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ELECTRICALLY ELICITED NEUROSECRETORY AND ELECTRICAL RESPONSES OF THE ISOLATED CRAB SINUS GLAND IN NORMAL AND REDUCED CALCIUM SALINES

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SUMMARY

The sinus gland (a neurohaemal organ) and its nerve have been isolated from the eyestalk of the crabs *Cardisoma carnifex* and *Portunus sanguinolentus* for studies correlating electrical responses recorded extra-cellularly from the sinus gland with hormone release. The appearance of erythrophore concentrating hormone (ECH) in the perfusate was followed by bioassay on leg segments of *Ocypode pallidula*.

Electrical stimulation of the sinus gland nerve (175 pulses in trains of 5 at 5/s, every 10 s) results in significant amounts of hormone appearing in the perfusate, provided that a propagated compound action potential is recorded from the sinus gland. Release is normally below assayable levels in equivalent unstimulated control periods. A single preparation will release in excess of the equivalent of 20 pg of synthetic ECH in response to the standard routine of 175 stimuli. Many such secretory responses can be obtained over periods extending to as long as 30 h.

Addition of tetrodotoxin (3×10^{-7} M) rapidly abolishes propagated electrical responses and secretion; the effects are reversible.

Perfusion with saline having 30% (7·5 mM) of normal (25 mM) calcium reduces hormone release to about 10%, while the electrical response is often augmented. In 10% normal calcium, release is further decreased, and in 1% is indistinguishable from unstimulated release; electrical responses are reduced. Reduced calcium salines also induce spontaneous unit potentials, which persist in 30% calcium saline but subside after 10 min or longer exposure to salines of lower calcium concentration. All the effects of reduced calcium salines are reversible.

Inhibition of secretion without reduction of electrical responses in 30% calcium saline provides evidence for a direct role of calcium in excitation-secretion coupling. In salines that are more calcium deficient, failure of terminal electrical responses may also contribute to reduction of secretion.

In the crab sinus gland, as in other neurosecretory systems, propagation of action potentials to the terminals causes hormone release for which the presence of external calcium is essential.

INTRODUCTION

This paper reports experiments on the physiology of neurosecretion which exploit the X organ-sinus gland system of the crab eyestalk. The aim is to provide evidence that the roles of neuronal electrical responses and of extracellular calcium in linking stimulation with secretion are sufficiently like those of other neurosecretory systems to justify considering the sinus gland as a model for further analysis of neurosecretory mechanisms. The sinus gland system offers some major advantages over other neurosecretory systems that have been studied. These include: (1) the secretory terminals are sufficiently large in certain species to permit intracellular electrical recording (Cooke, 1967, 1971, 1977); further, a few terminals are individually identifiable from preparation to preparation; (2) the neurohaemal structure (the sinus gland), its axon tract (the sinus gland nerve), and the X organ (which includes a major portion of the neuronal somata contributing terminals to the sinus gland) can be isolated without the inclusion of non-neurosecretory elements other than neuroglia and connective tissue; (3) a sensitive, rapid, and semi-quantitative bioassay for one of the neurohormones secreted, the erythrophore concentrating hormone (ECH), has been developed (Weatherby, 1976; Weatherby & Cooke, unpublished); (4) the isolated neurohaemal system (terminals and axons) remains in a stable, viable condition for periods extending to days as judged by electrical and secretory responsiveness to stimulation.

The X organ-sinus gland system was among the first neurosecretory systems to be recognized as such (Bliss, 1951; Passano, 1951). However, there appear to have been no previous studies on the mechanisms of hormone release from this system, although there is extensive information on its morphology and histology and on endocrine effects mediated by the system (reviews: Gabe, 1966; Carlisle & Knowles, 1959).

The sinus gland is a dense aggregation of bulbous axon terminals surrounding haemolymph channels; other than connective tissue and glial elements, there are no other cell types present. The sinus gland nerve consists of purely neurosecretory axons and represents the major, and probably the only, tract by which fibres enter the sinus gland (Potter, 1956; cf. Bliss & Welsh, 1952; Bliss, Durand & Welsh 1954). Each axon branches within the sinus gland to give rise to a number of terminals. Histological (Potter, 1954, 1956, 1958), histochemical (Rehm, 1959) and electron microscopical (Bunt & Ashby, 1967; Andrews, Copeland & Fingerman, 1971; Smith, 1974; cf. Shivers, 1967; Weitzman, 1969; Andrew & Shivers, 1976) studies provide evidence for at least five kinds of terminals distinguishable by their staining reactions or characteristics of the granules they contain. The coincidence of this number with evidence for biochemical (review: Kleinholz, 1976) and endocrinological studies for elaboration of at least five separate hormones from the sinus gland makes tempting the speculation that each morphologically distinct terminal type stores and releases a particular hormone.

The five hormones thus far separated are: (1) the hyperglycaemic hormone, (2) moult-inhibiting hormone, (3) a leucophore concentrating hormone (LCH), (4) a distal retinal pigment light adapting hormone (DRPH), and (5) erythrophore concentrating hormone (ECH) (review: Kleinholz, 1976). The latter two from

shrimp have been characterized and synthesized (Fernlund & Josefsson, 1972; Fernlund, 1974*a*, *b*, and as cited in Kleinholz, 1976), and are both relatively small peptides that are not inactivated by heating to 100 °C. The hyperglycaemic hormone is a polypeptide that is destroyed by heating (Kleinholz, 1976). LCH is probably a peptide. The moult-inhibiting hormone has not been characterized. ECH has striking similarities to vertebrate neurosecretory peptides such as oxytocin and vasopressin. In common with these, ECH is an octapeptide and has an aminoterminal pyroglutamic acid and a carboxyterminal amide.

In these experiments, we have followed hormone release from isolated sinus gland-sinus gland nerve preparations by assaying for ECH activity in perfusate. Synthetic shrimp ECH is used as a quantitative standard. This material is active in a number of widely different crustaceans (Fingerman, 1973). ECH activity of eyestalk extracts for different species behaves similarly to the shrimp synthetic hormone in column chromatography (Fingerman, Fingerman & Hammond 1974). While the synthetic hormone has not been observed to affect other chromatophores (Fingerman, 1973), there appears to be little information on whether red chromatophores may be caused to contract by other hormones of the eyestalk in analogy to cross reactivity of assays for the vertebrate neurohypophysial peptides. This possibility is relevant to evaluation of the experiments reported here, because stimuli are given which are expected to elicit release of all sinus gland hormones, while only ECH activity is assayed. However, the conclusions to be drawn from observations we report do not depend on resolution of this question.

These experiments have examined whether action potentials propagated in the neurosecretory axons lead to release of hormones from the terminals and whether extracellular calcium is essential for such stimulation to cause release. These are initial tests of whether this neurosecretory system conforms to predictions of the calcium hypothesis.

The calcium hypothesis has been proposed in explanation of observations on neurosecretion (Douglas & Poisner, 1964*a*, *b*) and of neurotransmitter release (Katz & Miledi, 1967*a*, *b*); it suggests that similar mechanisms may be involved (reviews: Katz, 1969; Rubin, 1974). Briefly, this hypothesis proposes that depolarization of nerve terminals (normally but not necessarily accomplished by the arrival of action potentials propagated to them by axons) leads to a change of permeability of their membrane, permitting the entry of extracellular calcium ion; this entry of calcium in turn leads to release of the neurohormone or transmitter.

In neurosecretory systems, tests using elevated perfusate potassium as a depolarizing stimulus have been the most extensively applied. A model for such experiments is the study of Douglas & Poisner (1964*a*) on isolated rat neurohypophyses. Uptake of ^{45}Ca during elevated potassium stimulation has been observed (Douglas & Poisner, 1964*b*; Ishida, 1967). The observation that removal of calcium inhibits hormone release by elevation of potassium from the neurohypophysis has been confirmed in a number of studies (e.g. Dicker, 1966; Daniel & Lederis, 1967; Ishida, 1967; Dreifuss *et al.* 1971; Uttenthal, Livett & Hope, 1971). Other neurosecretory systems in which inhibition of hormone release in response to elevation of extracellular potassium has been observed include the fish urophysis (e.g. Berlind, 1972), insect abdominal neurohaemal structures releasing diuretic hormone (Maddrell & Gee, 1974), corpora

cardiaca (e.g. Normann, 1974), and *Aplysia* bag cells releasing egg-laying hormone (Arch, 1972).

Relatively few studies have tested the predictions of the calcium hypothesis on neurosecretion elicited by the more physiological method of stimulation, i.e. stimulation of the axonal tract. Blocking of electrically elicited release of hormone by removal of calcium has now been demonstrated for the neurohypophysis (e.g. Mikiten & Douglas, 1965), for release of a cardioexcitor peptide from crab pericardial organs (Berlind & Cooke, 1968, 1971) and release of a cardioexcitor (but not a hypoglycaemic hormone) from cockroach corpora cardiaca (Gersch *et al.* 1970). With this latter exception, we are not aware of any system in which the predictions of the calcium hypothesis have not been met when tested.

