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ELECTROPHYSIOLOGICAL CHARACTERIZATION OF PEPTIDERGIC NEUROSECRETORY TERMINALS

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SUMMARY

Electrical activity recorded intracellularly from peptidergic neurosecretory terminal dilatations in the sinus gland of crabs (principally *Cardisoma guanhumi* and *C. carnifex*) is described. Recordings were made from the neurohaemal organ *in situ* on the neural tissue of the isolated eyestalk and from isolated sinus gland-sinus gland nerve preparations. Verification that electrodes penetrated terminals was obtained by dye marking. Resting potentials ranged between −30 and −80 mV. Overshooting action potentials of long duration (5–20 ms at 1/2 amplitude) relative to those of non-secretory axons (<2 ms) were recorded in approximately 70 % of stable penetrations. Action potentials occurred spontaneously at slow ($<0{\cdot}2\,s^{-1}$) rates in 75 % of penetrations. Sequential intra- and extracellular recordings with the same microelectrode, on the same terminal, indicate impulse generation by the terminal itself.

Extracellular stimulation of the axon tract evokes an all-or-none action potential at distinct threshold and latency. At rates of stimulation exceeding $5\,s^{-1}$, discrete fluctuations in the form of responses occur. Similar waveforms occur spontaneously and can be evoked by passing current through the electrode. They are interpreted to be electrotonically recorded activity of other parts of a complex axonal terminal arborization. Some, but not all, terminals exhibit impulse broadening (up to three-fold at 1/2 amplitude) during repetitive firing exceeding $1\,s^{-1}$. The same terminals show reduced impulse duration with hyperpolarization and broadened impulses with imposed depolarization. The changes are due to altered repolarization rates. Terminals sustain steady impulse firing at rates (up to $5\,s^{-1}$) linearly related to the imposed depolarizing current.

Regenerative potentials, though of reduced rate of rise and amplitude, can be evoked by depolarizing current passed through the electrode during perfusion with salines having 1/2 normal [Na^+], or containing tetrodotoxin (10^{-6} mol l^{-1}). However, these block axonal conduction. Nominally Ca-free saline causes increased spontaneity and depolarization

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of about 5 mV in half the preparations examined, but reaching 20 mV in the others, with resultant inactivation of regenerative activity. Impulses in low-Ca saline show alterations of the falling phase, it being faster initially and then slower than normal. Thus, while the action potentials of neurosecretory axons are Na dependent, the terminals support regenerative impulses mediated by both Na and Ca.

INTRODUCTION

Intracellular recordings from axon terminal dilatations of peptidergic neurosecretory cells of the crab eyestalk are described in this paper. Direct intracellular recording of electrical events at or near nerve terminals engaged in chemical synaptic transmission is available for a number of preparations, e.g. the giant fibre synapse of the squid stellate ganglion (Hagiwara & Tasaki, 1958; Katz & Miledi, 1967*a*, 1969*a*; Llinás, Steinberg & Walton, 1981*a*,*b*), the chick ciliary ganglion (Martin & Pilar, 1964), barnacle photoreceptors (Ross & Stuart, 1978), lobster neuromuscular junctions (Niwa & Kawai, 1982) and crayfish neuromuscular junctions (Wojtowicz & Atwood, 1984). However, none of these terminals is known to involve secretion of peptides.

The neurosecretory system of the crab eyestalk, known as the X-organ-sinus gland (XOSG) system (e.g. Bliss & Welsh, 1952), is anatomically discrete and easily visualized on the optic ganglia of the eyestalk by reason of the iridescent, white appearance of the tissue attributable to refraction from neurosecretory granules (Maynard & Maynard, 1962). The terminals are aggregated around a blood sinus to form an anatomically well-defined neurohaemal organ, the sinus gland (SG). The somata contributing at least 90 % of the terminals form a distinct cluster of about 150 cells, the X-organ; their axons form the sinus gland nerve. There may be up to a dozen axons of neurosecretory neurones, whose locations are undetermined, which join the SG nerve *via* the optic peduncle to terminate in the SG (Potter, 1956).

The XOSG system is the major crustacean neuroendocrine centre for control of diverse physiological functions (e.g. moulting, blood sugar levels, dispersion of hypodermal and retinal pigments; for reviews see Cooke & Sullivan, 1982; Keller, 1983). The system appears to be purely peptidergic; all hormonal factors that have been characterized have proved to be peptides. Of importance in evaluating the recordings presented here is morphological evidence, from both light (Potter, 1956, 1958; Rehm, 1959) and electron-microscopic (EM) studies (e.g. Bunt & Ashby, 1967; Andrews, Copeland & Fingerman, 1971; Smith, 1974; Silverthorn, 1975; Strolenberg, van Helden & van Herp, 1977; Weatherby, 1981), that there are five or six morphologically distinct terminal types. Two types, A and C, predominate in terms of their bulk in the *Cardisoma* sinus gland (Weatherby, 1981). EM studies are consistent with biochemical evidence that secretion from the sinus gland is exclusively peptidergic (Newcomb, 1983; Stuenkel, 1983*a*, 1985) in showing only large (90–310 nm) neurosecretory granules which, though

of several morphological types, are all in a size range and have a morphology (solid or reticular cores) thus far associated with peptidergic secretion.

A number of analogies exist with the vertebrate hypothalmic-neurohypophyseal system, including not only the role of both in homeostatic physiology, but also their roles as intermediaries between the CNS and endocrine function. Studies of secretion of the red pigment (erythrophore) concentrating hormone (RPCH or ECH, Fernlund & Josefsson, 1972) from the isolated XOSG system (Cooke, Haylett & Weatherby, 1977; Cooke & Haylett, 1984) extend the analogy to many details of their secretory physiology. These include: secretion in response to propagated action potentials and elevation of saline $[K^+]$; the dependence of secretion on the presence of Ca; uptake of ^{45}Ca during secretion (Douglas & Poisner, 1964*a*,*b*); 'inactivation' of secretion during sustained exposure to elevated $[K^+]$; and augmentation by reduction of saline $[Na^+]$ (e.g. Nordmann, 1976). The XOSG system of certain species of crabs appears to be unique in containing peptidergic nerve terminals sufficiently large to permit intracellular electrical recording and hence direct observation of the electrophysiological correlates of secretory regimes (Stuenkel, 1985). Electrophysiological characterization of X-organ cells of crayfish is available (Iwasaki & Satow, 1969, 1971; Iwasaki, Satow & Kuroda, 1973; Iwasaki & Ono, 1979). Recordings from the XOSG system of crayfish *in situ* have been briefly described (Glantz, Kirk & Aréchiga, 1983).

A number of the observations collected here have been previously reported in abstracts and review chapters (Cooke, 1967, 1971, 1977, 1981; Cooke & Sullivan, 1982).

METHODS

Animals

Most of the observations reported here were obtained from semi-isolated or isolated sinus glands of the semi-terrestrial crabs, *Cardisoma guanhumi* and *C. carnifex*. Limited observations on other species were also made and are sufficient to suggest that the observations may apply to the description of crab sinus glands generally. These include: *Grapsus grapsus, Ocypode ceratophthalmus, Gecarcinus lateralis, Portunus sanguinolentus, Scylla serrata* and *Podophthalmus vigil*. Except for the last two listed, the generally small size of terminal dilatations precludes routine intracellular recording from them in these species. This was the case with many other crustacean species examined.

Cardisoma (7–9 cm carapace width, 200–300 g) were collected locally (Bermuda) or air shipped (males only, *C. guanhumi* from Puerto Rico; *C. carnifex* from Fanning Island, Line Islands). They were held in a humidified room or in outdoor cages, provided with both fresh and sea water and fed Purina rat chow. Under these conditions, they survived indefinitely (observations to 1 year), but showed no evidence of preparation to moult.

Dissections

Semi-intact and isolated preparations of the sinus gland were utilized. An eyestalk was removed, the carapace cut, and the tissue removed under saline. Under the dissecting microscope, all muscle tissue and most of the superficial spongy connective tissue were removed. The sinus gland was then clearly visible as an iridescent, white structure about 1 mm in diameter, together with the initial portion of the sinus gland nerve on the surface of the medulla terminalis. The preparation was then moved to the perfusion chamber, pinned with cactus spines, and any remaining connective tissue overlying the sinus gland and the initial 1 mm of the sinus gland nerve was removed. This constituted the 'semi-intact' preparation. For the isolated preparation, dissection was continued to separate the sinus gland from the underlying ganglion, and the nerve was isolated by teasing the tissue from around it. In a few cases, the dissection was continued to also isolate the cluster of X-organ somata. Care was taken not to stretch the nerve and not to bring the preparation through the saline-air interface.

Electrophysiological arrangements

Intracellular recordings were made with non-filament-containing glass microelectrodes filled with 3 mol l^{-1} KCl having resistances of at least 40 MΩ. A preamplifier equipped with an active bridge circuit which ensured constant current despite alterations in electrode resistance (WP Instruments M4) permitted current passing and recording through the electrode. Current was monitored by means of a 100 KΩ resistor between a saline-KCl-Ag-AgCl bath electrode and ground. A saline-filled pipette, having a tip diameter of approximately 100 μm, equipped with internal and external chlorided silver wires was placed against the sinus gland nerve for extracellular stimulation of the neurosecretory axons. In isolated nerve-sinus gland preparations the proximal nerve stump was drawn into such a pipette. The viability of semi-intact preparations was monitored by recording from the proximal stump of the optic peduncle with a suction electrode. Preparations from which successful terminal recordings were obtained invariably showed multi-unit, spontaneous activity recorded from the optic peduncle. The same electrode was used, when needed, to stimulate the optic peduncle. In addition to the photographic recording from oscilloscopes, membrane potential and current were recorded with a two-channel penwriter. In some experiments, an FM tape recorder (Ampex FR 1300) was used utilizing a bandwidth of 5 kHz.

Salines

In the absence of a published saline for *Cardisoma*, the following saline, adapted from Dalton (1958) was utilized in the majority of experiments: (in mmol l^{-1}) Na, 470; K, 17·6; Ca, 25; Mg, 17; Cl, 551; SO_4, 8·8; buffered to pH 7·6 with HBO_3-NaOH, 9, or with HEPES, 2. Experiments with a number of alternative salines showed little sensitivity of the preparations to changes of K (10–20 mmol l^{-1}), Ca (13–25 mmol l^{-1}), the buffer utilized, nor to the osmolarity (diluted by 1/3).

Salines of altered K or Ca concentration were made by osmotically equivalent adjustment of NaCl. For reduced Na salines, the NaCl was substituted with Tris-Cl.

Following initial dissection, all preparations were kept under continuous perfusion in a chamber having just sufficient volume to ensure complete immersion of the tissue. A valve permitted introduction of altered salines without disruption or changes in the rate of flow. Flow rates were between 0·6 and 1·2 ml min^{-1}. Experiments were done at room temperature (21–25 °C). In the majority of experiments with altered salines, the perfusate passed through a heat exchanger equilibrated with running tap water (21 °C) just prior to entering the chamber.

RESULTS

Microelectrodes were directed primarily to spherical structures ('terminals') visible under the dissecting microscope at the surface of the sinus gland. Penetration was usually marked by sudden development of a resting potential and 'spontaneous' impulse firing. Injury was evidenced in some cases by a gradual decline in the potential accompanied by accelerating firing and diminishing impulse amplitude. The electrode, when raised, sometimes brought the impaled terminal with it, sometimes with continuing but waning impulse firing. Impulses recorded from sinus gland structures were distinguished by their long duration: from 5 to 20 ms at half amplitude. This is at least three times longer than the duration of impulses which were recorded from axons of the optic peduncle of the same preparation in several initial experiments. A majority (approx. 70 % in all) of terminals in which stable penetrations were made (over 100 for this study) showed overshooting (up to +40 mV) impulses. Except in terminals with very high resting potentials (more negative than −60 mV) impulses were followed by hyperpolarizing afterpotentials of up to 10 mV which returned to the baseline over hundreds of milliseconds. Resting potentials of units showing overshooting action potentials ranged from −30 to −80 mV. Over half (57 %) had resting potentials of −50 mV or more polarized, and a quarter were in the range of −50 to −60 mV. Morphological heterogeneity of terminal dilatations and evidence, to be described, of variability in electrophysiological behaviour make it likely that variation of resting potentials exists in the terminal population. Given variations in trauma produced by microelectrode penetration, effects of variable diffusion of KCl from the electrodes, and an artificial saline, the degree to which the observed resting potentials reflect normal values for the terminal population cannot be stated with confidence.

