A Persistent, TTX-Sensitive Sodium Current in an Invertebrate Neuron with Neurosecretory Ultrastructure

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In the CNS of the giant barnacle (*Balanus nubilus*) a single pair of large neuronal somata (cross-commissural, or CC, cells), located near the entry of the median ocellar nerve, occasionally displays a prominent whitish luster. These somata have ultrastructure typical of neurosecretory cells: numerous Golgi complexes and abundant, large dense-cored vesicles (DCVs; size range, 75–275 nm). Injection of a CC cell with cobalt tracer shows that it arborizes over a 7 mm length of the contralateral peripheral nerve out of which it projects. The processes of the arbor are profuse and varicose; the varicosities are packed with DCVs similar to those in the soma. Stimulation of a single CC cell causes a substantial decrease in the number of DCVs and increases the incidence of clusters of small electron-lucent vesicles, as well as the occurrence of large electron-lucent vesicles and membrane-bound cisternae.

We studied ionic currents flowing across this cell's somatic membrane with a single-electrode voltage clamp. Unusual among these currents is an inward current that is blocked by TTX but is essentially noninactivating. In current clamp, this "persistent" current causes the action potential to be prolonged (seconds) if opposing outward current is blocked with 4-aminopyridine. The inward current is carried by Na. Its amplitude depends on the external Na concentration, it is blockable by TTX, and it persists when the cell is bathed in Ca-free saline and/or Co. Other currents present in this cell include an outward current similar to molluscan A-current and a Ca current that contributes to the action potential (Stockbridge and Ross, 1986). The persistent Na current is partially activated at the cell's resting potential and, thus, may participate in determining the frequency of its impulse activity.

For many years, voltage-sensitive sodium channels were thought to comprise only two forms throughout the entire animal kingdom, one sensitive and one insensitive to TTX (Hille, 1984). Recently, sodium channels that lack inactivation have been described in nerve cells of both vertebrates and invertebrates (e.g., Grampp and Sjölin, 1975; Colmers et al., 1982; French and

Gage, 1985; Stafstrom et al., 1985). In some cases, this "persistent" sodium channel has a finite conductance at the cell's resting potential, and it has been suggested that it may influence the resting impulse activity of the neuron (French and Gage, 1985).

We describe here a persistent sodium channel in a pair of large, identifiable neurons (cross-commissural, or CC, cells) of the barnacle's nervous system that appear to be neurosecretory. These cells originally received attention because of the geometry of their processes and arbors, which is especially suitable for study with optical methods (Ross et al., 1986). Stockbridge and Ross (1986) also found that axons of CC cells have a calcium conductance so large that regenerative calcium-dependent voltage changes can actually propagate along them.

We present here ultrastructural observations indicating that CC cells are neurosecretory and that they release material from varicosities forming a plexus within a peripheral nerve. By voltage clamping their somata, we have observed the persistent Na current and a transient outward current resembling molluscan A-current (Conner and Stevens, 1971) in these cells. This constitutes the first report, to our knowledge, of a persistent sodium channel in a cell suspected to be neurosecretory.

Aspects of this work have been reported in abstracts (Davis and Stuart, 1985, 1986).

Materials and Methods

Specimens of the giant barnacle, *Balanus nubilus*, were obtained from Charles Eaton, Friday Harbor, Washington, and maintained in a recirculating aquarium at 10–15°C. Dissection involved removal of the CNS, including the supraesophageal ganglion (SEG), the median ocellus, median ocellar nerve (MON), and long lengths of the bilaterally symmetric antennular nerves (AN). In most respects the dissection was similar to that described by Hudspeth and Stuart (1977).

Light and electron microscopy. The dissected nervous system was fixed for 1.5–3 hr in 250 mM glutaraldehyde, 510 mM NaCl, 90 mM sucrose, 100 mM phosphate buffer (pH 7.4) at 3–5°C. It was immediately transferred to a postfix consisting of 30 mM OsO₄ and 80 mM glutaraldehyde in the same buffered saline for 1–1.5 hr at 3–5°C. After a water rinse, the tissue was dehydrated in a graded ethanol series and passed through acetone before embedding in a 3:4 Epon 812:Araldite 506 plastic mixture. Glass knives were used to cut 1 μ m sections for light microscopy and thin sections in the silver to gold range of interference colors. Electron microscopic sections were collected on uncoated copper grids, stained with aqueous uranyl acetate and lead citrate, and examined with a Zeiss EM-10 CA EM.

For marking with Co tracer, cells were injected with 125 mM cobaltic hexamine chloride (made up in distilled water). Depolarizing current pulses (500 msec; 20–90 nA) were delivered at 1 Hz for 40–90 min. The preparation was stored at 3–5°C for 12–24 hr to allow Co diffusion from the somatic injection site into the distal processes of the cell. For light microscopy, the Co was then precipitated with ammonium sulfide, processed with the Timm's silver-intensification procedure, dehydrated, cleared, and mounted (technique modified from Bacon and Altman, 1977). For electron microscopy of Co-filled processes, a 5 min ammonium sulfide precipitation step was carried out approximately 1–5 min into the glutaraldehyde fixation. Thick sections (1–2.5 μ m) were

Received June 16, 1987; revised Mar. 31, 1988; accepted Apr. 7, 1988.