The present observations show that in the isolated sinus gland system, hormone is released when electrical stimulation of the axons elicits propagated electrical responses of the sinus gland, and that the presence of calcium in the bathing saline is essential for this secretion. Thus, the mechanisms linking neuronal activity and secretion appear to be similar to those of most other systems studied and support the relevance of the sinus gland as a model system for further study of peptide neurosecretory mechanisms.

METHODS

Animals. The experiments were performed on isolated sinus gland-sinus gland nerve preparations from *Cardisoma carnifex* (Herbst) and *Portunus sanguinolentus* (Herbst). Large *Cardisoma* males, carapace width 9 cm, weight about 300 g, were obtained from Fanning Island. They are terrestrial crabs and survive for months kept in screened cages outdoors. They were supplied with pans of fresh and seawater and fed laboratory rat pellets. *Portunus* are shallow water marine crabs reaching sizes of 14 cm, measured laterally from spine to spine, and weights of 170 g. They were trapped locally and held for short periods (less than two weeks) in a filtered, recirculated seawater system and fed fish scraps twice a week. *Ocypode pallidula* Jacquinot (until very recently *O. laevis* Dana), for the bioassay, were collected from local beaches and held for up to a month in cages containing sand and providing access to flowing seawater. They were fed fish food pellets. The experiments were performed over a period of two years during nearly all months of the year.

Dissection. An eyestalk was cut from a crab and the exoskeleton removed with minimal disruption of the underlying tissue. Further dissection was done under chilled seawater in a petri dish with a blackened wax bottom. The sinus gland and usually the distal portion of the sinus gland nerve can be seen by reason of their iridescent white colour upon removing overlying muscles and connective tissue. By fine dissection under a binocular microscope, the sinus gland nerve and sinus gland are freed and transferred without bringing them through the liquid surface to a small (7 mm diameter) depression in the bottom of the same chamber. Here the sinus gland is pinned with cactus spines through fragments of adhering connective tissue. Pretreatment of the paraffin with petroleum jelly results in a small volume of fluid (ca. 0·1 ml) remaining in the chamber when the rest of the Petri dish is drained. The chamber is then perfused with the normal saline at room temperature (21–22°) at about 0·5 ml/min by means of inflow capillary and drained by a paper wick to a

deep trough in the wax. The time elapsed between removal of the eyestalk and commencement of perfusion is usually less than 45 min. The normal perfusion fluid is a modification of that developed by Dalton (1958). Its composition, in mM, is: Na, 468; K, 17·6; Ca, 25; Mg, 17; Cl, 552; SO_4, 9; it is buffered with 9 mM H_3BO_3, adjusted to pH 7·4 with 0·5 N NaOH (Welsh, Smith & Kammer, 1968). Reduced calcium salines are maintained osmotically equivalent by an appropriate increase of NaCl.

Recording and stimulating arrangements. Recording and stimulating electrodes consisted of saline-filled glass capillaries fitted with an internal and an external silver wire. For stimulation, the cut end of the sinus gland nerve was drawn into the capillary. Rectangular stimulus pulses of 0·3 ms duration were led from an electronic stimulator via an isolation unit. A mechanical counter could be set to stop stimulation after a selected number of stimuli. The recording electrode had a pore diameter of 50–70 μm. This was placed with the aid of a micromanipulator against the surface of the sinus gland; its placement in each preparation was recorded in a sketch. The leads were taken to a high input impedance, differential, a.c. preamplifier with gain of 1000 and half-amplitude frequency response filters set for 0·3 and 10 kHz. The output was taken to an oscilloscope and audiomonitor. An oscilloscope camera recorded traces synchronized with the stimulus or vertical deflexions of an unswept spot on continuously moving film.

Experimental routines. The experiments required that perfusion to the preparation be halted for a given time (usually 5–7 min), the preparation being left undisturbed in control periods or stimulated in experimental periods. Saline from the preparation chamber was then collected for bioassay. When an experimental series was to begin, perfusion was halted by redirecting the inflow pipette into the drain trough and removing the wick. Fluid was removed from the chamber with a 50 μl syringe and blunt needle, taking care not to lower the fluid level below the preparation. The chamber was refilled and fluid removed in this manner twice, and then a control period begun. This ensured that the perturbation to the preparation was similar for all collection periods. After a timed interval, fluid was withdrawn in a similar manner and the chamber refilled and fluid withdrawn twice more. The volume withdrawn, usually about 0·1 ml, was measured and deposited in a vial. Saline was then added to make a standard assay volume of 0·2 ml. Perfusion was restored to the preparation chamber for at least 1 min between collection periods. Assays were usually performed within 3 h. Collected perfusate activity is stable over periods in excess of this.

The standard electrical stimulation routine was a series of 35 stimulus trains given at 10 s intervals consisting of 5 stimuli at 5/s. Thus 175 stimuli were given in a period just under 6 min. The five responses to the 1st, 10th, 20th, 30th, and 35th trains were each photographed as superimposed traces from the oscilloscope. In some experiments, spontaneous activity between stimulus trains was monitored with continuously moving film. The preparation was left for 1 min after stopping electrical stimulation before beginning collection of the perfusate and two rinses as described. In *Cardisoma* experiments, unstimulated control collection periods were made just prior to and just after the electrical stimulation period (allowing, as always, 1 min perfusion between collections).

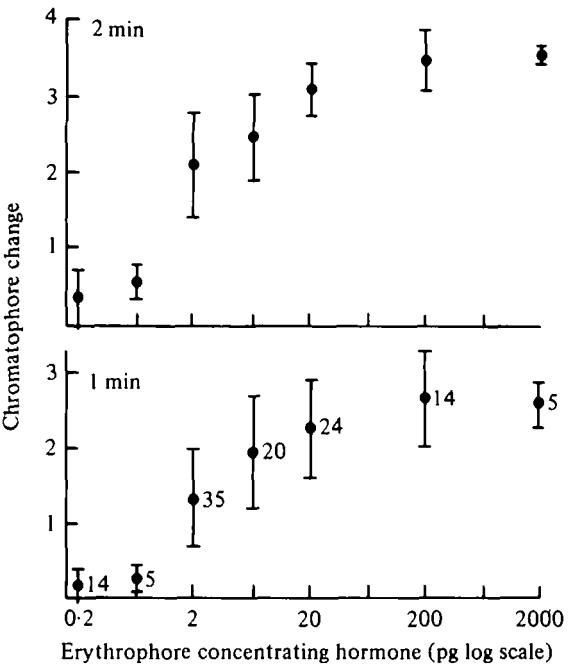

Fig. 1. Log dose vs. response curves for synthetic erythrophore concentrating hormone (ECH) on *Ocypode* erythrophores. Individual observations were the averaged change of stage of five erythrophores at 1 and at 2 min after introducing a 0·2 ml sample containing the total weight of hormone indicated into an isolated leg segment. Numbers give the number of such observations included in each averaged point and brackets indicate the standard deviation. Minimum assayable hormone is *ca.* 1 pg, or a solution having a concentration of 5×10^{-13} M.

When the effect of altered salines was to be tested, the new saline was brought through the perfusion tubing by allowing it to flow into the drain trough for 2 min before being introduced to the preparation chamber. The preparation was then perfused with the new saline for at least 1 min before halting perfusion for any assay period.

The bioassay. The assay system will be described in detail elsewhere (see, also, Weatherby, 1976). Isolated meropodite segments of the legs of *Ocypode pallidula* are perfused with the normal saline causing red chromatophores to become stellate. To perform an assay, perfusion is halted and the leg filled with the sample. A sample volume of 0·2 ml is used, as this is very slightly in excess of that required to displace all saline from the leg segment. The stages of five preselected chromatophores are noted according to the 5 point scale of Hogben & Slome (1931) at 1 and 2 min; stage 5 is fully stellate, stage 1 is completely punctate. No fractional scores are given. The scoring of stages is unambiguous to an experienced observer. (Nevertheless, to avoid subjectivity, assays were usually made by a worker unaware of the contents of the vials.) The scores for the five chromatophores were averaged, and the stage change used to compare with the dose-response curves (see Fig. 1), obtained by many assay trials on many legs, of dilutions of a stock solution of *Pandalus borealis* synthetic erythrophore concentrating hormone (Fernlund & Josefsson, 1972; Fernlund, 1974*b*). The dose-response curve (for 2 min observations) for dilutions of an aqueous

Extract of *Cardisoma* sinus glands and of *Ocypode* sinus glands are practically superimposable on that of the synthetic hormone over a concentration range of four log units. Although the chromatophore stages were chosen for unambiguous visual recognition, the log dose vs. response plot approximates a straight line for a change of up to two stages as observed at 1 min, and for changes of between 1 and 3 stages at 2 min. ECH concentrations causing faster or greater changes show evidence of saturating the response. Perfusate samples which caused saturating, indistinguishable responses at 2 min (i.e. a chromatophore change greater than 3) usually exhibited different degrees of chromatophore contraction at 1 min. Any individual assay of a given concentration may vary as much as $\pm$ one stage from the averaged assay response. For individual assay legs, however, the dose-response curves generally parallel that of the curve representing the averaged observations. Thus, when it is possible to make assays on the same leg, as was the case for stimulated collections in Fig. 2 and 4, the comparisons of relative activity between samples gain in confidence. Since in most experiments only a single assay was made, only an order of magnitude estimate of hormone present is available. However, the conclusions reached in these experiments are based on very large differences in the assay responses which were observed in several repetitions of each experiment. Results are expressed in terms of change of chromatophore stage at 2 min produced by the perfusate collected from the experimental chamber upon assay, unless otherwise stated.

