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IONIC CHANNELS AND HORMONE RELEASE FROM PEPTIDERGIC NERVE TERMINALS

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SUMMARY

Although there is considerable evidence that depolarization of nerve cell terminals leads to the entry of Ca^{2+} and to the secretion of neurohormones and neurotransmitters, the details of how ionic currents control the release of neuroactive substances from nerve terminals are unknown. The small size of most nerve terminals has precluded direct analysis of membrane ionic currents and their influence on secretion. We now report that it is possible, using patch-clamp techniques, to study stimulus–secretion coupling in isolated peptidergic nerve terminals.

1. Sinus gland terminals from *Cardisoma* are easily isolated following collagenase treatment and appear morphologically and electrically very similar to non-dissociated nerve endings. We have observed two types of single-channel currents not previously described. The first ('*f*') channel is activated by intracellular Na^+ and the second ('*s*') by intracellular Ca^{2+}. Both show little selectivity between Na^+ and K^+. In symmetrical K^+, these cation channels have mean conductances of 69 and 213 pS, respectively. Furthermore, at least three types of Ca^{2+} channels can be reconstituted from nerve terminal membranes prepared from sinus glands.

2. Nerve terminals can also be isolated from the rat neural lobe. These neurosecretosomes release oxytocin and vasopressin, in response to membrane depolarization, only in the presence of external Ca^{2+}. The depolarization of the nerve endings is associated with an increase in intracellular free Ca^{2+} concentration and this increase, measured using a fluorescent indicator, is abolished by Ca^{2+} channel blockers. Channels similar in their properties to the *f* and *s* channels also exist in rat neural lobe endings. Since these channels have not been found in other neurones or neuronal structures they may be unique to peptidergic nerve terminals.

INTRODUCTION

The pioneering work of Douglas and coworkers, on the mechanism of catecholamine release from the adrenal medulla (Douglas & Rubin, 1963) and neurohypophysial peptide release (Douglas & Poisner, 1964*a*,*b*) has established the basic hypothesis for the mechanism by which a molecule, enclosed in a granule (or vesicle), is released into the external medium. These authors showed that calcium

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ions play a major role in the process of stimulus–secretion coupling, and that neurohypophysial hormone release is triggered by depolarization of the nerve terminals. Accumulating evidence over the last 15 years suggests that the events leading to the release of neurohormones are essentially the same as those observed at synapses (Llinas, Steinberg & Walton, 1981).

The steps which link the increased calcium concentration to the release of the neurosecretory granule (NSG) contents are, as yet, unknown (for a review see Nordmann, 1983). However, biochemical and morphological studies have clearly demonstrated that release occurs by exocytosis and that endocytosis is tightly coupled to this release mechanism. It is of interest that, as early as 25 years ago, Douglas chose the neural lobe to demonstrate his stimulus–secretion coupling hypothesis. This is because, as is also true for the crustacean sinus gland, the nerve endings (i.e. the release 'site') can be easily separated from the transport 'site' (the axons) and from the site of synthesis (the neuronal cell bodies). Unfortunately, the relatively small size of neurosecretory nerve endings has not, until now, allowed direct measurement of their electrophysiological properties.

Recently, patch-clamp methods have been applied to a number of secretory systems, such as chromaffin cells (Fenwick, Marty & Neher, 1982*a*,*b*; Kidokoro, 1985), acinar cells (Maruyama & Petersen, 1982) and pituitary tumour cells (Hagiwara & Byerly, 1983), for both macroscopic current and single-channel recordings. We have reported that it is possible to apply these techniques to terminals obtained from a crustacean neurohaemal organ, the sinus gland (Lemos, Nordmann, Cooke & Stuenkel, 1986) and now extend this analysis to isolated peptidergic nerve terminals from the rat posterior neurohypophysis.

SINUS GLAND

The crustacean sinus gland has provided direct evidence that propagated action potentials invading the neurosecretory endings depolarize the terminal membrane and cause release of hormones (Cooke & Stuenkel, 1985). The isolated X-organ–sinus gland system is uncomplicated by the presence of non-neurosecretory or non-peptidergic neurones or of non-neuronal cellular elements other than connective tissue and glia. A clump of somata, the X-organ, sends its axons to form a profusion of terminals abutting blood sinuses in a discrete neurohaemal organ, the sinus gland (SG). In contrast to other neurosecretory systems, where targets have not been identified and release sites are inaccessible, the SG has been well characterized and the terminals can be recorded from *in situ* or easily dissociated.

Secretion can be monitored while continuously recording extracellularly from the axon tract and intracellularly from soma and terminal and has been shown to be Ca dependent (Stuenkel, 1985). There is no evidence for other than peptidergic secretion (Cooke, 1981). Furthermore, the activity, function and release mechanisms of this secretory structure are analogous to those of the vertebrate neurohypophysis (Cooke & Sullivan, 1982); thus findings in one system may be comparable or applicable to the other.

Intracellular recordings

It is the size of the terminals (up to 30 μm in diameter in *Cardisoma*) which has provided the possibility of employing intracellular recording techniques to study individual peptidergic terminal electrical responses (Cooke, 1967). SG nerve endings have special electrical properties (Fig. 2) which may have significance for secretion.

(1) Terminals continue to fire impulses without accommodation in response to a maintained depolarization (Cooke, 1977).

(2) Certain terminals burst spontaneously (Fig. 2; Stuenkel, 1985) or can be induced to burst by brief axonal stimulation (Cooke, 1981; Nagano & Cooke, 1983). Action potentials occurring in bursts are of particular interest because in many secretory systems, including the neurohypophysis, they have been shown to dramatically enhance secretion (Cazalis, Dayanithi & Nordmann, 1985).

(3) During repetitive firing and/or bursting in terminals there is an increase in action potential duration (Fig. 2C). A similar effect is induced by tetraethylammonium (TEA) application (Nagano & Cooke, 1981).

(4) There is an augmentation in terminals, as compared to axons, of the proportion of voltage-dependent calcium channels (Cooke, 1981).

Isolation of nerve terminals

Sinus gland terminals from the land crab, *Cardisoma carnifex*, are easily isolated (Lemos *et al.* 1986). In electron micrographs the isolated nerve terminals appear as circular profiles densely packed with neurosecretory granules (Fig. 1C). Dissociated neuronal terminals are morphologically very similar to non-dissociated nerve endings *in situ* (compare with Fig. 1A). The same morphologically distinguishable terminal types as those observed *in situ* (Weatherby, 1981) can be recognized among the isolated terminals (Fig. 1C). A single sinus gland yields hundreds of isolated terminals, many greater than 10 μm in diameter (Fig. 1B). Dissociated nerve terminals exhibit resting potentials between −30 and −50 mV (intact nerve endings have values of −50 to −60 mV; Stuenkel, 1985) and sometimes have spontaneous overshooting action potentials. These and other observations reported below show that the nerve terminals, after dissociation, are viable and comparable to nerve endings *in situ*.

Whole-cell currents in isolated nerve terminals

Following brief treatment with collagenase, terminals readily form >10 GΩ seals with fire-polished electrodes (Lemos, Stuenkel, Nordmann & Cooke, 1985), making feasible the application of patch-clamp techniques (Hamill *et al.* 1981) to characterize the ionic currents and channels of the terminal membrane. 'Terminal attached' patches can even be studied *in situ*. It has been more practical, however, to prepare isolated nerve endings (Lemos *et al.* 1986) of a size suitable for 'whole terminal' recording (Fig. 1B). In the whole-cell recording, the membrane within the pipette is ruptured, giving access with a low electrical resistance to the cell (terminal)

