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IONIC DEPENDENCE OF SECRETORY AND ELECTRICAL ACTIVITY EVOKED BY ELEVATED K^+ IN A PEPTIDERGIC NEUROSECRETORY SYSTEM

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SUMMARY

Secretion of the octapeptide erythrophore- (red pigment-) concentrating hormone (ECH, RPCH) and extracellularly monitored electrical activity were followed simultaneously from individual, isolated sinus glands (neurohaemal organs), of the crab *Cardisoma carnifex*. Following introduction of saline having elevated [K], 100–196 mmol l^{-1} (5–11× normal), secretion (bioassayed from 1-min fractions during continuous perfusion) increases from barely detectable (<1 fmol min^{-1}) to a peak, average 31 fmol min^{-1}, within 5 min, and immediately subsides. Additional responses are obtainable following a period, >30 min, of normal saline perfusion. Secretory responses to K are Ca-dependent. If Ca is restored (in high K) following perfusion in 0-Ca, high K, only a small secretory response is observed. Addition of Mn (10 mmol l^{-1}, normal Ca) reduces secretion to one-tenth. Increased net uptake of ^{45}Ca of 2·5- to 6-fold is observed in individual sinus glands exposed to 10× K compared to paired, unstimulated organs. The pattern and Ca-dependence of secretory responses to K are unaffected, but the amount of secretion is augmented in Na-deficient or TTX-containing salines. Intracellular recording confirms that brief (10–40 s) bouts of intense firing recorded extracellularly upon commencing a high K perfusion include repetitive firing by terminals, superimposed on rapid depolarization. Firing ceases as the membrane potential reaches a depolarized value (−18 to −15 mV for [K] 100–176 mmol l^{-1}), which is then maintained until restoration of normal saline, when slow repolarization ensues. In 0-Ca, spontaneous impulse firing is increased, resting potential depolarized by 5 to 15 mV, but the bout of impulse firing and the maintained depolarization in response to K are similar. Thus, mechanisms of secretion of a crustacean peptide neurohormone appear closely similar to those of other systems characterized: responsiveness to elevated K, dependence on Ca, depolarization-, but not secretion-dependent inactivation, and lack of dependence on Na inward current. Intracellular recording here permits direct observation of electrical responses of terminals.

INTRODUCTION

The crab sinus gland is a neurohaemal organ, consisting of a dense aggregation of bulbous axonal dilatations and terminals surrounding haemolymph channels

Key words: Crustacean sinus gland, neurosecretion, electrophysiology, K^+-stimulation, peptide.

(Weatherby, 1981). It functions as a storage and release site for neurohormones, apparently all of peptidergic nature, which produce a wide variety of physiological effects (reviews: Cooke & Sullivan, 1982; Keller, 1983). The sinus gland and its nerve can be isolated for *in vitro* studies of neurosecretion (Cooke, Haylett & Weatherby, 1977), and in some species the terminals are sufficiently large to permit intracellular recording (Cooke, 1967, 1971, 1977, 1981; Nagano & Cooke, 1981, 1983; Nagano, 1982; Stuenkel, 1983*a*). The preparation thus presents unique possibilities for correlation of secretory and electrophysiological responses. In order to evaluate whether such observations might be widely generalized, we have studied secretion of one of the hormones in response to elevation of saline [K] under a variety of conditions to permit comparison of the results with previous observations on other neurosecretory systems.

Conformity to the expectations of the calcium hypothesis provides an initial set of criteria for the presence of secretory mechanisms akin to other systems. Briefly, the hypothesis proposes that when the surface membrane of the cell or terminal is depolarized, its calcium permeability is increased, thus permitting the entry of extracellular calcium. The resulting increase in concentration of calcium at the inner surface of the membrane promotes secretion by mechanisms involving the exocytosis of the contents of vesicles or granules in which the secretory material is stored. Although originally proposed for neurosecretion (Douglas & Rubin, 1961; Douglas & Poisner, 1964*a*,*b*) and for neurotransmitter release (Katz & Miledi, 1967*a*,*b*), the hypothesis may also be generalized to non-neural tissue (see Douglas, 1968; Rubin, 1982).

Evidence that secretion by the sinus gland is consistent with the calcium hypothesis has been previously reported. Release of red pigment- (erythrophore-) concentrating hormone (RPCH, ECH, Fernlund & Josefsson, 1972; Fernlund, 1974*a*,*b*) in response to axonal stimulation is dependent on extracellular calcium (Cooke *et al.* 1977). The Ca-dependence of secretion in response to high K has also been demonstrated. The perfusate was shown to include the same 11 peptides found in sinus gland lysates (Newcomb, 1983*a*,*b*). Ca-dependent secretion of a number of these peptides as well as of radiolabelled proteins after biosynthetic radiolabelling has also been documented (Stuenkel, 1983*a*). ^{3}H-leucine-labelled peptides included ECH and hyperglycaemic hormone(s) (Keller, 1983).

Elevation of external K concentration appears to be a universal stimulus for secretion, apparently by reason of its effectiveness in depolarizing cell membranes. In the vertebrate neurosecretory preparations, secretion declines despite the continued presence of high K. In the adrenal medulla (Baker & Rink, 1975) and the neurohypophysis (Nordmann, 1975, 1976) two kinds of evidence indicate that the decline is not attributable to depletion of releasable material: application of a secretogogue results in an immediate restoration of release, and application of elevated K in the absence of Ca inhibits secretion when Ca is subsequently restored although no material had been previously secreted. Rapid decline of diuretic hormone secretion during high K application is observed in the insect *Rhodnius* (Maddrell & Gee, 1974), and secretion is immediately restored by addition of a Ca ionophore (Berlind, 1981).

A further characteristic of K-induced secretion is its independence of the presence of voltage-dependent Na conductance mechanisms; secretion occurs, and is even

enhanced, in highly Na-deficient saline (e.g. Douglas & Rubin, 1963; Douglas & Poisner, 1964*a*; Douglas & Sorimachi, 1971; Dreifuss, Grau & Bianchi, 1971; Nordmann & Dreifuss, 1972; Nordmann, 1976).

Enhanced exchange of calcium and net calcium uptake during K-evoked neurosecretion have been demonstrated for the vertebrate neurohypophysis (e.g. Douglas & Poisner, 1964*b*; Ishida, 1967; Nordmann, 1976; Nordmann & Zyzek, 1982) and adrenal medulla (e.g. Douglas & Poisner, 1961; Baker & Rink, 1975). We report here increased net uptake of ^{45}Ca in association with K-evoked secretion of ECH from the crab sinus gland.

The high rates of ECH release produced by elevated K saline together with the availability of a bioassay capable of detecting this hormone in amounts as small as 1 femtomole (approx. 1 picogram, Cooke *et al.* 1977) have made it possible to resolve the secretory response of single, isolated sinus glands to elevation of saline K at 1-min intervals. Because secretory mechanisms of the gland appear so similar to those of other preparations, it may prove possible to generalize the information on the parallel electrical responses which can be unambiguously recorded from the terminals in this preparation.

METHODS

Animals

Large male crabs, *Cardisoma carnifex* (Herbst) (carapace width approx. 9 cm, weight, approx. 300 g) were collected on Fanning Island, Republic of Kiribati (Line Islands District), flown to Honolulu and held for periods of a few days to several months in outdoor screened cages. The crabs had unlimited access to Purina rat chow pellets, and to both fresh water and sea water. For bioassay of ECH, ghost crabs [*Ocypode pallidula* (Jacquinot), formerly *O. laevis*] were caught on local beaches.

Dissection, perfusion and perfusate collection

An eyestalk was cut from the crab and subsequent dissection performed under chilled sea water (see Cooke *et al.* 1977). After isolation of the sinus gland (a roughly spherical structure of about 1 mm diameter) and an approximately 1 mm length of the sinus gland nerve, the preparation was pinned with cactus spines in a small depression formed in black wax. Perfusion (0·3 ml min^{-1}) with physiological saline at room temperature (23 °C) was begun within 45 min of removing the eyestalk. The extracellular recording electrode (see below) was placed on the surface of the sinus gland at this time, and then the preparation was left undisturbed under saline perfusion for at least 1 h before commencing any experimental observations.

ECH secretion from isolated sinus glands was monitored either by stopping perfusion, followed by collection, or by continuous flow and fraction collection.

The 'stopped-flow' regime was that of Cooke *et al.* (1977). Upon introduction of a different saline, 1 min of perfusion with the new saline was allowed before beginning a stopped-flow period. In collecting perfusate, care was taken not to allow the sinus gland through the fluid-air interface; an initial volume collected (approx. 0·2 ml) was replaced manually with appropriate saline again and collected; this rinse procedure

was repeated. These collections were pooled in a vial and saline added to make a volume of 0·6 ml for later bioassay. After a stopped-flow period, at least 1 min of perfusion with the appropriate saline intervened before commencing another stopped-flow period.

For continuous perfusion experiments, a peristaltic pump controlled the perfusion rate (usually 0·3 ml min^{-1}). A switch to a different saline could be accomplished without deadtime. The outflow from the chamber was accomplished by capillarity and gravity so that chamber volume remained constant at approx. 0·1 ml. The volume of fluid in the outflow channel amounted to about 0·15 ml, equivalent to about a 30 s deadtime in transfer from the chamber to a collecting tube, at the usual perfusion rate (see calibration of chamber washout, below).

The were some variations in the 'normal' and 'high K' salines used. 'Normal' saline, as used in all stopped-flow regime experiments, had the following composition (in mmol l^{-1}): Na, 470; K, 17·6; Ca, 25; Mg, 17; Cl, 551; SO_4, 8·8. 'Normal' saline used in most continuous flow experiments had the same composition, except that K was 35 mmol l^{-1}. All salines were buffered with 2 mmol l^{-1} HEPES, at pH 7·6. High-K salines were made by replacing an amount of Na with an equivalent amount of K: 176 mmol l^{-1} for stopped-flow experiments, and 193 mmol l^{-1} for most continuous perfusion experiments. Checks were made on individual preparations, including monitoring ECH release and intracellularly recorded terminal membrane potential, during changes from one normal saline to another without observing any changes. The effects of various [K] on ECH release are discussed in the Results. As seen in Fig. 8A, release rates were not significantly different for any of the salines having [K] of 113 mmol l^{-1} or greater, and hence we will refer to any of these as 'high K' saline. In 'Ca-free' or reduced Ca salines, Ca was omitted or reduced and an osmotically equivalent addition of NaCl was made. In low-Na salines NaCl was replaced with an equivalent amount of choline chloride; total Na amounted to 2 mmol l^{-1}, representing the buffer. Ba-containing salines omitted SO_4 and Ca.

Tetrodotoxin (TTX, Sigma) was diluted from vials containing lyophilysed powder with citrate; A-23187 (Cal Biochem) was dissolved in ethanol at 3×10^{-3} mol l^{-1}, and further dissolved to 3×10^{-5} mol l^{-1} with saline. No effects of ethanol at 1 % were observable on the preparations or the bioassays.

Calibration of tissue and chamber equilibration time

^{3}H-inulin was used to determine the rate at which material washed out of the chamber and appeared in the sample tubes, and the rates of permeation of material into or out of the sinus gland itself. After 30 or 60 min of incubation of a sinus gland in inulin-containing saline, perfusion with standard saline was begun at the same rate used in the release experiments. Samples were collected at 1-min intervals and counted on a scintillation counter. The number of counts per fraction declined exponentially ($T_{1/2} \approx$ 1·5 min) during the first 5 min to less than 1 % of those in the first sample (see Fig. 1A).