Tests were made to examine whether the altered salines by themselves caused chromatophore changes, or interfered with or changed the sensitivity to sinus gland extract. The presence of tetrodotoxin (5×10^{-7} M) did not change assay responses. Salines having calcium reduced to less than 10% did interfere with the chromatophore responses. However, since at least an equal volume of normal saline was added to the perfusate withdrawn from the chamber, calcium in the assay sample was never less than 50% normal. Assay responses were unaltered by reductions in calcium of this magnitude. For reduced calcium experiments, dilutions of the assay aliquots from periods in normal saline were made with the altered saline to be tested, thus making the assay control and test samples the same with respect to their saline ionic composition.

Criteria for successful isolation of a sinus gland. Before performing any experimental series on an isolated sinus gland, the preparation was tested by assaying hormone release from an unstimulated period and a period of stimulation by the standard regime at optimal voltage for recording an electrical response. Assays from unstimulated controls were required to be less than a one-stage chromatophore change, and from the stimulated period, at least a one stage greater change than the unstimulated control. Twenty-nine of 40 *Portunus* sinus glands dissected and 60 of 71 *Cardisoma* preparations met these criteria.

In experiments on the effects of reduced calcium salines, it was required that the original criteria also be met after return of the preparation to normal saline following the tests in altered calcium saline. Nineteen of 30 experiments with *Cardisoma* met the criteria; six were excluded because the preparations began to show significant rates of spontaneous release during exposure to the reduced calcium saline and continued to release spontaneously after return to normal saline. Five trials were excluded because stimulated release after return to normal saline was not assayed

(2 cases) or did not reach significant levels. Of 17 preparations of *Portunus* tested in reduced calcium salines, 5 met these criteria; most exhibited an increased level of spontaneous hormone release during exposure to low calcium salines which did not return to criterion levels on restoration of the preparation to normal saline.

RESULTS

Correlation of electrical responses with hormone release

Electrically elicited hormone release. Isolated sinus gland preparations were routinely tested before further experimentation for their ability to release hormone in response to electrical stimulation. A stimulus intensity was selected that gave a maximal propagated response recorded from the sinus gland. In 29 of the 40 *Portunus* preparations and 60 of 71 *Cardisoma* isolated sinus glands, hormone present after a period of stimulation (175 stimuli in trains of 5 at 5/s every 10 s, in most cases) exceeded that in saline from an unstimulated period of equal duration by at least one chromatophore stage when assayed. In the majority of the preparations there was undetectable hormone activity or a level giving less than one chromatophore stage change when the perfusate of the 'control', unstimulated period preceding the test period was assayed. When higher unstimulated levels of release were present, there was usually a poor electrical response; both may reasonably be attributed to damage in dissection. Roughly half of those preparations which did not show increased release during stimulation showed none or much lower amplitude of the compound electrical response than was observed in the majority of preparations. These provide a control group indicating an essential association between propagated electrical activity and hormone release.

As a test for the possibility that stimulus current may be capable of releasing hormone independently of stimulating axonal action potentials, nine *Cardisoma* preparations were given the standard repetitive stimulation routine at high current intensities with the electrode removed from the nerve but remaining in the 0·1 ml bath with the preparation. Two of eight preparations tested with a current strength of 400 μA showed an increase in hormone present in the perfusate over unstimulated control periods. Four preparations were tested with 1 mA pulses; two showed hormone release giving just significant assay levels; one continued hormone release in subsequent unstimulated periods, suggesting that damage may have occurred. None of the preparations showed release in response to stimulus trains using current strengths of 300 μA or less. Current strengths used in the other experiments here reported rarely exceeded this value and never exceeded 400 μA.

We did not observe any propagated electrical activity or change in spontaneous activity recorded from the sinus gland during periods in which current pulses were passed into the bath, with the exception of one preparation tested at 1 mA which exhibited increased spontaneous activity (but did not show detectable ECH release). Our recording electrode, however, samples a very small part of the sinus gland population of axons and terminals. Efforts to observe hormone release in the presence of tetrodotoxin (TTX), whether by stimulation with the electrode on the nerve (see further below) or in the bath, have never given evidence for release. This was true one preparation which did show detectable release during passage of 1 mA current

pulses into the bath in normal saline. Thus it seems likely that the few cases in which strong currents released detectable hormone resulted from the induction of action potentials, rather than direct effects on the release mechanism.

In each of the preparations used in these tests, the nerve was afterwards taken up in the stimulating electrode and levels of ECH assayed following unstimulated and stimulated periods with current strength much reduced to that giving just optimal propagated electrical responses. Stimulated release reached much higher levels than with the nerve not in the electrode. We conclude that at the current strengths used in our experiments, electrical stimulation causes hormone release by initiating action potentials in the neurosecretory axons, and not by a direct effect of electrical current.

Tetrodotoxin block of electrical and secretory responses. The conclusion that propagated action potentials cause hormone release is strengthened by the outcome of the experiments in which preparations were exposed to 3×10^{-7} M tetrodotoxin (TTX). Within 45 s of commencing TTX perfusion, all electrical activity, both spontaneous and in response to stimulation of the sinus gland nerve, disappeared. Perfusate from control and stimulated periods following the usual routine was collected and assayed. In all six preparations examined, hormone release during stimulation in TTX saline was not significantly different from unstimulated release. In all preparations, electrical responses were again observable and significant levels of ECH activity were released in response to stimulation when the sinus gland nerve was stimulated following an hour's perfusion with TTX-free saline.

These observations are consistent with others indicating that action potentials of sinus gland nerve axons are sodium-mediated (Iwasaki & Satow, 1971, 1973; Cooke, 1971, 1977). They strengthen our conclusion that hormone release in response to sinus gland nerve stimulation in these experiments is the result of action potentials propagated to the terminals and not due to a direct effect of the stimulus current.

Correlation of electrical and secretory response thresholds. A series of experiments examined the correlation between hormone release from the isolated sinus gland and electrical activity attributed to action potentials propagated by the neurosecretory axons to and into the terminals. In six preparations, the amount of ECH activity released to the perfusate during stimulation of the sinus gland nerve with the standard regime (5 stimuli at 5/s, every 10 s for a total of 175, about 6 min) was determined for a series of stimulus intensities and compared with the amplitudes of the electrical responses recorded extracellularly from the sinus gland. In three of the six preparations, when the stimulus was adjusted for a just detectable electrical response, subsequent assay showed no difference. In the experiment illustrated (Fig. 2), a doubling of current above threshold led to a noticeable increase in the amplitude of the recorded electrical activity and high levels of hormone release. The maximum response is approaching or exceeding the limits of the linear assay range for 1 min stage readings (see Methods). In all six experiments, stimulation at about twice threshold gave near maximal electrical response and high levels of hormone release.