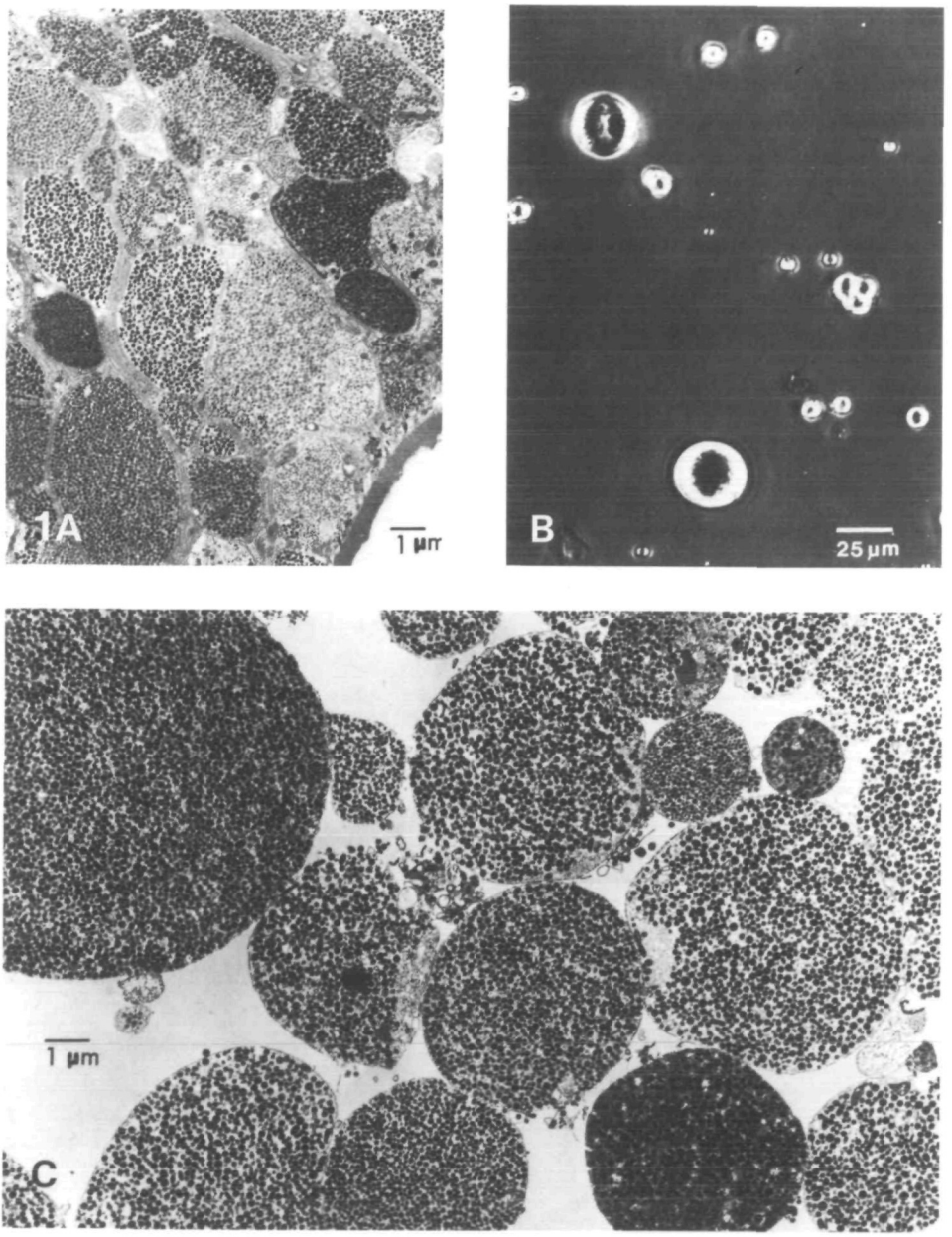

Fig. 1. Sinus gland terminals. (A) Electron micrograph of intact sinus gland from *Cardisoma* showing dense aggregation of terminals and of neurosecretory granules within terminal profiles. (B) Phase contrast micrograph of dissociated nerve terminals after isolation for patch-clamping experiments. (C) Electron micrograph of isolated nerve terminals. Note that they are membrane delimited and still densely packed with neurosecretory granules. (From Lemos, Nordmann, Cooke & Stuenkel, 1986.)

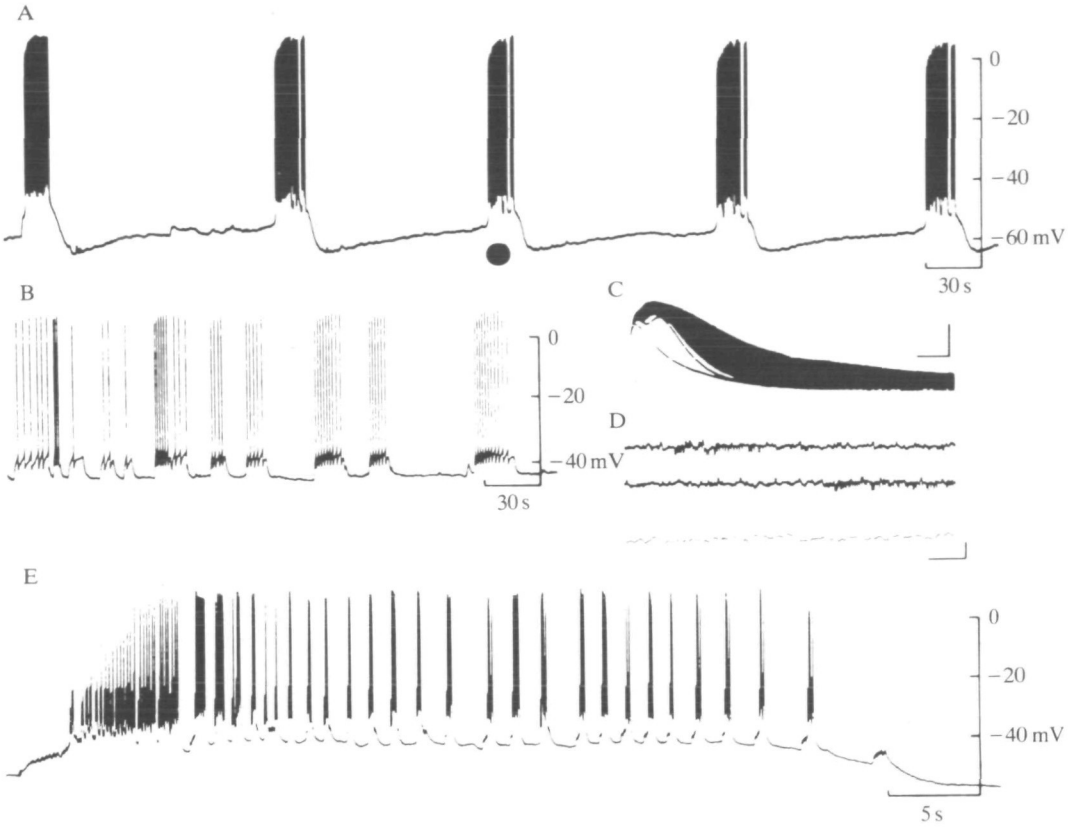

Fig. 2. Spontaneous bursting recorded from isolated *Cardisoma carnifex* X-organ–sinus gland systems. (A),(B) Intracellularly recorded bursting activity of sinus gland nerve terminals. Note the depolarizing plateau underlying each burst. (C) Spike broadening of successive impulses within a burst, recorded simultaneously with the early portion of the third terminal burst (●) shown in A; calibration 5 ms, 20 mV. (D) Burst of axonal impulses recorded extracellularly from the sinus gland nerve. Note bursts of impulses in a single unit; calibration, upper two traces, 0·1 s, 40 μV; bottom trace, 20 ms, 40 μV. (E) Bursting impulse pattern of sinus gland terminal demonstrating grouped impulse discharge within a single burst (courtesy of E. Stuenkel, 1983).

interior. This makes possible not only the study, under excellent voltage- and space-clamp conditions, of macroscopic transmembrane currents, but also, because there is rapid equilibration between the cell interior and the electrode solution, control of the internal milieu.

The currents, in response to depolarizing voltage-clamp commands, include initial inward followed by maintained, outward current (Fig. 3, top). In some nerve terminals, the inward current has the expected properties of a Ca^{2+} current (Hagiwara & Byerly, 1981): it is not blocked by tetrodotoxin (TTX) (Fig. 3), is reduced by external application of Cd^{2+} (Fig. 3) and begins to 'run down' after 30–40 min. The Ca^{2+} current relaxes more slowly in certain terminals and this could explain observed differences in release pattern. Release of red-pigment-concentrating hormone (Fernlund & Joseffson, 1972) is brief and is associated with a voltage-dependent Ca^{2+}

entry which inactivates (Cooke & Haylett, 1984); other peptides are released in a more prolonged manner and are probably associated with non-inactivating Ca^{2+} entry (Stuenkel, 1985). In some terminals a Cd^{2+}-resistant component (Fig. 3, bottom) could be blocked by TTX. Thus there are two inward currents: one carried by Na^{+}