This research was supported by National Institutes of Health Grant EY03347 to A.E.S. and NRS Grant EY05900 to R.E.D. We thank Laura Fleck and Lisa Pfrogner for unfailing technical assistance and Wilma K. Hanton for assistance with the EM. Dr. G. Frank Gwilliam originally injected CC cells with cobalt and helped us perfect the technique. Dr. John W. Moore provided invaluable guidance in the use of the voltage clamp. Finally, we wish to thank Milt Charlton for valuable comments on an earlier version of the manuscript.

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cut from embedded material and Timm's-intensified on coverslips using 5% AgNO₃ in Timm's reagent for 7–10 min [techniques modified from Phillips (1980), Tyrer and Bell (1974), and Bacon and Altman (1977)]. These sections were then reembedded, thin-sectioned, and treated for electron microscopy as described above.

Physiology. The dissected nervous system was pinned out in a Sylgard-lined chamber and superfused with normal barnacle saline: 462 mM NaCl, 8 mM KCl, 20 mM CaCl₂, 12 mM MgCl₂, and 10 mM Tris(hydroxymethyl)aminomethane-HCl buffer at pH 7.7. Temperature was maintained at approximately 10–12°C.

To facilitate removal of the ganglionic sheath and microelectrode penetration, the ganglion was immersed in 3 mg/ml pronase (No. 53702, from Calbiochem-Behring Corp., La Jolla, CA) for 2 min. It was then thoroughly washed in normal saline and the sheath removed with fine forceps. Stockbridge and Ross (1986) used 1 mg/ml pronase treatments of 8–10 min duration; they reported no obvious difference in the physiological properties of these cells (resting potentials, action potentials, and general activity) when the ganglion was desheathed with and without enzyme treatment. In animals prepared for electron microscopy, either no pronase was used, and the nervous system was immediately immersed in cold fixative upon removal from the animal, or, in the case of Co injections, a double-chambered bath was used and pronase treatment was applied only to the ganglionic compartment, sparing the antennular nerves.

Glass microelectrodes were filled with 3 M KCl and had resistances of 6–12 MΩ in the voltage-clamp experiments or 60–100 MΩ in current-clamp experiments. All intracellular recordings were made from cell somata.

A single-electrode voltage-clamp circuit (modified from Wilson and Goldner, 1975) built in the laboratory of Dr. John W. Moore (Dept. of Physiology, Duke University) was used for both current-clamp and voltage-clamp records. The voltage-clamp circuit employs a 50% duty cycle with a controllable clock period of 0.45–1.6 msec. Microelectrode capacitance compensation was carefully set to avoid potential capacitance-related artifacts. As is frequently the case in single electrode clamp situations, we are limited in the quantitative precision of our results since we are unable to constrain our cells to complete isopotentiality. Clamp speed limitations (in particular, the settling time of the clamp) require us to be cautious in interpreting the very fast (and frequently uncontrollable) currents at the onset of voltage steps; however, the prolonged and steady-state currents that were the principal objects of this study were easily resolved. Considerations relevant to the use of single electrode voltage clamps have been discussed elsewhere (e.g., Finkel and Redman, 1985).

A Nicolet digital oscilloscope provided analog-to-digital conversion and display of the current and voltage patterns. The data in the Nicolet memory was transferred to a PDP-11/23 computer (Digital Equipment Corporation) for analysis and plotting. For recording long-term activity, a Gould chart recorder was run in parallel with the above equipment.

Extracellular recordings of activity in the antennular nerve contralateral to the impaled soma were commonly made so as to monitor overall activity changes and confirm identification of the cell.

Co saline was made by replacing all of the Ca with Co. In salines with reduced Na, Tris replaced Na. 4-AP and TTX were obtained from Sigma Chemical Co. (St. Louis, MO).

Results

Morphology of CC cells

Topography of CC cells

The CC cells are a pair of bilaterally symmetric cells located on the anterior medial margin of the dorsal surface of the suprapharyngeal ganglion near the root of each antennular nerve (Fig. 1, A, B). Occasionally these cells exhibit a reflective opalescence under direct illumination that causes them to stand out noticeably from the other cells in the ganglion (Fig. 1A). This whitish luster has been seen in the soma alone (Fig. 1A) or also extending into the axon. The reflectance of CC cells varies on a continuum from that of the translucence of ganglion cells in general to an opaque whiteness that is highly distinctive. The consistent position, large size (60–100 μm), and characteristic pattern of synaptic and action potential activity of CC cells are the cues used to identify them when they are not in their white phase.

Injection of fluorescent dyes by Ross and colleagues (Ross et al., 1986; Stockbridge and Ross, 1986) has shown that the main axon of the CC cell, after forming a dense ipsilateral arbor near the soma, crosses the commissure connecting the hemiganglia. In the contralateral hemiganglion it branches to form another arbor and finally projects out the contralateral antennular nerve (Fig. 1B). Intracellular injection of a CC cell with Co tracer reveals that its axon terminates within the contralateral antennular nerve in a profusion of slender processes punctuated with numerous, small *en passant* varicosities (Fig. 1C). The primary axon (10–20 μm diameter) and its larger branches (>5 μm diameter) are restricted to the first 4–5 mm of the nerve (total antennular nerve length, 20–25 mm). The arborization of fine, varicose processes overlaps the primary axon and its branches, extending from approximately 1 mm to about 8 mm out the nerve.