The response recorded with a small (50–70 μm diameter) pore electrode applied to the surface of the sinus gland is in most cases predominantly monophasic as might be anticipated in recording from terminals, (e.g. Figs. 2, 9). The small increment in stimulus intensity required to increase the propagated electrical response from just detectable levels to maximal agrees with histological observations (Potter, 1956) of a

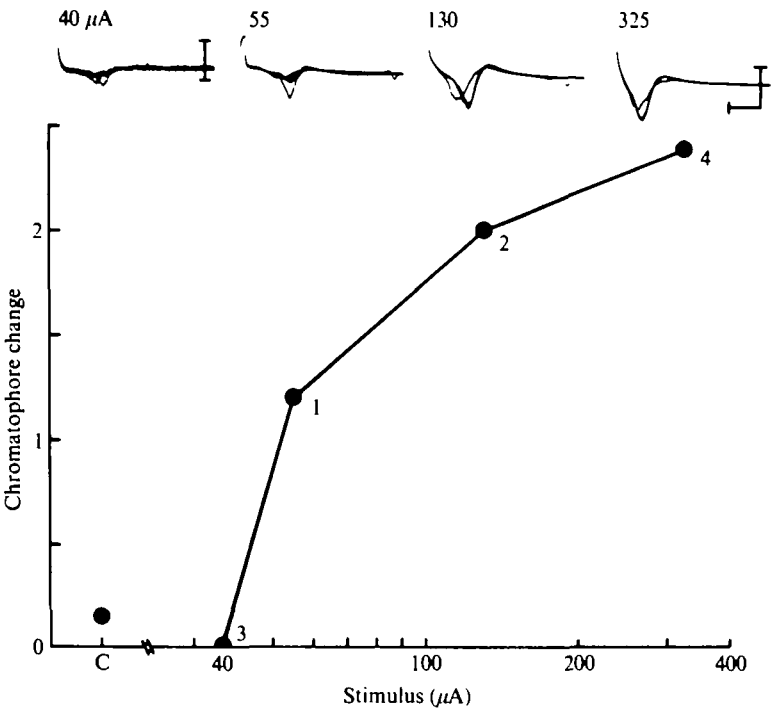

Fig. 2. Electrical responses and hormone assays for different intensities of sinus gland nerve stimulation. Superimposed responses (propagated compound action potentials) to a train of stimuli at 5/s at the voltage indicated are shown in the frames at the top (scales: 2 m/s, 1st frame, 0·1 mV; others 0·5 mV). The graph plots chromatophore stage change (at 1 min) in assays of fluid collected after standard patterned stimulation with 175 pulses *vs.* the stimulus current (log scale) (all assayed on the same preparation). Point *C* is the average of all control assay responses (fluid collected after 7 min in contact with the unstimulated preparation). Propagated electrical responses and hormone release show similar thresholds and increase with increasing stimulus current in parallel.

population of 'more than 200' axons of relatively uniform size (1–6 μm diameter). The responses to repeated stimuli, when superimposed, show fluctuations which vary in discrete increments. The fluctuations are interpreted to represent varying contributions to the compound potential of individual axons or terminals due to varying latency or responsiveness. These fluctuations are within the range of amplitudes of 'spontaneous' potentials recorded by the pore electrode (see further below). There was in many cases an increase in the amount of variability in the responses to individual stimuli within the trains and a decrease in the amplitude of the compound action potential with continuing stimulation in the 35-train standard routine. Observations made with intracellular electrodes show that individual terminal responses consist of overshooting action potentials with a half-amplitude duration of the order of 10 ms and in many cases an after hyperpolarization lasting hundreds of ms. Individual terminals fail to respond consistently to repetitive stimulation at frequencies exceeding 5/s. The intracellular observations will be presented in detail in a separate paper (see brief reports in Cooke, 1967, 1971, 1977). The pore electrode thus appears to sample electrical activity of a discrete population of axons and terminals localized near the tip, and not a massed response of the sinus gland as a whole.

Release following cessation of electrical stimulation. In the experiments with *Cardisoma* sinus glands, saline was assayed from an unstimulated control period just prior to electrical stimulation and from an unstimulated period of equivalent duration immediately following stimulation. In a majority of cases, these post-stimulus control assays showed higher levels of hormone present than the pre-stimulus controls. Although the level of hormone present in the pre- and most of the post-control periods was less than enough to give a change of one chromatophore stage (as, for example, in Fig. 4), there were enough observations in which the post-control amounts of hormone reached significant levels to indicate a consistent trend.

Possible explanations are (1) that hormone is released into the internal blood sinuses of the sinus gland and then continues to diffuse to the perfusate for some time after cessation of stimulation; and (2) that spontaneous electrical activity persists after a period of stimulation and results in hormone release. Both might contribute.

Concerning the suggestion of delayed diffusion, we have noted that effects on electrical activity of salines with reduced calcium appear within 30 s of the change of bath (see later). However, the recordings are from surface terminals. Assayable amounts of hormone (chromatophore change of more than one stage) have been obtained from *Portunus* after 25 stimuli when the perfusate was in contact with the preparation for just over 1 min. These observations are evidence against an important barrier to diffusion of hormone at the surface of the sinus gland. However, they do not exclude slow diffusion from the internal sinuses. If continued release after stimulation is due to delayed diffusion, the amount of hormone appearing during a given time after ceasing stimulation would be expected to be proportional to the amount released during stimulation. Our data are not adequate to test this prediction. The timing of the beginning of post-stimulus control perfusate collection would be critical and was not precisely controlled in our experiments, and the levels of hormone to be assayed are at the limits of the quantitative range of the assay.

A major contribution to continued hormone release after cessation of stimulation by continuing electrical activity of the sinus gland can almost certainly be ruled out: the heightened level of spontaneous electrical activity recorded immediately after trains of stimuli has always been observed to decline to pre-stimulation levels even before the collection of perfusate for the 'stimulated' period has begun. This electrical activity is described below.

Spontaneous electrical activity. The fluid-filled capillary applied to the surface of the sinus gland occasionally records brief electrical potential changes in the absence of stimulation. Spontaneous activity appears to represent action potentials of single axons or terminals as judged by the unique amplitude and shape of individual deflections. Such 'units' are sometimes seen to appear repetitively at a regular frequency; more commonly, the recurrence of a recognizable unit is at apparently random intervals. Unit potentials vary in amplitude from a size just detectable in the noise of the trace to as large as 1·5 mV. In ten cases (see e.g. Figs. 3, 7 and 8) a fortuitous electrode placement on the sinus gland detected tri-phasic potentials consisting of a rapid biphasic response immediately followed by a slower monophasic deflection. In the light of intracellular recordings to be reported separately (see also Cooke, 1977), this could reflect axonal conduction to a terminal of an action potential of normal

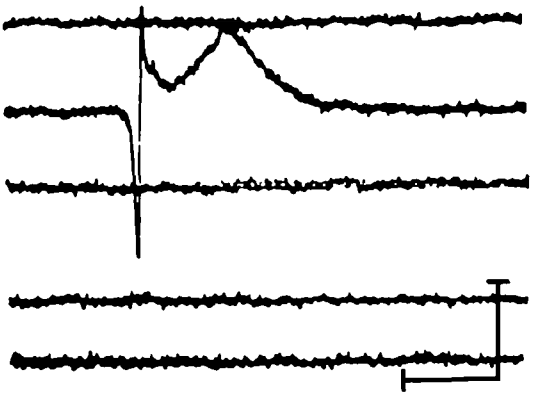

Fig. 3. Sinus gland spontaneous unit activity. Recorded during an unstimulated period by means of a capillary (50–70 μm) applied to the surface of the sinus gland, the rapid biphasic potential is always followed by the slower, monophasic potential. By reason of their time-courses, the rapid potential is interpreted as an axonal action potential and the slower potential, a terminal action potential. The 'unit' is recognizable by its consistent complex form and amplitude. The baseline was shifted following each sweep. Scales: 10 m/s, 200 μV. See also Figs. 7, 8.

duration followed by an active depolarizing response of the terminal lasting about ten times longer.

In normal saline, spontaneous electrical activity is infrequent in unstimulated preparations. However, during the 9 s intervals between the repetitive trains of stimuli used in our standard routine, increased levels of spontaneous activity were consistently observed. A particularly active preparation is illustrated in Fig. 8. Such activity consists of the appearance of 'unit' potentials of various amplitudes; usually at its maximum the activity reaches rates of about 6 units of various sizes per second. These observations are consistent with observations made in intracellular recordings from single terminals, where a period of increased spontaneous action potentials is frequently observed to follow repetitive stimulation. However, in neither case has the spontaneous activity lasted longer than 30 s to 1 min.

Durability of hormone release in response to electrical stimulation. The *Cardisoma* isolated sinus gland preparation is capable of releasing easily assayable amounts of ECH hormone from numerous periods of stimulation and for many hours after isolation. We have not studied in detail the time course of exhaustion of hormone release, but six preparations not otherwise treated were tested for hormone release in response to the standard routine of 175 repetitive electrical stimuli over a period of 7 h or more. Five of the six gave six or more periods of stimulated release without any evidence of a decline in the amount of hormone released. The sixth began to show high levels of release in unstimulated controls after 4 h, interpreted to represent deterioration of the preparation. One of the five showed some decline of stimulated release, in the 7th period of stimulation, 9 h after dissection.