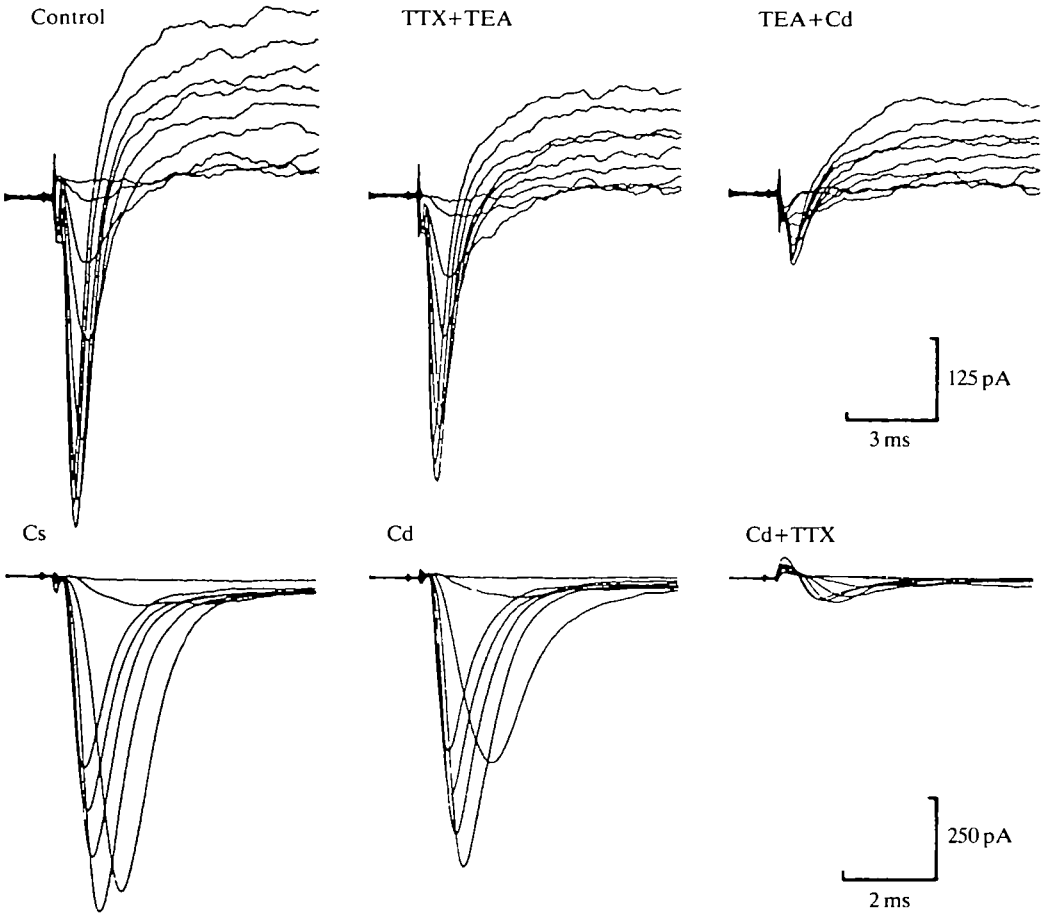

Fig. 3. Activation of whole-cell currents in dissociated sinus gland nerve terminal membrane during depolarizing voltage steps from a holding potential equal to normal resting potential (−50 mV). Control, appearance of both inward and outward currents during clamp steps to membrane potentials of −25 mV to +15 mV, incremented by +5 mV each time; 10 s between steps; 10 μm diameter neurosecretosome. (TTX+TEA) Note reduction of outward currents when the terminal is perfused with 40 mmol l^{-1} tetraethylammonium (TEA). There is only a small effect on inward currents with 6×10^{-7} mol l^{-1} tetrodotoxin (TTX). (TEA+Cd) Perfusion with 1 mmol l^{-1} Cd^{2+} of the same terminal (in 50 mmol l^{-1} TEA) causes a marked reduction of the inward currents. (Cs) Same protocol as in the control except that steps were from −25 mV to +35 mV in 10-mV increments, and K^{+} was replaced by Cs^{+} in the patch pipette resulting in an absence of outward currents; 15 μm diameter terminal. (Cd) 1 mmol l^{-1} Cd^{2+} has little effect on inward currents in this terminal. (Cd+TTX) Perfusion of 6×10^{-7} mol l^{-1} TTX blocks residual inward current in this nerve ending. All records are leak subtracted. (From Lemos, Nordmann, Cooke & Stuenkel, 1986.)

and the other by Ca^{2+}. This is expected from previous studies showing that most crab nerve endings exhibit overshooting action potentials having both Na^+ and Ca^{2+} components (Cooke, 1977; Nagano & Cooke, 1981). Outward currents were partially reduced by the application of TEA (Fig. 3, top), consistent with the increase by TEA of action potential duration in intact terminals, and were totally blocked if the terminal was internally perfused with Cs^+ (Fig. 3, bottom). These results indicate that the outward currents are carried by K^+. The magnitude of these currents is directly related to the size of the terminal (compare top and bottom of Fig. 3), presumably due to differences in their membrane surface area.

Single-channel currents

Single-channel currents were recorded from isolated terminals in cell-attached and inside-out patches. Two cation channels, not previously described, have been observed (Lemos *et al.* 1986).

f *channel*

One type of channel (*'f'*) shows brief (milliseconds) transitions to the open state, sometimes occurring in bursts, with long (seconds) intervals between openings (Fig. 4A). This channel, in symmetrical K^+, has a mean slope conductance of $69 \pm 3{\cdot}6$ pS (Fig. 4C). The single channel I/V curve (Fig. 4C) is not changed by substitution of K_2SO_4 or Na_2SO_4 for KCl on the internal face of the patch, but shows rectification when CsCl is substituted, indicating failure of the channel to pass Cs^+ or Cl^-. Channel currents in the presence of a salt gradient show nearly perfect selectivity for cations *versus* anions (Lemos *et al.* 1986). These observations argue against a significant anion permeability of the channels. Na^+ goes through the channel just as easily as K^+ since the reversal potential with equal concentrations ($310\,mmol\,l^{-1}$) of KCl outside and NaCl inside remains 0 mV. Furthermore, Na^+ concentrations between 78 and $310\,mmol\,l^{-1}$ on the inside of the patch cause this type of channel to remain open for longer periods (Fig. 4A, bottom) upon depolarization. This cation channel is observable in solutions having $[Ca^{2+}]$ buffered to $10^{-8}\,mol\,l^{-1}$ with EGTA, on the inside face.

s *channel*

The other type of channel (*'s'*) exhibits much longer (seconds) openings (Fig. 4B) and has a mean conductance of $213 \pm 6{\cdot}1$ pS in symmetrical K^+ (Fig. 4D). The openings appear to occur in bursts with rapid flickering back to the closed state. It is rarely observed in solutions having low $[Ca^{2+}]_i$, except during large voltage steps (Fig. 5), but is activated by increasing the internal free Ca^{2+} concentration above $1\,\mu mol\,l^{-1}$ (Lemos & Stuenkel, 1986). Higher concentrations of Ca^{2+} cause only a transient activation of channel activity. Ion substitution experiments indicate that this channel also has nearly equal permeabilities for Na^+ and K^+, but, unlike the *f* channel, allows Cs^+ to pass through (Fig. 4D). The *s* channel, therefore, has the characteristics of a Ca^{2+}-activated cation channel (Colquhoun, Neher, Reuter & Stevens, 1981), but exhibits a much larger slope conductance than has been observed

in other cells (Yellen, 1982; Maruyama & Petersen, 1982). It could be termed the 'maxi' Ca^{2+}-activated cation channel by analogy with Ca^{2+}-activated K^+ channels (Latorre & Miller, 1983).

The probability of opening for both the *f* and *s* channels appears not to be dependent on voltage (Lemos & Stuenkel, 1986). The P_o for the *f* and *s* channels is increased, however, by intracellular Na^+ and Ca^{2+}, respectively (Fig. 5).

