measuring the very small near-threshold currents, compounded by normalizing to the order-of-magnitude smaller peak inward current in the small cells compared with the veiling neurons,

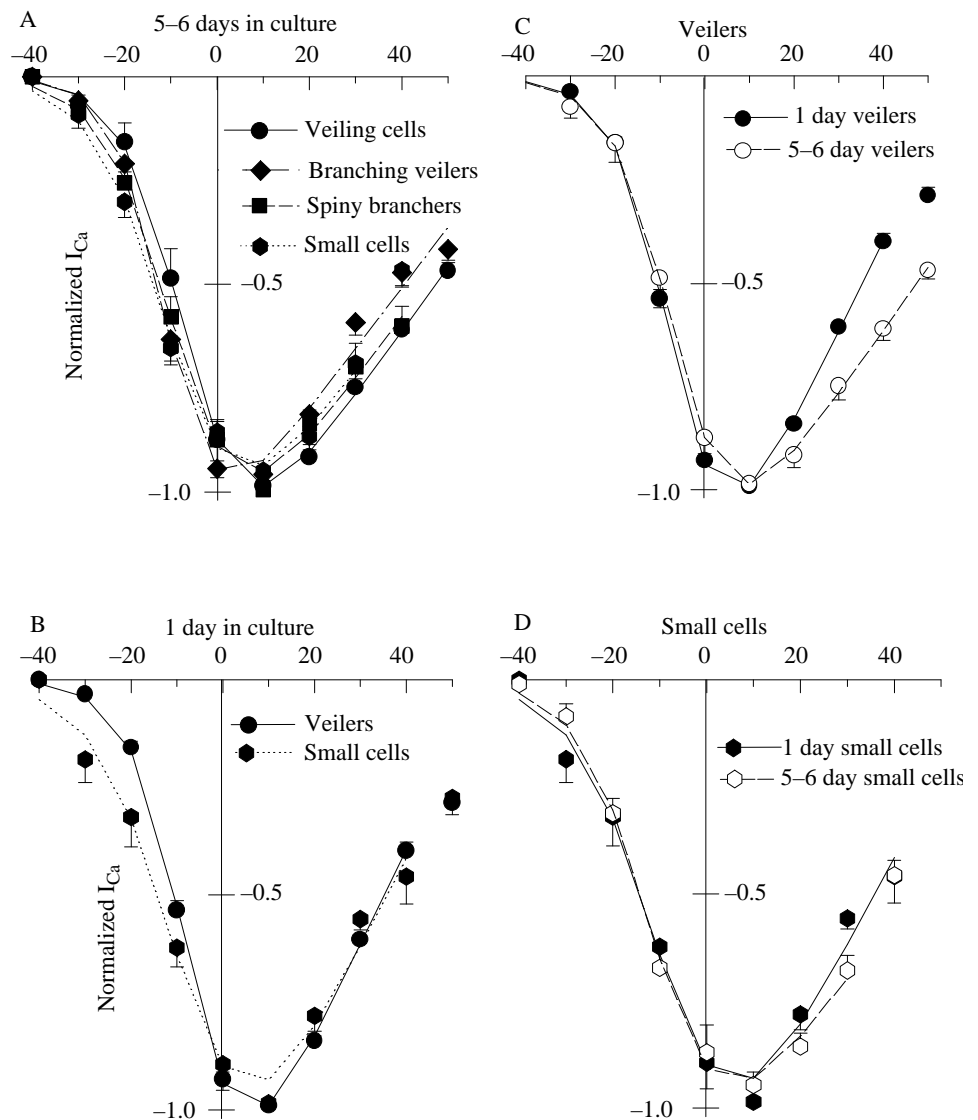


Fig. 7. Comparison of normalized $I_{Ca}(V)$ curves for neurons grouped by age and morphology. Measurements at each command voltage (V_c) for each cell were normalized to the largest current for that cell (usually occurring at +10 mV). Normalized values for each V_c were averaged for neurons of each group as indicated and are plotted (symbols) with bars showing standard errors ($N \geq 6$). Curves represent a fit of a modified Boltzmann equation to the averaged points (see Table 2 for equation and values). See text for statistical comparisons.