Six *Cardisoma* sinus gland preparations that had been used for other experiments during the initial six hours after dissection were left perfusing overnight, and hormone release in response to electrical stimulation was tested the following day, 24–31 h after dissection. With one exception (the same preparation which exhibited fatigue

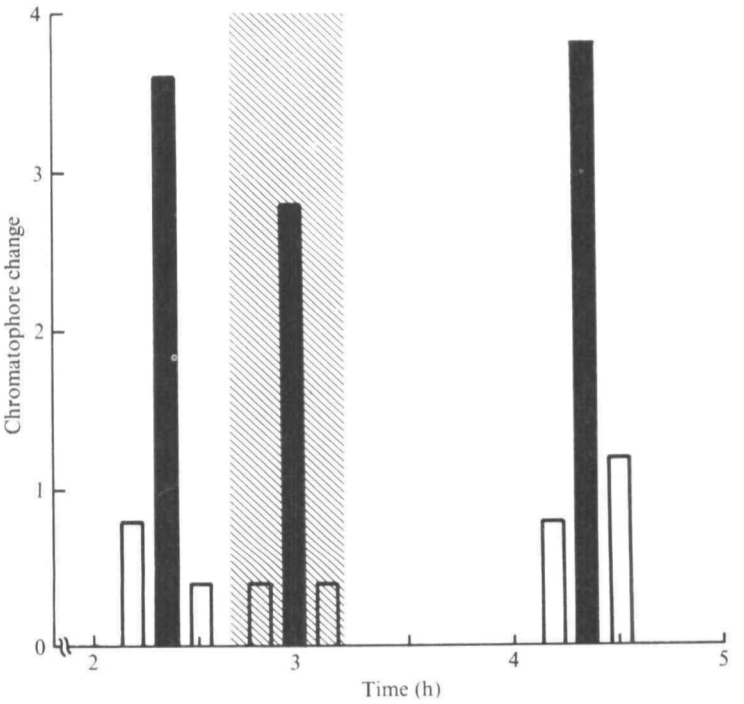

Fig. 4. Effects of reduced saline calcium on electrically elicited hormone release from a single sinus gland preparation. The bars show the chromatophore change (at 2 min) when fluid from the preparation chamber was assayed; empty bars, unstimulated; solid bars, electrically stimulated; they are placed on the horizontal axis to represent the time (relative to isolation of the preparation) that the assay aliquot was in contact with the preparation (usually a 7 min period). Cross-hatching indicates the period in 10% (2·5 mM) calcium saline. Reduction of saline calcium leads to reduction of hormone release in response to stimulation. Comparison of assays with Fig. 1 indicates that hormone released in 10% calcium saline was less than 10% that in normal saline. Electrical stimulation: 175 pulses of 0·3 m/s duration as 35 trains of 5 at 5/s every 10 s.

to repeated trials mentioned above), assays gave chromatophore responses within a half stage of those seen initially. Although the electrical responses recorded were not identical with earlier records, they were not noticeably reduced in amplitude. The changes in form were not any greater than are often observed between periods of stimulation given several hours apart during the same day. Stimulus intensities to give threshold and maximum electrical responses were in all cases increased; they are generally found to increase as a function of time since dissection.

These observations give us confidence that our preparations are in generally stable condition for the duration of the experiments and are not subject to exhaustion of releasable hormone by the number of tests used.

Effect of reduced calcium salines on electrically elicited hormone release

Effects on hormone release. A major premise of the calcium hypothesis is that entry of extracellular calcium into secretory terminals is a necessary prelude to release of the secretion. In a series of experiments, the isolated sinus gland preparation was held

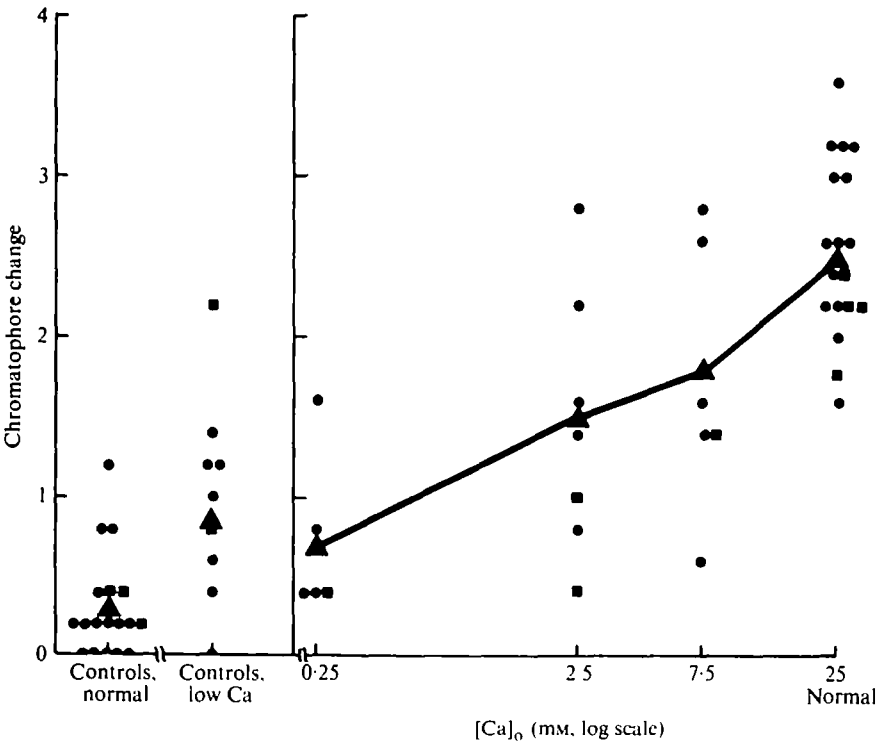

Fig. 5. Electrically elicited hormone release *vs.* saline calcium concentration. Assay results of 19 trials are plotted against calcium concentration (log scale); assays from unstimulated controls in normal and in the reduced calcium salines are also shown. ●, Individual trials; ■, second exposure of preparation to reduced calcium saline; ▲, average (excluding ■). Reduction of calcium leads to reduction of stimulated ECH release.

in saline of reduced calcium concentration long enough (20–30 min) to obtain unstimulated and stimulated collections of perfusate to be compared with assays of similar periods before and after recovery from exposure to the reduced calcium saline. Salines having 30, 10, or 1% of the normal calcium concentration (25 mM) were tested on different preparations. In a few cases, two altered salines were tested on the same preparation. The assay results of an experimental test of 10% calcium are shown in Fig. 4.

The assay responses of the 19 acceptable (see Methods) tests of reduced calcium saline are plotted against the saline calcium concentration (log scale) in Fig. 5. It will be seen that hormone release in response to electrical stimulation is reduced when the saline calcium concentration is lowered and, in saline having 1% of normal calcium, is indistinguishable from spontaneous release.

To demonstrate that the reduction of hormone release occurred in every trial with reduced calcium saline, the assay results of the same 19 experiments are replotted as shown in Fig. 6. Here the score observed on assay of the perfusate collected after stimulation in the reduced calcium saline minus the score from the preceding stimulation in normal saline is plotted against the calcium concentration (log scale). There is a reduction of hormone release in every case; on average, the reductions amount to an assay difference of one or more chromatophore stages.

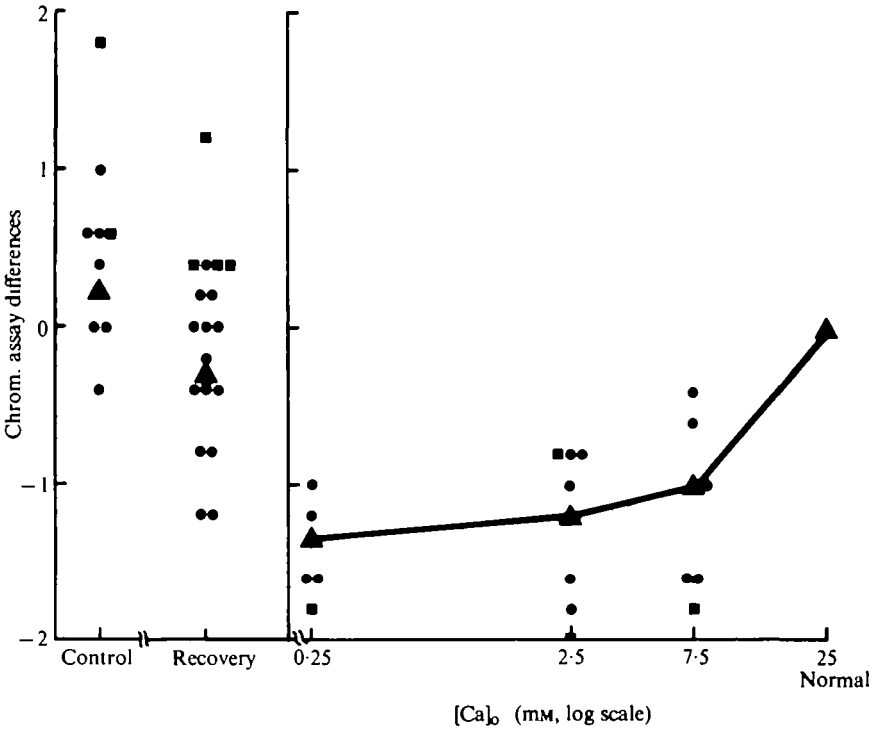

Fig. 6. Difference in hormone release in reduced calcium and normal saline for each trial. Also plotted are the differences in assays of unstimulated control periods in normal and reduced calcium saline, and of stimulation after return to normal saline minus stimulation before exposure to reduced calcium saline ('Recovery'). Second trials are excluded from averages. Symbols as in Fig. 5. Hormone release in reduced calcium saline is in every test less than in normal saline.