Washout of the same chamber, but without a preparation, followed the same time course during the initial period. The $T_{1/2}$ was 0·6 min. A more gradual decline, not following a single exponential, ensued, with counts declining to within 2× background at 20 min in the case of the empty chamber, and 25 and 30 min respectively

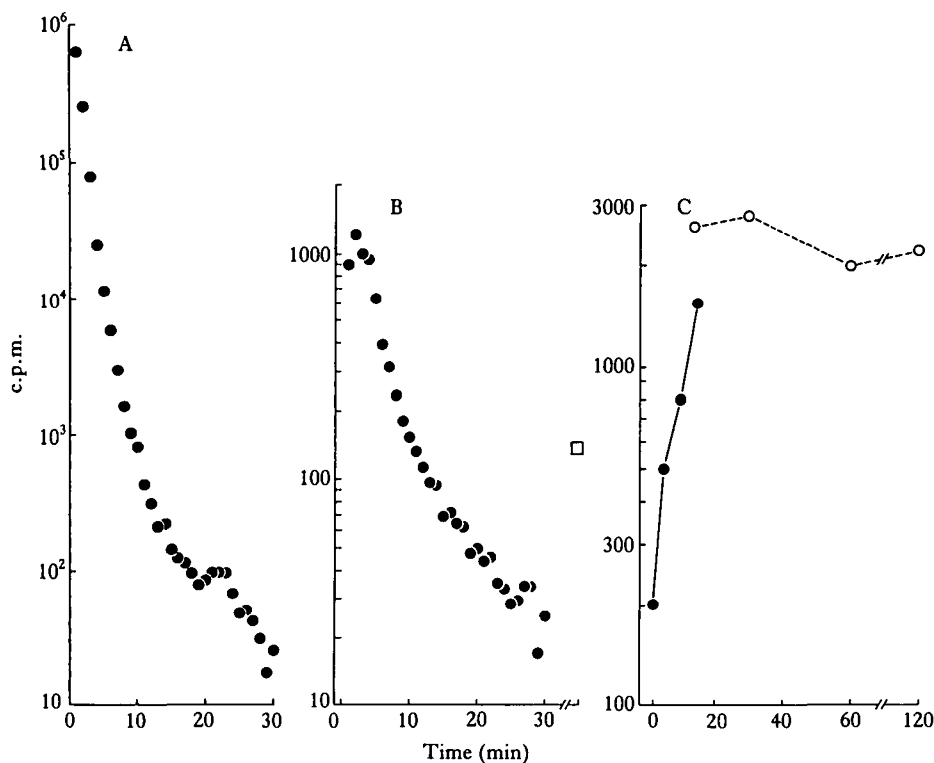

Fig. 1. Calibration of the tissue and chamber equilibration times using ^{3}H-inulin. (A) Washout curve of ^{3}H-inulin from the experimental chamber containing a sinus gland. After 30 min incubation of the sinus gland in ^{3}H-inulin (10^6 c.p.m. in 0·1 ml), saline perfusion was begun and 1-min samples collected for 30 min and counted. An approximately exponential initial decline in counts occurs over the first 20 min. The discontinuity occurring at 20–25 min is also observed in Fig. 1B and at 10–15 min in the rate of ECH release during high-K exposure (Fig. 3). $T_{1/2}$ for the early phase of the washout was about 1·5 min. Background (43 c.p.m.) has been subtracted from all values. (B) Washout curve of ^{3}H-inulin from a sinus gland exposed for 30 min to ^{3}H-inulin (10^7 c.p.m. in 0·1 ml saline) then rinsed in saline and placed in the experimental chamber. Perfusion was begun immediately and perfusate collected in 1-min samples as in A. The initial $T_{1/2}$ is similar to that in A; note discontinuity at 26 min. Counts remaining in the sinus gland after 30 min perfusion are shown (□). Background (28 c.p.m.) has been subtracted from all values. (C) Uptake of ^{3}H-inulin into sinus glands. Individual sinus glands were incubated for 2–120 min in 10^7 c.p.m. (in 0·1 ml) ^{3}H-inulin, briefly rinsed, solubilized and counted. The amount of inulin present increased with exposure to inulin for periods of up to 15 min then remained at a constant value indicating that it takes approximately 15 min to equilibrate the extracellular spaces of a sinus gland with inulin. Different symbols represent different experimental series. Background (50 c.p.m.) has been subtracted from all values.

for two preparations held 30 and 60 min initially. The slower phase of inulin washout was followed in more detail by soaking a sinus gland in ^{3}H-inulin in a separate chamber for 30 min, passing it through saline without inulin and then placing the gland in the experimental chamber and commencing perfusion and sample collection (Fig. 1B). Dye studies had shown that it required 30 s to clear the dead space between the chamber and the sample tube. Hence the maximum counts appeared in the second sample, and then declined exponentially with a $T_{1/2}$ of 2·5 min. At 9 min, the decline began to follow a slower but still exponential decline with a $T_{1/2}$ of 6 min. At the 9 min break in the curve, the number of counts had declined to about 10 % of the maximum. It is possible that inulin is not limited to extracellular space, but is taken

into cells, and that the complexities of the washout curves reflect transcellular movements.

Accumulation or permeation of inulin into sinus gland preparations was studied by soaking a series of individual glands for various periods in ^{3}H-inulin and after a 30 s saline rinse, placing them in vials for counting. The amount of inulin retained reached an apparent plateau at between 15 and 30 min (Fig. 1C).

Bioassay

Erythrophore-concentrating hormone activity present in samples of perfusate was determined by bioassay on isolated meropodite segments of large walking legs from *O. pallidula* as described by Cooke *et al.* (1977; see also Weatherby, 1976). An aliquot of 0·2 ml was introduced into a leg having fully expanded red chromatophores (stage 5, Hogben & Slome, 1931), and the change of stage of five chromatophores at 2 min was averaged, and compared with a standard curve obtained from assays of synthetic *Pandalus borealis* ECH (Fernlund & Joseffson, 1972). We have expressed the result of this comparison in most cases as femtomoles (fmol) of ECH secreted per minute.

In the stopped-flow experiments, three assays of each sample were made and averaged. In the experiments employing continuous perfusion, the volume of the samples and number to be assayed made impractical more than one assay of each sample. The practical lower limit of ECH detectable in the continuous perfusion experiments was approx. 1 fmol (= 1 picogram) min^{-1}. Variability attributable to the assay itself (for single samples) may be equivalent to a three-fold difference in ECH concentration.

For each altered saline, tests were made for effects on the bioassay, both by perfusing the leg with the altered saline itself, and by comparing responses of the leg segments to an extract of a fresh sinus gland assayed in normal saline and in the altered saline. Of all the altered salines, or salines to which drugs had been added, only the salines containing the highest concentrations of K caused a change of chromatophore responsiveness. Since saline having $<100\,mmol\,l^{-1}$ K did not affect the assay, the samples containing elevated-K saline were diluted with an equal volume of saline from which K was omitted. All other samples were routinely diluted with an equal volume of normal saline before assay. During an experiment, collected samples were held in an ice-bath; at the end of the experiment they were held for 2 min in a boiling water bath and then capped and placed in a freezer until assay (usually 2 days to 1 month later). Comparison of assays of aliquots of the same sample made before and after a period in the freezer showed no differences.

Extracellular recording

Electrical activity was monitored during most of the experiments on rate of hormone secretion by means of a saline-filled glass capillary fitted with an internal silver wire. The capillary, with a pore diameter of 50–80 μm, was placed against the surface of the sinus gland with the aid of a micromanipulator. Recordings, with reference to a bath electrode, were made with a differential a.c. preamplifier (Grass P15) having a gain of 1000× and filters set for half-amplitude at 0·3 Hz and 3 kHz. The output was taken to an oscilloscope and an audiomonitor.

Intracellular recording

Intracellular recordings utilized conventional techniques: $3\,mol\,l^{-1}$ KCl-filled electrodes (30–60 MΩ), a preamplifier equipped with a constant-current-passing 'bridge' circuit (WP Instruments, M4) permitting current passing while recording through the electrode, and simultaneous recording on a penwriter (Brush 220 or 280), and photographically from oscilloscopes. Some preparations used for intracellular recording from terminals included the complete neurosecretory system (the somata of the X-organ) as well as the axon tract and terminals (sinus gland). A Vaseline bridge across the axon tract permitted extracellular recording of impulse traffic (see Fig. 14) using a differential a.c. preamplifier (Grass P15). The recordings used different chambers and perfusion systems from those used for following secretion. Thus, while perfusion was continuous and approximated the rates used in the release studies, chamber volumes, perfusion dead-space on changing solutions, and rates of bath exchange differed.

RESULTS

Hormone release by elevation of saline potassium concentration

Time course of ECH release during continuous exposure to high potassium

An initial series of experiments explored the rate and time course of ECH secretion evoked by exposure to $176\,mmol\,l^{-1}$ [K] saline under an experimental regime similar to that utilized in our previous experiments on electrically elicited secretion (Cooke *et al.* 1977). After collecting perfusate following a stopped-flow period in normal saline ('control'), collections from 6-min periods (including rinses), in high-K saline were made over a period of an hour or more (Fig. 2). Release rates of near and over $100\,fmol\,min^{-1}$ were observed in samples collected within the first 10 min of starting the high-K exposure. The amount of ECH present in the next sample was in every case greatly reduced, being roughly one-tenth that in the first. Further samples showed a continuing decline of release until at the end of an hour the rates were only slightly greater than (perhaps double) the unstimulated rates.

It subsequently proved possible to follow the changes of secretion rate with much better resolution by assaying fractions collected at 1-min intervals during continuous perfusion with high-K saline (Haylett & Cooke, 1981). A characteristic pattern of ECH secretion following a switch of the perfusion from normal saline to a high-K ($193\,mmol\,l^{-1}$) saline has been consistently observed and may be seen in the assay results from one preparation plotted in Fig. 3 (see also Fig. 7, right side). The amount of ECH in the 1-min samples begins to increase immediately and rises to a sharp peak within the first 5 min. Peak secretion rates were 15 to more than $100\,fmol\,min^{-1}$ in this experimental series. ECH secretion declines following the peak nearly as rapidly as it rose, the levels being one-tenth or less of the peak value 5 min later. The preparations were perfused with high-K saline for an hour for comparison with the stopped-flow experiments reported above. Each of these exhibited a second small, more rounded peak of ECH release reaching levels of $5–10\,fmol\,min^{-1}$ 10–15 min after introducing high-K perfusion; release then gradually declined over the next 30 min to 1 h to levels close to unstimulated release rates.

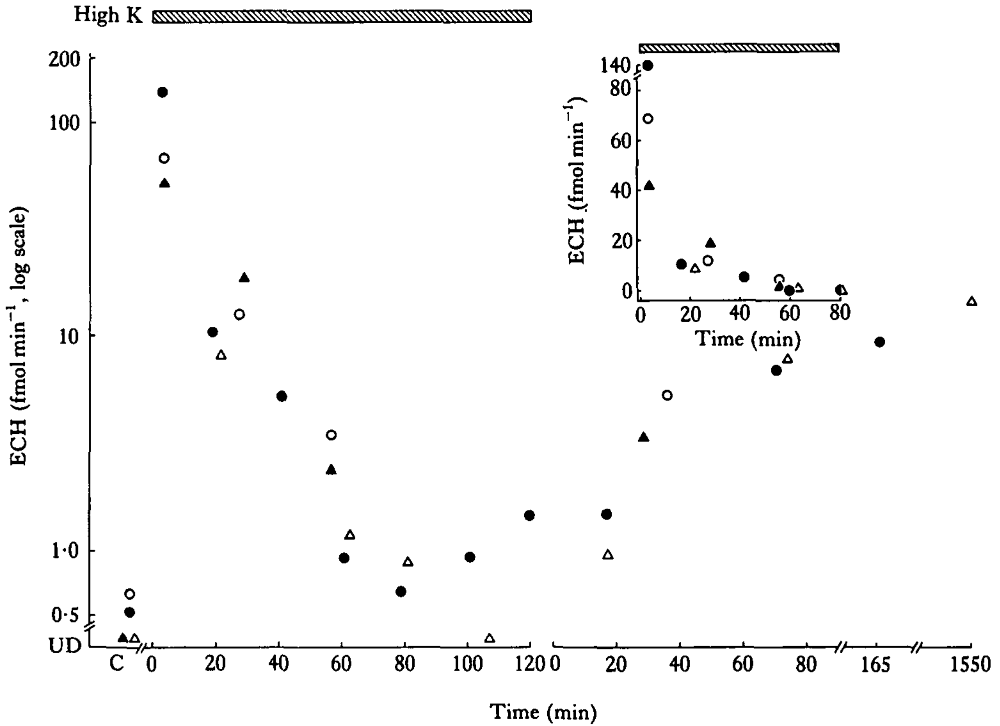

Fig. 2. Rate of ECH release from single sinus glands during 6-min periods of stopped flow during otherwise continuous perfusion with saline having 176 mmol l^{-1} K concentration. Each point represents the average of three bioassays of perfusate collected from an individual sinus gland preparation (each symbol represents a different preparation). Initially high rates of ECH release decline to 1 fmol min^{-1} or below by 1 h. Inset: the same data plotted on a linear vertical scale. Recovery, measured by assay of collections of perfusate from 6-min pulses of high K, shows increasing ability to release ECH with increasing time of perfusion with normal saline (time 0, return from high-K to normal saline). ECH release $<0·5$ fmol min^{-1} is below the detectable level of the assay system. UD, undetectable; C, control, i.e. rate of release in normal saline. Hatched bar marks period of continuous exposure to high-K saline.

The pattern of rapid onset and decline of release means that over 80 % of the ECH secreted during an hour-long exposure to high-K saline is released during the initial 10 min. For this reason, a majority of our further experiments utilized 10-min periods of perfusion with high-K saline. The consistency of the pattern of ECH secretion during continuous perfusion with high-K saline can be appreciated from the graph inset in Fig. 3. Data from 23 individual sinus glands exposed to high-K saline without previous experimental manipulation are plotted as the percentage of ECH present in each 1-min fraction relative to the fraction containing the maximum observed. It will be seen that, on average, none of the fractions contained more than about 30 % of the ECH present in the peak fraction.

The peak rates of secretion in this group of 23 preparations in which there were no previous manipulations ranged from 8 to 270 fmol min^{-1}, average $31·3 \pm 11·1$ (S.E.M.). Total amounts of ECH released during a 10-min period of high-K perfusion ranged, in this group, from 21 to 447 fmol, average $70 \pm 14·6$ (S.E.M.).