Evidence that recordings are from terminals

The structures penetrated with microelectrodes were localized by injection of potassium-ferricyanide and development with the Prussian Blue staining reaction. Comparison of histological sections of the marked structures (Fig. 1) with EM sections (Weatherby, 1981) indicates that they were major dilatations from

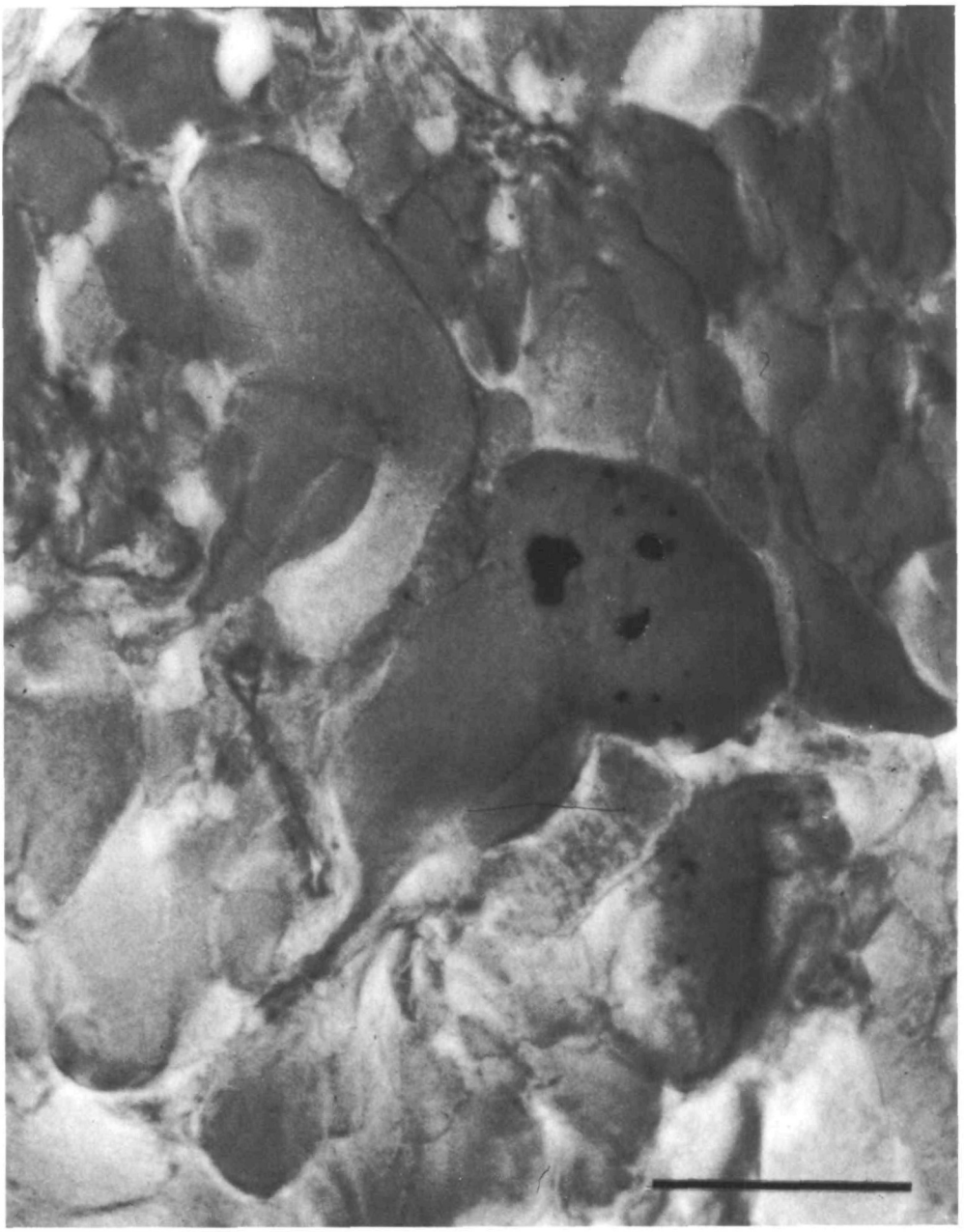

Fig. 1. Intracellularly stained terminal dilatation in the sinus gland of *Cardisoma guanhumi*. Dark material represents Prussian Blue precipitate marking the site of current passed from a microelectrode filled with potassium ferricyanide. Paraffin section stained with haemotoxylin-eosin. Note slender axonal process giving rise to the terminal. Scale bar, 25 μm. Histology by M. W. Goldstone.

much smaller axonal processes. The largest dilatations observed in EM sections have granules identifying them as type A or C (Weatherby, 1981). Indeed, the morphological studies reveal no structures in the sinus gland, other than granule-filled profiles, large enough to sustain penetration with a microelectrode. The electrophysiological behaviour, to be detailed below, is consistently interpretable as representing responses of an enlarged dilatation which is part of (or the termination of) a series of swellings along the branched course of a fine axon. Such a morphology has been observed by intra-terminal injection of Lucifer Yellow (Nagano & Cooke, 1983; M. Nagano, in preparation).

When a stable penetration was obtained, responsiveness of the unit to extracellular stimulation of the axon tract was tested. All of the units further characterized (over 90 for this study), responded at a distinct threshold with an all-or-none action potential. Such responsiveness excludes the possibility that a structure underlying the sinus gland might have been penetrated.

An occurrence, observed too frequently to remain unreported, is that as the intensity of the stimulus applied to the axon tract was gradually increased, the penetration was suddenly lost. This apparently mechanical response, with a threshold similar to that for eliciting action potentials in other terminals penetrated in the same preparation cannot be attributed to excitation of muscle by current escape, since all muscles were removed from the preparation.

In a number of isolated nerve and sinus gland preparations, microelectrode penetrations were obtained which were clearly not from terminals. These were distinguished by a substantial and stable negative potential (-70 to -80 mV) with no spontaneous activity observable. Current passing through the electrode indicated that the electrode was not blocked, and that the potential could not be altered by reasonable currents (up to 10^{-8} A). Tests for responsiveness to stimulation of the axonal tract showed, in some cases, a minute (1–5 mV) depolarizing response having the latency and time course of similarly evoked terminal impulses. These 'unresponsive' recordings were encountered with relatively deep penetrations, and were much less sensitive to adjustments of the electrode position than were terminal penetrations. In a given preparation, if one such penetration was encountered, it could be repeated numerous times. Efforts made to inject fluorescent dyes (Procion Yellow, Procion Rubine and Lucifer Yellow) following such penetrations were without success in achieving visualization of any structure. A suggestion for the interpretation of these observations is that the electrode was in a portion of the system of internal blood sinuses; through haemolymph clotting they could have become sufficiently isolated to have developed an electrochemical potential with respect to the external perfusing saline.

Terminals support action potentials

Evidence that the terminals themselves undergo regenerative impulse activity (rather than merely reflecting electrotonically conducted axonal action potentials) was obtained in several (6) recordings. A small dislocation of the electrode

resulted in loss of the resting potential and a change in the spontaneous activity from overshooting action potentials to small hyperpolarizing potentials. Such recordings are most easily interpreted as representing extracellularly recorded action potentials being generated by the previously penetrated terminal. The small distance over which the extracellular current of an action potential can be recorded and the small diameter of the axon (and the current it produces) relative to the terminal argue forcefully that the extracellularly recorded potentials represent impulses generated by the terminal.

Other evidence which also supports the conclusion that terminals support regenerative action potentials can be briefly summarized at this point: (1) overshooting impulses are followed (except in units having very high resting potential) by hyperpolarizing afterpotentials of slow time course; (2) axon-terminal invasion delays and failure are observed during repetitive axonal stimulation (see further below); (3) all-or-none block of terminal invasion of an axonally initiated impulse can be produced by passing hyperpolarizing current in the terminal; (4) perfusion of reduced-Na saline or tetrodotoxin (TTX) blocks terminal responses to axonal stimulation but leaves regenerative responses to depolarizing current passed through the recording electrode (see below). I thus conclude that the terminals themselves support regenerative action potentials.

Spontaneous electrical activity

Initial studies were made on preparations of the entire eyestalk nervous system, only overlying muscle and connective tissue having been removed. In later experiments the sinus gland and a length of sinus gland nerve were isolated. A further series utilized dissection of the entire neurosecretory system: the X-organ (somata), sinus gland nerve (axons), and sinus gland (terminals). With the exception of the appearance of bursting in the latter, the types of spontaneous or evoked activity recorded did not differ in any obvious way among these preparations.

A description of 'spontaneous' electrical activity must be prefaced by noting the difficulty of evaluating the degree to which dissections, an arbitrary saline composition, and the microelectrode penetration have influenced it. All but 5 of about 150 penetrations in sinus glands of several crab species showed impulse-like activity upon penetration and 70 out of 109 showed continuing spontaneity after a steady baseline was present.

A number of examples (12) were recorded in which there was a shift of resting potential, usually from a relatively low value (less polarized than -50 mV) to one 20–30 mV more polarized. Such shifts occurred most often from 5 to 30 min after penetration and may represent sealing of terminal membrane around the electrode. Spontaneous firing usually ceased with the increase in resting potential. Two examples of a shift in the depolarizing direction, accompanied by commencement of steady impulse firing, were also recorded, and two examples were noted of repeated slow changes of the baseline accompanied by spontaneous firing at depolarized values and quiescence at a more polarized resting potential. In a

subsequent study utilizing *in vitro*, intact XOSG preparations, various types of patterning of spontaneous impulse firing (bursting) have been observed (Nagano & Cooke, 1981; M. Nagano & I. M. Cooke, in preparation).

Spontaneous firing rarely exceeded $0{\cdot}2\,s^{-1}$. Highly regular firing (see e.g. Fig. 13, 'normal') was associated with pacemaker-like slow depolarization growing from the hyperpolarizing afterpotential of each impulse to initiation of the next. Such activity was most often seen in units with resting potentials less polarized than -45 mV. In view of subsequent observations (Nagano & Cooke, 1983) that in intact XOSGs impulse activity arises in the proximal neurite region, the presence of pacemaker-like potentials may be attributable to injury current. Other units (e.g. Fig. 6B) exhibited irregular spontaneous impulse firing. The lack of prepotentials suggests their propagation from a distant site of initiation.

A number of sinus gland recordings (e.g. Fig. 2) showed the presence of repeated, non-overshooting depolarizations, sometimes of several different and consistent forms, either recurring arythmically, or sometimes (as in Fig. 2) steadily but each at a different rate, and independently of an over-shooting impulse (if present). The possibility that these represent postsynaptic responses was considered but rejected by reason of the following observations.

(a) Passage of current through the recording electrode influenced their rate of occurrence but not their amplitude. Thus, sustained hyperpolarizing current led to their cessation while depolarizing current increased their rate of occurrence.

(b) The effects of Ca-free saline on non-overshooting potentials paralleled that on the overshooting impulse (see below, Fig. 13); spontaneous firing frequency of the impulse was increased and the rate of occurrence of non-overshooting potentials was also increased (though the rates were independent). Their amplitude was decreased only if that of the overshooting impulse was also decreased.

(c) The responses of the terminal during repetitive stimulation of the axon tract (see Fig. 2) would exhibit an overshooting action potential at low frequencies of stimulation, but at frequencies approaching $5\,s^{-1}$ could be fragmented to a variety of waveforms within which those of the spontaneous, non-overshooting depolarizations were distinguishable, at non-varying latency.

(d) Recordings in which the terminal showed several waveforms were obtained from isolated sinus gland and nerve preparations which included no morphologically demonstrable points of synaptic interaction (chemical or electrotonic) (Weatherby, 1981; T. M. Weatherby, personal communication).

An interpretation consistent with these observations draws on morphological studies of the sinus gland (e.g. Potter, 1956; Weatherby, 1981; Nagano & Cooke, 1983) indicating that each axon branches repeatedly and gives rise to a complex series of dilatations with intervening narrowings. An electrode in one dilatation would record, in electrotonically decremented form, regenerative activity in more distant parts of the axonal arborization. Changes in diameter as well as electrical properties would account for 'fragmentation' of a summed response present at low rates of axonal stimulation by increased rates of repetitive stimulation, if various dilatations have slightly differing durations of relative refractoriness.

Differing rates of spontaneous firing in different axonal branches would account for the independent spontaneous rates of various non-overshooting depolarizations observed. Spontaneous independent terminal firing may well result from injury, as it was rarely observed in recordings utilizing bevelled electrodes (M. Nagano & I. M. Cooke, in preparation).

Electrically-evoked activity

Two methods were routinely used to test for regenerative responses: extracellular stimulation of the axon tract, and passage of current through the recording electrode.

Responses to axon stimulation

Single and repetitive stimuli. Stimulation of the axon tract resulted in an overshooting, long duration action potential in most (68 %) stable penetrations (Fig. 2). The appearance of this response had a distinct threshold and a finite latency. A variety of complexities of the waveform in response to near-threshold stimulation are interpretable as invasion delays occurring in the complex terminal arborization. Most frequently observed was a notch on the rising phase interpretable as sequential axon-terminal invasion, analogous to the A-B notch in motor neurone recording. Increasing stimulus intensity in some cases altered the form of the rising phase, causing disappearance of the notch, if present, and reducing the latency between stimulus and peak. Direct stimulation at the recorded terminal may have occurred. It did not alter the extent of the overshoot or the form of the repolarizing phase.

The use of pore or suction electrodes for axon stimulation and uncertainty about the length of axon, due to the tortuous path seen in histological sections, makes measurement of conduction velocities uncertain; they can be placed in the range of 0·1 to 0·25 $m\,s^{-1}$.

Overshooting responses of consistent form could be observed for stimuli repeated at rates up to $5\,s^{-1}$ (Fig. 2). At higher repetition rates, responses fluctuated in amplitude and sometimes showed 'fragmentation', with certain recognizable waveforms recurring repeatedly, as discussed above. In a few preparations, high stimulus rates revealed a non-varying 'residual' response having the form of an electrotonically decremented impulse of short duration (<5 ms at 1/2 amplitude). When present, this response followed stimulus repetition rates up to $20\,s^{-1}$ (higher not tested) without change of shape. This is interpreted to represent the axonal impulse, invasion of the terminal being blocked by refractoriness. The observation indicates that axonal action potentials have a shorter duration than terminal responses. Recordings in axons have now shown this directly (Nagano & Cooke, 1983).

