J. exp. Biol. 139, 195–231 (1988)
Printed in Great Britain © The Company of Biologists Limited 1988

OPTICAL STUDIES OF THE SECRETORY EVENT AT VERTEBRATE NERVE TERMINALS

BY BRIAN M. SALZBERG AND ANA LIA OBAID

Department of Physiology, University of Pennsylvania School of Medicine, A-201 Richards Building, Philadelphia, PA 19104-6085 and *the Marine Biological Laboratory, Woods Hole, MA 02543, USA*

Summary

Potentiometric probes are small (300–500 M_r) amphipathic molecules that bind to, but do not cross, cell membranes and behave as fast linear transducers of membrane voltage. Their optical properties, particularly absorbance and fluorescence, respond to changes in potential in less than 2 μs, and they may be used to follow electrical events in membranes which are inaccessible to microelectrodes. We have used these dyes to study the properties of the action potential in the neurosecretory terminals of vertebrate neurohypophyses and, in particular, to investigate the behaviour of the local population of calcium channels. These channels are sensitive to the peptide toxin ω-conotoxin GVIA, derived from the venom of the marine snail *Conus geographicus*, but insensitive to dihydropyridine channel modulators. In the neurohypophysis of the mouse, it is possible to demonstrate that the calcium channels that are blocked by ω-conotoxin are those that are required for secretion of peptide hormones.

In the terminals of the neurohypophysis, excitation is coupled to secretion, and the secretory event is accompanied by large and rapid changes in light scattering. These intrinsic optical signals provide a millisecond time-resolved monitor of events in the terminal that follow the entry of calcium, and may precede the release of hormones. We will consider how the changes in light scattering can be related to secretion, and how the extrinsic (absorption) and intrinsic optical signals may provide complementary information about excitation–secretion coupling.

Introduction

The study of the secretory event *per se*, as well as the excitation that provokes it, has proved especially difficult at the nerve terminals of vertebrates. This, of course, is largely the result of the small size of the structures of interest and, consequently, much of our mental imagery, if not our understanding, related to the release of transmitter substances comes from the electron microscope (Heuser *et al.* 1979; Heuser & Reese, 1981). As far as electrophysiology is concerned, we have depended for our understanding largely upon a series of elegant studies of synaptic transmission at the frog neuromuscular junction (Katz, 1969), in which

Key words: optical recording, nerve terminals, light scattering, potentiometric probe, calcium channels.

most measurements were actually made on the postsynaptic element, and upon analogies drawn from that most useful of invertebrates, the Atlantic squid, *Loligo*.

Patch-clamp methods have begun to provide important tools for the study of secretion (Fernandez, Neher & Gomperts, 1984; Lindau & Fernandez, 1986) in some systems (e.g. mast cells), but their reliable application to vertebrate nerve terminals is only beginning (Mason & Dyball, 1986; Lemos & Nowycky, 1987). Optical techniques for the measurement and analysis of transmembrane electrical events have also found a wide range of applications over the past decade because, although they often provide less detailed information than does the voltage clamp, they can offer certain advantages over more conventional measurements. Because the membranes of interest are not mechanically violated, the methods are comparatively non-invasive. Spatial resolution is limited only by microscope optics and noise considerations; it is possible to measure changes in membrane potential from regions of a cell with linear dimensions of the order of 1 μm, and the use of optical sectioning, with elimination of scattering by means of confocal imaging, may soon permit optical recording from small regions of cells in intact ganglia or brains. Temporal resolution is limited by the physical response of the probes and by the bandwidth imposed upon the measurement, again usually by noise considerations, and response times faster than typical membrane time constants may be achieved. Because mechanical access is not required, unusual latitude is possible in the choice of preparation, and voltage changes may be monitored in membranes which are otherwise inaccessible. Also, since no recording electrodes are employed, and the measurements are actually made at a distance from the preparation – in the image plane of an optical apparatus – it is possible to record changes in potential simultaneously from a large number of spatially separated sites. Several recent reviews have surveyed the literature on optical measurement of membrane potential (Cohen & Salzberg, 1978; Waggoner, 1979; Freedman & Laris, 1981; Salzberg, 1983; Grinvald, 1985) and they should be consulted by the reader interested in the details of these techniques.

The evidence that optical methods are equivalent, at least in a limited sense, to electrode measurements has been detailed elsewhere (Cohen *et al.* 1974; Ross *et al.* 1977; Salzberg *et al.* 1977; Cohen & Salzberg, 1978; Gupta *et al.* 1981). Indeed, under certain conditions (Salzberg & Bezanilla, 1983), an optical measurement of membrane potential is superior to an electrode measurement. Our purpose here, however, is to illustrate the application of optical techniques to the coupled problems of excitation and secretion at vertebrate nerve terminals, problems for which optical methods are particularly well suited. The direct optical recording of the action potential from the nerve terminals of a vertebrate and the study of its ionic basis (Salzberg, Obaid, Senseman & Gainer, 1983; Obaid, Orkand, Gainer & Salzberg, 1985) provide information that is presently unobtainable by other means. A variety of linear potentiometric probes can now be used to make this kind of measurement, by exploiting the extrinsic absorption or fluorescence signals that result when the potential varies across a membrane stained with these dyes. We shall begin by describing some of the properties of the

nerve terminal action potential, as revealed in the light of the tungsten–halogen bulb. Analysis of optical recordings of the action potential in the nerve terminals of a mammal has disclosed the presence of at least two large and rapid changes in the intrinsic optical properties of the neural lobe, in addition to the extrinsic absorption changes that report changes in membrane potential, and one of these, a change in light scattering, has now been shown (Salzberg, Obaid & Gainer, 1985) to be intimately associated with the physiological events that attend the release of the neuropeptides arginine vasopressin (AVP) and oxytocin. After describing the properties of these intrinsic signals, and detailing some of the evidence that this optical signal is related to the secretory event, we will conclude this chapter by discussing some of the pharmacology of the calcium channels that are required for the release of neuropeptides in the neurohypophysis.

The vertebrate hypothalamo-neurohypophyseal system represents an extremely attractive model for the study of excitation–secretion coupling at nerve terminals. Magnocellular neurones located in the hypothalamus (supraoptic and paraventricular nuclei in mammals, preoptic nucleus of lower vertebrates) project their axons as bundles of fibres through the median eminence and infundibular stalk to terminate in the neurohypophysis, where the neurohypophyseal peptides (oxytocin and arginine vasopressin, or their homologues) and associated proteins (neurophysins) are secreted into the circulation. Indeed, the neurohypophysis has been a classical *in vitro* preparation for measuring the calcium-dependent release of peptide hormones under various conditions of stimulation (Douglas, 1963; Douglas & Poisner, 1964), and extracellularly recorded compound action potentials are readily measured in this organ (Dreifuss, Kalnins, Kelley & Ruf, 1971). The analysis of excitation–secretion coupling in the neurohypophysis has been compromised, however, by the circumstance that these nerve terminals are too small for intracellular measurement of the electrophysiological events that affect release.