When we compare the amounts of ECH released by high-K stimulation in ou

stopped flow experiments with those in the continuous perfusion experiments, we find that significantly more was released in the stopped-flow regime. The average released during the first collection period (6 min) was 433 fmol (Fig. 2).

Calcium dependence of potassium-stimulated hormone release

Whether tested by the stopped-flow method, or by sampling during continuous perfusion, the sinus gland preparations exhibited no increase of ECH secretion rate upon introduction of a Ca-free, elevated-K saline, provided the K challenge was preceded by a period of at least 20 min in Ca-free saline.

A series of experiments (utilizing the stopped-flow regime) explored the secretion rate of ECH in response to elevation of saline K in relation to a range of saline [Ca].

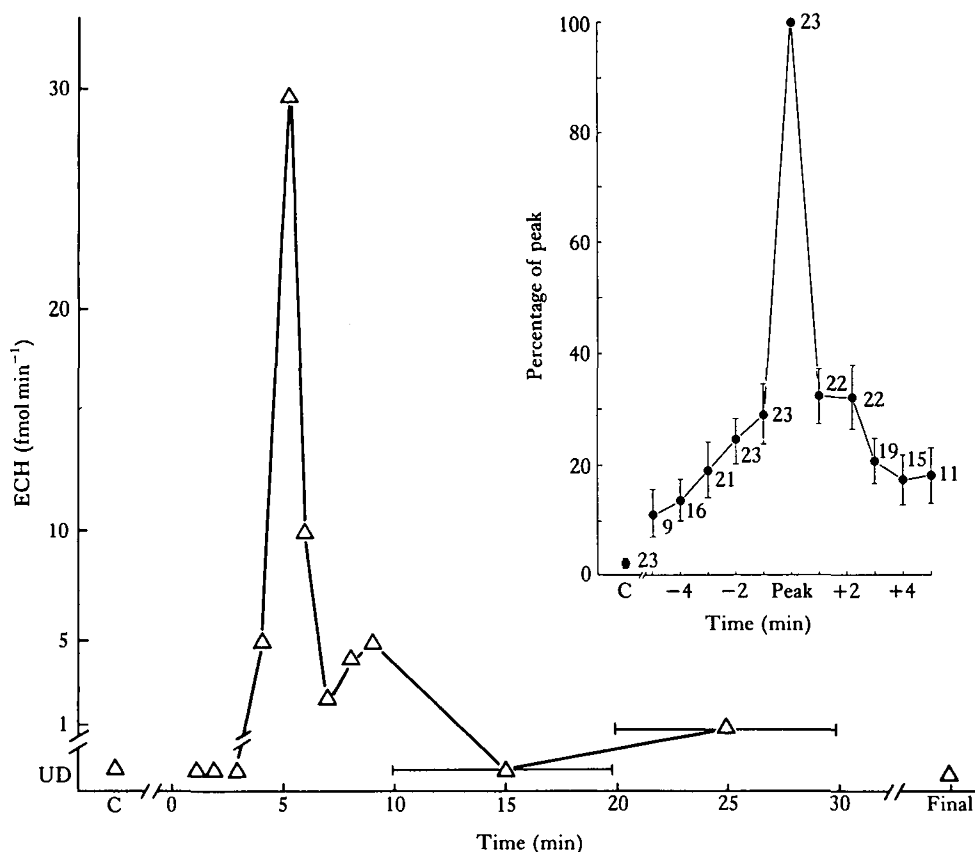

Fig. 3. Rate of ECH release from a single sinus gland under continuous perfusion with 193 mmol l^{-1} K saline. Bioassays of 1-min collections of perfusate show a rapid increase of release to a sharp peak within the first 5 min of perfusing with high-K saline. Release then rapidly decreases to below 10 fmol min^{-1} then slowly continues to decline. (|—|), Ten-min collection period, averaged. Releases <0·9 fmol min^{-1} are below reliably detectable limits of the assay. UD, undetectable; C, controls. Inset: consistency of the pattern of ECH release from sinus glands during 10 min of high-K saline perfusion. Peak rates of release during perfusion with 193 mmol l^{-1} K saline from each of 23 sinus glands were aligned and release rates before and after that of the peak fraction were plotted as a percentage of that in the peak. Average value for peak release rate is 31·3 ± 11·1 (S.E.M.) fmol min^{-1}. The number of observations is given at each point. Bars are standard errors.

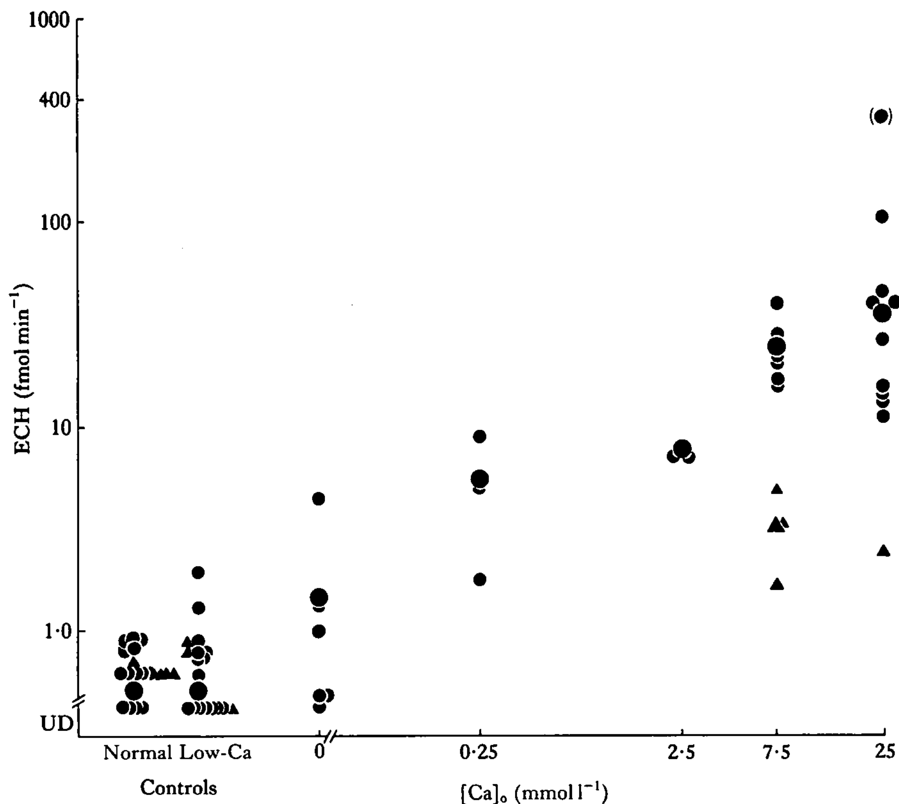

Fig. 4. ECH release from the sinus gland in response to high-K ($176\,mmol\,l^{-1}$ K) stimulation in the presence of various concentrations of saline Ca. After salines containing normal ($25\,mmol\,l^{-1}$) or $7·5\,mmol\,l^{-1}$ Ca were perfused over the sinus gland for at least 20 min, release in response to 5-min pulses of high K were in the usual range, all above $10\,fmol\,min^{-1}$. However, releases in $2·5\,mmol\,l^{-1}$ (1/10 normal) and $0·25\,mmol\,l^{-1}$ (1/100 normal) were $<10\,fmol\,min^{-1}$. In nominally Ca-free saline, the average release was just slightly above control levels. Two preparations were tested for the effect of $10\,mmol\,l^{-1}$ Mn in normal ($25\,mmol\,l^{-1}$) and reduced-Ca ($7·5\,mmol\,l^{-1}$) saline. The results, shown as ▲, indicate a 10-fold decrease in release evoked by high-K stimulation in the presence of Mn. Large symbols are averages. Small symbols are individual tests. UD, undetectable. Point in parentheses represents an assay response exceeding the reliable range of the bioassay.

Exposure to high-K salines was limited to the minimum time necessary to obtain a 6-min stopped-flow perfusate sample. Significant responses to elevation of K were observed in two of three preparations tested in $0·25\,mmol\,l^{-1}$ Ca (1 % of that released by the same preparations in normal [Ca]) (Fig. 4). Release was nearly as great in $7·5\,mmol\,l^{-1}$ Ca as in $25\,mmol\,l^{-1}$ Ca, suggesting that the process in which $[Ca]_o$ is linked to secretion is fully operating at $[Ca]_o$ above $7·5\,mmol\,l^{-1}$.

The presence of $10\,mmol\,l^{-1}$ Mn, an inhibitor of voltage-dependent Ca conductance, reduced K-evoked secretion to about one-tenth that in its absence in saline having $25\,mmol\,l^{-1}$ (normal) or $7·5\,mmol\,l^{-1}$ Ca (Fig. 4).

We thus conclude that the presence of extracellular Ca is essential for stimulation of hormone release by elevation of K, as it is for release elicited by electrical stimulation (Cooke *et al.* 1977).

Net uptake of ^{45}Ca by the sinus gland during high-K-evoked secretion

We have sought further evidence concerning the role of Ca in secretion by examining whether there is a net uptake of Ca associated with hormone release. In three experiments, a pair of sinus glands from the same crab were left unperfused for 30 min or 1 h in ^{45}Ca (141–155 mCi mol^{-1} Ca), one gland in normal saline and one in high-K saline. The incubation media were collected for bioassay of ECH and then a series of 10-min washes of the sinus glands was collected for liquid scintillation counting in order to ensure that superficial ^{45}Ca had been washed out. Counts were close to background levels by the third rinse (30 min) and showed very slow further decline with additional rinses. The sinus glands were finally solubilized in a scintillation cocktail and counted.

The total amount of ECH activity released (as moles, compared with synthetic ECH) may be compared with the net moles of Ca taken up (Fig. 5). It is not possible to compare directly the amount of release with the net Ca uptake because we assay for only one of several hormones released from sinus gland terminals. In these experiments, the sinus glands exposed to high-K saline released more than 10 times as much

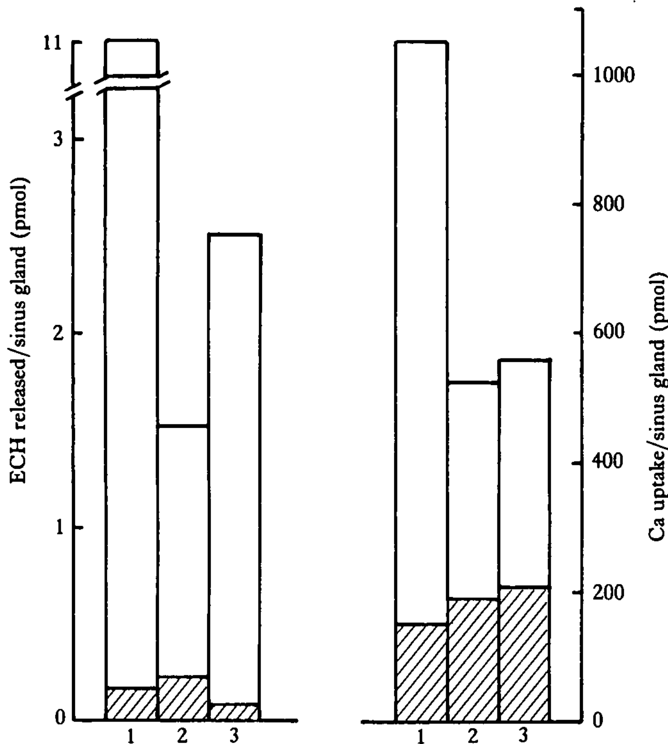

Fig. 5. Net ^{45}Ca uptake compared to ECH release evoked by high-K saline. Bars show ECH release (pmol, left side) and net Ca uptake (pmol, right side) by single sinus glands. Observations from glands held for 1 h (preparations 1 and 2) or 30 min (preparation 3) in high K (176 mmol l^{-1}) are shown by open bars; the other sinus gland from the same crab was held in normal saline for a similar period (hatched bars). Organs treated with high K show 2·5 to 6 times as much net Ca uptake as controls and more than 10 times as much ECH release as controls.

ECH as the saline controls. The net amount of Ca taken up by the secreting preparations was about 2·5 to 6 times that of the controls. Some of the ^{45}Ca initially taken up has undoubtedly been lost during the washout period. In view of the decline in rate of ECH release during continuous exposure to elevated K, documented above, it is not surprising to find that the total ECH activity released by the preparation held in high K for 30 min (preparation 3) is not greatly different from those held for 1 h. At the same time it is reassuring to note that the net uptake of ^{45}Ca is not doubled in the preparations exposed to the radiotracer for twice as long. These preliminary results are consistent with the hypothesis that Ca entry is linked with hormone release, but much additional work is needed to quantitate the relationships.

Depletion of releasable ECH by high potassium

The rapid decline of hormone release during exposure to elevated K raises the question whether the decline reflects an exhaustion of releasable hormone stores or an inactivation of some part of the mechanism by which stimulation is coupled to release, for example, the admission of external calcium (Baker & Rink, 1975; Nordmann, 1975, 1976). We first attempted to evaluate the time course and extent of recovery of the ability to secrete ECH following an hour's exposure to high K (in the stopped-flow regime) by briefly challenging the preparation (6 min in high K) at intervals after restoring normal perfusion. As may be seen in the data plotted at the right in Fig. 2, recovery of responsiveness requires 30 min or more of normal perfusion (a time corresponding to that necessary for repolarization of terminal membrane potential, see below). The rates of release observed during second or additional exposures to high K are an order of magnitude less than during the initial challenge.