It is difficult to determine the precise extent of the arbor. The number of stained varicose branches diminishes over the last 2 mm of this arborization (Fig. 1D). Further out, although no Co staining is observed, thin sections show occasional profiles with ultrastructure similar to that of CC cell (described below); thus, it is possible that the arbor is not completely filled by the Co. On the other hand, extracellular recordings show that the CC cell's extracellular spike is not recorded beyond 8–10 mm out from the nerve, suggesting that the Co fills may reflect the total extent of the arbor.

Unlike neurosecretory cells in many other invertebrates (for reviews, see Gupta, 1983; Orchard and Loughton, 1985), CC cell processes do not terminate in a neurohemal organ, nor do they end selectively in or near the sheath surrounding the nerve in which they travel. From viewing successive focal planes through nerve whole mounts, one has the impression that Co-filled processes and varicosities occur throughout the depth of the nerve. Cross sections of the nerve examined in the EM corroborate this finding (see Fig. 3A).

Ultrastructure of the CC cell's soma

The whitish luster of its soma, and its diffuse arbor within the nerve, suggested that the CC cell might be neurosecretory. Indeed, its ultrastructure is typical of neurosecretory cells. Low-power electron micrographs of the soma reveal a dense cytoplasmic matrix marked by abundant small-diameter mitochondria and dense-cored vesicles (DCVs). Even in thick (1 μm) sections this exceptional cytoplasmic density allows one to easily distinguish the CC cell within the ganglion. Numerous Golgi structures are seen in the CC cell's soma. Figure 2A shows a Golgi apparatus appearing to produce a DCV, an observation also made in many other neurosecretory preparations (see review by Orchard and Loughton, 1985). In one of the two thin-sectioned somata, Golgi structures and nearby DCVs occurred in consistent, close association with small patches of glycogen (Fig. 2B).

Ultrastructure of the antennular nerve in the region of the CC cell's arbor

Cross sections of the antennular nerve approximately 4 mm from the ganglion reveal numerous DCV-loaded profiles distributed throughout its depth. Large, DCV-laden profiles approximating the sizes of the main axon and primary branches of Co-filled CC cells were observed. Figure 3A shows a profile (large arrow) whose diameter is approximately that of a primary branch of the CC cell. The profile has dense cytoplasm like that

Figure 1. Position of CC cell somata and arbors in the supraesophageal ganglion (SEG) and antennular nerves (AN). *A*, Whole mount of the ganglion showing medial position of CC cell somata (arrowheads) on each side of the commissure connecting the 2 hemiganglia. The ganglion is illuminated directly, causing the cells to have a whitish appearance. The 2 antennular nerves exit the ganglion to the right and left of the median ocellar nerve (MON). Scale bar, 250 μ m. *B*, Schematic drawing illustrating the general morphology of one of the CC cells: The position of its soma in the ganglion and of its axonal ramification in the antennular nerve. *C*, light micrograph of a portion of the cell's arborization in a whole mount of the antennular nerve approx. 3.3–3.9 mm from the ganglion, as illustrated in *B*. The cell was filled with Co. The point at which the main axon subdivides is shown; in this area are many fine branches with varicosities. Blurred dark images are processes and varicosities that are not in this

of the CC cell's soma and numerous, large DCVs congregated toward the periphery of the profile. A smaller-diameter profile containing similar vesicles and approximating the size of varicosities in Co-filled whole mounts is also seen (small arrow). This micrograph shows the typical relationship of such vesicle-marked profiles to the overall topography of the nerve: They lie within the nerve's fascicles, among glial and other neuronal profiles. Longitudinal sections through the nerve (Fig. 3*B*) reveal that the vesicle-containing neurites shrink at intervals into thin necks connecting expanded varicosities. In most of these longitudinal profiles, the varicosities are packed with DCVs, while the intervening neurite contains few or no vesicles (Fig. 3*B*). Occasionally the vesicles are lined up, as if along a microtubule or neurofilament (Fig. 3*C*).

The DCVs are membrane-bound (Fig. 3*D*) and range in diameter from approximately 75 to 275 nm. The size and appearance of these vesicles are similar to those found in neurosecretory cells of other organisms (e.g., Price and McAdoo, 1979; Haskins et al., 1981; see reviews by Chaigneau, 1983; Orchard and Loughton, 1985; Sedlak, 1985). A few smaller, clear vesicles (approximately 20–60 nm in diameter) and mitochondria are also seen in these profiles (Fig. 3*D*). Though extremely rare, omega-forms have been observed in the membrane of the varicosities, indicating possible sites of release (Fig. 3*D*, arrow).

There is no obvious postsynaptic target for the material suspected of being released from the DCV-filled varicosities. Postsynaptic densities or widened clefts, which would indicate synapses, are not seen (Fig. 3*D*). There are no obvious channels, such as enlarged extracellular spaces surrounding these profiles, to conduct released material out of the nerve to the hemolymph (Fig. 3*A*). It appears that material may simply be released locally into the extracellular space of the nerve itself.