In order to be certain that reduction in homone release observed in reduced calcium saline was not the result of exhaustion of the releasable hormone stores of the sinus gland or of deterioration of the preparation, the preparations were returned to normal saline after each trial in reduced calcium saline and tested for the amount of ECH activity released in response to the standard stimulation regime. As mentioned in Methods, it was a condition for accepting a reduced calcium experiment as valid that, on return to normal saline, the preparation meet the critiera of low levels of unstimulated hormone release (assay less than 1 chromatophore stage change) and stimulated release exceed unstimulated by a chromatophore stage change of at least one. Eleven of 30 experimental runs were rejected because the necessary tests were not completed or these criteria were not met. Of the 19 trials accepted, assays of the stimulated period following return to normal saline were greater or within a half chromatophore stage of those before exposure to low calcium saline with four exceptions. The observations are shown as a separate set of points in Fig. 6. In all but one experiment (a 30% calcium trial), the sinus glands released more hormone on stimulation after return to normal saline than they did in reduced calcium saline.

Assays of perfusate collected during unstimulated periods in the reduced calcium salines generally gave greater chromatophore changes than assays from unstimulated

control periods in normal saline from the same preparation (see Figs. 5, 6). The amounts of hormone were in most cases at the limits of reliable detection, and hence only the consistency of the observation lends it significance. Possible correlation with spontaneous electrical activity is considered in the next section.

Experiments on isolated sinus gland preparations of *Portunus* in reduced calcuim salines gave results consistent with those for *Cardisoma*. However, only 5 of 17 experiments can be considered complete; many of the preparations increased their level of unstimulated hormone release during exposure to low calcium saline and continued high spontaneous release rates after return to normal saline. Effects on spontaneous and propagated electrical activity of low calcium on the *Portunus* preparations were also comparable to those observed in *Cardisoma* (see below.)

Effects of reduced calcium salines on electrical activity. The electrical activity that could be monitored extracellularly from the surface of the sinus gland, both that occurring spontaneously and that in response to stimulation, was recorded during the same periods that perfusate was being collected for assay in the experiments with reduced calcium salines described above. Since the reduced calcium salines affect spontaneous and propagated electrical activity in characteristic ways, the question arises whether the effects on hormone release are secondary to effects on electrical activity.

The first change observed on commencing perfusion of an isolated sinus gland with saline of reduced calcium concentration is a commencement or a marked increase in the level of spontaneous activity (Figs 7, 8). In normal saline, as mentioned previously, spontaneous activity is absent (as was the case in both the preparations illustrated) or very infrequent. Between 30 s to 1·5 min after beginning perfusion with a low calcium saline there was in every experiment a very marked increase in the frequency and number of individual units firing 'spontaneously'. It reaches a maximum within 5–10 min of beginning the low calcium perfusion and then usually declines. In the case of 30% normal calcium, spontaneous activity continues at levels greater than in normal saline (see e.g. Fig. 8). In 10% normal calcium, the activity declines sharply, with occasional episodes of high activity; in half of the preparations it ceased completely within 16–21 min of starting the low calcium perfusion. In the others, it continued at low levels. In 1% calcium, the increased levels of activity are more pronounced, but the activity declines more abruptly and ceases completely within 5–20 min.

As mentioned earlier, unstimulated control periods in reduced calcium salines gave consistently greater assay responses than perfusate of unstimulated control periods in normal saline. The control collection periods were begun 1 min after beginning perfusion with the low calcium saline and hence coincide with the period during which spontaneous activity is at a maximum. The increased release may thus reflect secretory responses to electrical activity occurring before full equilibration of low calcium saline within the tissue has resulted in inhibition of release. Consistent with this suggestion is the finding that in 30% calcium, spontaneous activity continues and unstimulated assays taken later in a trial consistently give significant assay responses. Our stimulated assays in 30% calcium indicate that a reduced but significant hormone release response is maintained at this calcium concentration. In the salines having 10% or 1% normal calcium, the later unstimulated assays were no greater or were less

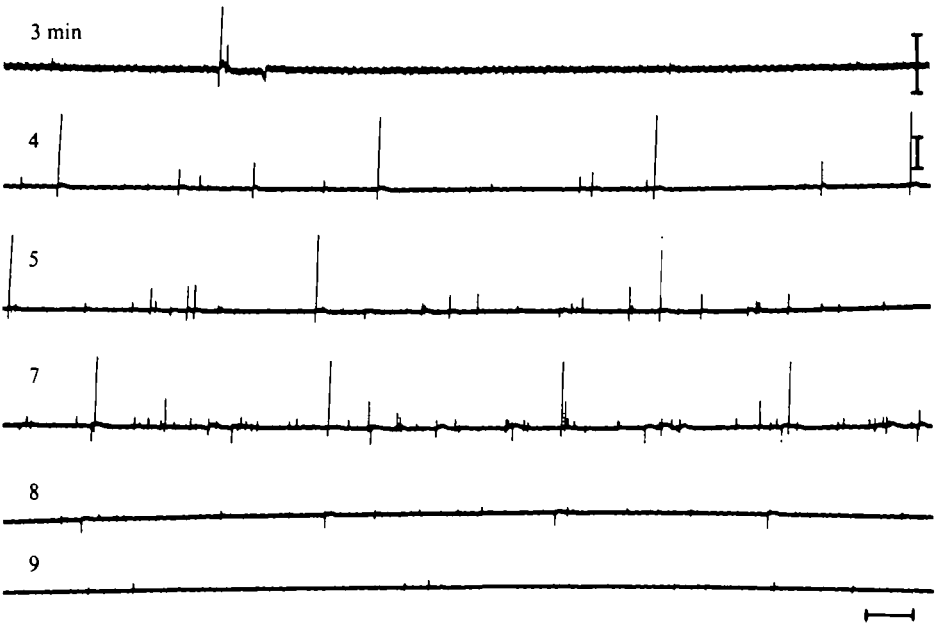

Fig. 7. Spontaneous activity during exposure to saline with reduced (10% normal) calcium. Each trace represents *ca.* 2 s continuous recording at the time (min) indicated since beginning perfusion with 2·5 mM calcium saline. This preparation showed very infrequent spontaneous activity in normal saline (not shown). At 3 min, a triphasic, large unit is recorded (compare Figs. 3, 8, other preparations). At 4 min (note decreased gain), this unit is firing regularly, and other units, including another triphasic one, have appeared. After 5 min, the number of units has increased, and the monophasic portion of the large triphasic unit is failing. At 7 min, it has disappeared; the number and rate of firing of units was about maximal for this test. A new triphasic unit is present. After 8 min, activity has declined sharply, though the triphasic unit of the 7 min trace continues to fire. After 9 min in low calcium little spontaneous activity remains. Scales: 0·1 s; 500 μV (retouched).

than the earlier ones. In eight preparations placed in nominally zero calcium saline, unstimulated controls showed reduced or no change in spontaneous hormone release compared with levels in normal saline.

Four of five preparations in which triphasic unit potentials (see, e.g. Fig. 3) could be observed showed characteristic changes in these during exposure to 10 or 1% calcium salines (as in the preparation giving the records of Fig. 7, see at 3 min). It will be recalled that such potentials have been interpreted as representing an axonal followed by a terminal action potential. As exposure to the reduced calcium saline continues, the 'terminal' potential first shows an increase in duration (4 min, Fig. 7) and then disappears from the records (5 min); the remaining 'axonal' potential ceases suddenly without any decline in amplitude a few minutes later (8 min). One preparation showed persistence of a triphasic unit without alteration of form for 18 min after commencing 10% calcium saline perfusion; it then ceased, together with other spontaneous activity.