We have also evaluated the ability of sinus gland preparations, under continuous perfusion, to respond with ECH secretion to repeated high-K stimulation, given intervening recovery periods. Both the total amount of ECH released and the peak rate of release show marked decline during repeated 10-min exposures to high K (Fig. 6).

Evidence that the decline in rate and amount of ECH releasable during repeated exposures to high K are not the result of an adverse effect of the high-K salines is provided by the secretory responses observed from preparations which were tested following perfusion with Ca-free saline (20 min) and Ca-free, high-K saline for an hour or more. As previously described, no ECH release occurs under these conditions. However, after a period of 30 min or more in normal saline, all of these preparations responded to elevation of K with rates and amounts of ECH release equivalent to those of preparations stimulated for the first time without previous manipulation.

Observations on preparations tested after 4 h or more under perfusion, but which had not been caused to secrete, showed ECH release rates in the same range as preparations tested an hour after dissection. Thus the reduction in secretion observed with repeated stimulation is not the reflection of a time-dependent deterioration of the preparation, but more likely the result of depletion of the releasable hormone pool.

Control assays taken during the recovery periods, whether after high K in normal or in Ca-free saline, show no significant unstimulated release to be occurring (not illustrated).

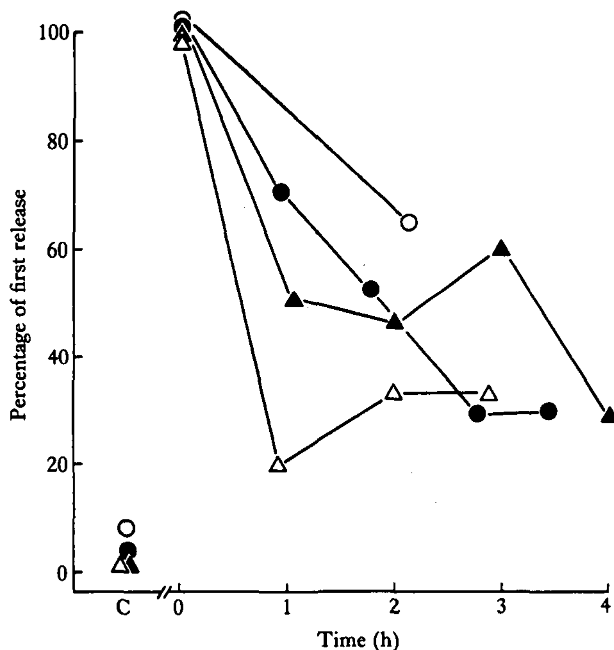

Fig. 6. ECH release during repeated 10-min exposures to high-K saline. Each point represents the total ECH released during 10 min of 193 mmol l^{-1} K saline perfusion, collected as 1-min fractions, assayed, release totalled and plotted as a percentage of the total released during the first high-K exposure for that preparation. Amounts released during the second stimulation periods were 20–70 % of the first period, while releases during third and subsequent stimulations were at low but variable levels. Symbols represent different preparations. C, controls.

Application of the presumed secretogogues, Ba and a Ca ionophore, A-23187, at a time when ECH secretion had declined during application of high-K saline was tested with a view to obtaining further insight on whether secretory decline was the result of depletion or inactivation of a secretory mechanism. It was unexpected to find that neither greatly augmented secretion, either when applied in normal saline, or in the presence of elevated K. The ionophore was tested at concentrations up to 5×10^{-5} mol l^{-1}. Ba was tested at 25 mmol l^{-1} and 40 mmol l^{-1} in Ca-free salines. The addition of Ba-containing saline (with normal K) caused no increase in ECH release. In the presence of Ba, elevation of K did not produce a secretory response. Further, in none of the preparations was a secretory response to normal elevated-K saline obtained following washout of Ba saline by perfusion for an hour or more with normal saline. Thus Ba appears to have irreversible inhibitory effects on the secretory process.

Inactivation of secretory responses

The preparations which had been exposed to elevated K in the absence of Ca were tested for their immediate response to restoration of Ca, without an intervening exposure to normal saline, as well as subsequent to a recovery period in normal saline. A stopped-flow collection period was begun 1 min after switching perfusion from Ca-free, high-K saline to normal-Ca, high-K saline. The release rates observed, about

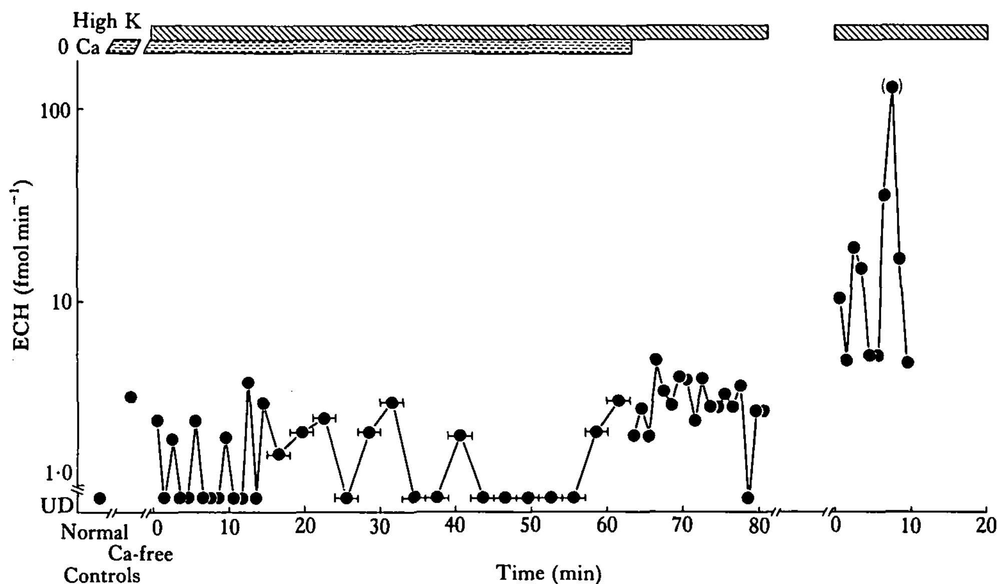

Fig. 7. Absence of secretion in Ca-free saline and inhibition by high-K saline. Results of bioassays on 1-min samples of the continuous perfusate from a single sinus gland. A period of 20 min perfusion in Ca-free saline precedes the introduction of 193 mmol l^{-1} K in Ca-free saline at time 0. No change in ECH secretion rate occurs. At 60 min, Ca (25 mmol l^{-1}) is reintroduced in the continuing presence of 193 mmol l^{-1} K; a slight increase in rate of secretion may have occurred. Breaks in the horizontal axis after 80 min represent a period of 1 h perfusion with normal saline, after which, at the second time 0, 193 mmol l^{-1} K is introduced and produced a secretory response whose rate is comparable to that shown by preparations exposed for the first time to high-K saline. Diagonally hatched bar denotes period in 10× K saline. Horizontally hatched bar is period in Ca-free saline. UD, undetectable. Point in parentheses represents assay response exceeding the reliable range of the bioassay. (├─┤), 3-min collection period, averaged.

10 fmol min^{-1} (one exception), were an order of magnitude greater than in the Ca-free saline, but about 10 % of the release rates exhibited by the same preparations after recovery periods in normal saline of 25 min or more. The 'recovered' rates of release were comparable to those observed from other preparations upon their initial exposure to high K in normal Ca.

In five preparations, the continuous perfusion technique was used to follow with improved time resolution the effect on secretion of restoring Ca after a long exposure to high-K saline. During the 10 min immediately following restoration of Ca to the high-K saline, there appeared to be a small increase in ECH release (Fig. 7). In none of the preparations were peak rates in excess of 10 fmol min^{-1} observed. The same preparations, when tested with high-K saline after an hour's perfusion with normal saline, gave secretory responses comparable to other preparations challenged for the first time with high K.

Two of the preparations were also tested for release in response to high K after intermediate periods of normal perfusion of 20 and 40 min following the 1-h exposure to Ca-free, high-K saline. The preparations showed an increase in the peak rate of ECH release as the recovery time increased. The release rates when tested 2 h after terminating Ca-free saline perfusion were comparable to those of the other preparations, despite the extra challenges.

The ^{3}H-inulin observations indicate that equilibration of the preparations with normal saline following the long exposure to Ca-free saline should be complete within 20 min, and intracellular recording data (to be described below) indicates that repolarization of terminals following high-K depolarization is nearly complete within

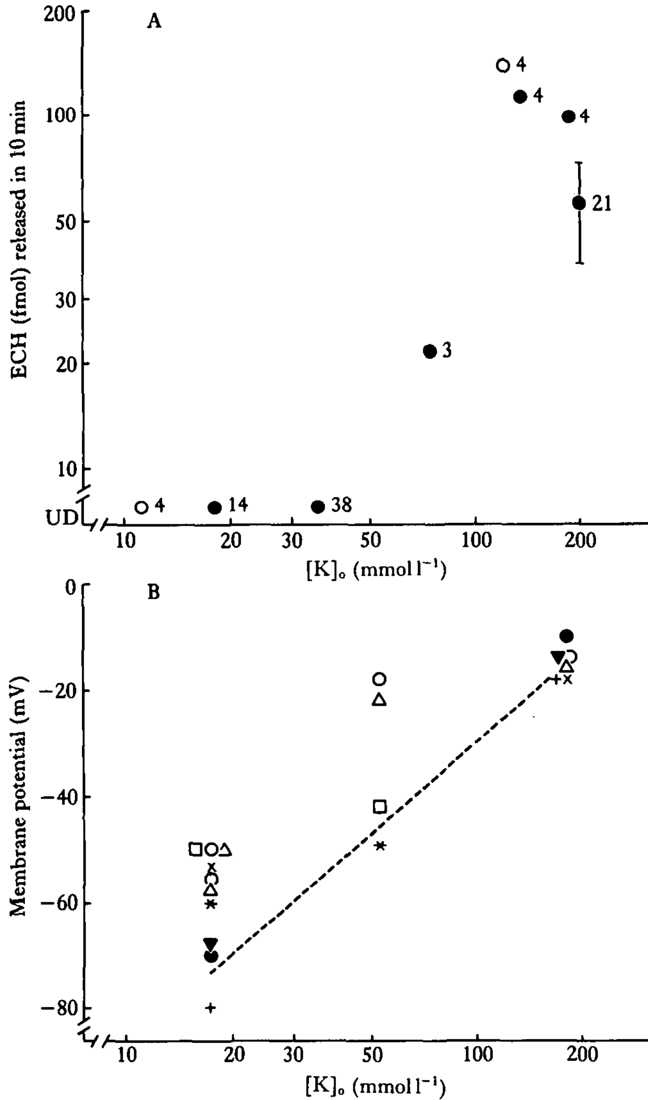

Fig. 8. (A) ECH released during 10-min periods of perfusion with saline having various concentrations of K. Increasing saline K to 70·4 mmol l^{-1} produces a small increase in release, while levels of K above 100 mmol l^{-1} all cause large amounts of release. Open symbols show data from E. Stuenkel (1983*a*, unpublished) utilizing saline with 13 mmol l^{-1} Ca. Numbers give the number of observations included in each averaged point; bar is standard deviation. UD, undetectable. (B) Intracellularly-recorded terminal membrane potential plotted against log K concentration of the saline. Observations from seven terminals (identified by different symbols) obtained during continuous recording following a switch in perfusion from normal to elevated-K saline. Records from terminal (△) are shown in Fig. 13. The dotted line indicates the Nernst relation, 59 mV depolarization per 10-fold increase in [K]. Preparations ○, △ were tested in 176 mmol l^{-1} K before 53 mmol l^{-1} K.

20 min of restoring normal perfusion. Thus, these observations of an extended recovery period indicate that prolonged exposure to Ca-free, high-K saline produces an inhibition of the release mechanism not related to actual release of hormone, nor, perhaps, to the membrane potential of terminals.

Two isolated sinus gland preparations were tested (with the stopped-flow regime) to establish whether any ECH release occurs in response to restoring Ca to the saline (at normal K concentration) after holding the preparation for a period of 1 h or longer in Ca-free saline. Release rates were in all cases near the minimum detectable levels (less than 1 fmol min^{-1}), and showed no significant changes on reintroducing Ca. Each preparation was subsequently shown to be responsive to elevation of K.

Effect of $[K]_o$ on pattern and amount of ECH secretion

ECH secretion was monitored, under continuous perfusion conditions, in salines having various concentrations of K (Fig. 8A). The amounts released in salines having $[K]_o$ less than 40 mmol l^{-1} were all near or below the detection limits of the bioassay.