Equivalence of DCV-containing profiles with those of the CC cell

To determine whether the profiles containing DCVs belong to the CC cell, the arbors of cobalt-filled CC cells were examined. Dense cobalt-silver precipitates occurred selectively over certain large and small profiles. The larger of these profiles (>5 μ m), the axon and primary branches of the CC cell, were filled with the dense precipitate that often obscured the ultrastructure. In the smaller profiles (1–5 μ m), the ultrastructure was not obscured and DCVs were obvious, intermingled with the clumped precipitate (Fig. 4). These vesicles are of the same size range (75–275 nm) and general appearance as those seen in profiles from unfilled preparations (Figs. 2, 3).

In preparations in which the CC cell had been filled with Co, precipitate also was found in the tissue surrounding DCV-containing profiles (Fig. 4), but appeared to be nonspecific since the granules were smaller, not clumped, and evenly distributed (except for a curious absence from the deep interior of profiles of adjacent neurons).

To determine whether the CC cell accounts for all DCV-containing profiles in the nerve, we counted profiles in cross sections of one preparation containing clumped precipitate and DCVs. Of 24 profiles containing DCVs, 16 (or 67%) also contained precipitate. While uneven distribution of the tracer could

Figure 2. Ultrastructure of the CC cell's soma. *A*, Golgi complex from soma of CC cell. Numerous such complexes are seen terminating in dense-cored buds. Scale bar, 250 nm. *B*, Lower-power view of Golgi from another soma illustrating the close association of these complexes with dense-cored vesicles and glycogen particles (*gly*). Scale bar, 250 nm.

result in CC cell profiles that showed no precipitate, these results suggest that there may be other neurons in the antennular nerve containing DCVs. In contrast, 9 out of 10 DCV-laden profiles showed ultrastructural changes when the CC cell was stimulated (see below), suggesting that most or all of the DCV-containing profiles belong to the CC cell or cells responding to its stimulation.

Changes in the ultrastructure of DCV-containing profiles with stimulation

As has been shown for other neurosecretory cells (e.g., Haskins et al., 1981), it is possible to deplete the varicosities of a large portion of their DCVs by electrical stimulation. A CC cell's soma was stimulated intracellularly at a frequency intended to mimic spontaneous bursting activity (see Fig. 5 for stimulus

focal plane. Scale bar, 50 μ m. *D*, Photomicrograph of the same nerve further out (approx. 6 mm from the ganglion), where the meshwork is less dense. A fine varicose process is marked by an arrow. Scale bar, 50 μ m.

Figure 3. Ultrastructure of profiles containing dense-cored vesicles in the antennular nerve. *A*, Cross section of a fascicle of the antennular nerve 4 mm from the ganglion. The profile indicated by the *large arrow* contains dense-cored vesicles around its periphery. The *small arrow* indicates a smaller profile containing dense-cored vesicles. Scale bar, 2 μ m. *B*, Longitudinal section showing 2 varicosities connected by a thin neurite. Vesicles are characteristically absent or present in very small numbers in the neck connecting varicosities. Scale bar, 1 μ m. *C*, Longitudinal sections occasionally reveal varicosities with vesicles arranged in what appear to be linear arrays. Scale bar, 1 μ m. *D*, Cross section of a vesicle-containing varicosity. Large, membrane-bound dense-cored vesicles and small clear vesicles are apparent. *Arrow* indicates omega-form where large vesicle appears to be releasing its contents into the extracellular space. Scale bar, 250 nm.

details). Fixation within 1–10 min of stimulation resulted in profiles with fewer DCVs (Fig. 5*A*) than in DCV-filled profiles from the contralateral antennular nerve (cf. profiles belonging to the unstimulated CC cell in Fig. 5*D*).

Stimulated profiles also had a higher incidence of electron-lucent vesicles than unstimulated ones. While these vesicles included all sizes between small “microvesicles” (approx. 20–60 nm) and large clear vesicles approximating or even slightly

Figure 4. CC cell profile in antennular nerve identified by Co tracer. Co was injected into the soma and allowed to diffuse throughout the cell. Profile is from cross section of nerve roughly 4 mm from the ganglion. Clumped precipitates of cobalt-silver are located over this profile, which is filled with large dense-cored vesicles. With the exception of the large neuronal profiles whose interiors are devoid of precipitate (*), the surrounding tissue shows non-clumped, evenly distributed precipitate. Scale bar, 0.5 μ m.

larger than the size of the DCVs (up to 300 nm), the two most prominent populations were the small and large vesicles. Figure 5, *A* and *C*, illustrates the smaller ones, which were distinct because they frequently occurred in clusters within varicosities. The large clear vesicles, while observed in the small varicosities, appeared to be even more prominent in profiles larger than the typical varicosity ($>3 \mu$ m; Fig. 5*B*), up to and including neurites approximating the primary axon in diameter. Around the periphery of the interior of the large clear vesicles a grayish substance may be seen, and in the center sporadic fine densities. Large membrane-bound cisternae were also frequently observed in the stimulated varicosities (Fig. 5*C*).