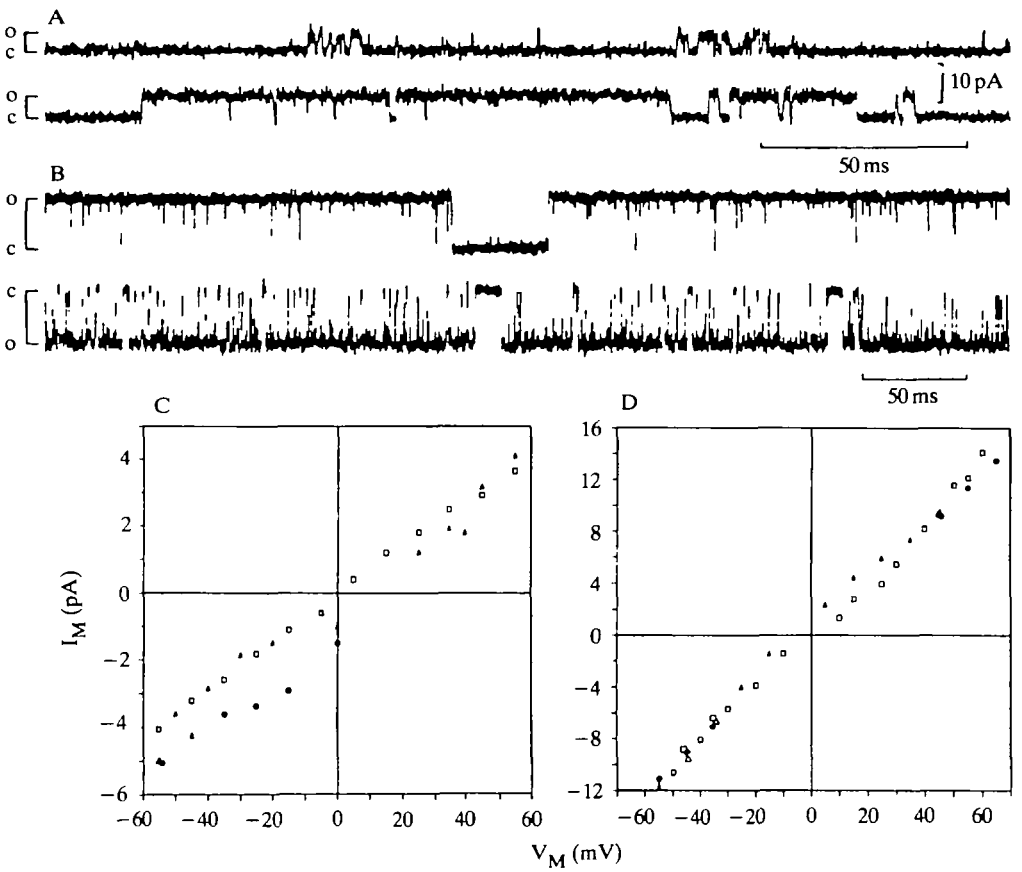

Fig. 4. Single-channel currents from inside-out patches showing two cation channel types observed in isolated sinus gland nerve terminals. (A) First (*f*) channel type: (top) patch held at +55 mV in symmetrical KCl. Upward openings (o) indicate outward current. (Bottom) Same patch held at +45 mV with 310 mmol l^{-1} KCl outside and 210 mmol l^{-1} Na_2SO_4 inside. (B) Second (*s*) channel type: (top) patch held at +55 mV with 310 mmol l^{-1} KCl inside and 210 mmol l^{-1} Na_2SO_4 outside and with 3 μmol l^{-1} $[Ca^{2+}]_i$. (Bottom) Same patch as above but held at −40 mV. Downward openings indicated inward current. All records are at the same gain to show difference in size of two channel types. c, closed. (C) I/V relationship of the *f* channel. Channel openings, in symmetrical KCl (□), of small amplitude (I_M) and short duration (see A) were plotted against patch potential (V_M). (D) I/V relationship of the *s* channel. Channel openings, in symmetrical KCl (□), of larger amplitude and longer duration (see B) were plotted against patch potential. Other symbols indicate I/V relationship when KCl is replaced by Na_2SO_4 (△), (▲) or CsCl (●). (From Lemos, Nordmann, Cooke & Stuenkel, 1986.)

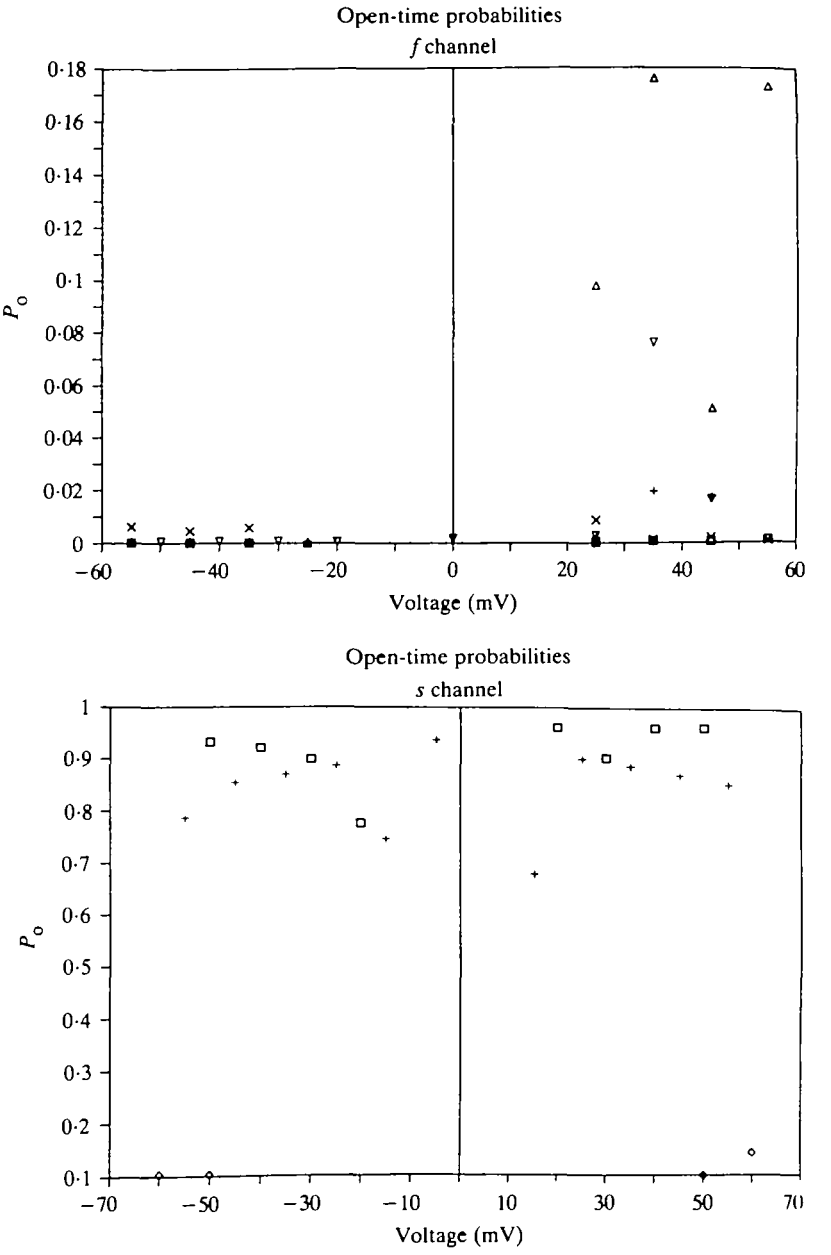

Fig. 5. Probability of opening. (A) Summary plot of relationship between probability (P_o) of *f* channel opening *vs* membrane potential. Data from inside-out patches with 310 mmol l^{-1} KCl in pipette and either 310 mmol l^{-1} KCl (□) (◇) (×) or 210 mmol l^{-1} Na_2SO_4 (+) (△) (▽) on the inside of the patch. (B) Summary plot of relationship between probability (P_o) of *s* channel opening *vs* membrane potential. Data from experiments with different concentrations of free $[Ca^{2+}]$ on the inside of the patch: <0·1 μmol l^{-1} (◇), 3 μmol l^{-1} (+) or 50 mmol l^{-1} (□). (From Lemos & Stuenkel, 1986.)

A scheme can be imagined in which Na^+ and/or Ca^{2+} entry activates the channel(s) which then become inactivated by increased $[Ca^{2+}]$ after a period of seconds. These channels might be responsible for the long-lasting plateau potentials which underlie bursting (see Fig. 2) in these terminals (Cooke, 1981; Cooke & Stuenkel, 1985). Certain terminals can be induced to burst by brief axonal stimulation, and it could be the entry of Ca^{2+} and/or Na^+ during the subsequent terminal spikes or depolarization that activates the cation channels. Repolarization may be mediated by both Ca^{2+} inactivation of the channels and a Ca-dependent K^+ current, which appears to exist in these terminals (Nagano & Cooke, 1983). Inward current events having the characteristics of the *s* channel are observable in whole-terminal recordings, under voltage-clamp, with crab saline (Pantin, 1948) in the bath.

Calcium channels

It is important to characterize the entry of Ca^{2+} into nerve terminals to understand how release is regulated. The study of Ca^{2+} channels, which in other material have generally proved to have unitary currents of <1 pA, and to show rapid transitions and bursting behaviour, is greatly facilitated by the use of 'tip-dip' methodology (Coronado & Latorre, 1983) and of Ba^{2+} as the charge carrier in the electrode, since it increases single Ca channel current amplitudes (Lux & Brown, 1984) and prevents inactivation (Tsien, 1983).

Three distinct types of unitary currents can be resolved in recordings (Fig. 6) from crab sinus gland nerve terminal membranes reconstituted into an exogenous lipid bilayer. The approximate single-channel conductances for the three types of channels, in symmetrical 200 mmol l^{-1} $BaCl_2$, are (a) 14 pS, (b) 27 pS and (c) 43 pS, respectively. The different types of Ca channels can also be distinguished by their voltage activation and sensitivity to nifedipine derivatives. Types b and c are activated by Bay K 8644, which greatly prolongs Ca^{2+} channel open times (Nowycky, Fox & Tsien, 1985), and blocked by NS-202 (J. R. Lemos, in preparation).

NEURAL LOBE

The posterior pituitary gland (neural lobe) is a convenient model for the study of the release of peptide neurohormones since it contains the distal parts of oxytocin- and vasopressin-containing neurones. The hormones are synthesized, as precursors, in the cell bodies, packaged into neurosecretory granules and transported down their axons to the posterior pituitary where they are released. The neural lobe (NL) contains, on average, $3{\cdot}4\times10^7$ nerve terminals. Each cell body gives rise to an average of $1{\cdot}8\times10^3$ nerve terminals, whose mean diameter is about 2 μm although much larger (approx. 8–10 μm) endings can be observed. Arginine-vasopressin (AVP) and oxytocin (OT) are the two major peptides found in the neurohypophysial secretory granules (molar ratio >1000 compared with other molecules).