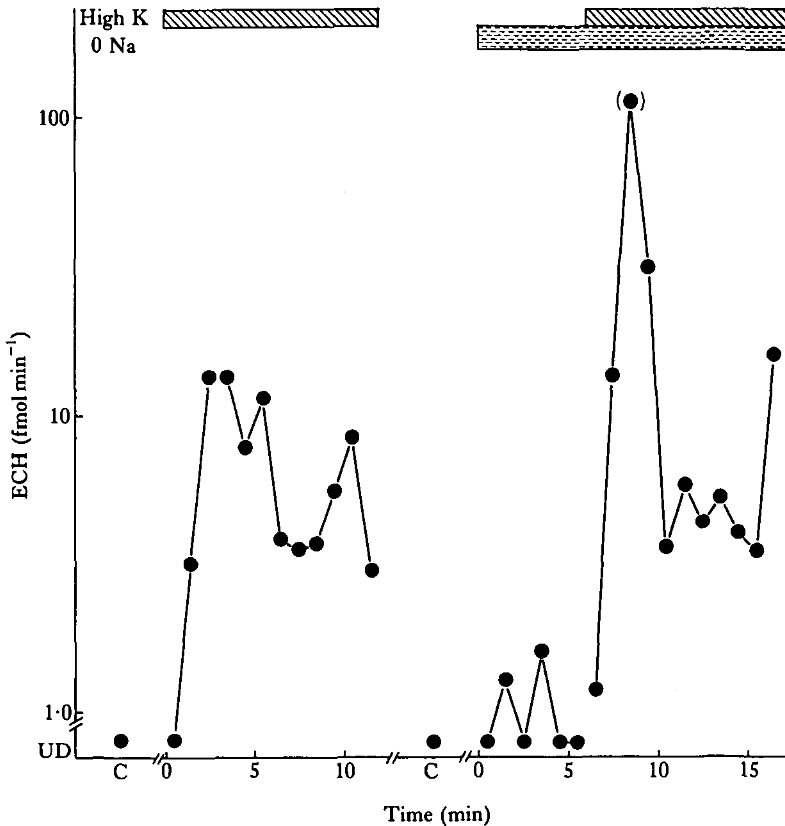

Fig. 9. Release of ECH in response to high-K stimulation during perfusion with reduced-Na (2 mmol l^{-1}) saline. Assays of a typical experiment are plotted in response to high-K (193 mmol l^{-1}) stimulation in normal-Na saline followed after 1·5 h of perfusion with normal saline by a test for release in reduced-Na, 193 mmol l^{-1} K-saline. The reduced-Na release shows a peak higher than the normal-Na, high-K test. Diagonally hatched bars denote period in high-K saline. Horizontally hatched bar is the period in reduced-Na saline. Point in parentheses exceeds reliable limits of assay. UD, undetectable; C, control.

The pattern of release in response to introduction of 70 mmol l^{-1} K saline differed from the pattern described above for response to high-K salines in lacking a single, prominent peak. Instead, there was an increase in rate of release during the initial 5 min of exposure to levels of up to 10 fmol min^{-1}; this level then slowly declined over a period of 20 min toward unstimulated levels.

Responses of sinus glands exposed to salines having 100 mmol l^{-1} K or greater were all very similar to those already discussed. In view of the uncertainty of the bioassay (a three-fold change in fmol released min^{-1} is required to be considered significant), and the relatively small number of experiments in 113 and 130 mmol l^{-1} K, the somewhat greater release seen in these concentrations compared with 193 mmol l^{-1} K cannot be considered significant. In the 113 mmol l^{-1} K saline, the [Ca] is 13 mmol l^{-1} (open circles); in all others, 25 mmol l^{-1}. The 113 mmol l^{-1} K data are provided by E. Stuenkel (1983*a*) who, besides obtaining comparable total amounts of ECH release, also observed the same pattern of release consisting of a single sharp peak followed by rapid decline. Thus, neither the total released nor the pattern of ECH secretion are highly sensitive to differences in the manner of dissection or the particular

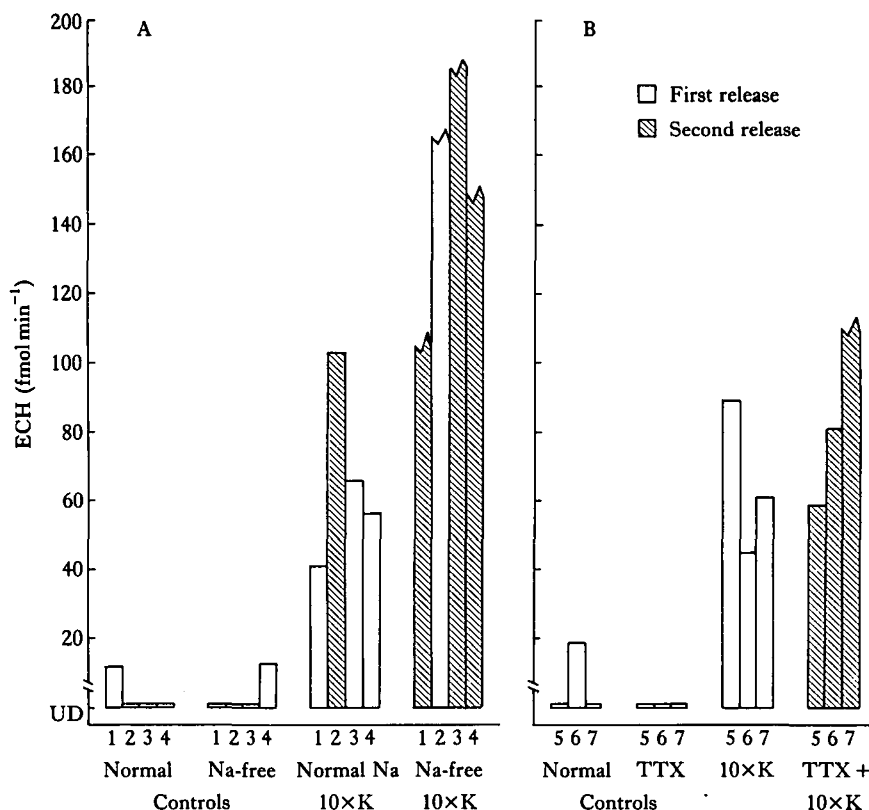

Fig. 10. Total ECH released over a 10-min period in response to high-K in the presence of reduced-Na or TTX-salines. (A) ECH release in 10 min in four preparations in normal-Na and 2 mmol l^{-1}-Na salines. (B) Total 10-min ECH release in three preparations in high K with and without 3×10^{-7} mol l^{-1} TTX. Open bars are first releases, cross-hatched bars are second releases. Irregular tops of bars indicate one or more assays above the reliably detectable limits of the assay. Numbers below bars indicate individual preparations. Preparations 5, 6 and 7 have some points extrapolated due to missing assays. UD, undetectable.

perfusion chamber in use. The similar levels of release in the two concentrations of Ca are consistent with observations from the stopped-flow experiments (Fig. 4) showing similar levels of ECH release for $[Ca]_o$ of 7·5 mmol l^{-1} and greater.

Dependence of ECH secretion on sodium

The ability to secrete ECH in response to elevation of saline K in a reduced-Na (2 mmol l^{-1}) (choline substituted) saline was tested six times in four preparations (e.g. Fig. 9). The pattern of secretory response was unchanged, but the peak was two to eight times higher than the peak observed during perfusion of the same gland with high-K saline having normal Na, whether the reduced-Na saline was applied before or at the same time as the high-K. The total amount of release during a 10-min application of reduced-Na, high-K saline was approximately double that obtained during high-K-evoked releases in normal Na from the same preparations (Fig. 10A). The ECH release in reduced-Na saline containing normal K remained at or a little above control levels.

Another series of sinus gland preparations was tested for secretory responses in the presence of 3×10^{-7} mol l^{-1} TTX. In two of three preparations, peak rates of release attained in TTX-high-K saline were about double, in a third, one-half that of the peak release rate observed in normal high-K saline, despite the tests following those in normal high K. The total of ECH released during the 10-min perfusion with high K in the presence of TTX (see Fig. 10B) was 70 %, 161 % and an estimated 209 % of the amount released by the three preparations in normal, high-K saline. Since the tests in TTX were the second exposure of the preparation to elevated K, it would be anticipated from the data presented in Fig. 6 that ECH release would be decreased by at least 25 %. While one showed such a decline, the others showed marked enhancement of response to elevated-K saline in the presence of TTX.

Four sinus glands were tested for ECH secretion during perfusion with Ca-free saline having normal and elevated K in the presence of TTX. The results are similar to the Ca-free experiments described above. We conclude that the presence of TTX does not inhibit ECH secretion in response to elevated-K saline, nor does it alter the Ca-dependence of release or the inactivation of release resulting from exposure to high-K saline in the absence of Ca.

Electrical activity of isolated sinus gland preparations during perfusion with salines of various compositions

Extracellularly-recorded activity

During most of the experiments discussed in this report, electrical activity of the sinus gland was monitored by means of an extracellular capillary electrode placed on the surface of the sinus gland. Such an electrode appears to sample regenerative potentials of a limited group of pre-terminal axons and terminals lying near the surface. Nearly all of the preparations exhibited infrequent electrical events of up to 400 μV in amplitude during perfusion with normal saline. Potentials of consistent waveform and, in some cases, recurring at regular intervals, are interpreted to represent activity of a single 'unit'. In many preparations, such units were characterized by a rapid, biphasic impulse, immediately followed by a much slower, smaller monophasic potential (e.g. top record, Fig. 11). In presenting similarly recorded

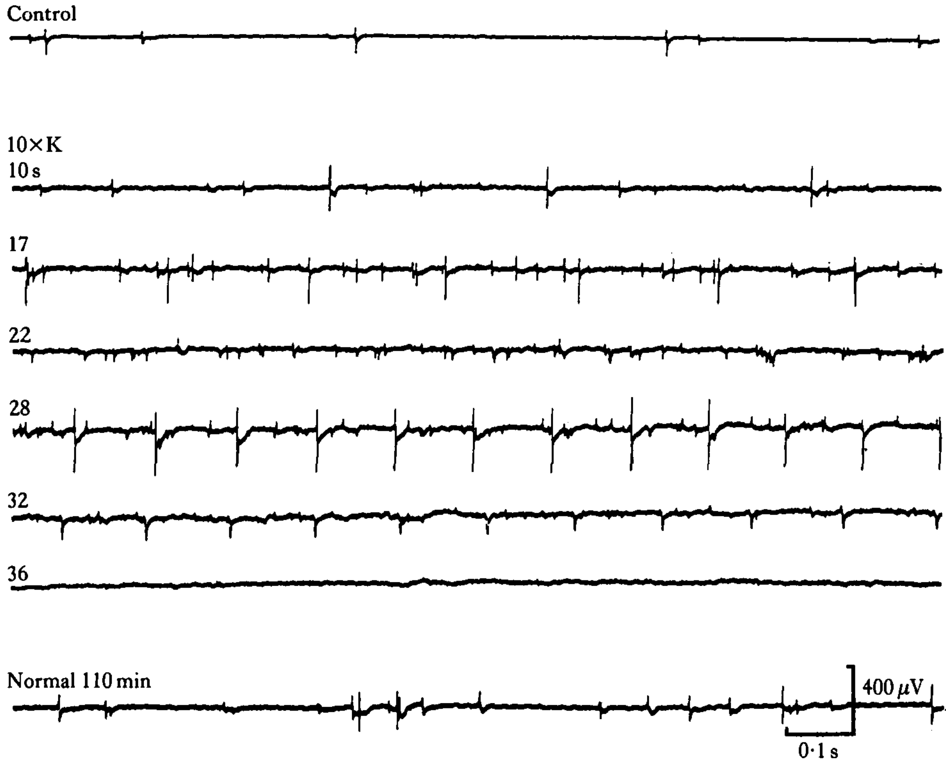

Fig. 11. Spontaneous electrical activity recorded extracellularly during normal and 193 mmol l^{-1} K saline perfusion. Within 10 s of the time the high-K saline reached the sinus gland spontaneous activity began to increase, and remained at a high level for about 20 s when it began to decline. At 28 s in this preparation, one large triphasic unit was firing regularly. The preparations are silent by 36–40 s and remain so until at least 10 min after normal saline perfusion has resumed, and then gradually resume low rates of spontaneity after 45 min to 1 h of normal perfusion. A higher rate of spontaneous activity following high-K exposure, as seen here at 1 h 50 min, after return to normal saline is common. The fast initial phase of the triphasic potentials is interpreted as the axonal impulse and the slow phase as representing a terminal impulse. Retouched.

activity previously (Cooke *et al.* 1977), we suggested that the rapid biphasic 'spike' represents an axonal impulse and the monophasic potential, the slower action potential of a terminal.