Optical recording of action potentials from nerve terminals in the frog *Xenopus*

Resting and slowly changing transmembrane voltages in synaptosomes prepared from rat brain homogenates had already been measured using cyanine (Blaustein & Goldring, 1975) and merocyanine (Kamino & Inouye, 1978) dyes a decade ago, and action potentials have been recorded from the growth cones of tumour cells in tissue culture using an oxonol dye (Grinvald & Farber, 1981). However, it was not until 1983 that it became possible to record the action potential from intact nerve terminals in a vertebrate, with good fidelity to the time course of the transmembrane potential change (Salzberg *et al.* 1983). This was achieved in the neurohypophysis of *Xenopus*, where we took advantage of the extraordinary qualities of this preparation for optical recording. In particular, the homogeneity of the neurohypophysis, the tremendous proliferation of nerve terminals, and the relative lack of excitable membrane that is not involved in secretion make this tissue nearly ideal for optical measurement. In addition, there is no postsynaptic membrane to

confound the interpretation of the optical signals. Because the optical recordings of the action potential in the nerve terminals of the frog neurohypophysis could be obtained with extraordinary signal-to-noise ratios, we could manipulate the shape of the action potential by changing extracellular calcium concentration and with other agents known to alter the release of neurohormones and neurotransmitters (Salzberg *et al.* 1983). Organic, as well as inorganic calcium channel modifiers could be studied (Salzberg *et al.* 1983; A. L. Obaid, R. Flores & B. M. Salzberg, in preparation), and we could also show that when voltage-sensitive Na^+ channels were blocked by tetrodotoxin (TTX) and K^+ channels were blocked by tetraethylammonium (TEA^+) (Katz & Miledi, 1967), direct electric field stimulation of the nerve terminals evoked large active responses that reflected the same inward Ca^{2+} current that is associated with hormone release from these nerve terminals (Obaid *et al.* 1985).

Fig. 1B shows an optical recording that represents the intracellular potential changes during the action potentials in a population of synchronously activated nerve terminals in the neurohypophysis of the frog, *Xenopus laevis*. This recording was photographed from an oscilloscope screen without signal averaging. The technique used here, multiple site optical recording of transmembrane voltage (MSORTV), is an extension of the method used to record extrinsic absorption changes in the squid giant axon and in many other preparations (Salzberg *et al.* 1977, 1983; Grinvald, Cohen, Lesher & Boyle, 1981; Senseman, Shimizu, Horwitz

Fig. 1. Multiple site optical recording of transmembrane voltage (MSORTV) from nerve terminals in the neurohypophysis of *Xenopus*. (A) Schematic diagram of the optical portion of the MSORTV system. Collimated light from an incandescent source is made quasi-monochromatic with interference and heat filters and focused on the preparation by means of a bright-field condenser with numerical aperture matched to that of the objective. A high numerical aperture water-immersion objective projects a real image of a portion of the preparation onto the central 124 elements of a 144-element photodiode array, whose photocurrent outputs are converted to voltages, a.c. coupled and amplified, multiplexed, digitized and stored in a PDP 11/34A computer under direct memory access (DMA). A full frame is recorded, with an effective resolution of 18 bits, every 800 μs. (B) A photograph of an oscilloscope recording of the change in the transmitted intensity monitored by one channel (element E5 of the array) of the MSORTV system, following a brief shock to the hypothalamus. This element monitored intensity changes from a region of the posterior pituitary entirely within the pars nervosa. The fractional change in intensity during the action potential was approximately 0·25 %. Single sweep; 722 ± 21 nm. Rise time of the optical system (10–90 %) 1·1 ms; a.c. coupling time constant, 1·0 s. (C) Drawing of the region of the posterior pituitary imaged on the photodiode matrix array, showing the positions of the individual detector elements with respect to the tisssue. Drawn from a photomicrograph of the preparation and a transparent overlay representing the detector array. Objective 20×, 0·33 numerical aperture. (D) Extrinsic absorption changes obtained in a single trial, following stimulation of the hypothalamus, superimposed on corresponding elements of the photodiode matrix array. The five elements in each corner of the array were not connected. The largest signals represent fractional changes in transmitted intensity of approximately 0·3 %. (After Salzberg, Obaid, Senseman & Gainer, 1983.)

& Salzberg, 1983; Salzberg, 1983) and has been described in detail in these reports and in an article by Cohen & Lesher (1986). The MSORTV system is illustrated schematically in Fig. 1A. Briefly, light from a tungsten–halogen lamp is collimated, made quasi-monochromatic with a heat filter (KG-1, Schott Optical Co., Duryea, PA) and an interference filter (700 nm; 70 nm full width at half maximum), and focused by means of a bright-field condenser onto the neurohypophysis (pars nervosa). The neurohypophysis had been excised together with the