Physiology of CC cells

Action potentials

Like many neurosecretory cells from invertebrates, CC cells frequently give rhythmic, patterned bursts of impulses. Often a slow ramp potential leads to one or more spikes (Fig. 6*A*), although in some cells the firing is more steady (Fig. 7, *A* and *B*). The resting potential of the cell is low (-40 to -50 mV), as also observed by Stockbridge and Ross (1986). Spontaneous inhibitory synaptic potentials often occur during the cell's activity (Figs. 6*A*; 7, *A*, *B*) but do not participate in generating bursting rhythms. The offset of light can also transiently inhibit the cell.

Ross and Stockbridge (personal communication) have observed that spontaneous CC cell spikes recorded intrasomatically are noticeably longer in duration than somatically recorded spikes in many other neurons of the barnacle. Our recordings of spontaneous or evoked spikes in the region of the CC cell's antennular nerve arborization reveal that the CC cell's extracellular spike exhibits similar broadness, being 2–3 times the duration of typical non-CC cell spikes in the nerve (Fig. 6*B*).

The rising phase of the action potential has been shown to have both a Na and Ca component (Stockbridge and Ross, 1986). Figure 6*C* presents a typical action potential elicited in normal saline by a short intracellular current pulse. When the

Ca in the saline is replaced by Co, the cell still gives an impulse; when 4-AP is added to the Co saline to block outward current (see below), this impulse is prolonged into a depolarization lasting several seconds (Fig. 6*D*). In Co/4-AP saline, we have also observed spontaneous depolarizations of very long duration (20–30 sec). The prolonged depolarization elicited by a current pulse is reversibly abolished by TTX (Fig. 6*E*), as are the spontaneous depolarizations. These results indicate that the prolonged depolarizations, elicited or spontaneous, are due to a Na current (since Co is replacing external Ca) that must inactivate extraordinarily slowly or not at all.

The addition of TTX to the saline frequently leads to a hyperpolarization (approximately 10 mV in the cell of Fig. 6*C–E*; see also Fig. 7*A*). A likely explanation of this observation is that a TTX-sensitive current contributes to the resting potential (see below). We localize this current to the CC cell itself (and not to a TTX-sensitive tonic presynaptic input to CC cell) since the current can be demonstrated in CC cells when synaptic transmission has been blocked by Co (cf. the current-clamp experiment of Fig. 6*C–E* and voltage-clamp experiments described below; see Fig. 9). As would be expected if the TTX-blockable current is carried by Na, reduced (50%) Na also hyperpolarizes the cell (Fig. 7*B*). Prior to complete block of the spikes either by TTX or reduced Na, a gradual decline in the spontaneous rate of spiking frequently occurs.

Outward A-currents

The somata of CC cells were voltage clamped (with a single electrode) with the aim of observing the TTX-blockable current predicted from the current-clamp experiments of Figure 6. In voltage clamp, the most obvious current evoked by moderate depolarizations (to -30 or -40 mV) is an early transient outward current (Fig. 8), which is similar to the A-current described in molluscan neurons (Connor and Stevens, 1971; Neher, 1971; see review by Thompson and Aldrich, 1980). Depolarizations to more positive potentials evoke a strong inward current lasting 10–20 msec (which cannot be controlled by the clamp) and a

Figure 5. Stimulation of CC cell with an intracellular electrode causes dramatic changes in ultrastructure of its processes compared with those from its unstimulated twin (*D*). All sections are longitudinal. The cell was stimulated for approximately 30 min at 0.5 Hz with 200 msec trains of 20 msec depolarizing pulses (10–20 nA; 20 msec interpulse interval) to mimic a typical spontaneous bursting pattern. All scale bars, 250 nm. *A*, Representative varicosity of the stimulated cell showing diminished numbers of large dense-core vesicles and increased incidence of clusters of small clear vesicles (arrow). *B*, Profile from the stimulated cell showing large, relatively clear vesicles of a diameter comparable to the dense-core vesicles. These large clear vesicles are rarely seen in unstimulated preparations (cf. Figs. 3 and 5*D*). *C*, Varicosities of the stimulated cell often show membrane-bound cisternae (arrow). *D*, Representative dense-core vesicle profile from the contralateral antennular nerve, containing the arbor of the unstimulated CC cell.

Figure 6. Recordings of spontaneous and evoked responses in current clamp. All intracellular recordings were made intrasomatically. *A*, Example of the spontaneous repetitive bursting, punctuated by IPSPs, seen in many CC cells. *B*, Injection of a brief intracellular depolarizing pulse evokes an intrasomatic spike (*lower trace*) and a resultant extracellularly recorded spike in the contralateral antennular nerve (*upper trace*). The extracellular CC cell spike is significantly longer in duration than a typical non-CC cell spike recorded just subsequent to it. *C*, Action potential evoked in normal saline by a brief current pulse. *D*, In the presence of 20 mM Co (Ca-free) and 5 mM 4-AP, the peak of the action potential is significantly reduced, and a prolonged plateau response is elicited (note change in time scale from *C*). *E*, Plateau potential is abolished when 1 μ M TTX is added to the Co, 4-AP saline. Note that the membrane potential becomes less negative in Co/4-AP saline and more negative in TTX. Stimulus was 7 msec and the same amplitude in *C*–*E*.