Table 2. Values used to fit the modified Boltzmann equation to I(V) curves

	<i>g</i>	<i>V</i> _{1/2}	<i>S</i>	<i>V</i> _r	<i>N</i>
Veilers					
1 day veilers	0.027	-6.15	6.16	57.8	8
5-6 day veilers	0.015	-5.94	6.65	79.8	7
5-6 day branching veilers	0.015	-10.31	5.57	74.7	12
Other					
1 day small cells	0.021	-7.94	8.47	59.9	6
5-6 day spiny branchers	0.015	-8.17	7.31	78.4	6
5-6 day small cells	0.015	-9.30	7.79	77.1	9

Lines drawn to the averaged normalized (to peak) *I(V)* curves of Fig. 7 were fitted to the equation $I = g(V - V_r) / (1 + \exp[(V_{1/2} - V)/S])$, where *g* is conductance in normalized units (mV^{-1}), *V* is clamp potential (mV), *V_r* is reversal potential (mV), *V*_{1/2} is the potential for half-activation (mV) and *S* (mV) is the slope for *I* in normalized current units; *N*, number of neurons averaged.

dictates caution regarding interpretation of the difference between their *I(V)* curves.

A comparison of the veilers and small cells maintained in culture for 1 day (ANOVA) showed no significant differences in either the combined averaged responses or the responses at individual voltages ($P > 0.19$).

Neurons of each of the morphological types were examined for differences in the *I*_{Ca} responses to voltage clamps from *V_h* values of -80 mV and -40 mV, as shown, for example, in Fig. 5. No differences of more than approximately 10% were observed. Previous observations on cells maintained in culture for up to 2 days also indicated no differences between morphological categories (Meyers *et al.* 1992).

In conclusion, our analyses failed to provide clear evidence for differences in the biophysical characteristics of the voltage-activated Ca²⁺ channels present in morphologically distinct neurons that have been in culture for a similar time.

Changes in the *I*_{Ca}(*V*) curve with time in culture

Plots of the normalized current responses averaged for each *V_c* (Fig. 7B) and fits to the Boltzmann equation were constructed for the 14 cells examined after 1 day in culture and are compared in Fig. 7C,D with similar plots for the veiling neurons and small cells from the same cultures left to grow for 5-6 days. Statistically significant differences for both the overall averages and individual responses ($P < 0.02$) were observed when comparing 1 day with 5-6 day veiling neurons (Fig. 7C): most of these differences result from the reduction of *I*_{Ca} with increasingly depolarized commands, which was less in the 5-6 day veiling neurons, leading to a more depolarized value of their extrapolated *V_r*.

Small cells maintained in culture for 1 day and 5-6 days did not show statistically significant differences in their overall averaged responses or responses to specific commands

($P > 0.3$); as in the case of the veilers, the small cells cultured for 5-6 days showed a more depolarized *V_r* than those cultivated for 1 day. Both groups of small cells (Fig. 7A,B) showed a greater activation with small depolarizations from *V_h* compared with veilers.

In conclusion, we have found no evidence from these analyses for the emergence of new classes of Ca²⁺ channels with time in culture, nor any evidence for the presence of more than one type of Ca²⁺ channel. The less depolarized values of *V_r* indicated from fits to the Boltzmann equation for cells that had been cultured for 1 day relative to older cells were unrelated to their morphological type.

Discussion

Cells dissociated from the cluster of peptidergic secretory neurons in the crab eyestalk referred to as the X-organ and plated in defined medium show immediate, robust outgrowth that generally reaches stable morphology in about 7 days (Cooke *et al.* 1989; Grau and Cooke, 1992). This study has examined the biophysical properties of the voltage-activated Ca²⁺ currents of these cells to determine whether these properties change with time in culture and whether any differences in the characteristics of the Ca²⁺ currents can be associated with neurons that differ morphologically. The emphasis on the Ca²⁺ currents reflects our interest in their role in the control of hormone secretion. Our results have demonstrated that there are significant differences in *I*_{Ca} densities in neurons of different morphology, with neurons previously shown to contain the hormonal peptides (Keller *et al.* 1995) having the highest current densities. However, within the limits of our experimental paradigm, the biophysical characteristics of the Ca²⁺ currents of neurons with different morphologies were indistinguishable and did not change with time in culture.