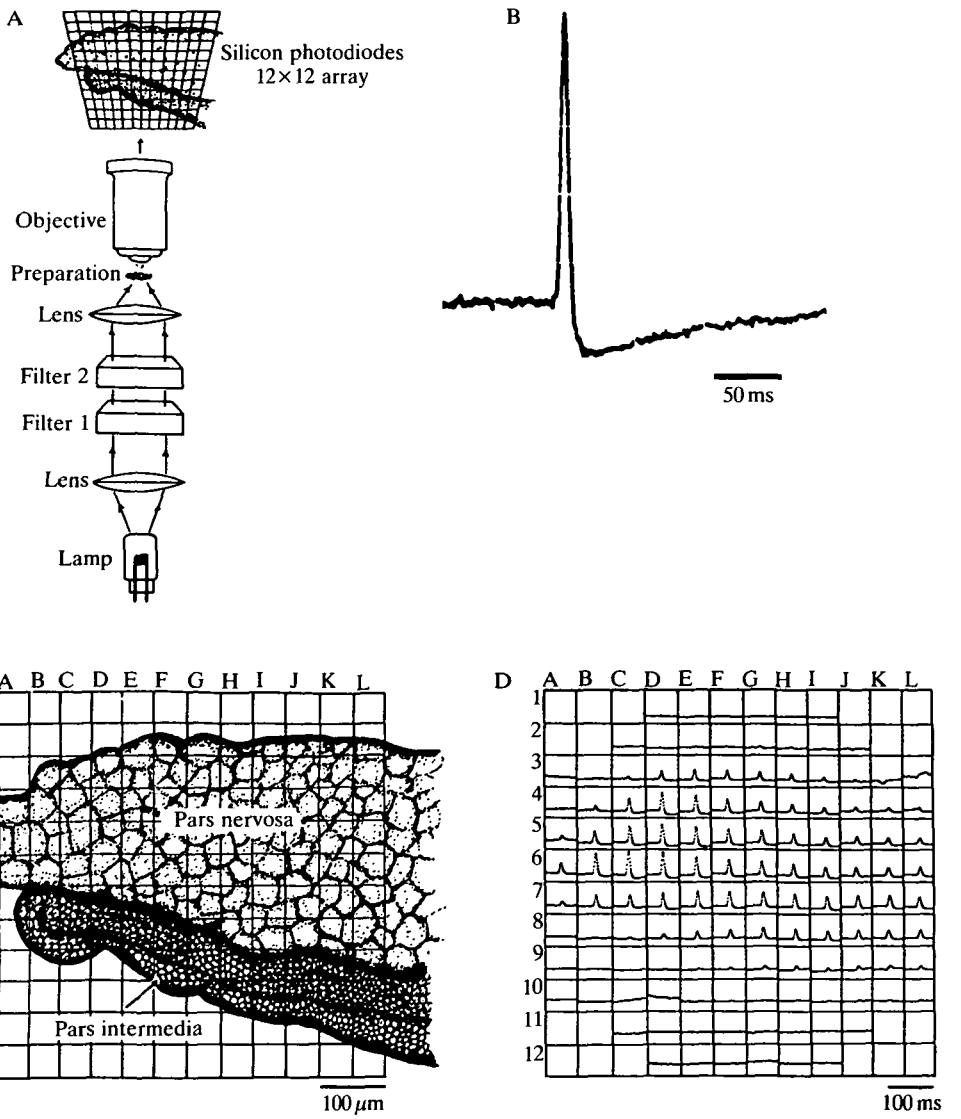

Fig. 1

hypothalamus and the infundibulum, and the entire preparation was vitally stained by incubating it for 25 min in a 100 $\mu g\,ml^{-1}$ solution of the merocyanine–rhodanine dye, NK 2761 (Nippon Kankoh Shikiso Kenkyusho Inc., Okayama, Japan) (Kamino, Hirota & Fujii, 1981; Gupta *et al.* 1981) in *Xenopus* Ringer's solution (112 $mmol\,l^{-1}$ NaCl; 2 $mmol\,l^{-1}$ KCl; 2 $mmol\,l^{-1}$ $CaCl_2$; 15 $mmol\,l^{-1}$ Hepes, 33 $mmol\,l^{-1}$ glucose; pH adjusted to 7·35). Light transmitted by the stained preparation was collected by a high numerical aperture water-immersion objective, which formed a real image on a 12×12-element silicon photodiode matrix array (MD 144-0; Integrated Photomatrix Inc., Mountainside, NJ) located in the image plane of a compound microscope (UEM, Carl Zeiss, Inc., Oberkochen, FRG). The photocurrents generated by the central 124 array elements were separately converted to voltages and amplified as described earlier (Salzberg *et al.* 1977; Grinvald *et al.* 1981). In most of the experiments, all the amplifier outputs were passed to a data acquisition system based on a PDP 11/34A computer (Digital Equipment Corp., Maynard, MA) capable of acquiring a complete frame every 0·8 ms with an effective resolution of 18 bits. This data acquisition system is similar to that described previously by Grinvald *et al.* (1981) and employed by Grinvald, Manker & Segal (1982), Senseman *et al.* (1983) and Salzberg *et al.* (1983). In some experiments, the temporal resolution was markedly improved (17 μs per point for a single detector element) when selected outputs of the current-to-voltage converters were also passed in parallel to a 16-channel signal averager (TN 1500; Tracor Northern Inc. Middleton, WI). These digitized output signals could be stored on magnetic tape for later display and analysis. A map of the preparation was obtained by superimposing a transparent overlay representing the photodiode array elements on a photograph taken through the trinocular tube of the microscope after removing the photodiode array housing. The experiment shown in Fig. 1A employed a 20×, 0·33 numerical aperture water-immersion objective (Nikon) to image part of the neurointermediate lobe (neurohypophysis plus pars intermedia) onto the photodetector array. Fig. 1C is a drawing of the projected region of the gland, prepared from the photomicrograph. Fig. 1D shows the MSORTV display of the action potentials recorded optically, in a single sweep, from different areas of the pars nervosa, following a single brief stimulus applied to the hypothalamus. The optical signals are invariably confined to elements of the grid that correspond to the neurohypophysis; note that in this instance, a small portion of the neurohypophysis lies under the pars intermedia (row 9). The optical signal illustrated in Fig. 1B represents the analog output from channel E5. This record, which is typical, illustrates the large signal-to-noise ratio that may be achieved in these experiments. A variety of control experiments showed that these signals represent voltage changes; e.g. no signals could be recorded in the absence of illumination or in white light; the signals could be inverted at shorter wavelengths (Senseman & Salzberg, 1980).

Evidence that the optical signals shown in Fig. 1 do, in fact, represent the membrane potential changes associated with the invasion of the nerve terminals in the neurohypophysis by the action potential was provided indirectly by some

morphometric observations. In the rat, it is estimated that the 18 000 magnocellular neurones in the hypothalamus give rise to approximately 40 000 000 terminal endings in the neurohypophysis, and morphometric data suggest that here secretory endings and swellings comprise 99 % of the excitable membrane area, with axons making up the remaining 1 % (Nordmann, 1977). Comparably detailed morphometric studies do not yet exist for the neurohypophysis of *Xenopus*, but the structural resemblance of the anuran neurohypophysis to that of the rat (Gerschenfeld, Tramezzani & De Robertis, 1960; Rodriguez & Dellman, 1970; Dellman, 1973) suggests that, in *Xenopus*, it is also very heavily enriched with nerve terminals. Thus, we concluded that the optical signals shown in Fig. 1, originating in the lateral regions of the neurohypophysis, are, at the least, dominated by the membrane potential changes in the terminals of the magnocellular neurones (for additional arguments, see Salzberg *et al.* 1983).

Properties of the action potential in the nerve terminals

The nerve terminal action potentials shown in Fig. 1 exhibit two rather unexpected features. First, they have relatively long durations (i.e. full width at half height of about 6 ms) compared with those recorded from vertebrate axons (e.g. $\ll 2$ ms in the frog, Stämpfli & Hille, 1976). There must be, of course, some temporal dispersion in the population recorded by a single photodetector element, and 6 ms represents, therefore, an upper bound on the spike width. However, internal evidence suggests that, in fact, very little temporal dispersion is present, since the spike width is constant over a considerable range of objective magnifications. Also, the value for the width of the action potential is in good agreement with the 5–6 ms duration reported for the action potentials measured in magnocellular neurone somata (Poulain & Wakerly, 1982) and for the compound action potential in the neurohypophysis (Dreifuss *et al.* 1971). Perhaps the more remarkable characteristic of the nerve terminal action potential illustrated in Fig. 1 is its conspicuous after-hyperpolarization that is reminiscent of the after-hyperpolarization frequently associated with a calcium-mediated potassium conductance (e.g. snail neurones, Meech & Strumwasser, 1970; rat myotube, Barrett, Magleby & Palotta, 1982; guinea pig Purkinje cells, Llinas & Sugimori, 1980*a*,*b*). We found that it was possible to alter dramatically the after-hyperpolarization recorded optically, by changing the ionic composition of the bath. Fig. 2 shows the results of a series of experiments designed to examine the calcium dependence of this long-lasting hyperpolarization. The traces shown here represent typical outputs from single channels of the 124-channel MSORTV system; each trace was recorded in a single sweep. In each row, the optical signal on the left was recorded in normal Ringer's solution which contained 2 mmol l^{-1} Ca^{2+}. These control records are normalized to the same peak height. The middle trace in Fig. 2A shows the effect of a 10-min exposure to elevated (5 mmol l^{-1}) extracellular Ca^{2+} concentration; the after-hyperpolarization increased by approximately 90 %. This probably represents a lower limit since it is likely that some dye was bleached