During the initial experiments on the complete eyestalk nervous system, responsiveness of each terminal penetrated and held was tested to stimulation of the optic peduncle as well as the sinus gland nerve. Two preparations showed responses to optic peduncle stimulation. In both examples, single stimuli produced a slow, rounded depolarization of approximately 3 mV. Repetitive

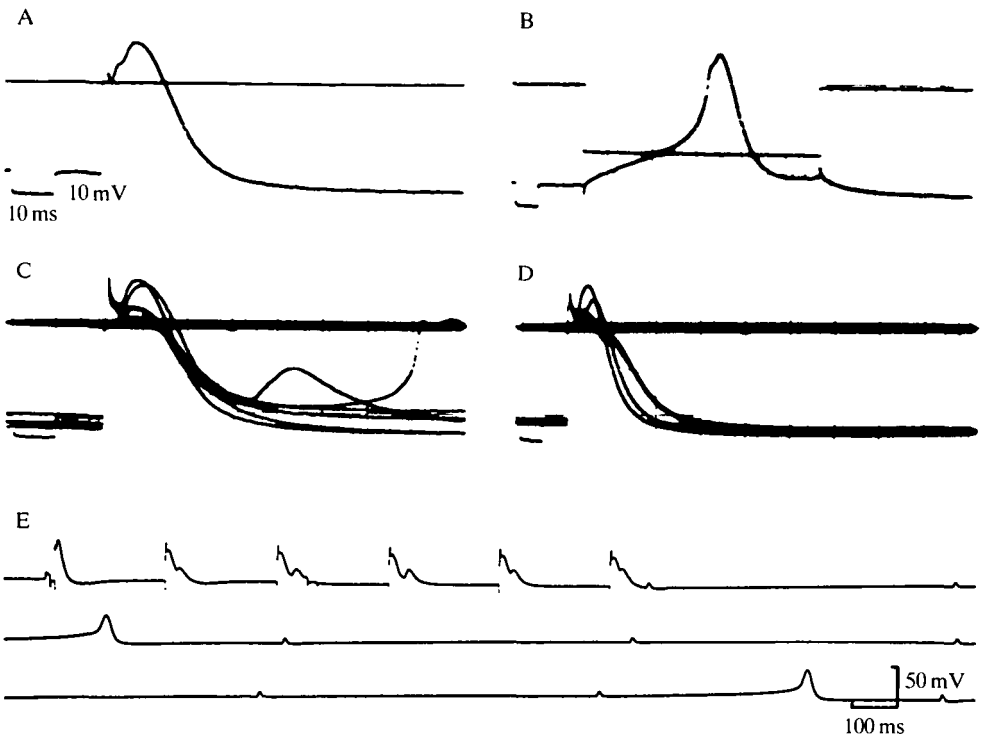

Fig. 2. Complex terminal responses to stimulation. (A) Single stimulus given to sinus gland nerve *via* a pore electrode. Note notch between axonal spike (emergent from stimulus artifact) and double peak of terminal response. (B) Response to depolarizing current (0·8 nA) through the recording electrode. Note similarity of response, including double peak. (C) Superimposed responses to a train of six axonal stimuli at $4\,s^{-1}$. Note three sizes of depolarizing response after initial notch, presence of delayed 'spontaneous' activity. (D) Stimulation as in C displayed at same sweep speed as B (1/2 of that in A and C, current monitor at 0 mV). (E) Unswept spot, moving film recording of responses to a train of axonal stimuli (six at $4\,s^{-1}$, as in C) and ensuing continuing activity (the three lines are continuous, and represent 6·2 s of recording, FM tape playback). Note independent repetition rate of the two waveforms. Different waveforms are interpreted to represent activity recorded electrotonically from different elements of a complex terminal arborization. *Cardisoma carnifex*, isolated X-organ-sinus gland.

stimulation at a slow rate ($0{\cdot}5$–$2\,s^{-1}$) resulted in a series of essentially unchanged responses, but a regenerative, overshooting impulse arose, seemingly at any point of the small response, after three or more stimuli. The observations are consistent with chemically-mediated synaptic activation of the neurosecretory axon and terminal by stimulation of a presynaptic axon in the optic peduncle. However, they are also explainable as direct stimulation of the neurosecretory axon in the optic peduncle, branching and invasion delays being responsible for electrotonically decremented or invading and overshooting responses. Potter (1956) describes, in *Callinectes sapidus*, a small number of axons which join the sinus gland nerve *via* the optic peduncle and thence terminate in the sinus gland.

In the series of recordings reported here, three isolated complete XOSG preparations provided examples of patterned, repetitive firing (bursting). In each case, the repetitive activity continued spontaneously following one or a series of

extracellular stimuli delivered at intervals of 1 s or more to the axon tract. In one terminal the bursting appeared, both spontaneously and following single stimuli, while the preparation was being perfused with saline having three times normal $[K^+]_o$. In these examples, the burst of impulses rides on a sustained underlying depolarization of 15–20 mV from the resting potential.

The records shown in Fig. 3 are interesting for the evidence they provide of a persistent plateau potential. The plateau potential appears to arise in advance of the commencement of repetitive firing and to persist after its cessation. The plateau responses arose spontaneously and could also be initiated by a single stimulus to the axon tract. Later stimuli appear to initiate repolarization.

Numerous examples of spontaneous bursting activity have been recorded from terminals in further studies on complete XOSG preparations (Cooke, 1981; Nagano & Cooke, 1981; Stuenkel, 1983*b*; M. Nagano & I. M. Cooke, in preparation).

Patterned stimuli. Because patterned activity (i.e. firing in bursts with intervening periods of quiescence) has been observed in a number of neurosecretory systems, including this one, I examined the responses of terminals to short trains of repetitive axonal stimulation. Five of this series were recordings from a terminal ('Stella') identifiable, in different preparations of *C. carnifex*, by its superficial location, large size and orange-tinged iridescence. This terminal, as did three of the six others, exhibited progressive broadening of the impulse duration with repetition of the stimulus (Fig. 4). The increase in duration was the result of slowing of the falling phase (see Fig. 10 in Cooke, 1977, also 'Stella'). Electrical differentiation of the rising phase confirmed that the rate of rise and overshoot were unaltered except at the higher repetition rates (approaching $5\,s^{-1}$). In some terminals, broadening was observable at repetition rates as slow as $0{\cdot}1\,s^{-1}$. The extent of broadening (up to a tripling of 1/2 amplitude duration, see Fig. 5) appeared to be a characteristic of a particular terminal, and required, for maximum development, a combination of the number of impulses and the time over which they occurred. Thus, for example, broadening in response to five stimuli at $2\,s^{-1}$ was similar to that observed after 10 stimuli at $4\,s^{-1}$. Return to minimal impulse duration required an unstimulated pause of tens of seconds.

Terminals which exhibited impulse broadening showed changes of the form of impulses when the holding potential was altered by passing steady current through the recording microelectrode; terminals which did not show broadening were less sensitive. The changes took tens of seconds to develop. When the depolarizing current resulted in repetitive firing (see the example in Fig. 5), impulse broadening by slowing of the repolarizing phase occurred, as in response to repetitive axonal stimulation.

Analysis of the effect of altered holding potential is documented for another terminal in Fig. 6. Hyperpolarization (6 mV in this case) resulted in reduced impulse duration as a result of an accelerated repolarization; rate of rise and overshoot were unaltered. Depolarization increased the impulse duration, but reduced the rate of rise and overshoot as well as the rate of repolarization.

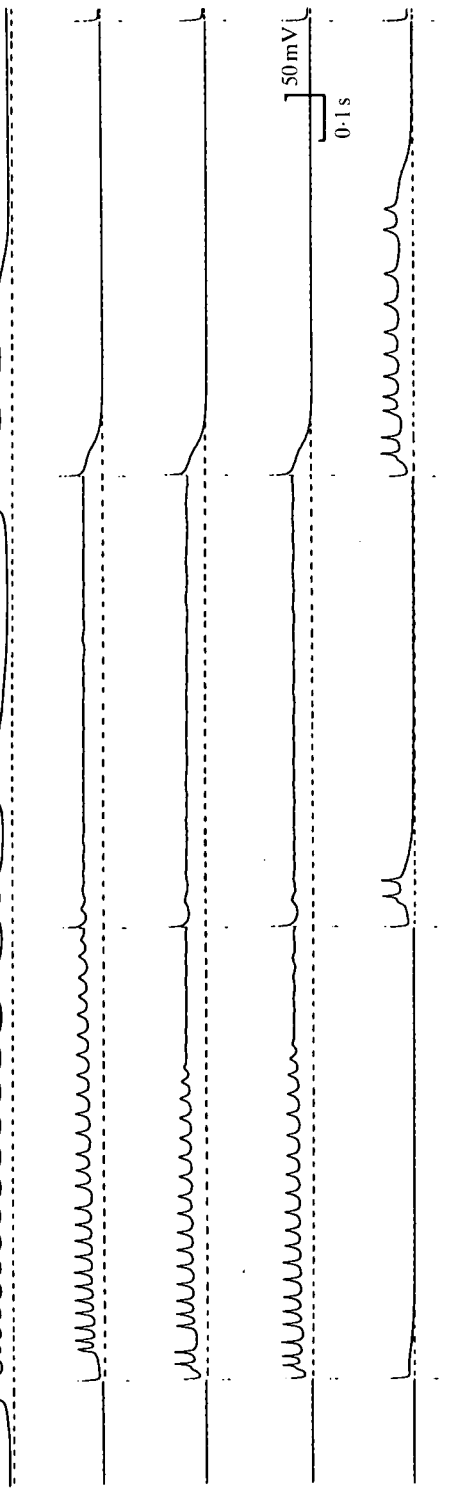

Fig. 3. Spontaneous and evoked plateau responses recorded from a terminal. Top trace: spontaneously occurring plateau with superimposed impulse firing lasting approximately 2·5 s. Remaining traces: responses to brief extracellular stimuli applied to the sinus gland nerve at 1 s^{-1} (artifacts), 0·75 s of the record is omitted between each trace; there were no responses to the stimuli occurring at the end of each trace. Stimuli initiate the plateau and 2 s later appear to terminate it. The stable plateau level is 25 mV depolarized from resting level (broken lines). Replay from FM tape recording. *Cardisoma guanhumi*, semi-intact preparation.

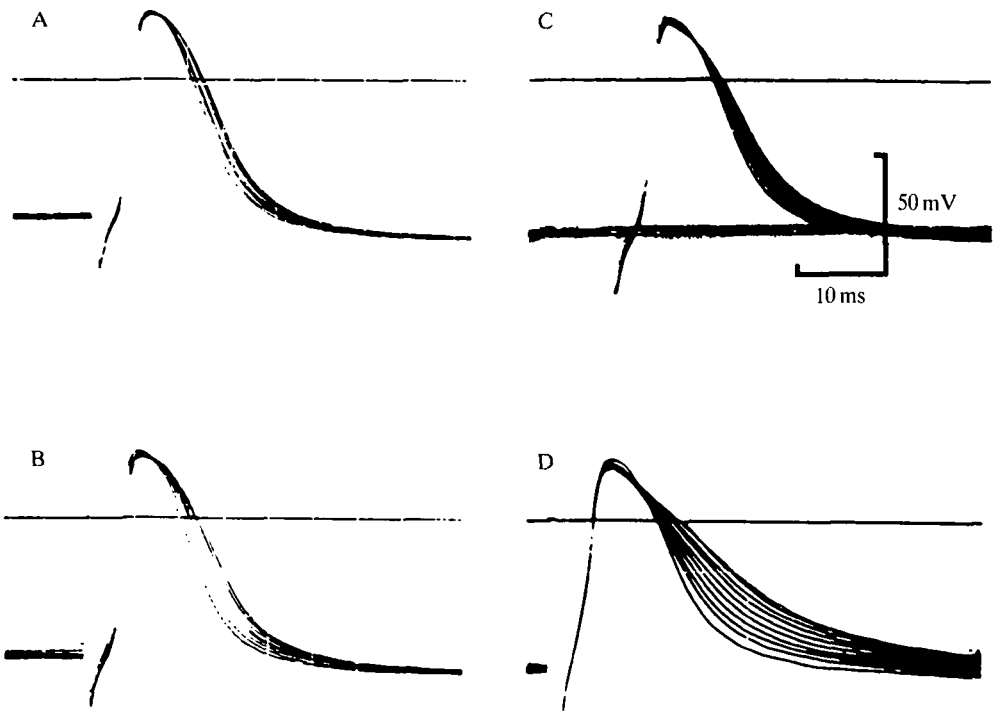

Fig. 4. Impulse broadening during repetitive stimulation. (A) Ten superimposed responses to stimulation of the sinus gland nerve at 0·5 s^{-1}. (B) 10 stimuli at 1 s^{-1}. (C) 10 stimuli at 4 s^{-1}. (D) A sequence of traces recorded during a train of 25 stimuli at 5 s^{-1}. Reference line at 0 mV. From the identifiable terminal, 'Stella'; *Cardisoma carnifex*, isolated sinus gland.

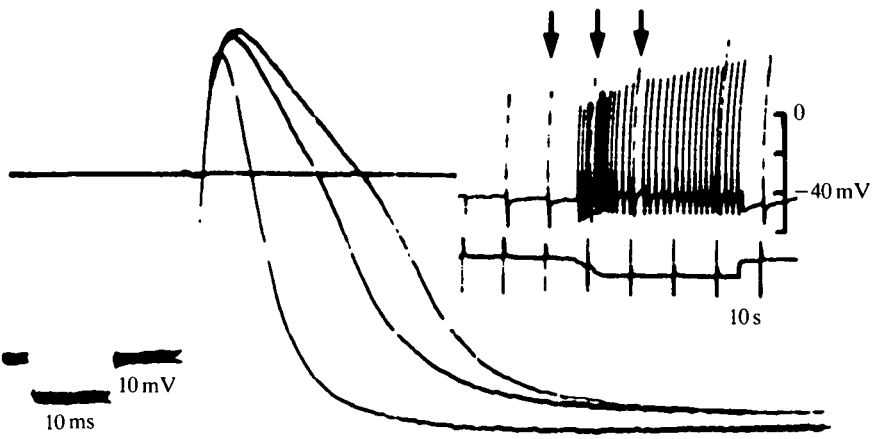

Fig. 5. Impulse broadening during passage of depolarizing current and resulting repetitive firing. Inset: penwriter record showing response of terminal to depolarizing current (0·2 nA). The impulse amplitudes are variably attenuated by the slow pen response. Larger deflections result from axonal stimulation, repeated every 10 s. Arrows indicate the three responses superimposed in the photo. Frame: the shortest impulse was recorded before current-passing; middle, 5th impulse, 4 s after commencing depolarization (approx. 4 mV); longest, 18th impulse, after 14 s of depolarization. Half-amplitude duration increased from 7 ms to 21 ms. Reference trace at 0 mV, resting potential, −44 mV. *Cardisoma guanhumi*, isolated sinus gland.

Recovery upon removing the depolarizing holding current was immediate for the rate of rise and overshoot, but required tens of seconds for the falling phase. Terminals examined in this way were exhibiting spontaneous impulse firing, and the rate of this was altered by the current passing (slowed or halted by hyperpolarization, speeded by depolarization). Thus it remains unclear to what extent the effects of the current passing result from changes of firing rate or from the altered resting potential.

Evidence suggesting that alteration of the resting potential affects the duration of terminal impulses in the absence of spontaneous firing was obtained from one of the preparations examined during perfusion with salines having reduced $[K]_o$ (Fig. 7, see further below). Impulses evoked by axonal stimulation during hyperpolarization in response to low-K saline showed a comparable reduction in duration at a given membrane potential to those evoked later, when a similar range of membrane potentials was achieved by passing depolarizing current. As seen in Fig. 7, there was a roughly linear relationship between impulse duration and membrane potential. Values, whether obtained in normal saline or reduced-K saline, with or without current passing, fell close to the same line. The observations are limited in the hyperpolarizing quadrant by failure of the impulse to invade the terminal at membrane potentials more polarized than -74 mV. The linear relationship continued for depolarization beyond the normal resting potential (approx. -61 mV) to membrane potentials at which extra impulse firing occurred. Markedly greater impulse broadening than predicted by extension of the linear relationship was observed in the presence of repetitive firing. In the reduced-K salines, threshold for impulse firing evoked by the depolarizing current was 8 or more mV more depolarized.