Biochemical and morphological knowledge of the hypothalamo–neurohypophysial complex provides insight into the mechanisms of synthesis, transport, release and storage of the neuropeptides synthesized by the supraoptic and paraventricular

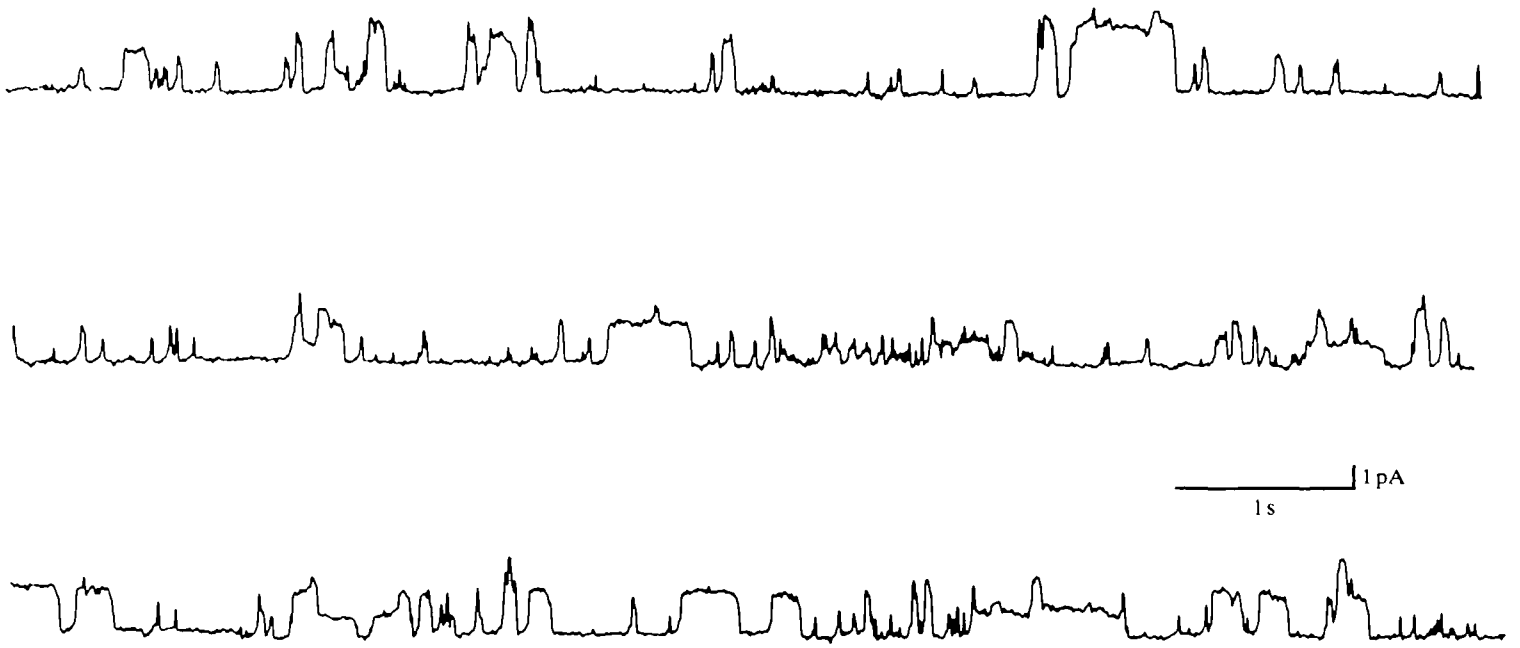

Fig. 6. Calcium channels from crab terminal membranes. Three distinct types of unitary currents can be resolved in this continuous recording from crab sinus gland nerve terminals reconstituted into an exogenous lipid (phosphatidylethanolamine/phosphatidylserine, 3/1) bilayer. The patch was held at +70 mV in symmetrical 200 mmol l^{-1} $BaCl_2$, 20 mmol l^{-1} Hepes (pH 7·4). Penwriter record, thus bandwidth is approximately 150 Hz.

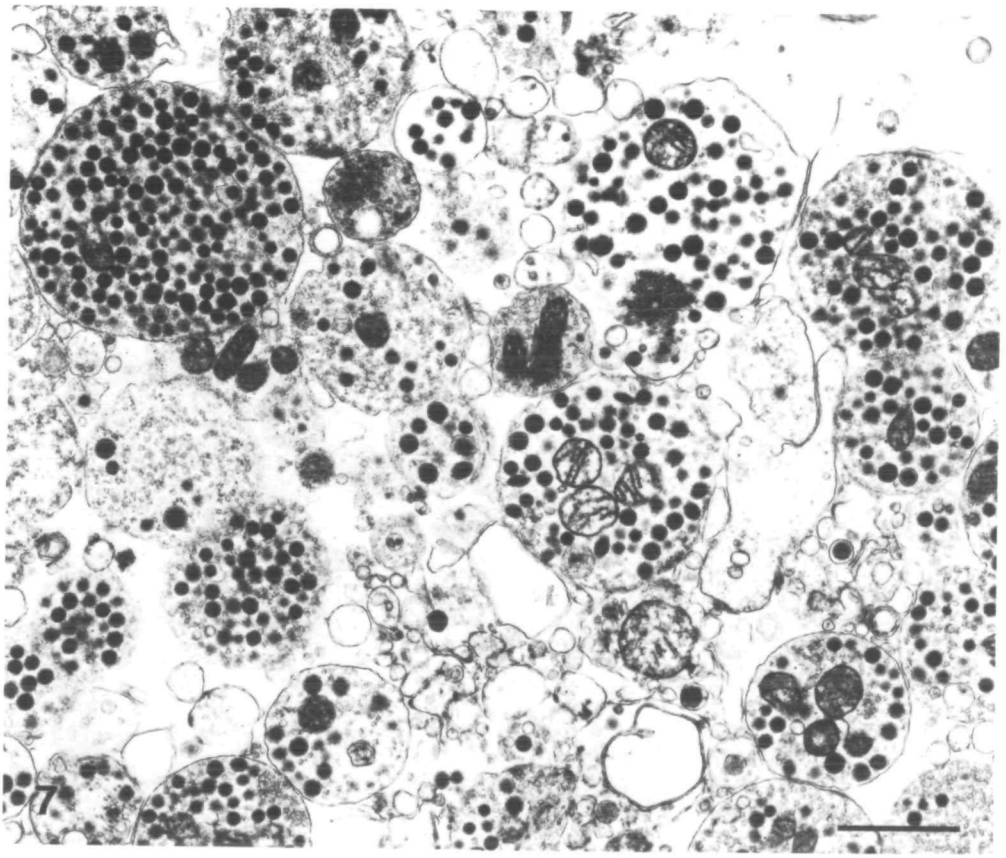

Fig. 7. Rat neural lobe terminals. Electron micrograph of dissociated nerve terminals from the rat neurohypophysis. Note variety of terminal sizes and abundance of neurosecretory granules and mitochondria within terminal profiles (J. J. Nordmann & F. D. Shaw, unpublished micrograph). Scale bar, 1 μm.

nuclei. In addition, much is known about the electrophysiology of these magnocellular neurones (for a review, see Poulain & Wakerley, 1982). Oxytocin neurones are characterized by their synchronous high frequency discharge during suckling, which leads to the pulsatile release of OT and subsequent milk ejection. Vasopressin neurones are characterized by their asynchronous phasic activity (bursting) during prolonged, 'trickle' AVP release and regulation of water balance. In both cases it is the clustering of action potentials which facilitates hormone release (Cazalis *et al.* 1985), albeit with different time courses.

To analyse the mechanisms by which membrane depolarization is linked to the release of AVP and OT, we have developed a technique for isolating neural lobe terminals (Nordmann, Desmazes & Georgescault, 1982; D. Brethes, G. Dayanithi, L. Letellier & J. J. Nordmann, in preparation). These neurosecretosomes consist almost entirely of isolated nerve endings as judged by electron microscopy (Fig. 7) or by immunocytochemistry of the neuropeptides and neurophysins (J. J. Nordmann, unpublished observation).

Stimulus–secretion coupling

The following steps in stimulus–secretion coupling, in the neural lobe, have been indirectly demonstrated (for a review see Nordmann, 1983). The arrival of action potentials induces the depolarization of the nerve terminals which have been shown to have in their plasma membrane both Na (Nordmann & Dyball, 1978) and Ca channels (Dreifuss, Grau & Nordmann, 1973; Russell & Thorn, 1974). The electrically induced depolarization promotes the entry of calcium into the nerve terminals which then triggers, by an *unknown* mechanism, the release of the NSG contents.