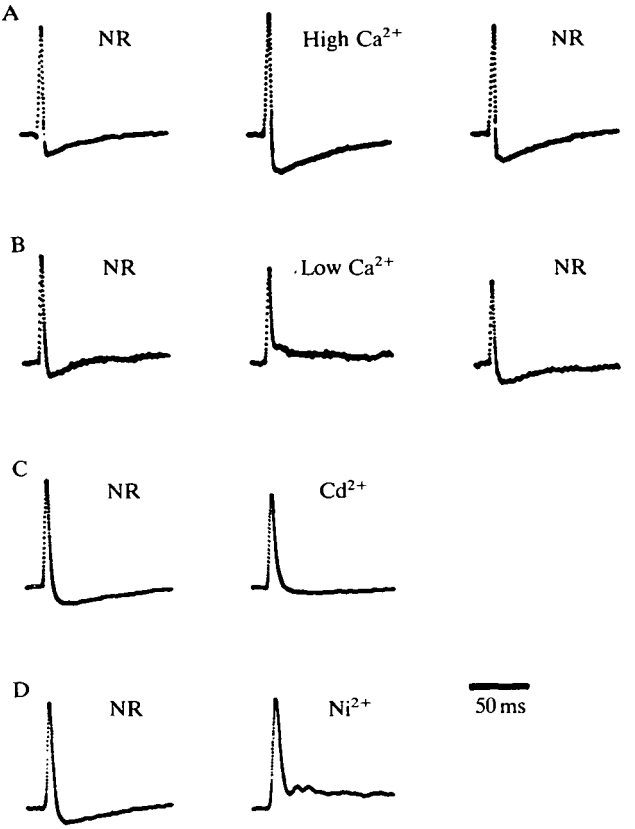

Fig. 2. Calcium-dependence of the long-lasting after-hyperpolarization of the action potential recorded from nerve terminals of the neurohypophysis. (A) Effect of elevated Ca^{2+} concentration on the nerve terminal action potential. Lefthand trace shows the nerve terminal action potential, recorded in normal Ringer's solution (NR; 2 mmol l^{-1} Ca^{2+}). This, and all the records that follow it, were obtained in single sweeps from either a single element, or from a small number of contiguous elements of the photodiode array monitoring potential changes from nerve terminals in the neurohypophysis. The action potential shown in the middle trace was recorded after 10 min in a Ringer's solution containing 5 mmol l^{-1} Ca^{2+}. The righthand trace shows the nerve terminal action potential 10 min following the return to normal Ringer's solution. (B) Effect of lowered extracellular Ca^{2+} on the shape of the nerve terminal action potential. The lefthand trace shows the control action potential. The action potential in the middle trace was recorded 10 min after replacement of the bathing solution with one containing 0·1 mmol l^{-1} Ca^{2+} and 2 mmol l^{-1} Mg^{2+}. The righthand trace shows the nerve terminal action potential 10 min following return to control Ringer's solution. Some bleaching is evident from the reduction in overall signal size. (C) Effect of 0·2 mmol l^{-1} Cd^{2+} on the shape of the nerve terminal action potential. Lefthand trace shows the action potential in normal Ringer's solution. Righthand trace shows the action potential 2 min following the addition of Cd^{2+} to the normal Ringer's solution. This effect could not generally be reversed. (D) Effect of 1 mmol l^{-1} Ni^{2+} on the shape of the nerve terminal action potential. Lefthand trace shows the control action potential. Righthand trace shows the action potential 2 min following the addition of Ni^{2+} to normal Ringer's solution. This effect was only partially reversible. Single array element. (After Salzberg, Obaid, Senseman & Gainer, 1983.)

during the measurement, and some diminution of the signal is almost inevitable. We always found (Salzberg *et al.* 1983) a large increase in the size of the after-hyperpolarization, although the magnitude of the effect was variable. An increase in the height of the action potential (14 % in Fig. 2A) was often noted; this finding suggests that an inward Ca^{2+} current might contribute to the normal rising phase of the action potential (see below). The record on the right in Fig. 2A shows the optical signal from the same region of the neurohypophysis 10 min after the return to 2 mmol l^{-1} Ca^{2+}. The optical traces in Fig. 2B show the effect of reducing extracellular Ca^{2+} to 0·1 mmol l^{-1} by replacing the Ca^{2+} with Mg^{2+}. The middle trace shows that after 10 min the undershoot is completely absent, replaced by a long-lasting depolarization. The magnitude of this effect also was variable from one experiment to the next; however, a reduction of extracellular calcium to 0·1 mmol l^{-1} or less never failed to reduce the after-hyperpolarization. Further lowering of the extracellular Ca^{2+} concentration occasionally eliminated the action potential entirely, but we consider this to be a consequence of steady-state depolarization to which the optical measurements are insensitive. The right-hand trace in Fig. 2B again illustrates the recovery (10 min following return to 2 mmol l^{-1} Ca^{2+}). In this experiment, reduction of the magnitude of the signal resulting from bleaching was more apparent. Although the effects of changes in extracellular Ca^{2+} concentration were always reversible, this was not the case with other ionic substitutions. Cadmium, for example, is known to block calcium currents in a variety of preparations (e.g. Standen, 1981) and Fig. 2C shows that after 2 min in 200 μmol l^{-1} Cd^{2+} the after-hyperpolarization was almost entirely eliminated. This effect was only partially reversible. Longer exposure to cadmium eliminated the after-hyperpolarization entirely. The peak height was also considerably reduced but, in the absence of recovery, it is impossible to know whether this is, at least partially, an effect of bleaching. Fig. 2D shows that nickel, another inorganic calcium blocker (Kaufmann & Fleckenstein, 1965) at millimolar concentrations, also blocks the spike after-hyperpolarization. In some experiments, Ni^{2+} produced a small but consistent increase in the spike height. The origin of this effect is not clear, and, although a decrease in sodium inactivation is possible, a change in resting potential cannot be ruled out. All the experiments illustrated in Fig. 2 suggest that calcium plays a significant role in shaping the action potential in the vertebrate nerve terminals studied here. Experiments with other inorganic calcium antagonists such as manganese, cobalt and lanthanum are consistent with this idea, although their potency seems to be less than that of cadmium.

Ionic basis of the depolarizing phase of the action potential

Because the entry of calcium plays an important role in excitation–secretion coupling, we attempted to determine whether an inward calcium current contributed measurably to the depolarizing phase of the action potential in the terminals of the neurohypophysis. *A priori*, calcium is unlikely to be the dominant carrier of

the inward current in these nerve terminals because the very large surface-to-volume ratio in these small secretory endings would then result in the imposition of a large, probably intolerable calcium burden. Fig. 3 illustrates an experiment designed to examine this point. Because calcium antagonists leave the depolarizing phase of the action potential largely intact, sodium appears to be carrying the bulk of the inward current. If so, it should be blocked by tetrodotoxin (TTX), and a positive result would provide evidence that the rising phase of the action potential is due primarily to the opening of voltage-dependent sodium channels. To test this possibility, it was necessary to change the stimulation conditions, since, in the presence of TTX, block of the sodium action potential in the axons of the infundibulum would prevent the excitation of the nerve terminals in the neurohypophysis by blocking spike conduction. We were able to circumvent this by resorting to a form of field stimulation in which the lateral tips of the pars nervosa were held by suction electrodes, and very brief (200–500 μs) currents were passed between the two electrodes. In this fashion, the terminals could be excited