Responses to high-K saline. The pattern of changes in electrical activity seen upon introduction of high-K saline was similar in all of the preparations, whether studied under the stopped-flow or continuous-perfusion protocol (Fig. 11). There is infrequent spontaneous electrical activity observable in normal saline. Within 30 s to 1 min in stopped-flow experiments, and 10 s in continuous perfusion experiments, following the introduction of high-K solution to the experimental chamber, there is a sharp increase in the amount of electrical activity recorded: there is an increase in frequency of appearance of particular waveforms (units) as well as an increase in the number of units distinguishable. Both biphasic spikes and slow monophasic potentials are seen during the period of increasing activity following introduction of the high-K saline. High frequency, multi-unit activity gives way shortly to reduced numbers of units with repetitive firing in a recognizable few units. A few seconds later, no further

activity is recorded. The entire period, from beginning of increased spontaneous activity to silence lasts from 20 s to 40 s in preparations of both types. The slower time to onset of the response in the stopped-flow preparations compared to those under continuous perfusion is attributable to the larger chamber volume used for the stopped-flow preparations and a resulting slower exchange of the bath with the high-K saline.

When normal saline is reintroduced, there is a gradual return of occasional spontaneous activity after 10 min or more. Because it is so infrequent, it is hard to document the time of its first reappearance.

Responses in Ca-free saline. The effects of reduced-Ca salines upon electrical activity were similar to those obtained previously (Cooke *et al.* 1977). An increase in spontaneity begins from 15 s to 1 min after introducing the Ca-free saline, reaches a maximum within 5–8 min and then declines until no activity is present after 9–13 min. Not noted in the previously reported low-Ca experiments is that as perfusion with Ca-free saline is continued, there is a slow waxing and waning of spontaneous activity, the periods of quiescence or of activity being of the order of several minutes in duration.

The effects of introducing Ca-free, high-K saline, after a 20-min period in Ca-free saline, were very similar to those observed in normal-Ca salines. An increase in activity begins within 5–8 s of introducing the elevated K (in continuous flow experiments), accelerates, and finally declines. During the period of decline, repetitive activity of a few identifiable units is often evident. The response to the elevated-K saline lasts a total of 20–30 s. During this K-evoked activity, the slow components of unit responses, compared to the unit activity seen during initial perfusion with Ca-free saline, are absent. This may be related to slower rates of rise and fall of terminal potentials (see Fig. 14), or their failure, observed during intracellular recording in Ca-free salines (Cooke, 1977).

When perfusion was changed from Ca-free, high-K saline to normal-Ca, high-K saline, no spontaneous electrical activity was observable. Extracellular recordings made an hour after return of such a preparation to normal saline showed occasional spontaneous events, as seen initially.

Responses in Na or TTX salines. Changes of the extracellularly-recorded activity due to introduction of reduced-Na or TTX-containing salines were similar. Within 1–2 min of introducing the altered saline (with normal K), spontaneous activity ceased. The addition of high K to the altered saline resulted, in about half the preparations, in the appearance within 10 s of a brief bout (6–10 s) of small, slow monophasic potentials (see Fig. 12). These suggest by their form that the regenerative firing of terminals without participation of an axonal impulse was being recorded. Within 40 min to an hour after returning preparations to normal saline perfusion, spontaneous activity having the triphasic waveform was observable.

Responses in salines containing Ba or A-23187. The addition of Ba (24 or 40 $\mathrm{mmol\,l^{-1}}$) in Ca-free, normal-K saline produced distinct increases in the rate of spontaneous activity recorded extracellularly, and a marked increase in the prominence and duration of the slow, monophasic portion of unit triphasic potentials. The spontaneity reached a maximum about 2 min after introduction of the Ba, and then declined within the next 2 min to a level only slightly above the initial frequency

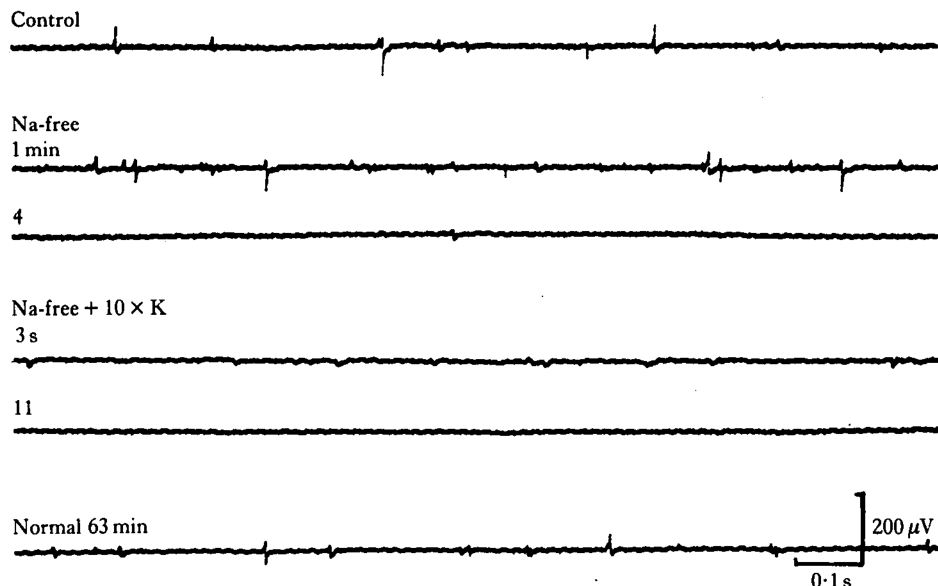

Fig. 12. Spontaneous electrical activity recorded during perfusion with nominally Na-free and Na-free, 193 mmol l^{-1} K saline, recorded with an extracellular pore electrode. Spontaneous activity, including triphasic potentials, continues for at least 1 min after Na-free saline reaches the preparation but gradually disappears in the next minute. Less than 2 s after the addition of 10× K to the Na-free saline, slow potentials were recorded in this preparation followed by complete silence by 11 s. By 1 h after return to normal saline, electrical activity had returned to control levels.

The preparations responded to elevation of K, in the continued presence of Ba, with the usual sequence of increased activity followed by decline and cessation. Potentials of unusually large amplitude and slow time course were prominent during this activity.

The exaggeration of amplitude and duration of the slow, monophasic portion of unit extracellular responses by Ba lends support to our interpretation of this phase of the potential as due to the action potential of terminal dilatations in the light of intracellular recordings from terminals during Ba saline perfusion (M. Nagano & I. M. Cooke, unpublished observations). In Ba, terminals exhibit spontaneous impulses followed by oscillating depolarized plateaus of several hundred ms duration.

The addition of the Ca ionophore, A-23187, to otherwise normal saline did not produce any noticeable change in extracellularly recorded electrical activity. The addition of the ionophore in high-K saline, after the preparation had ceased electrical activity in response to perfusion with normal high-K saline, did not result in any electrical responses recorded extracellularly.

Intracellularly-recorded electrical activity

Characterization of the electrical activity of sinus gland terminals utilizing intracellular electrode techniques has been briefly described (Cooke, 1977, 1981; Nagano & Cooke, 1981) and will be the subject of separate reports. Here we summarize continuous recordings made from separate individual terminals during continuous perfusion upon making changes of saline composition. Although the

sequences of altered salines were similar to those in studies already described, the preparations were mounted in different chambers whose volume and exchange characteristics differ.

Responses to elevated-K salines. A continuous recording from an individual terminal during a switch in perfusion from normal saline to one with elevated [K] was obtained in eight different preparations. In six, the terminal was also held for a period after return to normal perfusion (i.e. recovery). The arrival at the chamber of the high-K saline was marked by the beginning of depolarization (Fig. 13). At a potential of about −45 mV, repetitive impulse firing began. As membrane depolarization continued, the rate of firing accelerated. The size of impulses declined and became a series of small oscillations as the membrane potential stabilized at about −25 mV. A gradual further depolarization (to −18 mV) occurred over the next 10 min. The impulse activity during the rapidly depolarizing period of response started with overshooting (by about 35 mV) action potentials of long duration, typical of those recorded from sinus gland terminals (Cooke, 1977, 1981). These occurred with increasing frequency as the membrane depolarized. Also present were very small (about 5 mV) depolarizing deflections interpreted on various criteria (see Cooke, 1977, 1981) to be electrotonically decremented activity occurring in other terminal processes. These also increased in frequency in the early part of the depolarizing response; they cease to be visible in the later part of the record, probably as a result of decreasing input resistance of the terminal with depolarization (see further below) and a decrease in their size. The overshooting impulses, interpreted to reflect activity in the recorded terminal itself, became reduced in size as the depolarizing response to elevated K continued; there was a reduction of the overshoot and disappearance (middle of the second trace) of the hyperpolarizing afterpotential. Both the rise and fall time became slower, and the potentials ceased to overshoot as depolarization continued, until, after a series of waxing and waning oscillations, the membrane potential became nearly steady. The time from the first depolarization-induced impulse to the end of the period of oscillation was slightly over 1 min in the example shown in Fig. 13.

In the lower part of Fig. 13, superimposed oscilloscope traces show responses to depolarizing and hyperpolarizing current passed through the recording electrode before and during 176 mmol l^{-1} K perfusion, and after recovery in normal saline. Small depolarizing currents (less than 0·3 nA in this case) produced overshooting impulses; in the 'before' record, electrotonically decremented, small depolarizations were also evoked. Hyperpolarizing current produced hyperpolarization which increased slowly while the current remained on. This behaviour is typical of undamaged terminals examined with current clamping. Because the current clamp does not produce responses that reach a steady state, it is difficult to make a quantitative assessment of the terminal input resistance. By contrast, in elevated-K saline, the membrane response to an applied current pulse reached a plateau within 20 ms; there was no evidence of active responses to depolarizing current. One of the preparations was exceptional in continuing to show decreased, but still non-plateau responses to hyperpolarizing currents and in showing a slight active response on release from strong hyperpolarization. It is clear that input resistance is markedly reduced during depolarization by elevated-K salines.

Among the six preparations for which records such as those illustrated were

obtained, variations were minor. In all cases, the depolarization was within 3 mV of its maximum within 4 min; it then drifted to slightly more or less depolarized values as high-K perfusion continued. The high-K perfusion was continued for 20 to 45 min in this series. The rate of repolarization was in all cases much slower than the

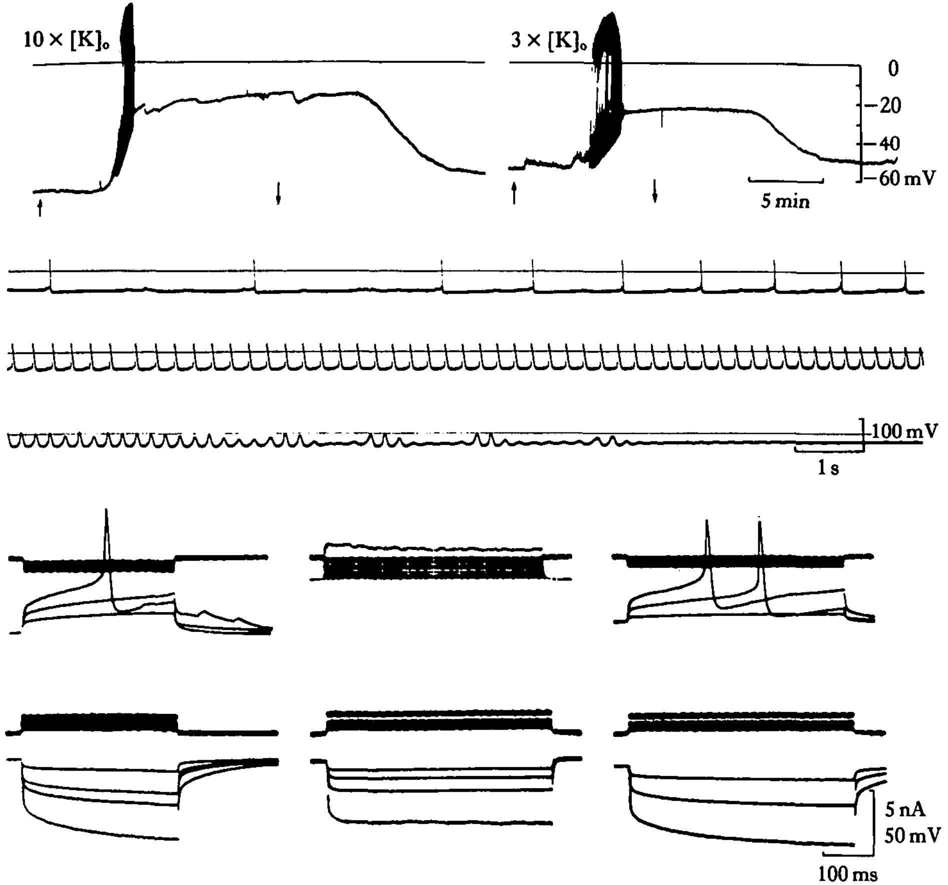

Fig. 13. Responses recorded intracellularly from neurosecretory terminals during changes of [K] in the perfusing saline. Isolated sinus gland – sinus gland nerve preparation under continuous perfusion. Top. Penwriter records at slow speed show continuous intracellular recording from a sinus gland terminal while the perfusing saline is switched (at upward arrow) to one containing 176 mmol l^{-1} K (10× K) (left) or 53 mmol l^{-1} K (3× K), (right), and then (at downward arrows) back to normal saline. A period of approx. 25 min of normal perfusion intervenes between these records. Perfusion deadspace results in approx. 4 min delay before the changed saline reaches the chamber. Elevated potassium results in depolarization accompanied by impulse firing which declines, leaving a steady, depolarized membrane potential (irregularities are probably due to mechanical disturbance, sharp deflections are from current passing shown in middle frames, below). Middle. Samples of a continuous oscilloscope recording (moving paper, unswept spot) taken during the bout of impulse firing in 10× K. Each line is continuous, the first taken at the commencement, the second near the middle, and the third, at the end of activity. Note the disappearance of hyperpolarizing afterpotentials and impulse broadening (second line); the decline of overshoot and waning of active responses (third line). Below. Frames, each having several superimposed sweeps, showing membrane potential response (lower traces) to depolarizing and hyperpolarizing current (upper traces, depolarizing current downward) passed through the recording electrode. Left, before; middle, during stable, depolarized potential in 10× K (sharp deflections in penwriter record); right, in normal saline following repolarization. Note lack of active responses and flatter trajectories in elevated K, suggesting increased membrane conductance.

depolarization, taking 10 to 20 min for membrane potential to be within 10 mV of the initial value; the rate of repolarization was not apparently dependent on the duration of the exposure to high K. In two preparations, membrane potential assessed an hour after returning to normal saline remained depolarized by 6 and 8 mV relative to values before exposure to high K.