TEA-sensitive current which contributes to the net outward current (data not shown) and to the repolarization of the action potential (Stockbridge and Ross, 1986).

4-AP reversibly blocks the early transient outward current in CC cells (Fig. 8*A*) as it does the A-current in molluscan neurons (Thompson, 1977). Like the molluscan A-current, the transient outward current in CC cells may be inactivated: depolarizing steps to a fixed voltage from reduced (depolarized) holding potentials result in inactivation of the outward current (Fig. 8*B*), which is complete when the cell is stepped to -40 mV for 1 sec or longer.

Na current

To attempt to isolate the Na current, we added 4-AP to the saline to block the A-like current and Co to block inward Ca currents. In this saline, depolarizing steps still evoke net outward current (Fig. 9*A*). When TTX is added to this saline, the net outward current increases, indicating that the TTX is blocking an inward component of the current (Fig. 9*A*). Subtraction of the current obtained in the presence of TTX from that obtained in its absence reveals the isolated inward TTX-sensitive current

(Fig. 9*B*). This current sags slightly during the pulse, perhaps due to a very slow inactivation process or to a slow increase in I_K .

The dependence of the persistent TTX-sensitive component on voltage (Fig. 9*C*) was determined from a family of currents measured at "steady state" just before the end of the 1.5 sec pulse. This current activates in this case at approximately -60 mV, a value negative to the normal resting potential of the cell (-40 to -50 mV), and consequently is partially activated at rest. While values of activation voltage varied from preparation to preparation, due either to real variation or to a difference in the adequacy of the space clamp, this observation is consistent with that of Figure 7*A* showing that TTX hyperpolarized the cell. Such a hyperpolarization would be expected if a steady current was blocked by the TTX.

When $[Na]_o$ is reduced by 50%, the amplitudes of the persistent currents in a family evoked by depolarizing voltage steps are reduced (Fig. 10). A rough estimate of E_{Na} in normal saline and in 50% Na was made by extrapolating the current-voltage relations taken in the 2 salines. In 50% Na there was an approximately 20 mV reduction in E_{Na} , a value close to 17 mV

Figure 7. Effect of TTX (*A*) and 50% Na (*B*) on membrane potential of the CC cell. *A*, Partial, reversible block of activity in the CC cell with TTX leads to a hyperpolarization of the CC cell (here 3–4 mV; cf. Fig. 6*C–E*). Block was achieved by a 30 sec pulse of TTX (1 μM) added to normal saline. The spontaneous action potentials were truncated by the chart recorder and therefore the trace does not reflect their true amplitude. *B*, 50% Na (substituted with Tris) also hyperpolarizes (5–6 mV) the CC cell. *A* and *B* are from separate cells.

calculated from the Nernst relation. We conclude that the persistent inward current is carried by Na since it is reduced in 50% Na, is sensitive to TTX, and persists in Co.

The persistent Na current may also be seen in normal saline

(no added Co or 4-AP) by subtracting currents taken in the presence of TTX from those taken in its absence (Fig. 11, *A* and *B*). Small depolarizing steps were used to minimize activation of the initial fast inward currents and also the early transient

Figure 8. Voltage-clamp records demonstrating the outward current, resembling molluscan A-current, evoked by depolarizing voltage steps. *A*, Depolarizing voltage steps (lower traces) activate an early outward transient current that is reversibly blockable by 5 mM 4-AP. Stronger depolarizing steps than those shown here elicit a pronounced inward current, blockable with Co and TTX. *B*, Stepping to a fixed voltage from increasingly depolarized holding potentials results in inactivation of the I_A , which is complete at approximately -40 mV (not shown).

Figure 9. TTX-sensitive, persistent inward current demonstrated by subtraction of currents in the presence of TTX ($1 \mu\text{M}$) from those in its absence. Co (20 mM) and 4-AP (5 mM) were added to the salines to block Ca and early transient outward current. *A*, Currents (upper traces) evoked by voltage steps (lower traces) in the absence and presence of TTX. The net outward current observed in saline containing Co and 4-AP increased when TTX was added. When hyperpolarizing voltage steps were applied, there was little difference between the evoked inward currents in the two salines. Measurements of steady-state currents were made late in the pulse (indicated by arrowhead). *B*, TTX-sensitive, persistent inward current obtained by subtraction of the net outward currents in *A*. *C*, Current-voltage plot for steady-state currents obtained from the experiment shown in *A*. V_h indicates holding potential.

outward current. The current-voltage relation (Fig. 11C) shows that this current activates below -50 mV in normal saline and is therefore finite at the normal resting potential.

Discussion

Two main points are made by the work presented in this paper. First, ultrastructural observations indicate that CC cells are neurosecretory and that they release material, packaged in dense-core vesicles, into the peripheral (antennular) nerve in which they arborize. Second, voltage-clamp experiments reveal a Na conductance that is essentially noninactivating and has a voltage threshold negative to the resting potential. Because this conductance is finite at the resting potential, it participates in controlling the level of activity of the cell: When it is partially blocked with TTX or when E_{Na} is made less positive by reducing $[\text{Na}]_o$, the cell hyperpolarizes and changes its firing frequency.