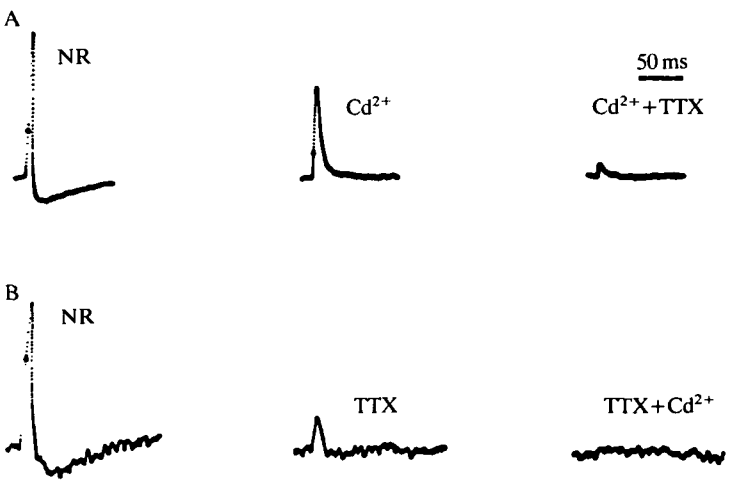

Fig. 3. Effects of sodium and calcium channel blockers on the rising phase of the nerve terminal action potential elicited by direct-field stimulation of the neurohypophysis. (A) Lefthand trace shows the nerve terminal action potential in normal Ringer's (NR) solution. The middle trace shows the action potential 18 min after the addition of 0·2 mmol l^{-1} Cd^{2+} to the normal Ringer's solution. The righthand trace shows the optical signal recorded from the same population of nerve terminals 6 min after the addition of 5 μmol l^{-1} tetrodotoxin (TTX) to normal Ringer's solution containing 0·2 mmol l^{-1} Cd^{2+}. (B) Lefthand trace shows the nerve terminal action potential in normal Ringer's solution. The middle trace shows the residual potential change 6 min following the addition of 2 μmol l^{-1} TTX to the Ringer's solution bathing the preparation. The righthand trace shows the elimination of this TTX-resistant response when calcium channels are blocked by Cd^{2+}. For this record, the preparation had been bathed in a Ringer's solution containing 2 μmol l^{-1} TTX and 0·2 mmol l^{-1} Cd^{2+} for 5 min. All traces are single sweeps, recorded by a single representative element of the array; 700 nm. Rise time of the light-measuring system (10–90 %) was 1·1 ms. (After Salzberg, Obaid, Senseman & Gainer, 1983.)

directly, without the requirement for axonal transmission. This technique was employed in the experiment shown in Fig. 3. The MSORTV record on the left in Fig. 3A shows the control, in 2 mmol l^{-1} Ca^{2+} Ringer's solution. The amplitude of the optical spike increased with field strength, as expected, since a large and potentially variable number of nerve terminals were stimulated and monitored. A maximal response was obtained typically with stimuli of 100–200 V and was largely independent of stimulus polarity. As with the optical signals elicited by stimulation of the hypothalamus, the amplitude of the response was approximately 0·3 % of the transmitted light intensity at 700 nm. The middle trace was recorded from the same population of nerve terminals following 18 min of exposure to 200 μmol l^{-1} Cd^{2+} to block any inward calcium current. The reduction in the magnitude of the action potential is obvious, along with a considerable broadening of the spike and the complete loss of the after-hyperpolarization, the latter effects probably resulting from the elimination of the large calcium-mediated potassium conductance. The extent of the reduction in the height of the action potential may be exaggerated, however, by factors other than blockage of calcium channels (e.g. dye bleaching). The righthand trace in Fig. 3A shows that 6 min after application of 5 μmol l^{-1} tetrodotoxin (TTX), the Cd^{2+}-resistant portion of the terminal action potential was completely eliminated. The residual signal seen in this record is the passive depolarization produced by the field stimulation; it was reversed with reversal of stimulus polarity, and it exhibited the same wavelength-dependence as that of the optical recording of the normal action potential. (It should be noted, however, that the size of the passive electrotonus was very variable from element to element.)

The complementary experiment is shown in Fig. 3B. Here, the sodium current was first blocked with TTX (middle trace), and the small residual active response was eliminated by Cd^{2+} (righthand trace), and substantially decreased in size by 1 mmol l^{-1} Ni^{2+} (not shown). Thus, the experiments illustrated here, and others, suggest that the rising phase of the nerve terminal action potential, in the neurohypophysis of *Xenopus*, is mediated predominantly by sodium, although these data also suggest the presence of at least a small calcium component. The magnitude of this calcium component is difficult to assess quantitatively, particularly because the TTX blockade of the normal regenerative sodium depolarization may limit the opening of voltage-dependent calcium channels. The important role for calcium entry in excitation–secretion coupling is fully consistent with its apparently small contribution to the terminal action potential (Katz & Miledi, 1969); in adrenal chromaffin cells, for example, where calcium influx is the critical event in excitation–secretion coupling (Douglas, 1978), the Ca^{2+} component of the action potential is also small (Brandt, Hagiwara, Kidokoro & Miyazaki, 1976).

Nonetheless, under conditions in which the terminal membrane depolarization is prolonged, sufficient inward calcium current may develop to give rise to a relatively large calcium action potential. The records shown in Fig. 4 are from one of many neurohypophyses in which tetraethylammonium (TEA^+) was added, in addition to TTX, to block the voltage-dependent potassium conductance and,

thereby, prolong the depolarization of the nerve terminal membrane. Fig. 4A shows a control action potential from a population of nerve terminals. The action potential exhibits the characteristic rapid upstroke that we attribute primarily to the effect of a fast inward sodium current, a rapid repolarizing phase resulting from K^+ efflux, largely through the delayed rectifier, and the typical after-hyperpolarization resulting from a Ca^{2+}-mediated increase in K^+ conductance. When 5 mmol l^{-1} TEA^+ was added to the Ringer's solution (not shown), the spike was prolonged and, in most experiments, a hump appeared on the falling phase, possibly due to repetitive firing of some of the terminals. The addition of a high concentration of TTX, sufficient to block voltage-sensitive sodium channels, abolished the hump and unmasked a large after-hyperpolarization that presumably results from the enhanced activation of a Ca^{2+}-mediated K^+ conductance. Fig. 4B shows the optical response recorded from the same population of terminals represented in Fig. 4A, following 20 min of exposure to 5 mmol l^{-1} TEA^+ and 2 μmol l^{-1} TTX. The regenerative response illustrated is smaller and slower than the normal action potential, and appears to depend entirely upon the influx of calcium and the subsequent rise in a TEA^+-insensitive K^+ conductance. The further addition of 0·5 mmol l^{-1} Cd^{2+} to the bath (Fig. 4C) completely blocked the remaining active responses. (We have already seen, in Figs 2, 3 that such low levels of cadmium do not block the action potentials in the nerve terminals of the neurohypophysis in the absence of TTX.) The residual optical