In Fig. 8B the steady values of membrane potential observed in this series of experiments are plotted against the log of the saline [K]. Whereas resting potential values in normal saline vary between −50 and −80 mV, the values in 176 mmol l^{-1} K vary much less and cluster near −15 mV. The line is drawn according to the Nernst relation for a K equilibrium potential, 59 mV/10× increase in $[K]_o$. Two of the points at 50 mmol l^{-1} K fall close to this line, two others are clearly above it. It may be significant that the two above the line had been previously exposed to 10× K, while the other two had not. In separate experiments it has been found that decreases in saline [K] produce much smaller changes in resting membrane potential than do increases. In their dependence of resting membrane potential on saline [K], the sinus gland terminals are similar to most excitable cells that have been examined.

The extracellular electrical recordings made during high-K hormone release experiments can now be interpreted in the light of the intracellular recording studies. The period of suddenly accelerating and then declining activity corresponds to the intense impulse firing and subsidence occurring during the initial rapid depolarization as the extracellular K concentration increases.

Ca-free and Ca-free, high-K salines. Recordings from terminals during introduction of nominally Ca-free saline showed a variety of responses, perhaps reflecting different properties of terminals subserving secretion of different hormones. In general, terminals showed augmented levels of spontaneous firing and decreased threshold for impulse initiation to intracellularly-applied depolarizing current. Some degree of depolarization occurred, varying from less than 5 mV, as in the example illustrated in Fig. 14, to more than 15 mV. If depolarization brought the resting

Fig. 14. Intracellularly-recorded responses to Ca-free salines. X-organ, nerve and sinus gland preparation. Independent perfusion of X-organ and sinus gland. (A) Penwriter record of intracellular potential during continuous perfusion with a succession of different salines as indicated next to arrows marking time of changes (altered salines applied to sinus gland only). The three lines are continuous, and represent 2 h of recording (top trace drawn at 0 mV, 1-min marks, note changes of chart speed). In normal saline, this terminal showed irregular impulse activity in bursts. Activity evoked by depolarizing current through the electrode is indicated by (●) under the record. Letters identify the time of recording the frames shown in B; numbers, the records shown in C. Introduction of Ca-free saline (0-Ca) results in slight depolarization and increased spontaneity. The switch to 0-Ca, 10× K (176 mmol l^{-1}) leads to depolarization and a brief episode of continuous impulse firing during which impulse amplitude declines (B*d*; C4,5); firing ceases leaving the membrane depolarized. Restoration of normal Ca in the continued presence of elevated K (second line) causes no change of membrane potential. Repolarization follows restoration of perfusion with normal saline (second line). Impulse firing, at first continuous (C10), spontaneously becomes organized into bursts as membrane potential approaches normal (B*e*; C11,12; B*f*). (B) Frames from oscilloscope recorded at corresponding points indicated in A. Impulses in *a*, *b* and *f* were evoked by current passing, the others are spontaneous. Lower trace, intracellular potential; upper trace, extracellular recording from Vaseline bridge across the sinus gland nerve. Note time-locked impulse attributable to axon of the recorded terminal. The polarity indicates initiation of activity proximally in all frames. (C) Moving film records of unswept oscilloscope spot. Each line is a 7-s sample taken at the time indicated by the corresponding number in A. Extracellular trace (below) is reversed in polarity relative to B. Figure kindly provided by M. Nagano.

potential to a value less polarized than −35 mV, inactivation of regenerative responses occurred. Effects on the form of terminal impulses vary from minor reduction in the prominence of the shoulder following the overshoot, to marked decrease in overshoot and rate of rise (Cooke, 1977).

A change of perfusion to saline with elevated [K] (in continued Ca-free conditions) in all cases led to further depolarization accompanied by a bout of accelerated firing (if the terminal was not already inactivated), in which there was a progressive decrease in amplitude of impulses, and finally cessation. The membrane potential reached steady depolarized values similar to those observed in normal-Ca, high-K salines (i.e. approx. −15 mV).

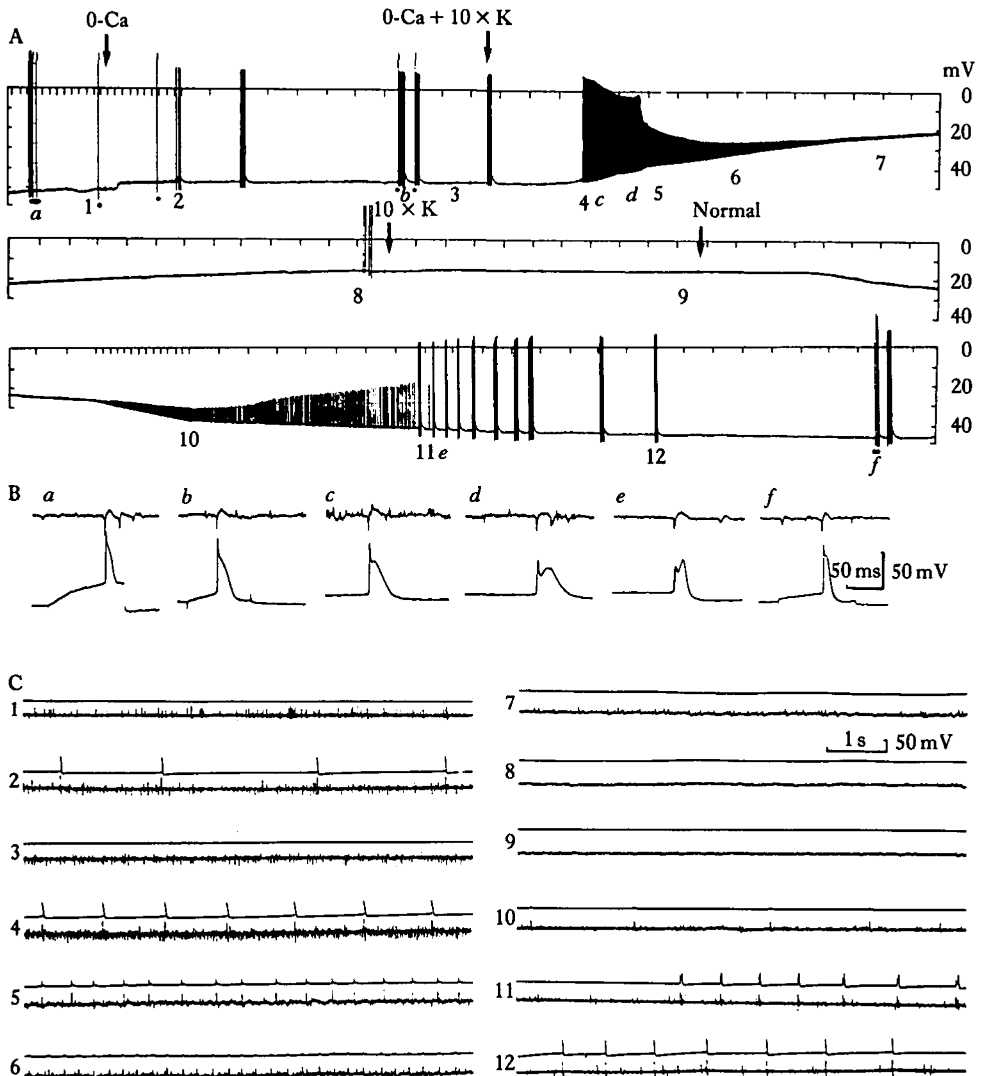

Fig. 14

If, after applying 0-Ca, high-K saline, the preparation was perfused with a high-K saline containing normal Ca, there was usually little change in the maintained, depolarized resting potential (six of nine preparations including Fig. 14). Two terminals hyperpolarized by 10 mV, and a third slightly depolarized.

The records of Fig. 14 represent one of three preparations in which recovery following a long period in Ca-free salines was documented. We have the impression that exposure to Ca-free saline, despite the presence of normal Mg (17 mmol l^{-1}) or the addition of Mg equivalent to the omitted Ca, alters the mechanical characteristics of the tissue, making the intracellular penetration difficult to maintain. After reintroduction of normal saline, the terminals repolarized and then became capable of regenerative responses, often showing spontaneous firing. Within 25 min, the resting potential was within 10 mV of its original value. Further repolarization occurred very slowly, if at all.

DISCUSSION

Support for the Ca hypothesis

Membrane depolarization

The dependence on the presence of extracellular Ca of secretion of ECH from the crab sinus gland in response to elevation of saline K concentration as reported in this study, and in response to electrical stimulation as previously reported (Cooke *et al.* 1977), places this neurosecretory system on the growing list of those secretory systems for which the Ca hypothesis (e.g. Douglas & Poisner, 1964*b*; Katz & Miledi, 1967*a*,*b*) has been proven to apply (review: Rubin, 1982). For the sinus gland preparation, the ability to record electrical activity intracellularly from the terminals (Cooke, 1977, 1981; Nagano & Cooke, 1981; Stuenkel, 1983*a*) permits relating terminal depolarization to $[K]_o$ and to secretion. The curve relating ECH secretion to $[K]_o$ (Fig. 8) suggests that there may be a threshold between 50 and 100 mmol l^{-1} K, maximal rates of secretion being reached with $[K]_o$ of 100 mmol l^{-1} or above; Stuenkel's intracellular recordings (1983*a*) show average membrane potential of terminals at this [K] is −18 mV. The data on secretion, depolarization and on the requirement for extracellular Ca, taken together, suggest that there is a threshold of depolarization of the order of −30 mV at which Ca permeability, and hence Ca entry through the membrane, is activated. For ECH secretion, inactivation, either of Ca entry or of another step of the secretory process, appears to follow quickly (see further below).

While our study provides the possibility of comparing the time courses of electrical and secretory responses during introduction of elevated K, there remains sufficient uncertainty about diffusion times for arrival of the new saline at ECH terminals and time needed for washout of secreted hormone to leave unanswered the question whether it is depolarization, *per se*, regardless of the presence of action potentials, which releases ECH. We have not, in this study, observed secretion under conditions which excluded Ca-mediated, regenerative potentials of the secretory terminals. An hypothesis, not excluded by our data, is that inactivation of regenerative Ca spikes and of ECH secretion during continuous perfusion with high-K saline reflect the operation of the same entity, for example, a voltage-dependent channel by which Ca enters th

terminal. However, it seems more likely that depolarization provides stimulation of secretion, perhaps through Ca entry, additional to or not involving regenerative action potential generation. First, we find that high K evokes secretion exceeding rates and amounts obtainable by electrically-elicited action potentials (see further, below). Secondly, there is evidence for more sustained release of other peptides from the sinus gland under the same conditions, in spite of the absence of sustained impulse firing (Stuenkel, 1983*a*).

Evidence that extracellular Ca entry is necessary for secretion

The relationship between secretion and $[Ca]_o$ (Fig. 4), the inhibition of secretion in response to electrical or elevated-K stimuli in salines having less than 2·5 mmol l^{-1} $[Ca]_o$ (Cooke *et al.* 1977), and finally, the evidence provided by use of ^{45}Ca that net uptake, and thus presumably influx of Ca, is increased during enhanced secretion (Fig. 5) all support the hypothesis that secretion requires the entry of extracellular Ca.

A possible role for Ca as an ion essential for terminal depolarization to occur can be ruled out by evidence from intracellular recording. The elevation of K in a Ca-free saline leads to a level of depolarization in terminals no different from that in normal Ca saline; further, the brief bout of impulse firing as the elevated K begins to depolarize is still observable, and even enhanced in rate, in many terminals during Ca-free perfusion (Fig. 14; also Stuenkel, 1983*a*).