Using a fluorescent probe to measure changes of membrane potential, we have shown that the neurosecretosomes can be depolarized with increasing external potassium concentration or with agents such as veratridine (Nordmann *et al.* 1982). The depolarization of the isolated nerve terminals is correlated with the release of AVP, OT and neurophysins. Depolarization-induced hormone release requires external calcium and is abolished by agents known to block Ca^{2+} channels (Co^{2+}, Mn^{2+}, D600, Cd^{2+}, Gd^{2+}, nitrendipine and nicardipine; M. Cazalis, G. Dayanithi & J. J. Nordmann, in preparation).

The entry of calcium and its homeostasis in the nerve terminals has also been studied (Douglas & Poisner, 1964*b*; Nordmann, 1976; Russell & Thorn, 1974). Only recently has it been possible to show, using vasopressinergic activity as the stimulus, that depolarization of the nerve endings is associated with an increased ionized calcium concentration in their cytoplasm (Fig. 8). Similarly, we have shown that this increase, measured with Fura-2, is abolished by Ca^{2+} channel blockers (D. Brethes, G. Dayanithi, L. Letellier & J. J. Nordmann, in preparation). As in the intact neural lobe, calcium can be replaced by strontium as a trigger for hormone release.

Permeabilized neurosecretosomes

Using detergent-permeabilized isolated nerve endings, hormone release can be observed (Fig. 9) with calcium concentrations in the micromolar range (Bicknell, Cazalis, Dayanithi & Nordmann, 1985; M. Cazalis, G. Dayanithi & J. J. Nordmann, in preparation). This is in contrast with 'normal' isolated preparations which release neuropeptides only when depolarized in the presence of millimolar concentrations of external calcium. Furthermore, the release mechanism is greatly potentiated by the presence of ATP. Other nucleotides have little or no effect on the secretion of AVP and OT. The permeabilized preparation is extremely useful for studying the steps which are hypothesized to link the entry of calcium to the exocytosis of the NSGs. We have found that trifluoroperazine, at high concentrations, only partially inhibits the calcium-dependent hormone release in this preparation (Fig. 10). A phorbol ester (TPA), on the other hand, stimulates hormone release from the permeabilized neurosecretosomes at low concentrations (M. Cazalis, G. Dayanithi & J. J. Nordmann, in preparation). These preliminary results suggest that C-kinase might be involved in the secretory response.

Single channels

The rat neurosecretosomes also readily form giga-ohm seals with fire-polished electrodes, making feasible the application of patch-clamp techniques to characterize the ionic channels of the terminal membrane. Single-channel currents from isolated rat NL nerve terminal inside-out patches show both fast (J. R. Lemos & J. J. Nordmann, in preparation) and slow channel types (Fig. 11). The slow channel has a slope conductance of 221 pS. Both Na^+ and K^+ permeate through the channel, but anions do not, indicating that this is a cation channel. Openings are only seen with internal Ca^{2+} concentrations above $1\,\mu mol\,l^{-1}$. Thus this neural lobe Ca^{2+}-activated cation channel seems comparable to the *s* channel found in SG nerve terminals (see Fig. 4). Since the *f* and *s* channels have not been found in other neurones or neuronal structures (such as X-organ somata; J. R. Lemos & B. Haylett, unpublished observations), they may be unique to peptidergic nerve terminals. More experiments, however, are necessary to establish this conclusion.

Neurosecretory granule channels

It is possible to separate and isolate neurosecretory granules on iso-osmotic gradients (Nordmann, Louis & Morris, 1979). The purity of such preparations makes

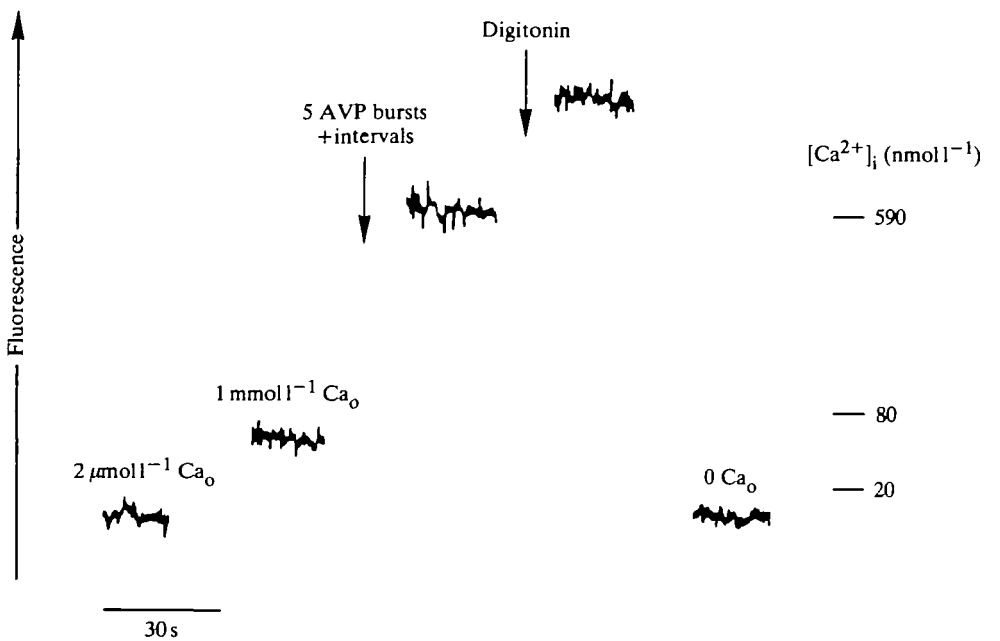

Fig. 8. Effect of electrical stimulation on $[Ca^{2+}]_i$ in isolated neurohypophysial nerve terminals. The internal calcium concentration was measured using the fluorescent indicator Fura-2. The nerve endings were stimulated with five bursts mimicking the electrical activity of vasopressinergic cells (for details see Cazalis, Dayanithi & Nordmann, 1985). The free internal calcium concentration was measured after addition of digitonin. The calculated free $[Ca^{2+}]_i$ is indicated on the right-hand side (D. Brethes, G. Dayanithi, L. Letellier & J. J. Nordmann, unpublished observations).

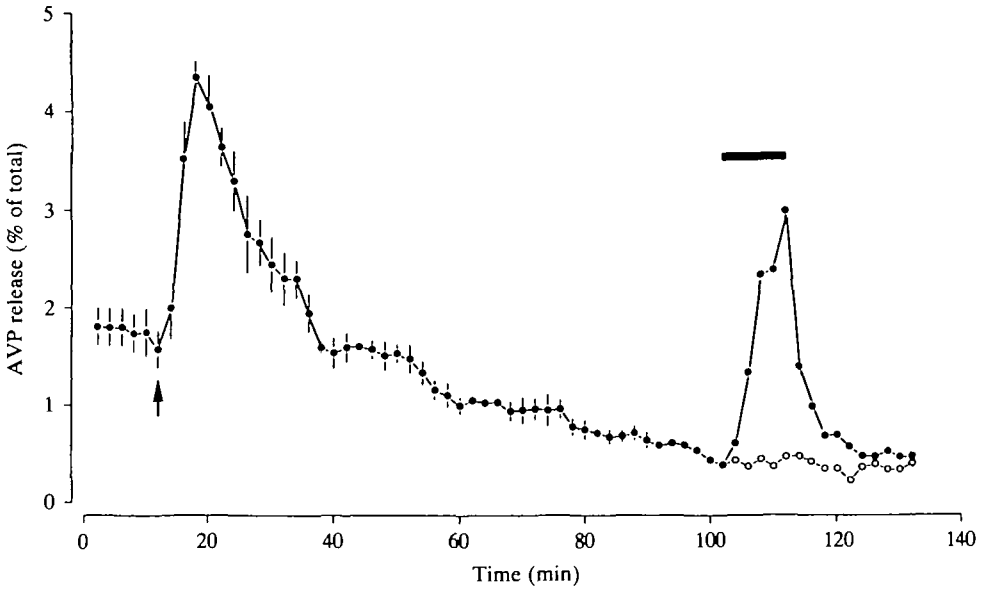

Fig. 9. Vasopressin release from rat neurosecretosomes. Release of arginine-vasopressin (AVP) was measured from neurosecretosomes stimulated by exposure to high ($100\,mmol\,l^{-1}$) external K^+ (at arrow) in the presence of $2{\cdot}2\,mmol\,l^{-1}$ $[Ca^{2+}]_o$ and then permeabilized by digitonin (during bar) in the absence (○) or presence (●) of $1{\cdot}1\,\mu mol\,l^{-1}$ $[Ca^{2+}]$. Digitonin concentrations were adjusted so that non-calcium-dependent release was less than 10% of total release. (From M. Cazalis, G. Dayanithi & J. J. Nordmann, in preparation.)