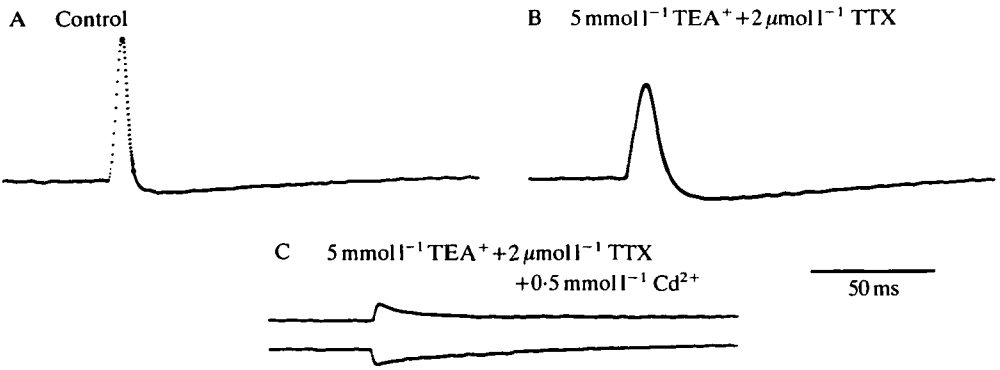

Fig. 4. Cadmium sensitivity of the active responses (calcium spikes) elicited by direct-field stimulation of the neurohypophysis in the presence of TTX and TEA^+. Optical recording of action potentials from nerve terminals of the *Xenopus* neurohypophysis following staining for 25 min in 0·1 mg ml^{-1} NK 2761. Single-sweep outputs of a single channel from the photodiode matrix array in the MSORTV system. (A) Action potential recorded in normal Ringer's solution. (B) Active response of the nerve terminals following 20 min exposure to 5 mmol l^{-1} tetraethylammonium (TEA^+) plus 2 μmol l^{-1} tetrodotoxin (TTX) in normal Ringer's solution. (C) Passive response (electrotonus) remaining 15 min after the addition of 0·5 mmol l^{-1} Cd^{2+} to the TEA^+/TTX Ringer's solution bathing the preparation, upon stimulation with normal and reversed polarity; 700 nm. Rise time of the light-measuring system (10–90 %) was 1·1 ms; temperature 18–22°C. (After Obaid, Orkand, Gainer & Salzberg, 1985.)

signals shown in Fig. 4C represent the purely passive electrotonus, as indicated by their symmetrical response to stimulus polarity. The combined effects of TEA^+ and Cd^{2+} could often be reversed, but it was never possible to reverse completely the effect of TTX.

Although the optical signal recorded in Fig. 4B has the appearance of a calcium action potential, it is possible that a major contribution to the rising phase of the response, in TTX, comes from the influx of Na^+ through Ca^{2+} channels, which are not blocked by the toxin. It was possible, in these experiments, to assess qualitatively the relative contributions of Ca^{2+} and Na^+ to the active response by recording optical signals at different $[Ca^{2+}]_o$ at high and low $[Na^+]_o$, and at different $[Na^+]_o$ at high and low $[Ca^{2+}]_o$. These experiments (Obaid *et al.* 1985), all carried out in TTX/TEA^+, suggested that, in low $[Ca^{2+}]_o$ ($0{\cdot}2\,mmol\,l^{-1}$), Na^+ might contribute significantly to the inward current conducted by cadmium-sensitive, TTX/TEA^+-insensitive channels. However, at normal $[Ca^{2+}]_o$, varying $[Na^+]_o$ had little effect on the rate of rise of the optical signal, as if sodium ions were unable to compete effectively with normal concentrations of Ca^{2+}.

Charybdotoxin (CTX) block of the after-hyperpolarization

Although the evidence presented above (Figs 2–4) indicates the presence of a prominent calcium-mediated potassium conductance in the terminal membrane, the inhibition of this conductance by a high-affinity blocker would provide important complementary evidence. Charybdotoxin (CTX), a protein toxin derived from scorpion venom, blocks calcium-sensitive potassium channels from skeletal muscle in planar lipid bilayers (Miller, Moczydlowski, Latorre & Phillips, 1985) with a K_i of $10\,nmol\,l^{-1}$. We have observed (Obaid & Salzberg, 1985; A. L. Obaid, R. Flores & B. M. Salzberg, in preparation) that charybdotoxin, at $50\,nmol\,l^{-1}$, specifically blocks the after-hyperpolarization of the nerve terminal action potential in *Xenopus*. In addition, both the width and height of the action potential are increased in the presence of CTX. Fig. 5 shows the effect of $50\,nmol\,l^{-1}$ CTX on the nerve terminal action potential recorded optically. Fig. 5A is the action potential elicited in normal Ringer's solution by direct-field stimulation, and Fig. 5B shows the complete elimination of the after-hyperpolarization following an 8-min exposure to CTX. (Note that there is a 10 % increase in the height of the action potential, in addition to the loss of the undershoot.) Evidence that charybdotoxin acts directly on a calcium-mediated potassium conductance, rather than indirectly by blocking calcium entry, is provided in Fig. 6. Fig. 6A shows the action potential in normal Ringer's solution, and Fig. 6B illustrates the calcium response obtained 8 min following the addition of $5\,mmol\,l^{-1}$ TEA^+ and $1\,\mu mol\,l^{-1}$ TTX to the Ringer's solution. This response also exhibits an after-hyperpolarization, similar to that seen in Fig. 3. Fig. 6C demonstrates the effect of 23 min of exposure to $50\,nmol\,l^{-1}$ charybdotoxin (in TEA^+/TTX Ringer's solution). The after-hyperpolarization is again eliminated, but calcium entry is actually enhanced. At the same time, the loss of an outward current prolongs the duration of the response. Evidence that the active response is

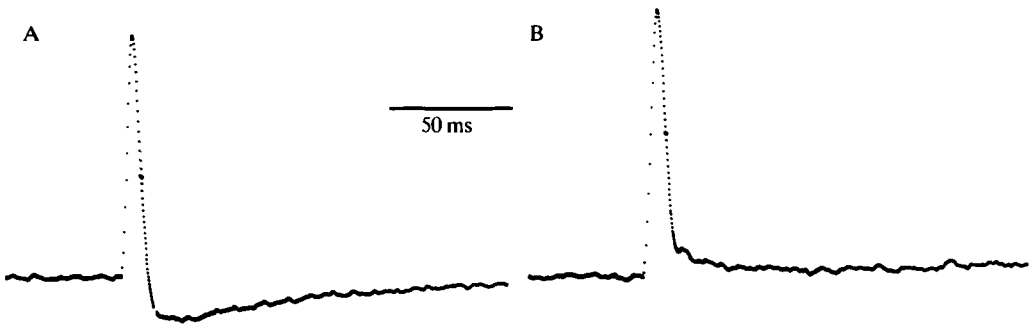

Fig. 5. Effect of charybdotoxin on the after-hyperpolarization of the nerve terminal action potential in *Xenopus*. Optical recordings of the action potential resulting from direct-field stimulation of the nerve terminals of the *Xenopus* neurohypophysis following staining for 25 min in 0·1 mg ml^{-1} NK 2761. Outputs of a single channel of the MSORTV system. (A) The action potential recorded in normal Ringer's solution. (B) The action potential recorded 8 min following the addition of 50 nmol l^{-1} charybdotoxin to the Ringer's solution bathing the neurohypophysis. The rise time (10–90 %) of the light-measuring system was 1·1 ms; single sweeps; 700 nm; a.c. coupling time constant was 3 s. (After A. L. Obaid, R. Flores & B. M. Salzberg, in preparation.)

mediated by calcium entry is given in Fig. 6D, where a 2-min exposure to 0·5 mmol l^{-1} Cd^{2+} entirely abolishes the active response.