The function of the CC cell

Is the CC cell neurosecretory?

Discoveries of recent years have blurred the distinction between a neuron making contact with a postsynaptic target and the classic neurosecretory cell, which releases a (usually aminergic or peptidergic) messenger that affects target cells some distance away (see review by Dismukes, 1979, and appended commentaries). In particular, neurosecretory-like "neuromodulatory cells" may not make a synapse or release messenger into the

general circulation, but instead release a molecule that has an immediate, local influence on a number of target cells (Orchard, 1982; Evans and Myers, 1986). The salient features of the "neurosecretory" cell remain, first, its lack of a clear postsynaptic target and, second, its large and abundant DCVs. In these respects the CC cell is a neurosecretory cell.

Figure 10. Current-voltage plot for steady-state currents in saline with normal extracellular Na and in saline with extracellular Na reduced to 50% (replaced by Tris). Co and 4-AP were present in both salines to block Ca and early transient outward current. The TTX-sensitive current was decreased in the 50% Na saline. Isolation of the inward Na current was accomplished by subtraction as in Figure 9. Measurements were made near the end of the 1.5 sec pulse.

Figure 11. TTX-sensitive persistent current demonstrated by subtraction of currents evoked in normal saline (NS) and TTX ($1 \mu\text{M}$). **A**, Pair of identical depolarizing and hyperpolarizing steps (*lower traces*) evoke currents in NS and TTX. Note the increase in the net outward current in TTX compared with NS for the identical depolarizing steps. Downward current deflections before and after the voltage steps are spontaneous events. Partial activation of the early transient outward current is indicated by the sagging current records. **B**, Computer subtraction of the TTX current record from the NS current record for the depolarizing steps shown in **A**, showing the TTX-sensitive current. **C**, I/V relation for a family of voltage steps including the records shown in **A**. Current values were measured in steady-state just before the end of the step. V_h indicates holding potential.

The output processes of the CC cells

Effect of arbor geometry on the propagating impulse

It was consistently observed that the extracellularly recorded action potential of the CC cell was considerably broader than other units in the antennular nerve. The cell's extensive meshwork of processes in the nerve might account in part for this observation: The extracellular electrode would be expected to record currents not only from the main processes but from smaller neurites that might conduct impulses out from the main neurite more slowly. The Ca component of this impulse is also inherently broader than the Na component in this and other cells (Lewenstein, 1983; Ross et al., 1986; Stockbridge and Ross, 1986); if it were to constitute the major component of the axonal spike, it would also be expected to contribute to the spike's broadness.

The highly branched, varicose axonal tree of the CC cell presents a load to an impulse propagating down the axon which could potentially cause impulse failure. Ross et al. (1986) have presented evidence for a high density of Ca channels in the soma, neuropilar and primary axonal processes of the cell that seems to aid the impulse in propagating despite this load. A high density of Ca channels might also be expected to occur out in the cell's arborization because of the numerous release sites

present there. Stockbridge and Ross (1986) have demonstrated that a propagating Ca spike is the functional correlate of this high Ca channel density, and it is interesting, as they have noted, that one of the few other instances of a propagating Ca spike also occurs in an identified neurosecretory cell (Orchard, 1976).

Release of substances from the DCVs at the varicosities

The varicosities of the unstimulated CC cell were in many respects similar to those described for another well-studied invertebrate neurosecretory system, the bag cells of *Aplysia* (Haskins et al., 1981). In both cases DCVs are packed densely in the varicosities, and often appear to be arranged in linear trains as though traveling along distinct intracellular channels, perhaps guided by cytoskeletal elements; omega figures suggestive of possible release sites were seen on rare occasions in each cell type.

The changes in ultrastructure of the CC cell's varicosities seen with stimulation are typical of regions of cells undergoing exocytosis and are similar to those seen in other stimulated neurosecretory cells (e.g., Haskins et al., 1981; see review by Nordmann, 1983). DCVs are reduced in number, and microvesicles, DCV-sized electron-lucent vesicles, and larger cisternae become prominent.

We do not know whether an actual numerical increase in the

microvesicles has occurred or if the clustering simply represents a redistribution of the vesicles. Microvesicles may serve to buffer $[Ca]$, by Ca accumulation and extrusion following stimulation-induced depolarization (Nordmann and Chevallier, 1980; Shaw and Morris, 1980; see review by Nordmann, 1983).

It has been suggested (Nordmann and Morris, 1976; see review by Nordmann, 1983) that an increase in the occurrence of DCV-sized electron-lucent vesicles following stimulation might be associated with endocytotic membrane retrieval. Neurosecretory cells, unlike most neurons, are thought not to recycle endocytosed membrane to form new secretory vesicles in the terminal (Winkler, 1977), but rather to transport this membrane back to their somata, where it is either recycled or broken down (Theodosis, 1982, 1983). It is possible that the large electron-lucent vesicles we have observed, particularly in the larger diameter processes, may represent such retrogradely transported membrane.

Heuser and Reese (1979) have noted that large cisternae (similar to those we have observed here) become more abundant in preparations intensely stimulated for prolonged periods. Their precise role in endocytosis, however, remains unclear.