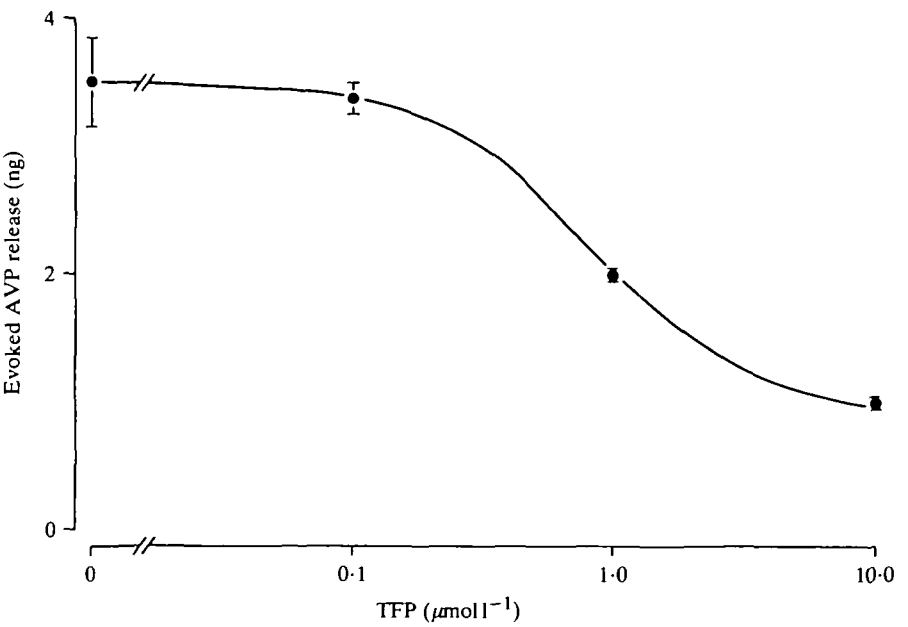

Fig. 10. Effect of different concentrations of trifluoroperazine (TFP) on release of vasopressin (AVP). AVP release was evoked with digitonin (see Fig. 9) in the presence of $1{\cdot}1\,\mu mol\,l^{-1}$ Ca^{2+}. (From M. Cazalis, G. Dayanithi & J. J. Nordmann, in preparation.)

possible the analysis of neurosecretory granule ion fluxes. We have been able to reconstitute neurosecretory granule membrane proteins and study their activity using tip-dip methods. Preliminary evidence indicates that the NSG membranes do not contain Ca channels but do exhibit at least two other ionic channels (J. R. Lemos & J. J. Nordmann, in preparation). One channel type is permeable to K^+ and activated by internal Ca^{2+}. It appears to be similar to a channel recently reported in secretory granule membrane from pituitary glands studied in planar bilayers (Stanley, Ehrenstein & Russell, 1986). It has been suggested that entry of Ca^{2+} into pituitary nerve endings activates this channel and that elevation of $[K^+]$ in the NSG causes subsequent entry of anions through hypothesized anion channel(s). We have observed such an anion channel (Fig. 12) in membranes prepared from isolated neural lobe NSGs. In asymmetrical salt gradients the channel appears to be

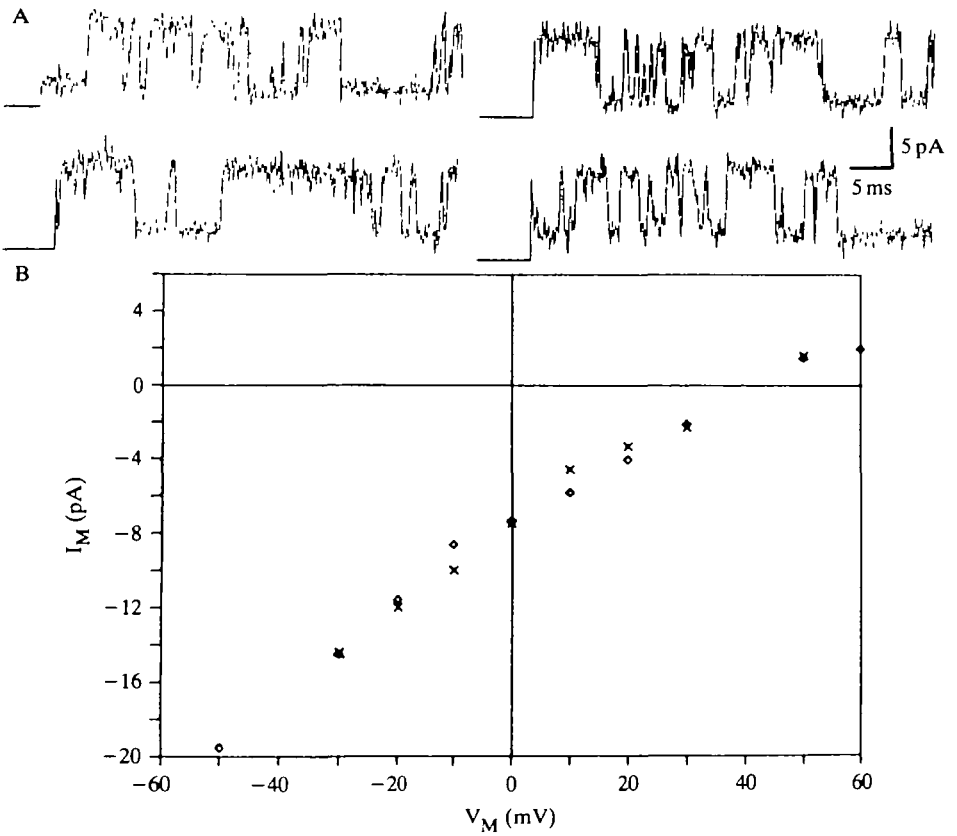

Fig. 11. Single-channel currents from inside-out patches (×) (◇) showing slow channel type observed in isolated rat neural lobe nerve terminals. (A) Patch held at −15 mV with 160 mmol l^{-1} KCl outside and Locke's solution on the inside (with 1·7 μmol l^{-1} free $[Ca^{2+}]_i$). Upward openings indicate inward current; 4 kHz bandwidth. (B) I/V relationship of the neural lobe slow channel. The amplitude (I_M) of large channel openings with long duration (see A) were plotted against patch potential (V_M). Calculated regression shows a slope conductance of 221 pS.

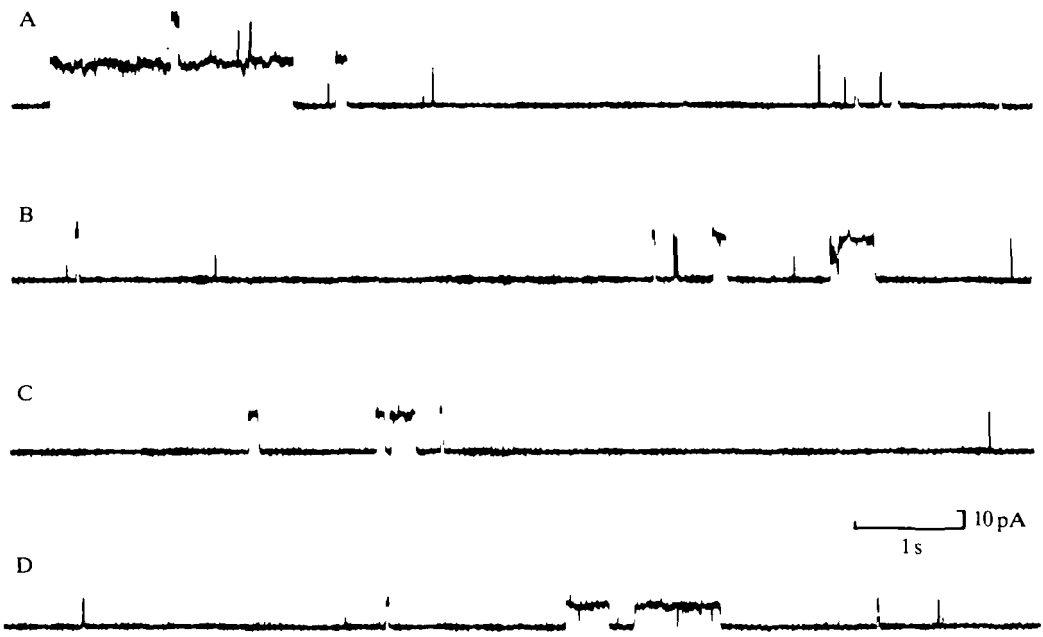

Fig. 12. Anion channels of neurosecretory granules from rat neural lobe. Current records from rat neural lobe neurosecretory granule membranes reconstituted into an exogenous lipid (phosphatidylethanolamine/phosphatidylserine, 3/1) bilayer. 200 mmol l^{-1} KCl on one side and 100 mmol l^{-1} KCl on the other. Patch was held at the following potentials: (A) +90 mV; (B) +75 mV, (C) +60 mV; (D) +45 mV; 3 kHz bandwidth.

permeable only to anions, such as Cl^-, and not to cations. This NSG anion channel, in symmetrical 200 mmol l^{-1} KCl, has a slope conductance of about 280 pS and opens even in the presence of only 10^{-8} mol l^{-1} free internal [Ca^{2+}]. The existence of these two channels in NSG membrane lends support to the theory (Cohen, Akabas & Finkelstein, 1982; Ehrenstein & Stanley, 1986) that Ca^{2+} entry could lead to swelling of the NSG and thus promote fusion with the plasma membrane and release of the NSG contents.