Earlier studies detailing characteristics of *I*_{Ca} in neurons cultured for 1-2 days (Meyers *et al.* 1992) or acutely isolated (Richmond *et al.* 1995) led to the characterization of the Ca²⁺ current of X-organ neurons as a high-voltage-activated current showing primarily Ca⁺-mediated inactivation. The failure of pharmacological agents (dihydropyridines, ω-conotoxins GVIA, MVIIV, ω-Agatoxin IVA) that have been used to define Ca²⁺ channels in other preparations (e.g. Randall and Tsien, 1995) to produce any effect on the crab Ca²⁺ channels (Richmond *et al.* 1995) suggests that a single type of channel generates *I*_{Ca} in our preparations. Characterization of *I*_{Ca} in dissociated terminals of X-organ neurons is consistent with the presence of a single type of Ca²⁺ channel of the same type (Richmond *et al.* 1996). Whether any of the agents mentioned would reveal subclasses of Ca²⁺ channels in neurons maintained for longer in culture remains untested, but this seems unlikely given that the currents recorded in the large number of neurons cultured for 5-6 days and examined in the present study were distinguishable only by an apparently more depolarized reversal potential (*V_r*) from those recorded both from neurons cultured for 1-2 days in this and previous studies (Meyers *et al.* 1992) and from acutely isolated X-organ

neurons (Richmond *et al.* 1995) or terminals (Richmond *et al.* 1996). One possible cause of an apparently less depolarized V_T may be contamination by outward current.

The outgrowth of the neurons in culture is reflected in the increased capacitance of the cells. The failure of peak currents to increase in those categories of cells that can be reliably compared between 1-day-old cultures and 5- to 6-day-old cultures reflects a decreased I_{Ca} density in older cells. It is tempting to conclude from this finding that the synthesis and insertion of functional Ca^{2+} channels during regrowth does not occur at a rate that is detectable using whole-cell recording techniques. In view of the simple defined medium that was used, it is entirely possible that the constituents necessary for producing functional channels are lacking.

We must, however, also consider the possibility that the failure to see increased current density reflects a spatial-geometric recording problem. Are we recording current only from the soma, while newly synthesized Ca^{2+} channels have been exported to processes where, because of spatial decrement, we fail to activate them or to record current through them? For the veiling neurons and branching veilers, this possibility seems highly unlikely: the process linking the growth cone region with the soma is stout (approximately 5 μm in diameter) and, even assuming a sixfold decrement in the effective membrane resistance during maximal inward current activation, modeling (D. K. Hartline, unpublished results) shows that the space constant is at least 1 mm. None of the neurons had processes extending even half this length. A direct demonstration of the adequacy of voltage control has been provided in leech neurons of similar size and complexity in culture using a voltage-sensitive dye (Ross *et al.* 1987). For neurons categorized as small cells and for some of the spiny branchers with very small-diameter processes, we can be less certain. However, the capacitance measurements indicate that large amounts of membrane additional to that accounted for by the soma are included in the membrane effectively charged by voltage commands and recorded from. In cells having fine processes, the inward current was very small or undetectable, and thus the increase in conductance during clamp steps (and the reduction in the space constant) should not be very great. We conclude, therefore, that neurons regenerating processes under the conditions of our simple, defined culture system do not add Ca^{2+} channels at a rate matching the addition of membrane, resulting in a decrease in the I_{Ca} density with time in culture.

Outward currents identifiable as I_A and I_K (Meyers *et al.* 1992) were separated and compared between 20 veiling neurons cultured for 1 day and 16 veilers and branching veilers cultured for 5 days (S. Duan, unpublished data). While a trend towards reduced current density was discernible for a subset of 10 cells showing the greatest outgrowth, none of the changes, including the ratio of I_A/I_K density, was statistically significant ($P > 0.4$, currents, $P = 0.11$ for capacitance increases of the 10 cells). No changes in the $I(V)$ relationships were observed. Thus, the apparent stability of the number and characteristics of voltage-gated Ca^{2+} channels in culture is also observable for at least two types of K^+ channels.

When cells kept for 5–6 days in culture were grouped according to their morphology, veilers and branching veilers could be distinguished from spiny branchers and small cells on the basis of a larger peak current. This separation was even more striking when current density was compared. These data, therefore, support the alliance suggested by the morphology, and supported in a number of cases by sequential observations of their development, that veilers and branching veilers represent neurons that show veiling outgrowth at 1 day in culture, while spiny branchers and small cells develop from cells with other morphologies. In fact, except for differences in soma diameters, spiny branchers and small cells are not easily distinguished, even after 5–6 days in culture. It should be noted, however, that, as with most morphologically based classification schemes, there are always exceptions. Four cells in this study presented a distinctly bipolar form (Fig. 1B); three were categorized as spiny branchers and one as a veiling brancher, but perhaps these should be placed in a separate category since they had thicker processes and higher current densities than the average for spiny branchers.