The observations summarized in Fig. 7 permit the conclusion that the rate of repolarization of terminal impulses is influenced by the prevailing membrane potential in the absence of repetitive firing. Further, the decrease of impulse duration with hyperpolarization relative to normal resting potential is not an artifact of current passing. They suggest that repetitive firing is much more potent in activating the mechanisms underlying impulse broadening than is subthreshold membrane depolarization. One such mechanism might be a time- and voltage-dependent inactivation of voltage-dependent K-conductance, analogous to that observed in certain molluscan neurones (Aldrich, Getting & Thompson, 1979).

In several (5) terminals, repetitive stimulation of the axon tract resulted not only in changes in form of the terminal impulses but in a prolonged, slowly developing and slowly subsiding depolarizing shift in the resting potential (Fig. 8). These terminals were characterized by having a high resting potential (-70 to -80 mV), and by having shown a spontaneous, rapid shift in resting potential early during the recording session from initial values near -50 mV. In the terminal providing the records shown in Fig. 8, each impulse initially repolarized to the baseline, then slowly (hundreds of milliseconds) developed a depolarized afterpotential of as much as 20 mV which declined over tens of

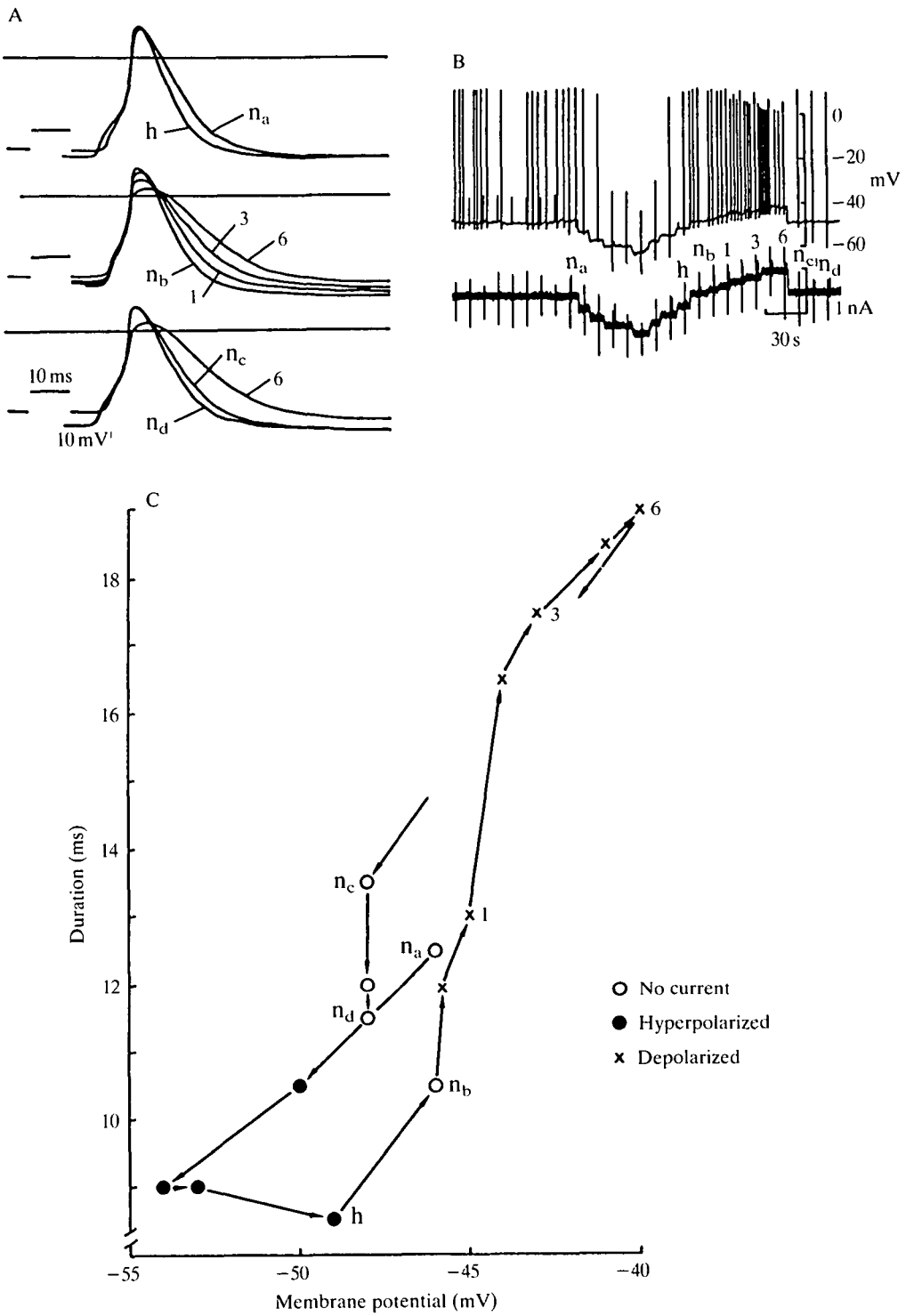
A
h
n_a
3
6
n_b
1
n_c
6
10 ms
10 mV
n_d
B
0
−20
mV
−40
−60
n_a
h
n_b 1 3 6
n_c n_d
1 nA
30 s
C
Duration (ms)
18
16
14
12
10
6
3
1
n_c
n_a
n_d
n_b
h
No current
Hyperpolarized
Depolarized
−55
−50
−45
−40
Membrane potential (mV)

Fig. 6

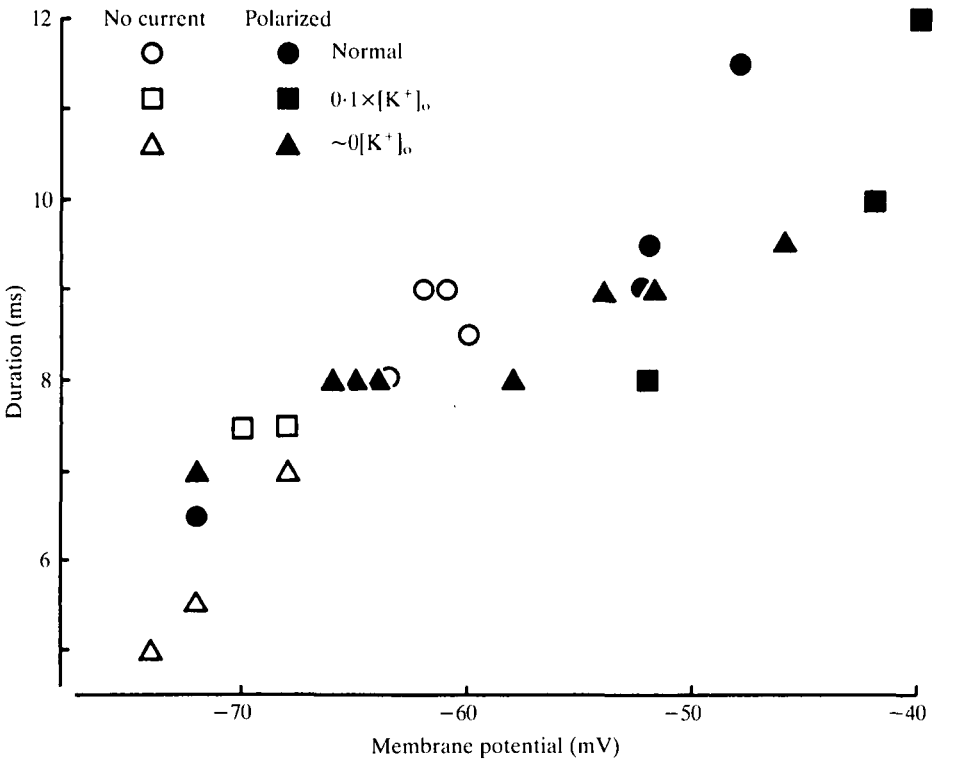

Fig. 7. Similarity in the effect on impulse duration of altering membrane potential by current passing or reduction of $[K^+]_o$. Duration of impulses (at half-amplitude) evoked by axonal stimulation (at $0{\cdot}1\ s^{-1}$) is plotted against membrane potential for measurements without current passing (open symbols) in normal (○), 0·1 × normal-$[K^+]_o$ (□), or nominally $0[K^+]_o$ saline (△), as well as during passage of hyperpolarizing (normal saline only) or depolarizing current (filled symbols). Sharply increased duration with depolarization is associated with extra impulse firing evoked by the current passing.

Fig. 6. Influence of membrane holding potential on the duration of impulses. (A) Oscilloscope records of impulses in response to sinus gland nerve stimulation with rising phases superimposed by hand tracing. The traces are identified by corresponding symbols in B and C; h, hyperpolarized by current passing through the recording electrode; n, no polarizing current; 1, 3, 6 depolarized by these amounts (mV). (B) Slow time-base penwriter record of membrane potential (upper trace) and current during period of recordings shown in A and measurements plotted in C. Spontaneous impulse activity is present in the absence of stimulation or current passing at the beginning of the record. Beginning at n_a, a brief shock to the sinus gland nerve is given immediately after the calibration pulse every 10 s and initiates an action potential propagated to the terminal. Hyperpolarization of more than 10 mV prevents invasion of the terminal leaving an electrotonically conducted axonal spike. Depolarization results in impulse firing in addition to responses to nerve stimulation. (C) Impulse duration (at half-amplitude) plotted against holding potential for impulses evoked at 10-s intervals during the sequence shown in B (arrows indicate the order, symbols identify corresponding traces in A and B). Note that broadening and recovery require tens of seconds. *Cardisoma carnifex*, isolated sinus gland.

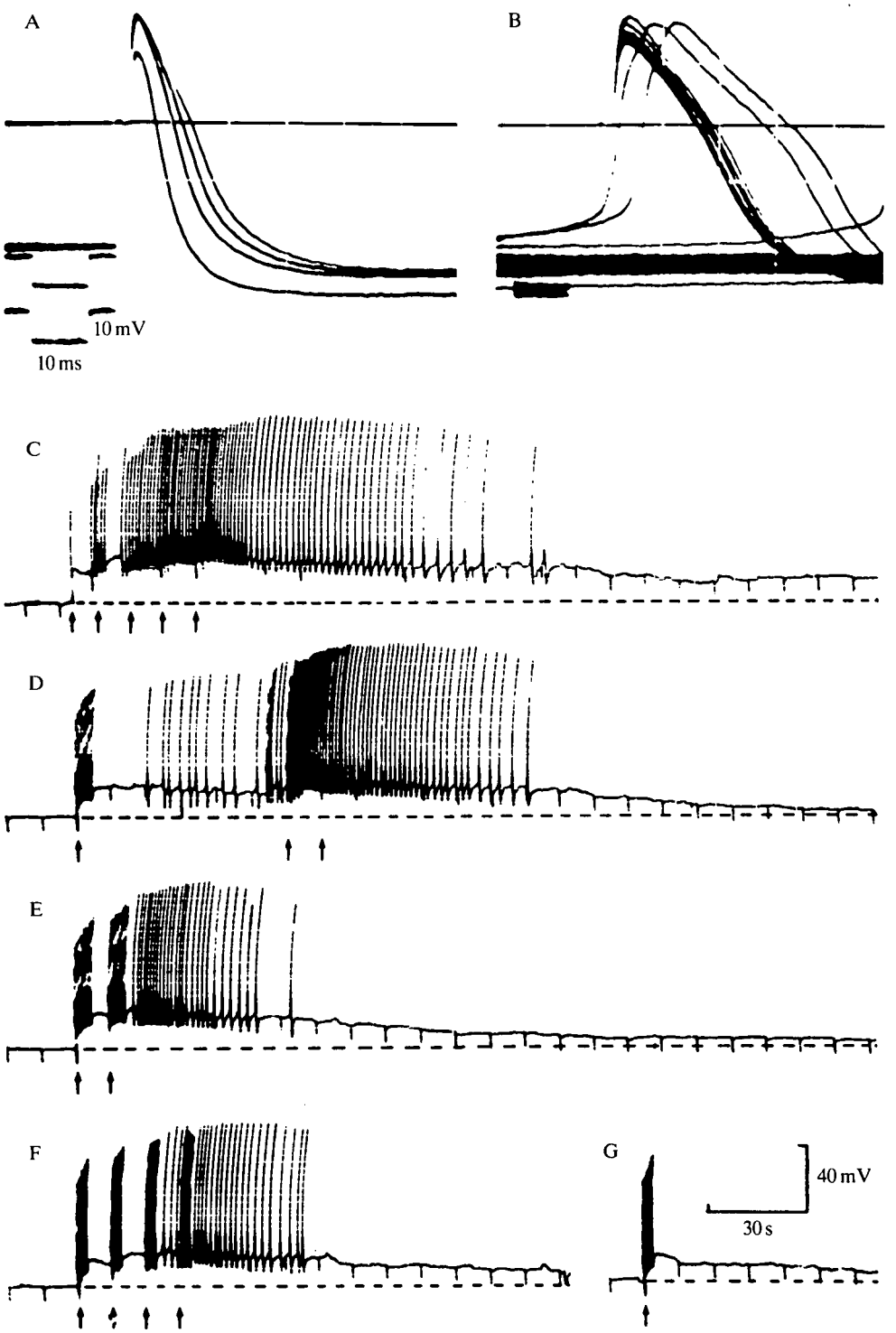

Fig. 8

seconds. During response to stimulus trains, these afterpotentials partially summed and reached a level resulting in sustained repetitive firing (for as long as 2 min). After cessation of firing, recovery to the initial membrane potential required 2–3 min. These observations are reminiscent of the depolarizing afterpotentials described for certain molluscan neurones by Thompson & Smith (1976). The depolarizing afterpotentials are distinguishable from the plateau potentials associated with bursting (Fig. 4) by the following: they follow impulses, and sustained firing requires stimulus initiation of a number of impulses; plateau potentials may occur spontaneously and may precede impulse firing; the plateau and burst may be initiated by a single stimulus; the plateau potential reaches its sustained value immediately, does not show fluctuation with impulse firing rate, and repolarizes much more rapidly.

Responses to intracellular current-passing

Responses to maintained depolarizing current. A consistent feature of the responses of terminals to maintained depolarization by steady current-passing through the recording electrode was their ability to take up and maintain a steady firing frequency with no evidence of accommodation to the stimulus. Measurements from the records illustrated in Fig. 9 showed a nearly linear relationship between the depolarizing current and the impulse firing frequency (up to a rate of $6\,s^{-1}$). The records illustrate that the terminals showed a slow approach to their steady firing frequency, in contrast to the rapid initial onset and subsequent decline observed in many neuronal preparations. During repetitive firing, spike broadening occurred, and there was a steady reduction in the extent of the repolarization occurring between impulses. Reduced repolarization and broadening may reflect the same process, inactivation of voltage-dependent K-conductance. Decline of the afterpotential was also seen during repetitive firing in response to the onset of depolarization upon introduction of saline having elevated $[K^+]$ (see below). Passage of too large a depolarizing current quite often resulted in loss of the penetration (e.g. Fig. 9). Osmotically-induced swelling of the terminal as a result of the injected material may have been a cause.