The calcium-mediated active responses, recorded optically and described here, appear to share some properties with the local subthreshold action potentials described by Hodgkin (1938) in unmyelinated crab nerve; with TTX blocking any propagated action potential in the axons of the infundibulum, the small calcium currents cannot excite a sufficient length of nerve to produce a propagated action potential. Nonetheless, their dependence on $[Ca^{2+}]_o$ and sensitivity to Cd^{2+} block indicates that they probably result from a voltage-sensitive calcium influx into the terminals (Kostyuk & Krishtal, 1977; Hagiwara & Byerley, 1981; Obaid *et al.* 1985) which mediates hormone release.

Organic modifiers of calcium channel behaviour

Aminoglycoside antibiotics, including neomycin, streptomycin and gentamicin, depress evoked transmitter release at the neuromuscular junction by competing with extracellular calcium (Fiekers, 1983). We were able to show (Parsons, Obaid & Salzberg, 1986) that neomycin (220 μmol l^{-1}) reduced the height of the upstroke of the nerve terminal action potential, increased the spike duration and abolished the after-hyperpolarization, in the neurohypophysis of *Xenopus*. These effects were reversible upon washing, and could be antagonized by raising $[Ca^{2+}]_o$ from 2 to 5 mmol l^{-1} (Parsons *et al.* 1986). When the effects of these agents on the regenerative calcium response were examined, we found that both neomycin (220 μmol l^{-1}) and gentamicin (190 μmol l^{-1}) decreased the height of the calcium spike, and practically eliminated the after-hyperpolarization. These observations

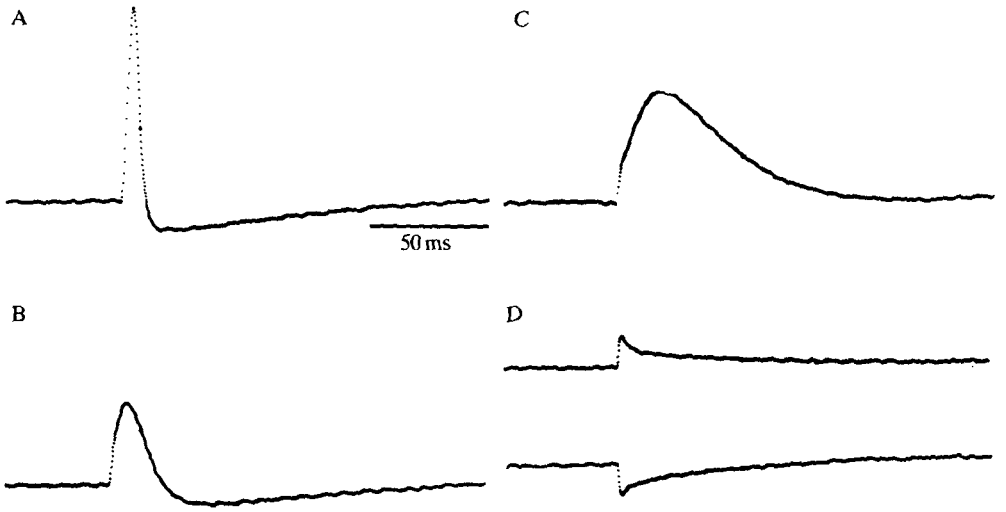

Fig. 6. Charybdotoxin (CTX) eliminates the nerve terminal after-hyperpolarization without blocking calcium entry. Optical recordings of the action potential resulting from direct-field stimulation of the nerve terminals of the *Xenopus* neurohypophysis following staining for 25 min in 0·1 mg ml^{-1} NK 2761. Outputs of a single channel of the MSORTV system. (A) The action potential recorded in normal Ringer's solution. (B) The action potential recorded 8 min following the addition of 5 mmol l^{-1} tetraethylammonium (TEA^+) and 1 μmol l^{-1} tetrodotoxin (TTX) to the Ringer's solution bathing the neurohypophysis. (C) The action potential recorded 23 min after the addition of 50 nmol l^{-1} CTX to the Ringer's solution already containing 5 mmol l^{-1} TEA^+ and 1 μmol l^{-1} TTX. The after-hyperpolarization is eliminated. (D) Purely passive response (electrotonus) 1 min after the addition of 500 μmol l^{-1} Cd^{2+} to the Ringer's solution containing 50 nmol l^{-1} CTX, 1 μmol l^{-1} TTX and 5 mmol l^{-1} TEA^+. The rise time (10–90 %) of the light-measuring system was 1·1 ms; single sweeps; 700 nm; a.c. coupling time constant was 400 ms. (After A. L. Obaid, R. Flores & B. M. Salzberg, in preparation.)

all supported the idea that aminoglycoside antibiotics antagonize the entry of calcium into vertebrate nerve terminals during excitation.

Other organic modifiers of calcium channel behaviour have been used by several researchers to distinguish different types of calcium channel in a variety of preparations (Miller, 1987). Previously, however, it has not been possible to use these pharmacological tools to study calcium channels in the intact nerve terminals of a vertebrate. Using the extrinsic absorption changes exhibited by the terminals of the frog neurohypophysis stained with the merocyanine–rhodanine dye NK 2761, we carried out a series of experiments (Salzberg, Obaid & Flores, 1987; A. L. Obaid, R. Flores & B. M. Salzberg, in preparation) that help to define the pharmacological profile of the calcium channels present in intact neurosecretory terminals of vertebrates. Because the $g_{K(Ca)}$-dependent afterpotential of the spike is such a sensitive indicator of calcium entry into the terminals of the neurohypophysis (Salzberg *et al.* 1983), we used this component of the optical signal to

examine the sensitivities of the calcium channels that are present in this population of vertebrate nerve terminals. We used the dihydropyridine compounds nifedipine and Bay-K 8644, at concentrations ranging from 2 to 5 μmol l^{-1}, and the peptide toxin ω-conotoxin GVIA (Olivera *et al.* 1985). In the frog neurohypophysis, neither dihydropyridine compound had any effect on the calcium-dependent components of the action potential (Figs 7, 8), the spikes remaining absolutely unchanged for up to 1 h in the presence of either nifedipine or Bay-K 8644. ω-Conotoxin GVIA (1 μmol l^{-1}), however, rapidly abolished the after-hyperpolarization of the normal action potential (Fig. 9) by blocking calcium entry (and not by reducing $g_{K(Ca)}$). These results demonstrated clearly the effect of ω-conotoxin on the calcium channels involved in the release of neuropeptides (see below) from the intact nerve terminals of vertebrates and, although this is the first direct evidence from measurements on intact nerve terminals, it is consistent with the findings of others using patch-clamp techniques on neuronal preparations (McCleskey *et al.* 1987; Fox, Nowycky & Tsien, 1987). The insensitivity to dihydropyridines exhibited by these same calcium channels is apparently at variance with some reports of dihydropyridine block of neuronal calcium channels (for a review, see Tsien, 1986). However, Rane, Holz & Dunlap (1987) concluded that chronic depolarization is required to achieve nifedipine block of calcium channels in chick DRG neurones, but is not required for Bay-K 8644 action (Holz, Dunlap & Kream, 1988). Our results with nifedipine are consistent with this interpretation, although our failure to observe any enhancement of calcium channel activity in the presence of Bay-K 8644 is not.