Previous immunostaining studies of the X-organ–sinus gland system using the antisera employed here have shown selective staining of large ($>30 \mu\text{m}$ in diameter) neurons of the X-organ, with approximately twice as many CHH-reactive neurons (approximately 30 in *Cardisoma carnifex*, B. Haylett and I. Cooke, unpublished data) as MIH-reactive neurons (Dircksen *et al.* 1988). The reliable association of CHH reactivity with cultured neurons from *Cardisoma carnifex* having the morphology here categorized as veilers and branching veilers has been established by performing quantitative assays on individual cells aspirated from cultures (Keller *et al.* 1995). The immunoblots examined in the present study are in accord with the findings of Keller *et al.* (1995). In contrast to the relatively large number of cells that show CHH reactivity, few of the neurons tested in the present study were reactive with MIH antisera. This is in agreement with the small number of MIH-reactive neurons seen histologically. With respect to the bipolar neurons discussed above, the immunoblot data combined with observations from staining populations of neurons in culture dishes (Cooke *et al.* 1989; S. Grau and I. Cooke, unpublished data), suggest that these cells are the most likely to show MIH immunoreactivity.

The small cells and the spiny branchers with very fine processes were not immunoreactive and had small or undetectable I_{Ca} currents and low I_{Ca} density. Two exceptions, showing MIH immunoreactivity, also had an exceptionally large inward current. The cells of these types examined by electron microscopy show a paucity of the large ($>90 \text{ nm}$) secretory granules that are abundant in veiling neurons (Cooke *et al.* 1989; T. Weatherby, S. Grau and I. Cooke, unpublished observations). It is, therefore, possible that most of these cells are non-secretory cells so closely associated with the X-organ cell group that they are co-cultured with the secretory cells.

The combination of size, robust I_{Ca} densities and immunoreactivity for sinus gland hormonal peptides confirms the veilers and branching veilers as neurosecretory cells. The

larger neurons of the X-organ are unusual among arthropod neurons in showing regenerative potentials, including a major Ca²⁺ component (Iwasaki and Satow, 1971; Stuenkel, 1985; Nagano and Cooke, 1987, Onetti *et al.* 1990). In contrast to these cells, the somata of most arthropod neurons lack sufficient inward current to produce regenerative responses. The very small or undetectable currents of some of the spiny branchers and many of the small cells is consistent with the possibility that they represent 'ordinary' non-secretory neurons.

Among numerous studies of neurons in culture, relatively few have examined regeneration of mature neurons in simple medium lacking serum or conditioning factors. The most directly comparable studies to this one are those on lobster stomatogastric ganglion neurons. These neurons show little or no electrical activity for 3 or more days after plating, but after this, neurons physiologically characterized before isolation recover their characteristic activity (Panchin *et al.* 1993; Turrigiano and Marder, 1993). This recovery of activity is accounted for by an increased density of Na⁺ and Ca²⁺ currents, and a decrease in density of outward currents (Turrigiano *et al.* 1995). The densities of I_{Ca} observed were as much as 25 μA cm⁻², a value approximately 10 times greater than that observed in the X-organ neurons in this study.

In conclusion, neurons categorized according to their different outgrowth morphologies showed significant correlated differences in I_{Ca}, and neurons showing immunoreactivity to CHH or MIH antiserum were also those with a higher I_{Ca} density. The biophysical characteristics of I_{Ca} were indistinguishable across morphological types and did not change with time in culture (acutely dissociated, 1–2 days or 5–6 days in culture). The biophysical data again suggest the presence of a single type of high-voltage-activated Ca²⁺ channel. The addition of new membrane during regenerative outgrowth of mature crustacean peptidergic neurons in defined medium was not accompanied by an addition of Ca²⁺ channels that could be resolved using whole-cell recording.

We thank P. Couvillon for help with statistical analyses, Jana Labenia for performing the immunoblots; S. Grau and Jana Labenia for technical assistance and R. Keller for gifts of antisera. The work was supported by grants from the Whitehall Foundation (J89-48), NSF (BNS 89-10432), NIH (RO1 NS 15453) and the Ida Russell Cades Fund of the University of Hawaii Foundation.

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