Conductances in the CC cell's membrane

Possible effect of K and Na conductances on the cell's activity

Most cells of the barnacle's supraesophageal ganglion have a distinctive pattern of impulse and synaptic activity when impaled by a microelectrode. In contrast, the activity patterns of CC cells are quite variable and include near quiescence, steady firing (Fig. 7), or firing in a bursting pattern (Fig. 6A), depending on the cell. The cell possesses at least two conductances, the transient outward current and the persistent Na current, which are in a position to influence the firing of the cell because they are likely to be partially activated at the cell's resting potential of -40 to -50 mV.

Transient outward currents with properties similar to the one we report here have been described in a number of preparations (see review by Thompson and Aldrich, 1980) and in particular in certain invertebrate neurons, where they are important in determining the duration of the interspike interval in repetitive firing (Conner and Stevens, 1971; Conner, 1978). The transient outward current described here is similar to the molluscan A-current in that depolarization to a particular potential causes the current first to activate and then to inactivate. Like the A-current, this current is also blocked by 4-AP. In many respects, our current is similar or identical to the A-current seen in other organisms; however, detailed kinetic studies and demonstration of K ion dependence remain to be done to make this identification certain.

Persistent (or slowly inactivating) Na conductances have now been described in a diversity of neurons: stretch receptors (Grampp and Sjölin, 1975; Gestrelus et al., 1981); giant neurons of *Aplysia* (Colmers et al., 1982); frog (Dubois and Bergman, 1975), squid (Shoukimas and French, 1980; Matteson and Armstrong, 1982), and cockroach axons (Yawo et al., 1985); cerebellar Purkinje neurons (Sugimori and Llinás, 1983); cortical (Stafstrom et al., 1985), neocortical (Connors et al., 1982) and thalamic neurons (Jahnsen and Llinás, 1984); hippocampal pyramidal cells (French and Gage, 1985), and even frog muscle (Almers et al., 1983). In many of these cases, the cells either fire spontaneously in repetitive fashion or can be induced to fire repetitively when stimulated. In most of these reports it has

been suggested that the activation of a persistent Na conductance could be important in controlling the rate of repetitive firing.

Since TTX or 50% Na changed the firing rate of an active CC cell, the persistent Na conductance may play a similar role in this cell type. This function is reasonable for the CC cells since the persistent Na current, partially activated at rest, is strongly activated in the range of membrane potentials where repetitive firing occurs.

In cells that burst, the depolarizing phase of the bursting pacemaker potential has been attributed to persistent, slowly inactivating I_{Na} or I_{Ca} or both (Johnston, 1976; Barker and Smith, 1978; Gorman et al., 1982). It is possible that CC cells rely on inactivation of the I_A and/or activation of the persistent Na current for the generation of the depolarization underlying their bursts. The current(s) responsible for repolarization of the depolarized burst potential in CC cells has not yet been determined.

Evidence for a diversity of Na channels is now beginning to appear based on differential sensitivity to TTX and other neurotoxins, immunological studies, etc. (Barchi, 1987). Na channels with fast (a few msec), slow (msec-sec), and ultraslow (min or more) inactivation have been described (e.g., Hodgkin and Huxley, 1952; Rudy, 1981; Almers et al., 1983), but these channels are not necessarily different types: Patlak and Ortiz (1986) have found that the same Na channel in frog muscle can give rise to transient early currents and to persistent or "late" currents.

In some preparations, persistent Na currents are partially activated at rest (e.g., French and Gage, 1985, and in CC cells), while in others, the activation voltage for the current seems to be at a somewhat depolarized value with respect to the resting potential (e.g., Stafstrom et al., 1985). Differences in threshold would be expected to produce differences in the precise function of the current. In the squid axon, a small population of Na channels that activate at low threshold but have slow closing kinetics (relative to the classical fast channels) has been described (Gilly and Armstrong, 1984); it has been suggested that their function may be to trigger the action potential. These "threshold channels," though only a small percentage of the total Na permeability, may thus play an important role in the control of firing frequency. Low-threshold Na channels, particularly if they are active at rest and do not inactivate over seconds or minutes, would seem to be in a unique position to control the cell's firing pattern particularly for long-duration burst periods. It is an intriguing possibility that neurotransmitters or neuromodulators could conceivably regulate this channel and thus exert dramatic effects on the activity of the cell (see Barker and Smith, 1978).

Might the persistent Na current affect release?

A number of studies indicate that an increase in $[Na]_i$ can augment transmitter release by inducing translocation of Ca from internal stores, thereby increasing $[Ca]_i$ (Lowe et al., 1976; Lev-Tov and Rahamimoff, 1980; Melinek et al., 1982; Atwood et al., 1983). Perhaps the persistent Na conductance of this cell leads to a higher $[Na]_i$ and, through translocation, a higher resting $[Ca]_i$, which increases the effectiveness of the Ca that enters during the action potential. Although we have no evidence that the persistent Na channel is located at the release sites in the CC cell, recordings from peptidergic neurosecretory terminals in the sinus gland of crustaceans suggest the presence of channels similar to those described here (see Fig. 11 in Nagano and Cooke,

1987). A possible effect of the Na current on release should be testable if either the released compound or a cell postsynaptic to the CC cell in the ganglion is identified.

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