Following return of the preparations to normal saline, the sequence of changes in spontaneous activity and in the form of the characteristic potentials occurs in reverse order. There is a period of increased spontaneous activity before the eventual return to infrequent or no spontaneous activity. The time course for recovery is of the order

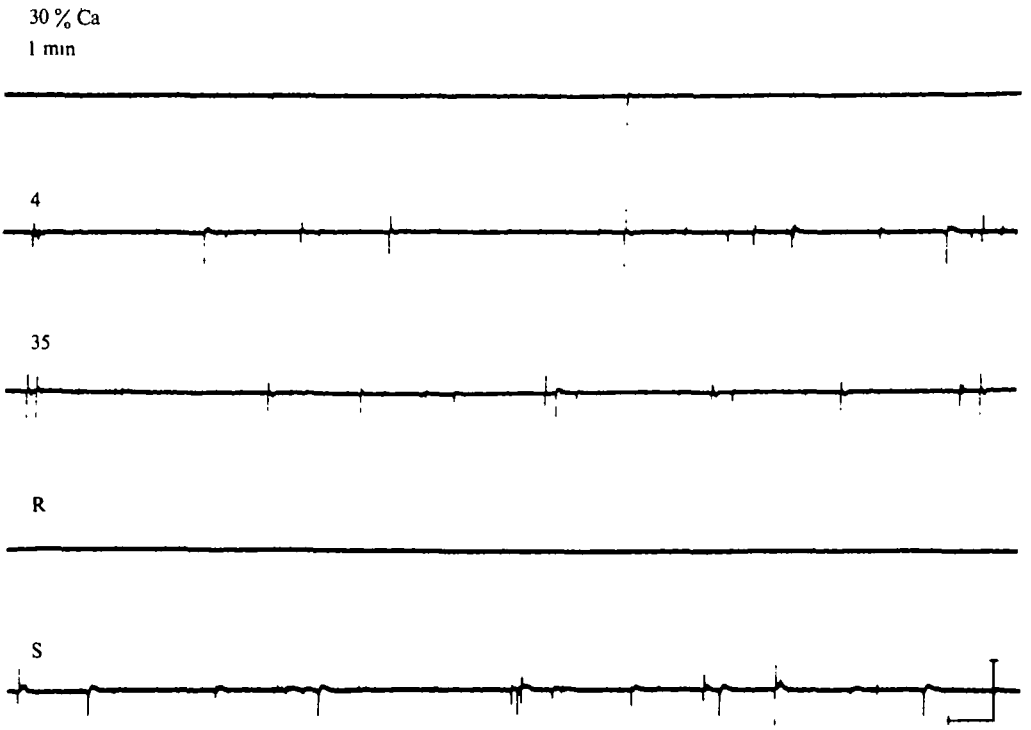

Fig. 8. Spontaneous activity during exposure to saline with 30% normal calcium and following trains of electrical stimulation. Activity increases during the first 4 min in 7·5 mM calcium saline and then remains approximately the same. At least three units having a slow monophasic component (interpreted to represent a terminal action potential) can be distinguished at 4 min. The same units are present and show little change of form after 35 min in reduced calcium (compare Fig. 7). On return to normal saline (R), spontaneous activity ceased within 1 min. *S* shows spontaneous activity following the 20th train of standard patterned electrical stimulation of the sinus gland nerve. It was recorded an hour after return to normal saline with unchanged electrode placement. Certain units appear to be the same as those seen during 30% calcium perfusion, though slightly changed in form. Scales: 0·1 s; 500 μV (not retouched).

of two or three times as long as for the onset of the effects of the low calcium salines.

Records from a preparation tested in 30% calcium saline that exhibited triphasic unit potentials are shown in Fig. 8. The significant differences to observations in 10% calcium or 1% calcium are (1) that the level of unit activity increases (from practically none, in this case) and then remains stable for the duration of 30% calcium exposure, rather than ceasing, and (2) units which show triphasic potentials retain the same wave form for over 35 min rather than in most cases gradually losing the monophasic portion. Restoration of normal saline results in almost immediate silencing of spontaneous activity. Records are included in the figure of spontaneous activity appearing between trains of electrical stimuli applied to the sinus gland nerve as recorded with the same placement of the recording electrode. Minor changes in waveform of otherwise similar unit potentials to those observed during 30% calcium perfusion may be the result of a slight change in the recording situation over time

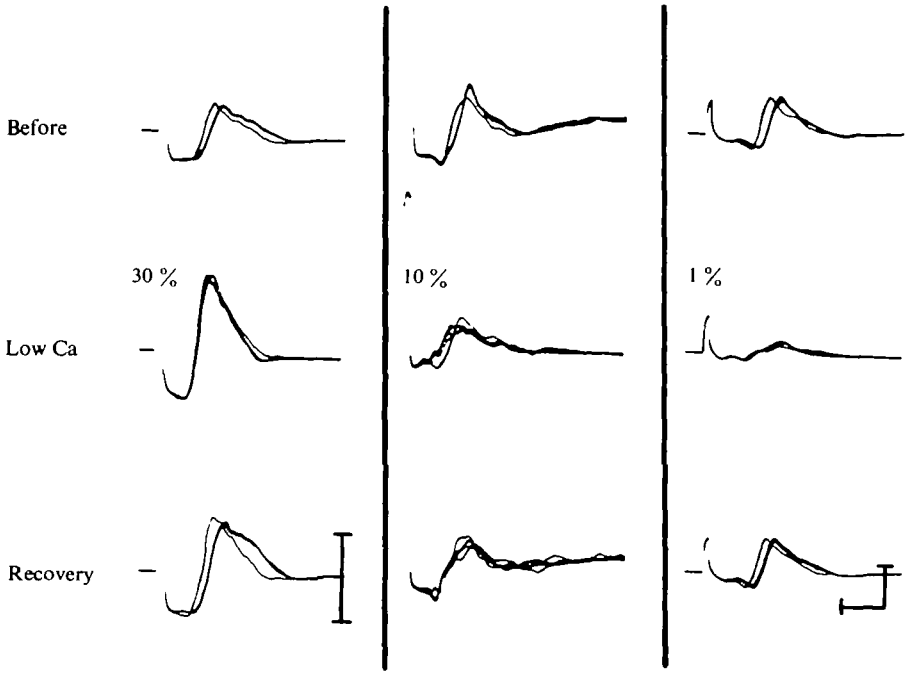

Fig. 9. Sinus gland electrical responses to nerve stimulation in reduced calcium saline. Each frame shows superimposed the propagated, compound action potentials in response to the 20th of 35 trains of 5 stimuli at 5/s; trains were given every 10 s. The sinus gland nerve was stimulated with a suction electrode at optimal voltage. The middle frame shows the response recorded after *ca.* 15 min in the saline with calcium reduced to the percent indicated. Frames above and below show responses in normal saline before and after. Separate traces, where seen, are responses to the first stimulus of the train. In 30% of normal calcium, the response shows reduced latency (*ca.* 30%) and increased amplitude; in 10% calcium, reduced latency, reduced amplitude and increased variability; in 1% calcium, the response is depressed. The effects are reversible. Scales: 2 ms 2 mV.

(1 h), different sites of initiation of the activity, or an effect of the change in calcium concentration.

Changes in the propagated responses to stimulation recorded from the sinus gland during treatment with reduced calcium salines in almost all tests include a reduction in conduction time (Fig. 9). In 30% calcium, there was increased definition of the peak of the response, and, in about half the preparations, increased amplitude, interpreted as reflecting reduced variability in response and conduction time and thus better summation of individual responses. Preparations in 10 or 1% calcium saline in most cases showed a reduced latency, 'peakier' response to the first few stimuli, after which the compound potential in some preparations declined and became quite variable from stimulus to stimulus. The records in Fig. 9 are taken after this initial decline and are representative of the electrical responses observed during the majority of the period during which perfusate collection is made. After reintroducing normal saline, a number of the preparations exhibited, for an hour or more, propagated responses of greater amplitude than those recorded before introduction of reduced [illegible]ium saline.

A number of the preparations showed little change in the amplitude of the response

to stimulation during exposure to reduced calcium saline. These cases may represent an electrode placement that monitors predominantly axonal and not terminal action potentials. This suggestion is supported by experiments (on *Portunus* preparations) in which activity was monitored simultaneously by two extracellular electrodes, one placed on the sinus gland nerve and one on the sinus gland. The responses recorded from the sinus gland nerve showed, in all reduced calcium salines, decreased latency, increased definition of the peak of the compound potential, and in many cases, increased amplitude; amplitude decreases were not observed. At the same time, responses monitored from the sinus gland were often reduced, as previously described for the *Cardisoma* preparations.

In summary, reduced calcium salines produced variable effects on the propagated electrical responses to stimulation. The variability may be the result of different electrode placements; the pore electrode monitors a very small part of the terminal population. The changes observed include enhancement of the compound action potential (most often seen in 30% calcium, never in 1%), little change in amplitude, or decrease in amplitude (most often seen in 1% calcium saline, never in 30%).

The extent of reduction of hormone release by low calcium salines was not clearly correlated with whether or not the propagated electrical responses were reduced. As shown previously (Figs. 5, 6), in all preparations providing adequate data, low calcium salines produced a significant reduction in hormone released in response to electrical stimulation.