Our data do not rule out the possibility that intracellularly sequestered Ca is released by depolarization to serve an immediate role in secretion, extracellular Ca being required as an initial 'trigger' for the release of this Ca (e.g. Fabiato & Fabiato, 1978, in vertebrate heart muscle), and in the longer term to replenish and maintain these stores. Earlier studies showed substantial, but incomplete, inhibition of secretion after 5 min exposure to Ca-free saline (Cooke, 1977). We feel the presence of sufficient Ca in internal blood spaces of the sinus gland to serve as an extracellular source offers a more likely explanation, in view of the relatively slow equilibration of inulin (Fig. 1), than a slow depletion, in Ca-free saline, of internal Ca stores.

Lack of dependence of secretion on sodium

Operation of the secretory process in the near absence of extracellular Na or in the presence of TTX, as reported here for the sinus gland, has now been established for a variety of preparations (review: Rubin, 1982), including the adrenal medulla (e.g. Douglas & Rubin, 1963), neurohypophysis (e.g. Douglas & Poisner, 1964*a*; Nordmann, 1976), frog neuromuscular junction (Katz & Miledi, 1967*a*) and squid stellate ganglion synapses (Katz & Miledi, 1967*b*; Llinas, Sugimori & Simon, 1982). In the case of the synapses direct evidence for voltage-dependent Ca channels exists (e.g. Katz & Miledi, 1969*a*,*b*; Llinas *et al.* 1982). Observation of regenerative action potentials in sinus gland terminals during perfusion with TTX (Cooke, 1971, 1977) or Na-free saline (Nagano & Cooke, 1981) provides direct evidence of voltage-dependent Ca conductance in peptidergic terminals. Further, there is evidence of specialization of the terminals for Ca-conductance, since sinus gland nerve axons lack regenerative Ca impulses. Observations showing block of conduction in the neurohypophysial stalk by TTX (Ishida, 1967; Dreifuss *et al.* 1971) and the ability of the isolated neurohypophysis to release hormone in response to direct electrical stimulation

in a Na-free, TTX-containing saline (Douglas & Sorimachi, 1971) are explicable by postulating a similar localization of Ca conductance channels in the terminals of the neurohypophysis. Voltage-sensitive dye techniques have now demonstrated TTX-resistant compound action potentials in response to direct electrical stimulation in the vertebrate neurohypophysis (Salzberg, Gainer, Obaid & Senseman, 1983), in strong support of this suggestion.

Convincing support for hypotheses for the mechanism by which removal of extracellular Na enhances neurosecretion has not yet been presented, to our knowledge. Reduced competition between monovalent and divalent ions for sites of entry has been proposed (e.g. Douglas & Rubin, 1963). However, in our experiments, release is enhanced in the presence of TTX, normal [Na] being present, although less so than in reduced-Na saline.

In the sinus gland, action potentials may be slightly increased in duration, but are decreased in amplitude, in Na-free saline or in TTX (Cooke, 1977; Nagano & Cooke, 1981). In this they are unlike the Ca-mediated driver potentials of crustacean cardiac ganglion neurones, which do show an increased amplitude in reduced-Na salines (Tazaki & Cooke, 1979). E. Stuenkel (unpublished) has obtained recordings from sinus gland terminals under Na-free perfusion regimes similar to those used in our experiments. Introduction of a Na-free (choline substituted), high-K ($113\,\mathrm{mmol\,l^{-1}}$) saline (following a period of 20 min in a Na-free, normal-K saline) leads to a rapid depolarization of the terminal accompanied by impulse firing and inactivation as the membrane potential reaches a steady level of approx. $-15\,\mathrm{mV}$. This, it will be recalled, is the same value observed for high-K saline of otherwise normal composition. Thus, the intracellular recordings do not provide new insight as to the mechanism by which reduction of saline Na enhances neurosecretion.

The time course of ECH secretion

Comparison with other neurosecretory responses

By contrast with synaptic transmission, in which an intimately localized assay system is available to monitor the secretory process, neurohormonal secretion requires collection of perfusate for biological, chemical or radiotracer assay. Observations for K-evoked secretion at 1-min time resolution are available for catecholamine release from bovine adrenal medulla (Baker & Rink, 1975), diuretic hormone release from *Rhodnius* neurohaemal structures (Maddrell & Gee, 1974), and for serotonin and dopamine release from lobster pericardial organs (Sullivan, Friend & Barker, 1977; Sullivan, 1978; see Cooke & Sullivan, 1982). The observations are all strikingly similar in showing a rapidly developed, sharp peak of secretion, showing decline within 1 min, and falling away to half the maximum rate within 5 min. Differences in the rate of onset and of decline of release for 5-hydroxytryptamine (5-HT) and dopamine measured in the same preparation under precisely similar experimental conditions, however, point to the interesting possibility that physiologically significant differences in secretory kinetics related to differences in roles of the released hormones might exist (Cooke & Sullivan, 1982). The sinus gland of *Cardisoma*, with six morphologically distinct types of terminals (Weatherby, 1981) and at least 11 peptides that are released during high-K perfusion (Newcomb, 1983*a*), may present

favourable material for observing such differences. Release of radioactivity following biosynthetic labelling with ^{3}H-leucine (Stuenkel, 1983*a*,*b* and unpublished), and of chemically detectable peptides (Newcomb, 1983*b*) is well sustained for 20 min and then declines during the next 20 min during perfusion with elevated K. It thus shows a time course similar to that seen in the release of vasopressin from isolated neurohypophyses (e.g. Nordmann, 1975, 1976), where $T_{1/2}$ is of the order of 20 min, compared with a $T_{1/2}$ for ECH of less than 1 min. The time course of secretion followed here may be considered highly specific for ECH. Tests of synthetic analogues of ECH show that any alteration in the structure of this peptide results in a minimum of a 100-fold reduction in effectiveness in causing contraction of red chromatophores (R. W. Newcomb, unpublished).

Inactivation or depletion?

In the cases of the adrenal medulla (Baker & Rink, 1975), the neurohypophysis (Nordmann, 1975, 1976) and insect abdominal neurohaemal organs (Maddrell & Gee, 1974; Berlind, 1981), the decline in secretion during continued exposure to elevated K has been attributed to inactivation of some part of the secretory mechanism (presumably entry of Ca) as a result of sustained depolarization, rather than to depletion of a readily releasable pool of hormone. Evidence for this conclusion includes (1) showing that after decline of secretion a secretogogue immediately restores secretion, and (2) that depression of secretion occurs during exposure to high K in a Ca-free saline in the absence of hormone secretion. The observations leading to this conclusion, when repeated on the sinus gland preparation, have produced less conclusive answers.

(1) In the case of the sinus gland, Ba, an excellent secretogogue on the vertebrate preparations (e.g. Douglas & Rubin, 1964), proved an irreversible inhibitor of secretion. Observations of enormously prolonged terminal action potentials in Ba saline (M. Nagano & I. M. Cooke, unpublished) suggest that in this tissue Ba can pass through Ca channels, but may fail to inactivate them or may inactivate or not activate outward currents, as found when Ba is tested in other neuronal preparations. Its failure to support secretion of enzymes by the exocrine pancreas (Case, 1978; Scheele & Haymovits, 1979) provides another exceptional example in which Ba does not activate a Ca-mediated response.

The responses to the ionophore, A-23187, were not sufficiently large to provide an unambiguous answer to the question whether some of the decline is due to depletion of a readily releasable pool of ECH. In a study of the effects of A-23187 on electrophysiological responses of crayfish X-organ somata, decreases of Ca-action potential amplitude and membrane resistance, attributed to increased $[Ca]_{in}$, were observed at concentrations as much as three orders of magnitude lower than those used here (Iwasaki & Ono, 1979). Whereas A-23187 is an effective secretogogue on *Rhodnius* abdominal neurohaemal organs (Berlind, 1981), it is not effective on rat neurohypophyses (Nordmann & Currell, 1975; Cochrane & Douglas, 1975).

(2) In the sinus gland preparations, the result of restoring Ca to a high-K saline after the preparation had been exposed for some time to Ca-free, high-K saline (Fig. 7) was slightly to augment the level of ECH secretion above that in the Ca-free, high-K saline; but secretion never approached levels observed during initial exposure of a

sinus gland to normal high-K saline. Since the rate of secretion remains low or even declines, it seems more likely that the reduced rates observed result from inactivation than from extended delay in the newly introduced Ca reaching the terminals.

In sum, our observations are consistent with the hypothesis that inactivation of a part of the secretory process occurs as a result of exposure to high-K saline, presumably by reason of the sustained depolarization this causes (Fig. 13), but cannot rule out a contribution to the decline of secretion rate of depletion of an immediately releasable pool of ECH.

Inactivation of voltage-dependent Ca channels as a result of increased [Ca] at the internal face of the membrane has been proposed from voltage clamping studies on molluscan neurones (Eckert & Tillotson, 1981). Ca-dependent inactivation has also been suggested for release of oxytocin from isolated rat neurohypophyses (Dyball & Shaw, 1981). We observed no difference in the rate of decline of ECH release in salines having 13 $mmol\,l^{-1}$ rather than 25 $mmol\,l^{-1}$ Ca. However, we saw both a lower peak rate of release and rate of decline when secretion was tested in 70 $mmol\,l^{-1}$ K rather than 113 $mmol\,l^{-1}$ or higher K. Together with observation of inhibition of release by previous exposure to high-K saline in the absence of Ca, our experiments support a depolarization-mediated inhibition of ECH secretion, although they do not exclude additional inactivation as a result of entry of Ca. Secretion of other sinus gland peptides is not inactivated by previous treatment with Ca-free, high-K saline (E. Stuenkel, unpublished).

Amount of ECH releasable by elevation of saline K

While measurement of the amount of ECH released under various forms of stimulation must be recognized to be imprecise as a result of the inherent variability of the bioassay, it permits comparisons to be made between K-evoked and neurally-evoked stimulation. The peak rates of release observed in high-K saline, an average of 433 $fmol\,min^{-1}$ in stopped-flow experiments (25–250 $fmol\,min^{-1}$, average 70 $fmol\,min^{-1}$, under the continuous perfusion regime) are an order of magnitude higher than those thus far observed under various regimes of electrical stimulation of the axon tract (2–5 $fmol\,min^{-1}$) (Cooke *et al.* 1977; I. M. Cooke & B. A. Haylett, unpublished observations). By contrast with high-K evoked release, ECH secretion in response to electrical stimulation of the axon tract (5 stimuli at 5 s^{-1} every 10 s) can be sustained for periods of hours, and does not show any evidence of the 'exhaustion' observed, for example, in similar experiments on crab (*Libinia emarginata*) pericardial organs (Cooke, 1964; Berlind, 1977). The amount of ECH released during a 3-h period of continuous electrical stimulation (about 2 $fmol\,min^{-1}$, 360 fmol total), is not very different from the total amounts released (200–300 fmol) during three or four repeated exposures of a sinus gland to 10-min periods of perfusion with high-K saline over a period of several hours (Fig. 6). The release rates in response to high K appear to have declined to small, but still significant levels, after three or more stimulation periods. We did not determine how many more such periods might have shown detectable release levels.

Comparing the largest amount of ECH released from a single sinus gland under high-K stimulation (approx. 1000 fmol) with the average value for the total amount of ECH present in a sinus gland (43 pmol) we conclude that we have never released

by physiological stimuli, more than approximately 2% of the ECH present. As discussed above, our data are consistent with a mechanism of inactivation of stimulus-secretion coupling by depolarization. However, they allow the suggestion that the depletion of a readily releasable pool may explain the reduction in response to successive high-K exposures. A possible morphological correlate of the depletion of a readily releasable pool is the observation in electron micrographs of terminal profiles depleted of granules abutting the neurolemma lining the blood sinuses when the sinus gland is fixed following exposure to high-K saline (e.g. Nordmann & Morris, 1980; T. M. Weatherby, unpublished; or electrical stimulation, Bunt & Ashby, 1968). Exocytotic profiles are exclusively observed at such sites, suggesting that hormone release occurs only from the points of contact between terminals and the blood sinus lining.

Among several neurosecretory systems examined for the proportion of total hormone present releasable by high-K or electrical stimulation (neurohypophysis, see e.g. Sachs & Haller, 1968; Lescure & Nordmann, 1980; *Rhodnius* neurohaemal organs, Maddrell & Gee, 1974; crab pericardial organs, Berlind, 1977) that proportion has never exceeded 12%; thus our observations for the release of ECH are not an exception. The only exception we are aware of is the report by Aréchiga, Huberman & Martinez-Palomo (1977) of achieving total depletion of the neurodepressing factor from crayfish sinus gland as a result of stimulation with the sinus gland drawn inside a suction electrode. Perhaps the explanation lies in a combination of mechanical trauma and excessive electrical current having broken down the tissue. For reasons discussed in Cooke & Sullivan (1982) the existence of a neurodepressing hormone cannot be accepted without confirmation (see also Mancillas, Leff & Selverston, 1980).

The crab sinus gland with respect to the physiology of neurosecretion of ECH appears to employ mechanisms indistinguishable from those observed in neurosecretion from a wide range of animal groups. It is important to recognize, however, that we have examined the secretion of only one of several peptide hormones produced by this heterogeneous collection of neurosecretory cells. Quite different mechanisms might be associated with the secretion of hormones, such as the moult-inhibiting factor, whose action, rather than being required acutely, must be maintained for periods of months.

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