Interaction between axonal stimulation and current passed into the terminal. When current is passed through a recording electrode to alter the holding

Fig. 8. Depolarizing afterpotentials. (C)–(G) Penwriter records to show sustained depolarization following single (1st arrow in C), or trains of stimuli applied to the sinus gland nerve (penwriter variably attentuates spike amplitudes). Stimuli at arrows: in C (except 1st arrow), trains of five stimuli at $1\,s^{-1}$ every 10 s; D and E, approximately 10 at $2\,s^{-1}$; F and G, approximately 10 at $4\,s^{-1}$. Note continuation of impulse firing after cessation of stimulus trains; individual impulses show initial hyperpolarizing afterpotentials. Regular downward deflections are 10 mV, 10 ms calibration pulses given every 10 s. Dashed line marks resting potential, −68 mV. (A) Superimposed responses sampled during stimuli shown in E; short impulse is 1st response after 6 min without activity; 2nd and 3rd sampled later in first train; longest (with depolarized calibration pulse), 1st response of 2nd train. (B) Superimposed responses to stimuli of the 3rd and 4th trains of F also showing two of the intervening spontaneous impulses (delayed peaks); note broadened spikes relative to those in A. *Cardisoma guanhumi*, isolated sinus gland.

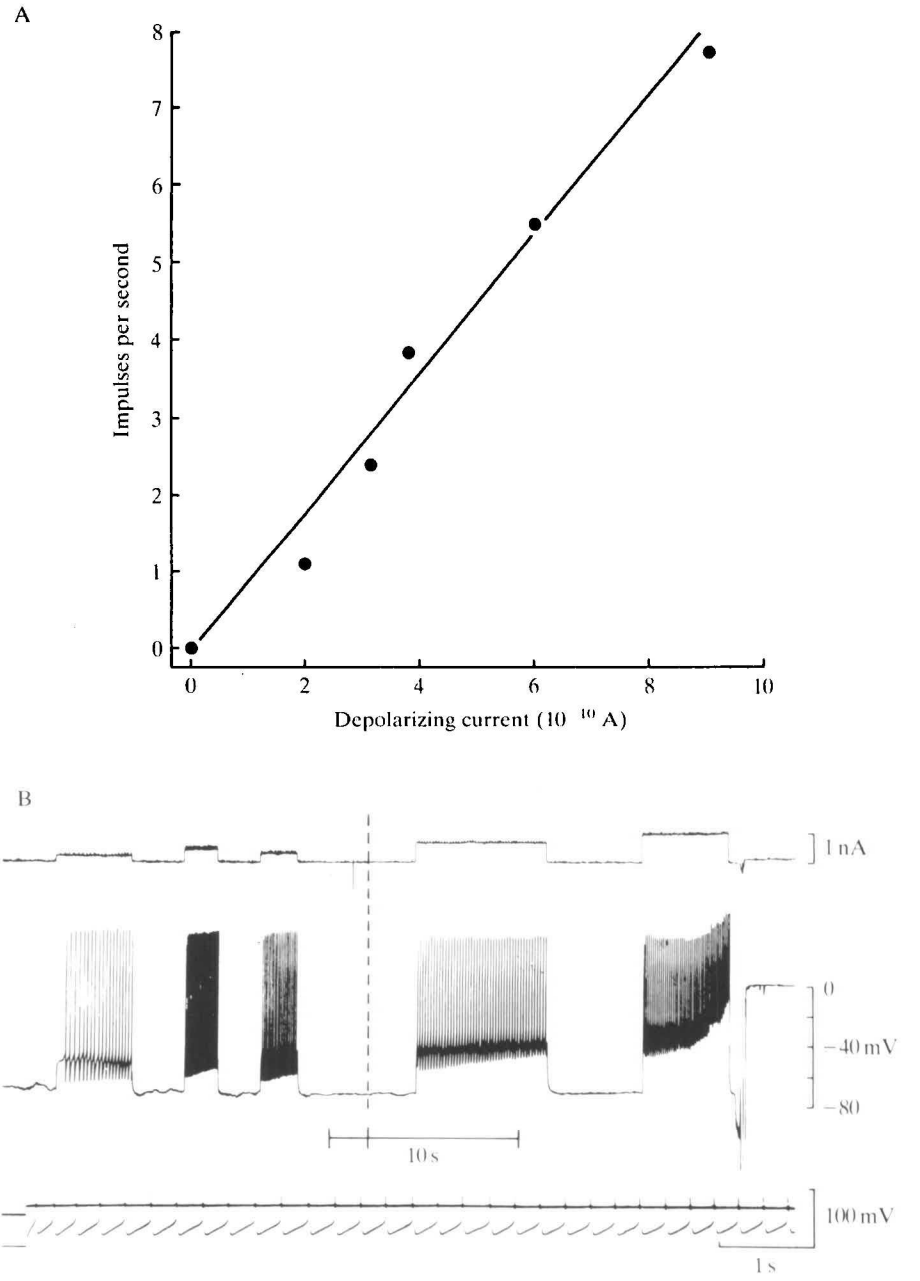
A
Impulses per second
0
1
2
3
4
5
6
7
8
0
2
4
6
8
10
Depolarizing current (10 ⁻¹⁰ A)
B
1 nA
0
−40 mV
−80
10 s
100 mV
1 s

Fig. 9

potential, the threshold to axonal stimulation *via* an extracellular 'pore' electrode is correspondingly changed, being increased for hyperpolarizing current and reduced for depolarizing current. The observations suggest that the electrotonic spread of current proximally, or the effective length constant, is much larger than would be suggested by the small diameter of the neurosecretory axons, as seen in histological sections. The distance separating the intracellular from the axonal stimulating electrode was of the order of 1 mm in *Cardisoma* preparations. Other observations (M. Nagano & I. M. Cooke, unpublished observations; Stuenkel, 1985) also indicate a long effective length constant in the proximal direction from the terminals.

Effects of hyperpolarizing current. Terminals were examined with hyperpolarizing current clamps with the intention of determining their input resistance. In the majority of terminals, except at very small current intensitites (less than 0·2 nA), the membrane potential did not reach a steady value even for pulse durations of several seconds (Fig. 10). The response to hyperpolarizing current was initially a nearly linear increase (hyperpolarization) in membrane potential at a rate related to the current intensity. The rate of relaxation of membrane potential after turning off the current was generally slower than had been the onset, suggesting that input resistance had been increased, relative to that at rest, during the hyperpolarization. In some terminals membrane potential did not simply return to resting level on turning off the current, but was partly depolarized, sometimes giving rise to one or a series of impulses. If the current intensity (generally $>0{\cdot}5<1$ nA) or pulse duration were sufficiently great, membrane potential, after reaching values of the order of -150 mV, would slowly begin to decline again. Terminals having a high (more negative than -60 mV) resting potential and noisy baseline exhibited these responses to hyperpolarizing current to a greater extent than did those with lower resting potentials.

The ionic bases of electrical activity recorded from terminals

Effect of alterations of $[K^+]_o$

Increased $[K^+]_o$. Observations on the effects of increased $[K^+]_o$ have been presented by Cooke & Haylett (1984), but will be reviewed briefly here (see also Stuenkel, 1985). In 10 times normal $[K^+]$ (176 mmol l^{-1}), the membrane potential depolarized and reached a maintained, steady potential of -15 mV on

Fig. 9. Relationship of impulse firing frequency to imposed depolarizing current. (A) Impulse firing rate is plotted against the value of steady depolarizing current passed through the recording electrode. This terminal showed capability to fire at faster rates than many, and a nearly linear relationship of frequency to current (line drawn by eye). (B) Records from which the plot was made; above, penwriter (current monitor, upper trace, change of chart speed at vertical broken line). Note delayed onset of spiking at smallest current (left), steadiness of firing rate with larger currents, marked reduction of undershoot with continued firing (pen does not accurately register impulse peaks). Loss of penetration is shown at the end of the record. Below: moving film record of vertically-deflected oscilloscope spot for the beginning of the second response shown above. Note that spike overshoot remains constant. Improved photographic registration during the course of the record is the result of spike broadening. *Cardisoma carnifex*, isolated X-organ – sinus gland.

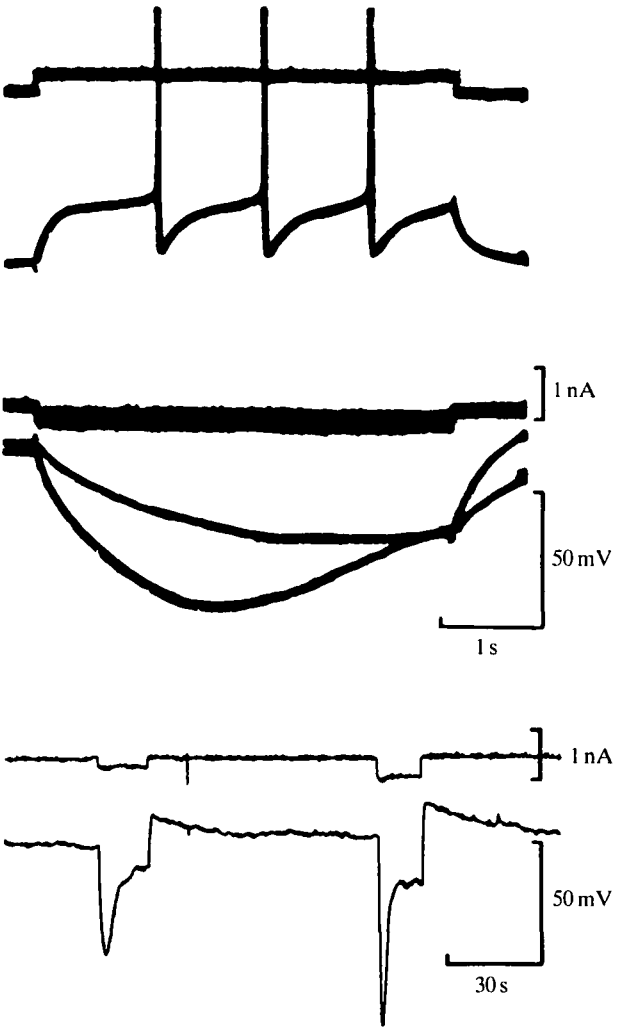

Fig. 10. Responses of a sinus gland terminal to current clamps. Depolarizing current (top) leads to non-adapting, steady impulse firing. Middle: two superimposed sweeps to two intensitites of hyperpolarizing current. Note slow approach to steady state for smaller current, relaxation during continued current at slightly larger current. Below: penwriter record of two current clamps held for approximately 15 s. Hyperpolarization is followed by partial relaxation during continued current passing and by depolarization at current off. All records from the same terminal. *Cardisoma carnifex*, isolated sinus gland.

average (range −18 to −10 mV, seven preparations). This range was much smaller than that of the normal resting potentials of these terminals, −80 to −50 mV. A line drawn according to the Nernst relationship, from −15 mV, falls within the normal resting potential range (−74 mV, for $[K^+]_o$ of 17·6 mmol l^{-1}). Of four observations of membrane potential at 3 times $[K^+]_o$, two fell close to the Nernst line, while two were much (25 mV) more polarized than expected. Those falling near the line had not been previously exposed to elevated-K saline, while those above it had previously been tested in 10 times $[K^+]_o$.

Responses of terminals during a change of the continuous perfusion from normal saline to saline having 10 times normal [K^+] and during return to perfusion with normal saline have been described and illustrated (Cooke & Haylett, 1984; Stuenkel, 1985). Briefly, as the depolarization begins, repetitive impulse firing at an accelerating rate occurs. The amplitude of the impulses declines at first as a result of reduced repolarization; impulse firing then gives way to a series of waxing and waning oscillations and finally to sustained depolarization. Preparations have been held as long as 40 min in elevated-K saline without showing any repolarization or change in the potential. Responses to current clamps during elevated-K perfusion indicate that input resistance is significantly reduced; depolarizing pulses result in nearly flat membrane potential trajectories, although hyperpolarizing current does not result in steady-state potentials. On returning to normal saline perfusion, repolarization occurs at a much slower rate than the depolarization. In most of the terminals, the resting potential, after as much as 1 h in normal saline, was 5–10 mV depolarized relative to the value before commencing high-K perfusion.

Effects of reduced $[K^+]_o$. A separate series of preparations was examined for changes in the electrophysiological parameters of terminals during perfusion with salines having 10 % of normal [K^+] or nominally 0 mmol l^{-1} [K^+]. Individual terminals showed hyperpolarization of their resting potential of from 5 to 30 mV and increases of apparent input resistance. Three individual terminals of three different preparations were held through a succession of changes in saline [K^+]. In two of the three (including the preparation providing the data of Fig. 7), reversible increases of apparent input resistance of 20–35 % were observed (using the current-voltage relationship plotted by measuring the voltage change observed at 1 s after imposing a current clamp). Initial apparent input resistance of these terminals was about 60 MΩ. A third terminal showed an increase of input resistance from 100 to 240 MΩ, and the increased resistance was maintained after return to normal saline perfusion. Because of the unevaluated effects of the changed membrane potential and the non-steady state of the membrane potential at the time of the measurements, it is not possible to use them to derive a quantitative statement about the extent of resting conductance represented by K^+. The observations are similar to those from many neural preparations in showing a much greater dependence of resting potential on $[K^+]_o$ for augmented relative to reduced $[K^+]_o$. They are consistent with a proportionately greater conductance to K^+ relative to other ionic species during sustained depolarization than at normal resting potential, and an increasing importance of conductance to depolarizing ionic species (Na^+, Ca^{2+}) in determining the resting potential at normal and hyperpolarized membrane potentials.

It is noteworthy that in none of the preparations tested in nominally K-free saline did intracellularly-recorded terminals show depolarization, as is the case in neurones in which an electrogenic Na-K pump contributes to the resting potential. K^+ present in blood sinuses may have provided enough K^+ to sustain operation of such a pump. However, addition of ouabain, or perfusion with saline

in which Li^+ is substituted for Na^+, results in changes of less than 5 mV in the resting potential (E. Stuenkel, personal communication).