DISCUSSION

Neuronal control of neurosecretion. The present observations add another neurosecretory system to those in which it has been shown that hormone release is under the control of action potentials propagated to the terminals by the axons of the neurosecretory neurons. The others include the vertebrate neurohypophysis (e.g., Mikiten & Douglas, 1965; Ishida, 1970; Dyball, 1971; Dyball & Dyer, 1971; Dreifuss *et al.* 1971), the insect brain-corpora cardiaca system (e.g. Kater, 1968; Normann & Duve, 1969; Gersch, *et al.* 1970), *Aplysia* bag cells (Kupfermann, 1970), and crab pericardial organs (e.g. Cooke, 1964; Berlind & Cooke, 1970). Although the evidence presented in this paper applies strictly to the release of erythrophore concentrating hormone (ECH), the broader conclusions can probably be generalized to the entire sinus gland system, for the following reasons. Propagated electrical responses to sinus gland nerve stimulation have not been observed to vary noticeably or in any systematic way with the location of the extracellular recording electrode. Intracellular recordings do suggest that individual terminals may have different electrical characteristics, such as greater or smaller resting potentials, tendency to spontaneity, and different sensitivity to withdrawl of extracellular calcium. However, they all show overshooting, long duration (10 ms) responses (action potentials) to stimulation of their axons and no evidence of synaptic interaction (Cooke, 1967, 1971, 1977). Their differences have not to this point been attributable to topographic location in the sinus gland. In histological preparations, terminals having different staining characteristics (and on a number of lines of indirect evidence, assumed to represent terminals storing different hormones) are grouped in locations which are consistent from preparation to preparation (Potter, 1956). Therefore, if terminals storing different hormones had very

different electrical characteristics, both extracellular and intracellular recordings which have been made from different sites on the sinus gland should have indicated this.

Unlike most of the other neurosecretory systems that have been studied, histology and electron microscopy (e.g. Potter, 1956; Bunt & Ashby, 1967; Weitzman, 1969; Andrews *et al.* 1971) reveal no non-neurosecretory neuronal elements in the sinus gland. There is no morphological evidence for synaptic (electrical or chemical) interaction among elements of the sinus gland (Andrews *et al.* 1971). Hence, there is no need to consider and exclude the possible participation of non-secretory elements in the hormone release process or interaction among terminals in order to conclude that hormone release from each terminal can be signalled by electrical activity of its axon. Our assay system measured a hormone whose physiological effectiveness (at least in the case of the assay crab, *Ocypode*) may be presumed to involve its occasional rapid (within seconds) release to the circulation in large amounts. Caution should guide the extension of the conclusions to the control of release of hormones, such as moult inhibiting hormone, whose physiological role presumably requires release in very small amounts over periods of months.

The physiological role of ECH in *Cardisoma* is unclear. *Cardisoma* has a heavy, opaque exoskeleton, and red chromatophores are sparsely present underlying the transparent, flexible membranes of the leg joints. Total amounts of ECH activity per sinus gland in *Cardisoma* are the same, within limits of our assay, as found in, for example, *Ocypode*, which exhibits colour matching to its background.

Although colour change is apparently unimportant in *Cardisoma*, ECH is stored in large amounts, and its release is controlled neurally as shown in this work. It seems safe to presume that ECH is serving an important physiological role other than, or in addition to, control of red chromatophores. It would be interesting to know the extent to which red chromatophores respond to other hormones of the sinus gland.

In isolated preparations, release of ECH activity normally occurs when electrical currents stimulate axonal action potentials in the sinus gland tract which propagate to and produce active electrical responses of the sinus gland terminals. Addition of TTX to the saline rapidly obliterates all electrical activity recordable, both spontaneous and in response to sinus gland nerve stimulation; no ECH release is detectable in the presence of TTX. The TTX effects are reversible. These observations strengthen our conclusion that propagated action potentials are the normal signal for hormone release from sinus gland terminals. They also confirm previous reports that sinus gland axons exhibit typical sodium-mediated action potentials (Iwasaki & Satow, 1971, 1973; Cooke, 1971, 1977).

Threshold for appearance of hormone in the saline corresponds with threshold for recording an electrical response (see Fig. 2), and the amount of hormone release increases sharply as the amplitude of the electrical response is augmented by increasing the stimulus intensity. The population of neurosecretory axons and terminals must be relatively homogeneous with respect to its responsiveness to electrical stimulation, as might be expected from the histology (Potter, 1956), since correlation of hormone release and electrical response did not depend on the location on the sinus gland of the recording electrode. Doubling of stimulus intensity was often sufficient to increase the compound response from threshold to maximal. We conclude that action potentials

propagated to and into the terminals of the sinus gland cause the release of neuro hormones.

'Spontaneous' release. Release of hormone in the absence of axonal stimulation is normally undetectable or at very low rates. Some evidence for increased spontaneous release following repetitive stimulation was obtained; it could represent delayed diffusion of material released into the internal haemolymph spaces or a neurosecretory equivalent of the increased rate of spontaneous quantal neurotransmitter release observed at some synapses after activity (e.g. del Castillo & Katz, 1954; Miledi & Thies, 1971).

A high rate of spontaneous hormone release is considered abnormal; it was usually associated with poor electrical responsiveness and could, in many cases, be related to trauma in the course of isolation or placement of electrodes. A number of preparations especially of *Portunus*, became 'leaky' after exposure to reduced calcium salines. Finally, deterioration of a preparation with time after dissection was apparently signalled by increasing spontaneous hormone release. This may represent fatigue (for lack of ATP) of calcium sequestering or expelling mechanisms (Thorn, Russell & Vilhardt, 1975). However, it should be emphasized that the *Cardisoma* preparations were generally remarkably durable: those that were tested responded with electrical activity and hormone release more than 30 h after isolation. Glycogen seen in supportive cells (Weitzman, 1969) may supply a source of metabolic energy; the saline included none.

The role of calcium in neurosecretion. The sinus gland neurosecretory system has been shown in these experiments to conform to the general predictions of the calcium hypothesis: hormone release in response to electrical stimulation is reduced compared with release in normal saline when calcium is decreased in the perfusing saline. The amount of hormone released in response to a given regime of stimulation is a positive function of the saline calcium concentration (Figs. 5, 6). A significant reduction is produced by a saline having 30% of normal calcium concentration, and release is not detectably greater than unstimulated release in saline having 1% normal calcium. These findings are essentially similar to those in several other neurosecretory systems that have been examined, as reviewed in the Introduction.

The sinus gland preparation, however, provides more detailed information on the effects of reduced calcium saline on propagated electrical activity than is available for any other neurosecretory system. The complex, compound potentials recorded by an extracellular electrode applied to the surface of the sinus gland comprise both axonal and terminal responses (Fig. 9). Reduced calcium salines were found to produce a variety of changes ranging from enhancement (primarily in 30% normal calcium saline) to marked reduction of responses (primarily in 1% normal calcium saline).

Examination of spontaneously occurring triphasic 'unit' potentials, which we have interpreted as including a distinct component attributable to a terminal action potential, suggests that terminal potentials persist in 30%, and sometimes in 10%, calcium, but not in 1% calcium saline. We have not had enough examples to allow forceful generalizations. Recordings with two extracellular electrodes show that axonal conduction persists in the reduced calcium salines.

Studies employing intracellular recording from *Cardisoma* sinus gland termin (Cooke, 1977, and unpublished) support the above interpretations, though the

number of observations is limited. Reduction of saline calcium leads to spontaneous action potentials and to alterations of form, most prominantly in the falling phase. In all those tested in 30 or 10% calcium, spontaneous activity and responsiveness to axonal stimulation persisted. In about half those tested in 1% or nominally calcium-free saline full action potential responses continued; the remainder ceased to show spontaneous or stimulated action potentials. In some of these, stimulation of the sinus gland nerve resulted in a small response interpretable as the electrotonically spread axonal action potential. The persistence of terminal responses in tetrodotoxin (unlike axonal conduction) and other evidence has led to the conclusion that the terminal action potential in many terminals includes a major component of inward calcium current (Cooke, 1971, 1977).

The available evidence indicates that in the sinus gland neurosecretory system, calcium plays a duel role in the physiology of neurosecretion: it participates directly in generating the ionic currents contributing to the terminal action potentials and at the same time, its entry results in hormone release. Evidence that these roles are separable comes from the experiments in 30% calcium. At this calcium level there is no evidence for decrement of electrical responses, yet hormone release is significantly inhibited. Additional inhibition of hormone release when calcium is further reduced may well reflect linked effects on terminal electrical responses and stimulus-secretion coupling.

In experiments to be reported separately, reduction of calcium is found to inhibit hormone release in response to elevation of saline potassium. Such stimulation causes release by depolarizing terminals directly without requiring terminal action potentials. The experiments thus support a direct role of calcium in hormone release mechanisms, as has been shown in many other systems.

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