CONCLUSIONS

Although there is considerable evidence that depolarization of nerve cell terminals leads to the entry of Ca^{2+} and to the secretion of neurohormones and neurotransmitters, the details of how ionic currents control the release of neuroactive substances from nerve terminals remain undetermined. This study presents two preparations in which stimulus–secretion coupling can be directly analysed. Much is already known about neurohormone release from both the neural lobe and the sinus gland, and the electrical activity of individual terminals has been well characterized.

Patch-clamping of the isolated neurosecretosomes has now allowed the elucidation of some of the macroscopic currents underlying nerve terminal voltage responses. The finding of two previously undescribed cation channels, possibly unique to nerve terminals, may have particular importance for bursting activity. Such patterns have been shown to have a facilitatory effect on calcium entry and hence on hormone release. We do not know exactly at which step of the stimulus–secretion coupling mechanism facilitation occurs, but recent data (Cazalis *et al.* 1985) suggest that the phasic pattern of discharge has some effects on Ca^{2+} channels. Plausible explanations are that it increases either (1) the number of channels activated at a given time or (2) the time for a channel to inactivate. The ability to reconstitute Ca^{2+} and NSG channels from nerve endings should allow us to answer such questions. Furthermore, the abundance of granules, known to contain peptide hormones, together with histological and biochemical evidence for release by exocytosis, suggest that membrane capacitance measurements (Neher & Marty, 1982) could be utilized in these preparations to study directly the events coupling depolarization of the nerve terminal membrane with the release of peptide hormones from neurosecretory granules.

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REFERENCES

BICKNELL, R. J., CAZALIS, M., DAYANITHI, G. & NORDMANN, J. J. (1985). Calcium-dependence of hormone release from isolated neurohypophysial nerve endings (neurosecretosomes) of the rat. *J. Physiol., Lond.* **369**, 166P.

CAZALIS, M., DAYANITHI, G. & NORDMANN, J. J. (1985). The role of patterned burst and interburst interval on the excitation-coupling mechanism in the isolated rat neural lobe. *J. Physiol., Lond.* **395**, 45–60.

COHEN, F. S., AKABAS, M. H. & FINKELSTEIN, A. (1982). Osmotic swelling of phospholipid vesicles causes them to fuse with planar phospholipid bilayer membrane. *Science* **217**, 458–460.

COLQUHOUN, D., NEHER, E., REUTER, H. & STEVENS, C. F. (1981). Intracellular current channels activated by intracellular Ca in cultured cardiac cells. *Nature, Lond.* **294**, 752–754.

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

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

COOKE, I. M. (1981). Electrical activity in relation to hormone secretion in the X-organ–sinus gland system of the crab. In *Neurosecretion: Molecules, Cells, Systems* (ed. D. Farner & K. Lederis), pp. 237–249. New York: Plenum Press.

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

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

COOKE, I. M. & SULLIVAN, R. E. (1982). Hormones and neurosecretion. In *The Biology of Crustacea*, vol. 3 (ed. H. Atwood & D. Sandeman), pp. 206–290. New York: Academic Press.

CORONADO, R. & LATORRE, R. (1983). Phospholipid bilayers made from monolayers on patch-clamp pipettes. *Biophys. J.* **43**, 231–236.

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

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

DOUGLAS, W. W. & RUBIN, R. P. (1963). The mechanism of catecholamine release from the adrenal medulla and the role of calcium in stimulus–secretion coupling. *J. Physiol., Lond.* **67**, 388–410.

DREIFUSS, J. J., GRAU, J. D. & NORDMANN, J. J. (1973). Effects of isolated neurohypophysial agents which affect the membrane permeability to calcium. *J. Physiol., Lond.* **231**, 96–98.

EHRENSTEIN, G. & STANLEY, E. (1986). A model for fusion based on the opening of calcium-activated potassium channels. *Biophys. J.* **49**, 18a.

FENWICK, E. M., MARTY, A. & NEHER, E. (1982*a*). A patch-clamp study of bovine chromaffin cells and their sensitivity to acetylcholine. *J. Physiol., Lond.* **331**, 577–597.

FENWICK, E. M., MARTY, A. & NEHER, E. (1982*b*). Sodium and calcium channels in bovine chromaffin cells. *J. Physiol., Lond.* **331**, 599–635.

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

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

HAGIWARA, S. & BYERLY, L. (1983). The calcium channel. *Trends Neurosci.* **6**, 189–193.

HAMILL, O. P., MARTY, A., NEHER, E., SAKMANN, B. & SIGWORTH, F. J. (1981). Improved patch-clamp techniques for high resolution current recording from cells and cell-free membrane patches. *Pflügers Arch. ges. Physiol.* **391**, 85–100.

KIDOKORO, Y. (1985). Electrophysiology of adrenal chromaffin cells. In *The Electrophysiology of the Secretory Cell* (ed. A. M. Poisner & J. M. Trifaro), pp. 195–218. New York: Elsevier.

LATORRE, R. & MILLER, C. (1983). Conduction and selectivity in potassium channels. *J. Membrane Biol.* **71**, 11–30.

LEMOS, J. R., NORDMANN, J. J., COOKE, I. M. & STUENKEL, E. L. (1986). Single channels and ionic currents in peptidergic nerve terminals. *Nature, Lond.* **319**, 410–412.

LEMOS, J. R. & STUENKEL, E. L. (1986). Two novel cation channels in nerve terminals. *Biophys. J.* **49**, 368a.

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

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

LUX, H. D. & BROWN, A. M. (1984). Patch and whole cell calcium currents recorded simultaneously in snail neurons. *J. gen. Physiol.* **83**, 727–750.

MARUYAMA, Y. & PETERSEN, O. H. (1982). Single channel currents in isolated patches of plasma membrane from basal surface of pancreatic acini. *Nature, Lond.* **299**, 159–161.

NAGANO, M. & COOKE, I. M. (1981). Electrical activity in the crab X-organ–sinus gland system. Site of initiation, ionic bases and pharmacology. In *Neurosecretion: Molecules, Cells, Systems* (ed. D. Farner & K. Lederis), pp. 501–502. New York: Plenum Press.

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

NEHER, E. & MARTY, A. (1982). Discrete changes of cell membrane capacitance observed under conditions of enhanced secretion in bovine adrenal chromaffin cells. *Proc. natn. Acad. Sci. U.S.A.* **79**, 6712–6716.

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

NORDMANN, J. J. (1983). Stimulus secretion coupling. *Prog. Brain Res.* **60**, 281–304.

NORDMANN, J. J., DESMAZES, J. P. & GEORGESCAULT, D. (1982). The relationship between the membrane potential of neurosecretory nerve endings, as measured by a voltage-sensitive dye and the release of neurohypophysial hormones. *Neurosci.* **7**, 731–737.

NORDMANN, J. J. & DYBALL, R. E. J. (1978). Effects of veratridine on Ca fluxes and the release of oxytocin and vasopressin from the isolated rat neurohypophysis. *J. gen. Physiol.* **72**, 297–304.

NORDMANN, J. J., LOUIS, F. & MORRIS, S. J. (1979). Purification of two structurally and morphologically distinct populations of rat neurohypophysial secretory granules. *Neurosci.* **4**, 1367–1375.

NOWYCKY, M. C., FOX, A. P. & TSIEN, R. W. (1985). Three types of neuronal calcium channels with different calcium agonist sensitivity. *Nature, Lond.* **316**, 440–442.

PANTIN, C. F. A. (1948). *Notes on Microscopical Techniques for Zoologists*. Cambridge: Cambridge University Press. 48 pp.

POULAIN, D. A. & WAKERLEY, J. B. (1982). Electrophysiology of hypothalamic magnocellular neurones secreting oxytocin and vasopressin. *Neurosci.* **7**, 773–808.

RUSSELL, J. T. & THORN, N. A. (1974). Calcium and stimulus–secretion coupling in the neurohypophysis. II. Effects of lanthanum, a verapamil analogue (D600) and prenylamine on 45-calcium transport and vasopressin release in isolated rat neurohypophyses. *Acta endocr., Copnh.* **76**, 471–487.

STANLEY, E., EHRENSTEIN, G. & RUSSELL, J. (1986). Evidence for calcium-activated potassium channels in vesicles of pituitary cells. *Biophys. J.* **49**, 19a.

STUENKEL, E. L. (1983). Peptide biosynthesis and secretion from a crab neurosecretory system. Ph.D. dissertation, University of Hawaii.

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

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

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

YELLEN, G. (1982). Single Ca^{2+}-activated nonselective cation channels in the neuroblastoma. *Nature, Lond.* **296**, 357–359.