The role of Na in electrical activity of terminals

Effects of reduced $[Na^+]_o$. A series of experiments tested the effects of perfusing the preparation with salines in which $[Na^+]_o$ was substituted by Tris, as a presumably impermeant ion, to maintain isotonicity of the saline. Reduction of $[Na^+]_o$ by 25 % produced little noticeable change, but perfusion with salines having 50 % normal $[Na^+]_o$, while not affecting the resting potential, resulted in cessation of spontaneous activity and a sudden failure of the terminal response to stimulation of the axon tract. The terminal continued to initiate impulses in response to depolarizing current passed through the recording electrode, however. In Fig. 11, threshold potential for initiation of an active response was increased by about 10 mV, and overshoot was reduced but still present (uncertainty due to the use of a bridge circuit for current passing makes quantitative statements unwarranted). Terminals were not held for longer than 15 min in reduced $[Na^+]_o$ salines because I wished to validate that the changes observed were due to reduced $[Na^+]_o$ and not to deterioration in the recording conditions or preparation by demonstrating the reversal of the effects on return to normal saline. Full reversal of effects of 50 %-$[Na^+]_o$ saline was documented for five terminals, all in different preparations of *C. guanhumi*. Regenerative responses of terminals perfused with Na^+-free saline are documented by M. Nagano & I. M. Cooke (in preparation).

Effects of tetrodotoxin. The effects of adding the selective Na^+-conductance inhibitor, TTX, to the saline closely parallelled those of 50 %-$[Na^+]_o$ saline. Fig. 12 shows an example of the effect of 10^{-7} mol l^{-1} TTX. As in 50 %-$[Na^+]_o$ saline, spontaneous activity ceased and there was apparently a sudden failure of axonal conduction, seen as an all-or-none failure of the terminal impulse to develop following a stimulus to the axon tract. At this time, little change in form of the terminal response had occurred. The terminal remained responsive to depolarizing current, but responses showed progressive increases in threshold and decreases in rate of rise and in extent of overshoot as perfusion with TTX-containing saline was continued. Responses persisted during periods of up to 15 min of perfusion with concentrations of 10^{-6} mol l^{-1} TTX. Longer exposures were not made because attempts were made to show reversibility. Reversibility was achieved in three terminals tested in 10^{-7} mol l^{-1} TTX and one tested in 10^{-6} mol l^{-1} TTX. One preparation was tested with hyperpolarizing current pulses before and in the presence of 3×10^{-7} mol l^{-1} TTX. It showed an increase of 25 % in the slope of the I-V relationship (determined at a 1-s pulse duration) during perfusion with TTX.

The observations in reduced-$[Na^+]_o$ salines and in TTX-containing saline indicate that regenerative responses of the terminals are sustained independently of the voltage-dependent Na^+-conductance mechanisms, while axonal conduction is highly dependent on Na^+-mediated conductance changes.

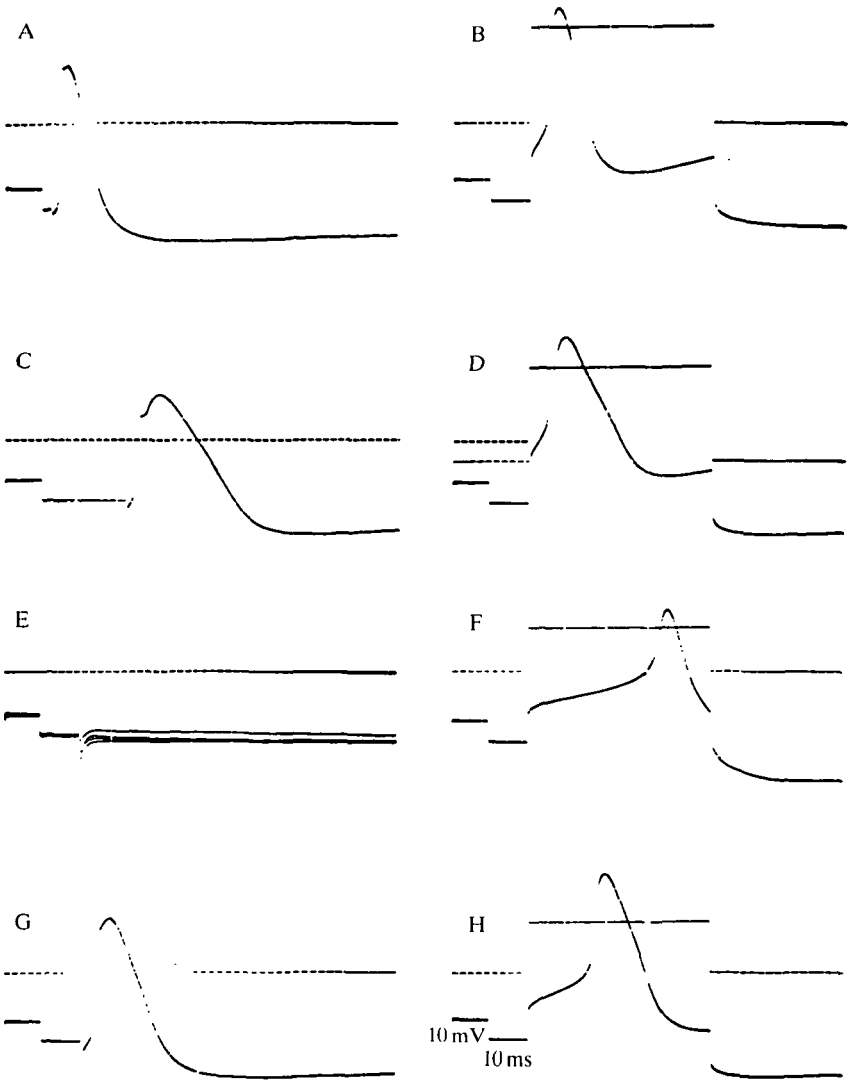

Fig. 11. Block of axonal conduction during perfusion with reduced-Na saline. Left frames, responses to stimulation of the axon tract with a pore electrode (reference line approximately at 0 mV); right, responses to depolarizing current passed through the recording electrode. (A),(B) In normal saline, (C),(D) during perfusion with saline with half-normal $[Na^+]_o$ (Tris-substituted), immediately before axonal conduction failure; (E) failure of axonal conduction in half-Na saline to stimulation at same and increased stimulus strengths (five sweeps); (F) continued response of terminal to direct depolarization (1 nA) after over 30 min in half-Na^+ saline; (G),(H) recovery of axonal conduction and decrease of impulse duration after returning to perfusion with normal saline. Calibration pulse, +10 mV, 10 ms, begins each sweep. *Cardisoma guanhumi*, semi-intact preparation.

Effects of reduced-$[Ca^{2+}]_o$ salines on electrical activity of terminals

Reducing $[Ca^{2+}]_o$ to less than 30 % of the normal value consistently produced an increase in general spontaneous activity of the preparation. This activity could be monitored in the optic peduncle with a suction electrode. Only terminals in

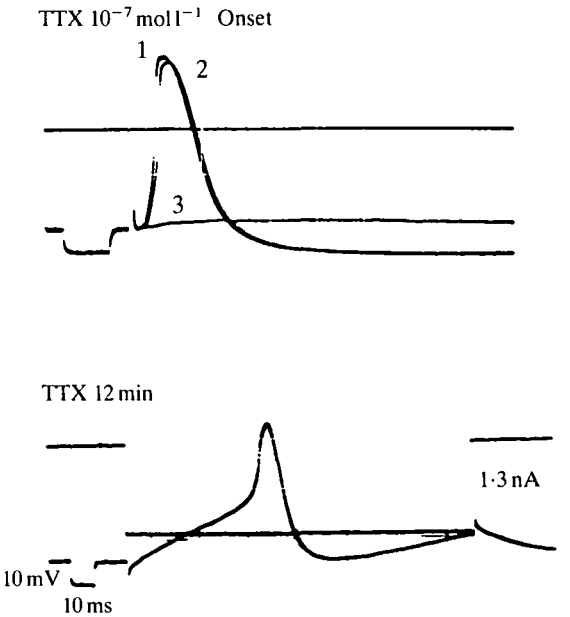

Fig. 12. Selective block by TTX (10^{-7} mol l^{-1}) of axonal conduction. Top: onset of TTX effect; responses to three successive stimuli applied to the sinus gland nerve at 10-s intervals; axonal conduction has failed on the third stimulus. Below: response to depolarizing current passed through the recording electrode recorded 12 min later (note slower time base). There has been an increase in threshold and impulse duration, and a decrease in the overshoot. TTX effects were reversed after 1 h of perfusion with normal saline (not shown). Calibration pulse: −10 mV, 10 ms. *Cardisoma carnifex*, isolated X-organ-sinus gland.

which the penetration was held through subsequent recovery in normal saline are considered here. All of the terminals recorded during perfusion with reduced-$[Ca^{2+}]_o$ salines could be unambiguously placed in one of two groups according to their degree of depolarization. Since the recordings were obtained from three species (*C. guanhumi, C. carnifex* and *Podophthalmus vigil*), from semi-intact and fully isolated sinus glands, and at various times of the year, it seems likely that they represent different terminal types present in a single sinus gland. Seven of 14 terminals for which recovery was obtained showed depolarization occurring gradually during the first 5 min in the presence of reduced-$[Ca^{2+}]_o$ saline and then reaching a steady state at a potential of about −30 mV. During this depolarization, spontaneous impulse firing increased in rate and sometimes showed a fragmentation to several discrete waveforms (Fig. 13) similar to that described under repetitive stimulation, above. The amplitude of the responses then gradually declined leaving the terminal showing slight oscillations or quiescent. In two of these preparations, steady current was passed through the recording electrode to repolarize the terminal, and this restored overshooting, spontaneous impulse activity.

In the other seven terminals, slow depolarization occurred, but did not exceed 5 mV. Spontaneous firing appeared or increased in rate. The form of impulses was altered, especially in the falling phase. The impulses appeared to have a

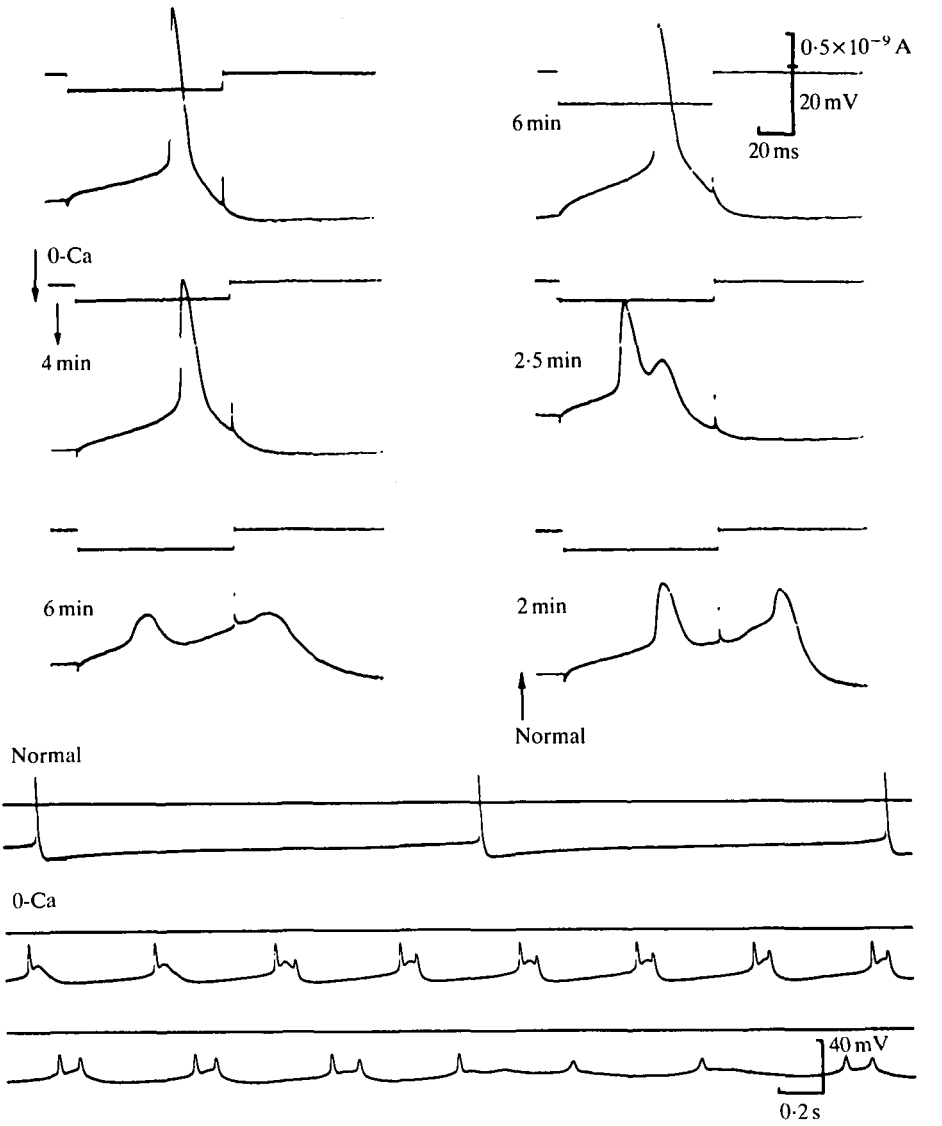

Fig. 13. Spontaneous and evoked potentials during perfusion with nominally Ca^{2+}-free saline. Below: oscilloscope recording (unswept spot, moving film) showing spontaneous activity of a terminal in normal saline (1st pair of traces, upper line indicates 0 mV), and within 30 s (middle) and 10 s (bottom) of cessation of activity (approx. 6 min after commencing 0-Ca^{2+} perfusion). Above, left: oscilloscope frames showing responses to depolarizing current (current monitor at 0 mV, depolarizing current downward) in normal saline (upper left), and at 4 min and 6 min after commencing 0-Ca^{2+} perfusion. Right: recovery after introduction of normal saline perfusion; lower right, after 2 min; middle, 2·5 min; upper right, 6 min. Note fragmentation of terminal impulse into two or more waveforms in both spontaneous activity and evoked responses in reduced $[Ca^{2+}]_o$, and the similarity between spontaneous and evoked waveforms. *Cardisoma guanhumi*, semi-isolated preparation.

sharper peak because the initial repolarization began rapidly; this then gave way to a slower than normal repolarization having a characteristic, flat trajectory (Fig. 14). Most terminals in reduced-$[Ca^{2+}]_o$ salines show no hyperpolarizing afterpotential. The duration of terminal impulses observed in reduced-$[Ca^{2+}]_o$ salines was longer than those observed in the same terminal at approximately the same resting potential in normal saline.

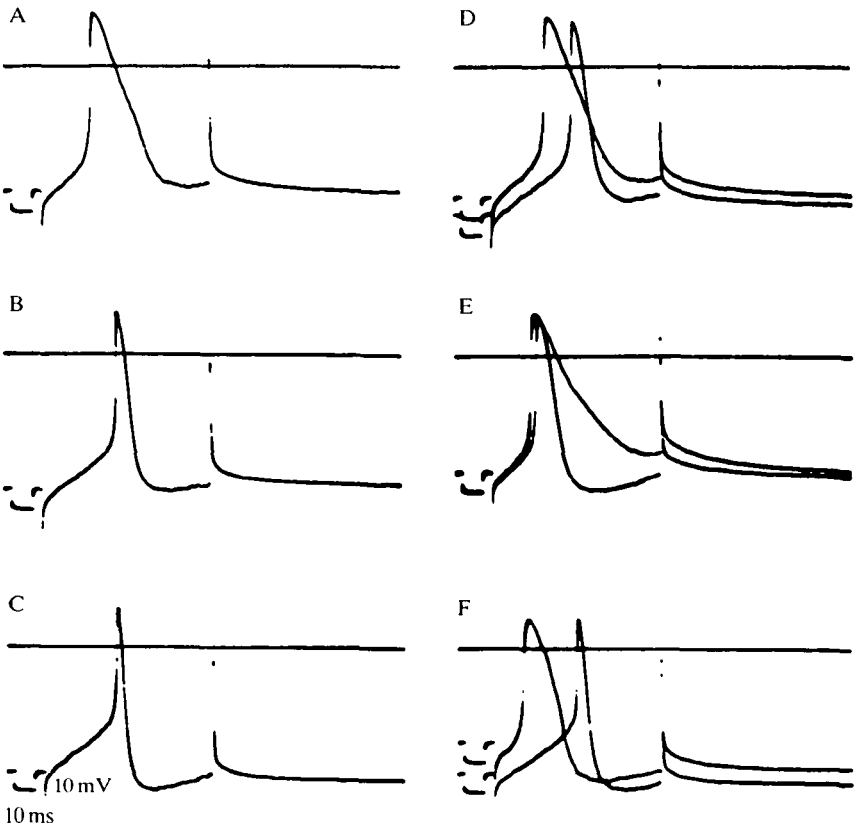

Fig. 14. Influence of reduced $[Ca^{2+}]_o$ and holding potential on the form of the impulse. Impulses are evoked by a 1-nA, 80-ms depolarizing current passed through the recording electrode (between artifacts). (A) After 9 min perfusion with saline having 7·5 mmol l^{-1} (30 % of normal), Ca, resting potential, −56 mV. Note flat trajectory of falling phase (compare with C). (B) 11 min in 30 %-Ca^{2+}, hyperpolarized to approximately −62 mV by imposed current. Note shortened impulse duration. (C) 22 min after restoring normal saline perfusion, resting potential, −58 mV. (D) After 8 min perfusion with saline having 10 % normal $[Ca^{2+}]_o$. The first impulse, with flattened falling trajectory, evoked at a resting potential of −58 mV during spontaneous firing at approximately 2 s^{-1}. Superimposed response was evoked during polarization to approximately −65 mV (spontaneous firing suppressed). (E) Reintroduction of normal saline. Faster rising, longer impulse recorded 2 min after switch to normal saline (spontaneous firing at approx. 3 s^{-1}). Superimposed response recorded 5 min after starting normal perfusion (spontaneous activity approx. 1 s^{-1}). Resting potential −53 mV. (F) After 20 min of normal perfusion, short impulse at a resting potential of −58 mV. Superimposed response during imposed depolarization to approximately −44 mV and spontaneous firing at approximately 1 s^{-1}. Impulse is broadened, but does not show the flat trajectory seen in reduced-$[Ca^{2+}]_o$. All records from the same terminal. Reference line at 0 mV. *Cardisoma guanhumi*, isolated sinus gland.

In one preparation, it was possible to observe the same terminal during perfusion with salines having 30 %, 10 % and 1 % of normal $[Ca^{2+}]_o$ (Fig. 14). in 1 %-$[Ca^{2+}]_o$, the terminal depolarized to approximately −30 mV and ceased to show regenerative activity, thus placing it in the group showing major depolarization in low $[Ca^{2+}]_o$. Impulse activity could be restored by hyperpolarizing current. During perfusion with 30 % or 10 % normal-$[Ca^{2+}]_o$ saline, depolarization occurred, but did not inactivate regenerative mechanisms. The alteration of impulse form, particularly the characteristically flat trajectory of the falling phase, was evident even in 30 %-$[Ca^{2+}]_o$ saline. This change of form was reversed by holding the terminal at more polarized potentials. It is possible that the presence of hyperpolarizing current rather than the change of holding potential was responsible. This terminal also displayed the characteristic of impulse broadening during repetitive activity and/or in response to depolarization. These broadened spikes had a much more rounded falling phase than impulses of similar duration observed in reduced-$[Ca^{2+}]_o$ saline (Fig. 14, F *vs* E). During restoration of normal saline after perfusion with 30 %- or 10 %-$[Ca^{2+}]_o$, the recovery of the form of impulses occurred before repolarization of the membrane potential (Fig. 14E). The reduction of impulse duration may in this case reflect the reduced rate of spontaneous firing, but the change of form is suggestive that the effect is attributable to the restoration of normal $[Ca^{2+}]_o$. The flattened trajectory of the falling phase and lack of hyperpolarizing afterpotentials of impulses recorded in terminals during exposure to reduced-$[Ca^{2+}]_o$ salines suggest that Ca^{2+}-mediated K-conductance may play a particularly important role in shaping the repolarizing phase of terminal impulses.

DISCUSSION

This report provides an initial description of electrophysiological events recordable with conventional intracellular microelectrode techniques from the neurohaemal structure of the crab eyestalk, the sinus gland. The neurosecretory products of this structure appear all to be peptides. Since the terminals were not isolated from their axons, the recordings reflect a mixture of the properties of axonal and terminal electrical behaviour. Nevertheless, a number of features of the recordings suggest specialized properties of the terminals related to their secretory function: (a) terminals support regenerative impulse activity; (b) the duration of impulses is three- to ten-fold longer than in non-secretory crustacean axons; (c) terminals are capable of regenerative impulse activity in reduced-Na^+ salines and in TTX when axonal conduction has been blocked, implying a major contribution of inward Ca^{2+} current to the normal terminal action potential; (d) terminals are capable of repetitive firing (although at low frequency only), without exhibiting accommodation, in response to a sustained depolarization; (e) a sub-population among the terminals exhibits broadening of their impulses as a result of repetitive firing; (f) several types of spontaneous impulse activity are routinely observed; patterned firing (bursting), apparently endogenously organized, has been observed.

The relevance of these properties to the secretory function of terminals is supported by experiments on the secretory physiology of *in vitro* sinus gland preparations (see Introduction). All of the observations place the crab sinus gland secretory system in conformity with the 'Ca-hypothesis' of Douglas & Poisner (1964*b*) and Katz & Miledi (1967*b*) which links terminal depolarization, Ca^{2+}-entry and secretion.

In the light of the Ca-hypothesis, regenerative, long-duration impulses and the presence of an enriched population of Ca^{2+}-channels in terminal membrane are seen as specializations to augment entry of Ca^{2+} at the terminal to promote secretion. The capability for sustained, repetitive activity may have relevance to hormonal secretion in which a given titre of hormone must be maintained over long periods.

Impulse broadening suggests a prolonged entry of Ca^{2+}; Na^{+} channels inactivate rapidly with depolarization in all neural preparations examined, while Ca^{2+} channels do not (for reviews see Hagiwara & Byerly, 1981; Tsien, 1983). Spike broadening might then be an electrophysiological correlate of facilitation. Broadening occurs during spontaneous bursting in sinus gland terminals (Cooke, 1981; M. Nagano & I. M. Cooke, in preparation). Patterned or bursting activity is observed in many neurosecretory systems (for review see Cooke & Stuenkel, 1985) and has been recorded from X-organ somata of crayfish (Iwasaki & Satow, 1969). In view of the demonstrated very large augmentation of vasopressin release from isolated neurohypophyses by patterned activity (Dutton & Dyball, 1979; Bicknell & Leng, 1981), it is attractive to suggest a functional linkage between bursting, impulse broadening and facilitation of hormone release. Efforts to provide direct evidence for facilitation of (RPCH) release from the sinus gland have been inconclusive, however; the assay can only confidently detect differences greater than three-fold (Cooke *et al.* 1977). It may be that terminals secreting RPCH are not among those showing broadening. A role of broadening in facilitation of transmission at a molluscan synapse is suggested on the basis of broadening observed at the soma (Klein & Kandel, 1978). Broadening is not observed during facilitation of transmission at a crustacean neuromuscular junction (Zucker & Lara-Estrella, 1979).

Considering the morphological (Weatherby, 1981) and biochemical (Newcomb, 1983; Newcomb, Stuenkel & Cooke, 1985) diversity of terminals in crab sinus glands it is perhaps more surprising that the recordings from terminals are so generally similar that they show differences. However, EM sections (Weatherby, 1981) suggest that two morphological terminal types, A and C, constitute the bulk of the sinus gland and are predominant among large structures. Recording is clearly biased in favour of large, surface terminals, and for large terminals in deeper portions. Thus, it seems likely that recordings from types A and C are predominant. More subtle responses are the most easily disrupted as a result of damage in penetration, as for example bursting. The morphological studies raise the question whether the large profiles are, in fact, final terminations, or pre-terminal storage zones. EM sections indicate that small

'fingers', 2 μm in diameter and 3–5 μm long extend from the large dilatations to contact the neurolemmal lining of the haemolymph sinus; only at these points of contact are exocytotic profiles observed, implicating these as the sites of secretion. The final digitations are sufficiently short and stubby to make it seem likely that electrical events in them would not differ from those observable from the adjacent dilatation.

The extent of the possible influence of electrotonically conducted events from other parts of a complex terminal arborization is best realized in the recordings (e.g. Fig. 2) in which fragmentation of a simple, overshooting impulse into complex but discrete waveforms occurs either as a result of repetitive axonal stimulation or of passing depolarizing current. Evidence for a long effective length constant proximally from terminals is the interaction between a current passed into the terminal region with axonal stimulation. A similar conclusion follows from observations of effects of localized application of K^+ (Stuenkel, 1985). Geometric complexity is not adequate to explain the slow, continuously increasing hyperpolarization observed during hyperpolarizing current-clamps. Hyperpolarizing inactivation of conductances present at normal resting potentials is implied. Other interesting and unexplained electrical behaviour includes the appearance of a plateau potential underlying bursting; this could be similar to plateau potentials recently observed in cultured hypothalamic neurones (Legendre, Cooke & Vincent 1982), in lobster cardiac ganglion neurones (Tazaki & Cooke, 1983) and in lobster stomatogastric neurones (Russell & Hartline, 1978). Since impulses in spontaneous bursts originate in the proximal axon (Cooke & Nagano, 1981; Cooke, 1981), a plateau localized in that region giving rise to the impulses may be postulated. Its amplitude, as observed in terminal recordings, would then be appreciably attenuated. The possibility of plateau potentials also arising at terminals is suggested by the records of Fig. 3. Similar observations have been made by E. Stuenkel (unpublished observations).

In exhibiting overshooting, long-duration action potentials mediated by both Na^+ and Ca^{2+} inward currents, the crab terminal activity resembles electrical activity recorded from the somata giving rise to sinus gland terminals in crayfish (Iwasaki & Satow, 1971) and in some, but not all, X-organ somata in crabs (Nagano & Cooke, 1983; Stuenkel, 1985). Hence, the suggestion of Klein & Kandel (1978) that the properties of soma membrane are reflected in those of a neurone's terminals must be generalized with caution. There is increasing evidence that dual Ca^{2+}- and Na^+-mediated action potentials are widespread in the somata of many animal groups; they are found in developing neurones and cultured neurones (for review see Spitzer, 1979). Indeed, it could be suggested that such regenerative mechanisms represent the more general, primitive impulse-forming machinery, and that the Na^+-dominated action potential of axons, apparently the case in the crab neurosecretory axons also, is a specialization of membrane for more rapid (and perhaps more frequent) impulse conduction.

A number of characteristics of terminal electrical activity imply the presence of the three major classes of K^+ conductance channels known from voltage clamping

of molluscan somata (for review see Adams, Smith & Thompson, 1980). Thus, delayed initiation of repetitive firing to imposed depolarization suggests the presence of I_A (Conner, 1975); rapid, hyperpolarizing afterpotentials are attributable to I_K; and the flattened trajectory of the falling phase of action potentials and reduction of hyperpolarizing afterpotentials in reduced-Ca^{2+} salines suggest a role of I_C in determining the form of impulses and the duration of relatively refractory periods. With, in addition, the presence of Ca^{2+}- as well as Na^+-channels, terminals appear to have a more complex endowment of ionic conductance channels than do axons (Nagano & Cooke, 1983; M. Nagano & I. M. Cooke, in preparation).

Trituration of an isolated sinus gland results in formation of membrane-bound, granule-filled spheres ranging in size up to 30 μm in diameter. These readily form giga-ohm seals with glass pipettes and have permitted application of patch-recording techniques. TTX-resistant inward current and outward current are observed under 'whole terminal' voltage clamp, and two classes of K-channels have thus far been characterized from isolated patches (Lemos, Stuenkel, Nordmann & Cooke, 1985). Characterization of ionic conductances unambiguously attributable to terminal membrane should be possible with these techniques.

Active invasion of synaptic terminals has now been documented in a number of preparations in addition to the previously known giant synapse of the squid stellate ganglion (Hagiwara & Tazaki, 1958). Evidence for enrichment of the terminal membrane by voltage-dependent Ca^{2+}-channels has been obtained by intracellular recording in several species (see Introduction) and by extracellular recording at others (e.g. the frog neuromuscular junction, Katz & Miledi, 1969*b*). The crayfish neuromuscular junction is thus far the only exception (Wojtowicz & Atwood, 1984). A number of observations on the mammalian neurohypophyseal system are suggestive of Na^+-dominated action potentials in the axons and of dual or Ca^{2+}-dominated action potentials in the terminals. These include failure of conduction following TTX treatment (e.g. Driefuss, Kalnins, Kelly & Ruf, 1971), and the ability to elicit release from rat neurohypophyses in Na^+-free, $CaSO_4$ saline by electrical pulses applied directly to the terminal region (Douglas & Sorimachi, 1971). Extracellular recording from the cat neurohypophysis reveals slower potentials at the periphery (where terminals are dense) and faster potentials centrally where axons pass (Zeballos, Thornborough & Rothballer, 1975). More direct evidence for regenerative Ca^{2+}-mediated impulses in the rat neurohypophysis has now been obtained by use of a voltage-sensitive dye (Salzberg, Obaid, Sensemann & Gainer, 1983). The additional density of voltage-sensitive Ca^{2+}-channels in the membrane of presynaptic and neurosecretory terminals would seem to be clearly related to the importance of Ca^{2+} entry for initiation of transmitter and neurohormone secretion.

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REFERENCES

ADAMS, D., SMITH, S. J. & THOMPSON, S. H. (1980). Ionic currents in molluscan soma. *A. Rev. Neurosci.* **3**, 141–167.

ALDRICH, R. W. JR., GETTING, P. A. & THOMPSON, S. H. (1979). Mechanism of frequency-dependent broadening of molluscan neurone soma spikes. *J. Physiol., Lond.* **291**, 531–544.

ANDREWS, P. M., COPELAND, D. E. & FINGERMAN, M. (1971). Ultrastructural study of neurosecretory granules in the sinus gland of the blue crab *Callinectes sapidus*. *Z. Zellforsch. Mikrosk. Anat.* **113**, 461–471.

BICKNELL, R. J. & LENG, G. (1981). Relative efficiency of neural firing patterns for vasopressin release *in vitro*. *Neuroendocrinol.* **33**, 295–299.

BLISS, D. E. & WELSH, J. H. (1952). The neurosecretory system of brachyuran Crustacea. *Biol. Bull. mar. biol. Lab., Woods Hole* **103**, 157–169.

BUNT, A. & ASHBY, E. (1967). Ultrastructure of the sinus gland of the crayfish *Procambarus clarkii*. *Gen. comp. Physiol.* **9**, 334–342.

CONNER, J. A. (1975). Neural repetitive firing: a comparative study of membrane properties of crustacean walking leg axons. *J. Neurophysiol.* **38**, 922–932.

COOKE, I. M. (1967). Potentials recorded intracellularly from neurosecretory terminals. *Am. Zool.* **7**, 732–733.

COOKE, I. M. (1971). Calcium dependent depolarizing responses recorded from crab neurosecretory terminals. *Proc. int. Union physiol. Sci.* **9**, 119.

COOKE, I. M. (1977). Electrical activity of neurosecretory terminals and control of peptide hormone release. In *Peptides in Neurobiology*, (ed. H. Gainer), pp. 345–374. New York: Plenum.

COOKE, I. M. (1981). Electrical activity in relation to hormone secretion in the X-organ - sinus gland system of the crab. In *Neurosecretion: Molecules, Cells Systems*, (eds D. S. Farner & K. Lederis), pp. 235–247. New York: Plenum.

COOKE, I. M. & HAYLETT, B. (1984). Ionic dependence of secretory and electrical activity evoked by elevated K^+ in a peptidergic neurosecretory system. *J. exp. Biol.* **113**, 289–321.

COOKE, I. M., HAYLETT, B. & WEATHERBY, T. (1977). Electrically elicited neurosecretory and electrical responses of the isolated crab sinus gland in normal and reduced calcium salines. *J. exp. Biol.* **70**, 125–149.

COOKE, I. M. & NAGANO, M. (1981). Bursting, Ca spikes and spike broadening recorded intracellularly from crab neurosecretory terminals. *Soc. Neurosci. Abstr.* **7**, 837.

COOKE, I. M. & STUENKEL, E. (1985). Electrophysiology of invertebrate neurosecretory cells. In *The Electrophysiology of the Secretory Cell*, (eds A. M. Poisner & J. Trifaro), pp. 115–164. Amsterdam: Elsevier.

COOKE, I. M. & SULLIVAN, R. E. (1982). Hormones and neurosecretion. In *The Biology of Crustacea*, Vol. 3, (eds H. Atwood & D. Sandeman), pp. 205–290. New York: Academic Press.

DALTON, J. C. (1958). Effects of external ions on membrane potentials of a lobster giant axon. *J. gen. Physiol.* **41**, 529–542.

DOUGLAS, W. W. & POISNER, A. M. (1964*a*). Stimulus-secretion coupling in a neurosecretory organ and the role of calcium in the release of vasopressin from the neurohypophysis. *J. Physiol., Lond.* **172**, 1–18.

DOUGLAS, W. W. & POISNER, A. M. (1964*b*). Calcium movement in the neurohypophysis of the rat and its relation to the release of vasopressin. *J. Physiol., Lond.* **172**, 19–30.

DOUGLAS, W. W. & SORIMACHI, M. (1971). Electrically evoked release of vasopressin from isolated neurohypophyses in sodium-free media. *Br. J. Pharmac.* **42**, 647.

DRIEFUSS, J. J., KALNINS, I., KELLY, J. S. & RUF, K. B. (1971). Action potentials and release of neurohypophysial hormones *in vitro*. *J. Physiol., Lond.* **215**, 805–817.

DUTTON, A. & DYBALL, R. E. J. (1979). Phasic firing enhances vasopressin release from the rat neurohypophysis. *J. Physiol., Lond.* **290**, 433–440.

FERNLUND, P. & JOSEFSSON, L. (1972). Crustacean color-change hormone: amino acid sequence and chemical synthesis. *Science, N.Y.* **177**, 173–175.

GLANTZ, R. M., KIRK, M. D. & ARÉCHIGA, H. (1983). Light input to crustacean neurosecretory cells. *Brain Res.* **265**, 307–311.

HAGIWARA, S. & BYERLY, L. (1981). Calcium channel. *A. Rev. Neurosci.* **4**, 69–125.

HAGIWARA, S. & TASAKI, I. (1958). A study of the mechanism of impulse transmission across the giant synapse of the squid. *J. Physiol., Lond.* **143**, 114–137.

IWASAKI, S. & ONO, T. (1979). Effects of divalent cation ionophores on the neuron membrane of the crayfish. *J. Membrane Biol.* **45**, 167–183.

IWASAKI, & SATOW, Y. (1969). Spontaneous grouped discharge of secretory neuron soma in X-organ of crayfish *Procambarus clarkii*. *J. physiol. Soc. Japan* **31**, 629–630.

IWASAKI, S. & SATOW, Y. (1971). Sodium- and calcium-dependent spike potentials in the secretory neuron soma of the crayfish. *J. gen. Physiol.* **57**, 216–238.

IWASAKI, S., SATOW, Y. & KURODA, T. (1973). On the hyperpolarizing inactivation of the Ca^{2+}-dependent action potentials. *Proc. Japan Acad.* **49**, 564–569.

KATZ, B. & MILEDI, R. (1967*a*). A study of synaptic transmission in the absence of nerve impulses. *J. Physiol., Lond.* **192**, 407–436.

KATZ, B. & MILEDI, R. (1967*b*). The release of acetylcholine from nerve endings by graded electric pulses. *Proc. R. Soc. Ser. B* **167**, 23–38.

KATZ, B. & MILEDI, R. (1969*a*). Tetrodotoxin-resistant electrical activity in presynaptic terminals. *J. Physiol., Lond.* **203**, 459–487.

KATZ, B. & MILEDI, R. (1969*b*). Spontaneous and evoked activity of motor nerve endings in calcium Ringer. *J. Physiol., Lond.* **203**, 689–706.

KELLER, R. (1983). Biochemistry and specificity of the neurohemal hormones in *Crustacea*. In *Neurohemal Organs of Arthropods*, (ed. A. P. Gupta), pp. 118–148. Springfield, IL: Charles Thomas.

KLEIN, M. & KANDEL, E. R. (1978). Presynaptic modulation of voltage-dependent Ca^{2+} current: mechanism for behavioral sensitization in *Aplysia californica*. *Proc. natn. Acad. Sci. U.S.A.* **75**, 3512–3516.

LEGENDRE, P., COOKE, I. & VINCENT, J.-D. (1982). Regenerative responses of long duration recorded intracellularly from dispersed cell cultures of fetal mouse hypothalamus. *J. Neurophysiol.* **48**, 1121–1141.

LEMOS, J. R., STUENKEL, E. L., NORDMANN, J. J. & COOKE, I. M. (1985). Ionic currents and channels in peptidergic neurosecretory terminals. *Biophys. J.* **47**, 446a.

LLINÁS, R., STEINBERG, I. Z. & WALTON, K. (1981*a*). Presynaptic calcium currents in squid giant synapse. *Biophys. J.* **33**, 289–322.

LLINÁS, R., STEINBERG, I. Z. & WALTON, K. (1981*b*). Relationship between presynaptic calcium current and postsynaptic potential in squid giant synapse. *Biophys. J.* **33**, 323–352.

MARTIN, A. R. & PILAR, G. (1964). An analysis of electrical coupling at synapses in the avian ciliary ganglion. *J. Physiol., Lond.* **171**, 454–475.

MAYNARD, D. M. & MAYNARD, E. A. (1962). Thoracic neurosecretory structures in Bracyura. III. Microanatomy of peripheral structures. *Gen. comp. Endocrin.* **2**, 12–28.

NAGANO, M. & COOKE, I. M. (1981). Electrical activity in the crab X-organ sinus gland system. Site of initiation, ionic bases, and pharmacology. In *Neurosecretion: Molecules, Cells, Systems*, (eds D. S. Farner & K. Lederis), pp. 504–505. New York: Plenum Press.

NAGANO, M. & COOKE, I. M. (1983). Morphology and intracellularly recorded activity of peptide neurosecretory somata, axons, and terminals: differences in ionic mechanisms. *Int. Union physiol. Soc. Proc.* **15**, 194.

NEWCOMB, R. (1983). Peptides in the sinus gland of *Cardisoma carnifex*: isolation and amino acid analysis. *J. comp. Physiol.* **153**B, 207–221.

NEWCOMB, R., STUENKEL, E. & COOKE, I. (1985). Characterization, biosynthesis, and release of neuropeptides from the X-organ - sinus gland system of the crab, *Cardisoma carnifex*. *Am. Zool.* **25**, 157–171.

NIWA, A. & KAWAI, N. (1982). Tetrodotoxin-resistant propagating action potentials in presynaptic axon of the lobster. *J. Neurophysiol.* **47**, 353–361.

NORDMANN, J. J. (1976). Evidence for calcium inactivation during hormone release in the rat neurohypophysis. *J. exp. Biol.* **65**, 669–683.

POTTER, D. D. (1956). Observations on the neurosecretory system of portunid crabs. Ph.D. thesis, Harvard University, Cambridge, Massachusetts.

POTTER, D. D. (1958). Observations on the neurosecretory system of portunid crabs. In *2, Internationales Symposium uber Neurosekretion*, (eds W. Bargmann, B. Hanstrom, E. Scharrer & B. Scharrer), pp. 113–118. Berlin: Springer-Verlag.

REHM, M. (1959). Observations on the localisation and chemical constitution of neurosecretory material in nerve terminals in *Carcinus maenas*. *Acta Histochem.* **7**, 88–106.

ROSS, W. N. & STUART, A. E. (1978). Voltage sensitive calcium channels in the presynaptic terminals of a decrementally conducting photoreceptor. *J. Physiol., Lond.* **274**, 173–191.

RUSSELL, D. & HARTLINE, D. K. (1978). Bursting neural networks: a reexamination. *Science, N.Y.* **200**, 453–456.

SALZBERG, B. M., OBAID, A. L., SENSEMANN, D. M. & GAINER, H. (1983). Optical recording of action potentials from vertebrate nerve terminals using potentiometric probes provides evidence for sodium and calcium components. *Nature, Lond.* **306**, 36–40.

SILVERTHORN, S. U. (1975). Neurosecretion in the sinus gland of the fiddler crab, *Uca pugnax*. *Cell Tissue Res.* **165**, 129–133.

SMITH, G. (1974). The ultrastructure of the sinus gland of *Carcinus maenas* (Crustacea: Decapoda). *Cell Tissue Res.* **155**, 117–125.

SPITZER, N. C. (1979). Ion channels in development. *A. Rev. Neurosci.* **2**, 363–397.

STROLENBERG, G., VAN HELDEN, H. & VAN HERP, F. (1977). The ultrastructure of the sinus gland of the crayfish *Astacus leptodactylus* (Nordmann). *Cell Tissue Res.* **180**, 203–210.

STUENKEL, E. (1983*a*). Biosynthesis and axonal transport of proteins and identified hormones in the X-organ sinus gland neurosecretory system. *J. comp. Physiol.* **153**, 191–205.

STUENKEL, E. (1983*b*). Peptide biosynthesis and secretion from a crab neurosecretory system. Ph.D. thesis, University of Hawaii, Honolulu, Hawaii.

STUENKEL, E. (1985). Simultaneous monitoring of electrical and secretory activity in peptidergic neurosecretory terminals of the crab. *J. Physiol. Lond.* **359**, 163–187.

TAZAKI, K. & COOKE, I. M. (1983). Neuronal mechanisms underlying rhythmic bursts in crustacean cardiac ganglia. In *Neural Origin of Rhythmic Movements*, (eds A. Roberts & B. Roberts). *Symp. Soc. exp. Biol.* **37**, 129–157.

THOMPSON, S. H. & SMITH, S. J. (1976). Depolarizing afterpotentials and burst production in molluscan pacemaker neurons. *J. Neurophysiol.* **39**, 153–161.

TSIEN, R. W. (1983). Calcium channels in excitable cell membranes. *A. Rev. Physiol.* **45**, 341–358.

WEATHERBY, T. M. (1981). Ultrastructural study of the sinus gland of the crab *Cardisoma carnifex*. *Cell Tissue Res.* **220**, 293–312.

WOJTOWICZ, J. M. & ATWOOD, H. L. (1984). Presynaptic membrane potential and transmitter release at the crayfish neuromuscular junction. *J. Neurophysiol.* **52**, 99–113.

ZEBALLOS, G. A., THORNBOROUGH, J. R. & ROTHBALLER, A. B. (1975). Neurohypophysial electrical activity in the anaesthetized cat. *Neuroendocr.* **18**, 104–114.

ZUCKER, R. S. & LARA-ESTRELLA, L. O. (1979). Is synaptic facilitation caused by presynaptic spike broadening. *Nature, Lond.* **278**, 57–59.