Intrinsic optical changes that accompany secretion from mammalian nerve terminals

The anatomy of the amphibian posterior pituitary provides a unique opportunity for the study *in vitro* of excitation–secretion coupling in the vertebrate: as

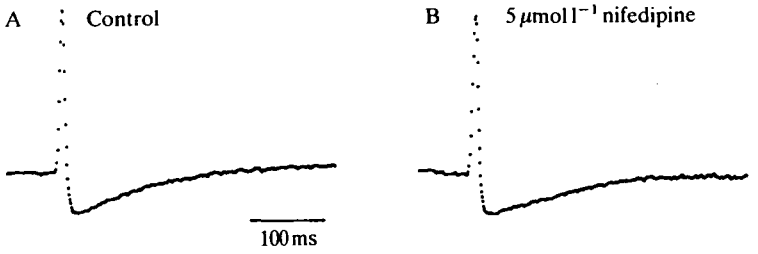

Fig. 7. The nerve terminal action potential in the neurohypophysis of *Xenopus* is unaffected by nifedipine. (A) Optical recording of the action potential in control Ringer's solution after staining with NK 2761. (B) Optical recording of the action potential following 30 min exposure to 5 μmol l^{-1} nifedipine (in 0·25 % ethanol). Spatial average of the digitized outputs of four contiguous elements of the photodiode array; single sweep; ×20; 0·33 numerical aperture; 700 ± 35 nm; infundibular stimulation; a.c. coupling time constant 400 ms; response time constant (10–90 %) 1·1 ms. (After A. L. Obaid, R. Flores & B. M. Salzberg, in preparation.)

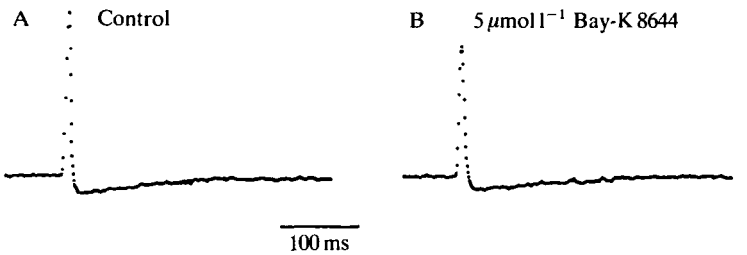

Fig. 8. Bay-K 8644 fails to enhance the calcium-dependent components of the nerve terminal action potential in the neurohypophysis of *Xenopus*. (A) Optical recording of the action potential in control Ringer's solution after staining with NK 2761. (B) Optical recording of the action potential following 32 min of exposure to 5 μmol l^{-1} Bay-K 8644 (in 0·25 % ethanol). Spatial average of the digitized outputs of three contiguous elements of the photodiode array; single sweep; ×20; 0·33 numerical aperture; 700 ± 35 nm; infundibular stimulation; a.c. coupling time constant 400 ms; response time constant (10–90 %) 1·1 ms. (After A. L. Obaid, R. Flores & B. M. Salzberg, in preparation.)

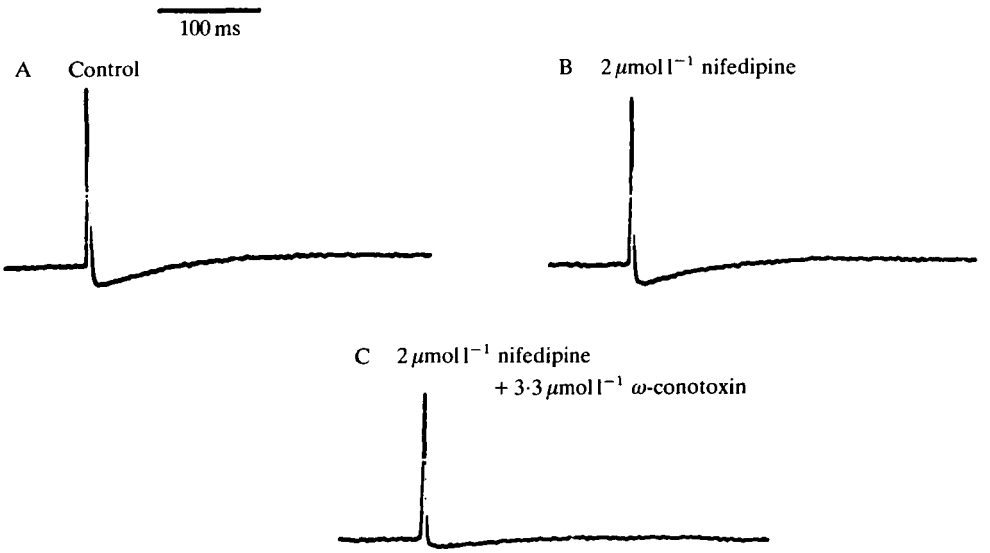

Fig. 9. ω-Conotoxin reduces the amplitude of the calcium-dependent components of the nerve terminal action potential in a preparation in which 2 μmol l^{-1} nifedipine had no effect. (A) Optical recording of the action potential in control Ringer's solution after staining with NK 2761. (B) Optical recording of the action potential following 30 min exposure to 2 μmol l^{-1} nifedipine (in 0·1 % ethanol). (C) Optical recording of the action potential 10 min after the addition of 3·3 μmol l^{-1} ω-conotoxin GVIA to the 2 μmol l^{-1} nifedipine Ringer's solution. Analogue output of a single representative element of the 124-element photodiode array; single sweep; ×10; 0·4 numerical aperture; 700 ± 35 nm; infundibular stimulation; a.c. coupling time constant 400 ms; response time constant (10–90 %) 1·1 ms. (After A. L. Obaid, R. Flores & B. M. Salzberg, in preparation.)

we pointed out earlier, there is no postsynaptic excitable membrane to confound the interpretation of the optical signals (Salzberg *et al.* 1983; Obaid *et al.* 1985) and, more important for biochemical studies, no barrier to the collection of its secretory products. However, relatively little is known about the secretion of amphibian neuropeptides and neurohormones, although an extensive literature has accumulated on the biochemistry of secretion in the mammalian, and particularly the rodent, neurohypophysis. Together with Hal Gainer, we felt that it would be important to be able to correlate the electrical events in the terminals with the kinetics of release of the secretory products, and we thought that this might be accomplished by means of optical recording of membrane potential combined with sensitive radioimmunoassays using the CD-1 mouse.

Fig. 10A shows an optical recording of a train of seven action potentials, stimulated at 16 Hz, in a population of nerve terminals in the neurohypophysis of the CD-1 (Charles River Breeding Laboratories) mouse. It represents the change in transmitted light intensity at 675 nm, monitored in a single sweep by one channel of the 124-channel MSORTV system. The preparation had previously been incubated in a Ringer's solution (in mmol l^{-1}: NaCl, 154; KCl, 5·6; $MgCl_2$, 1;

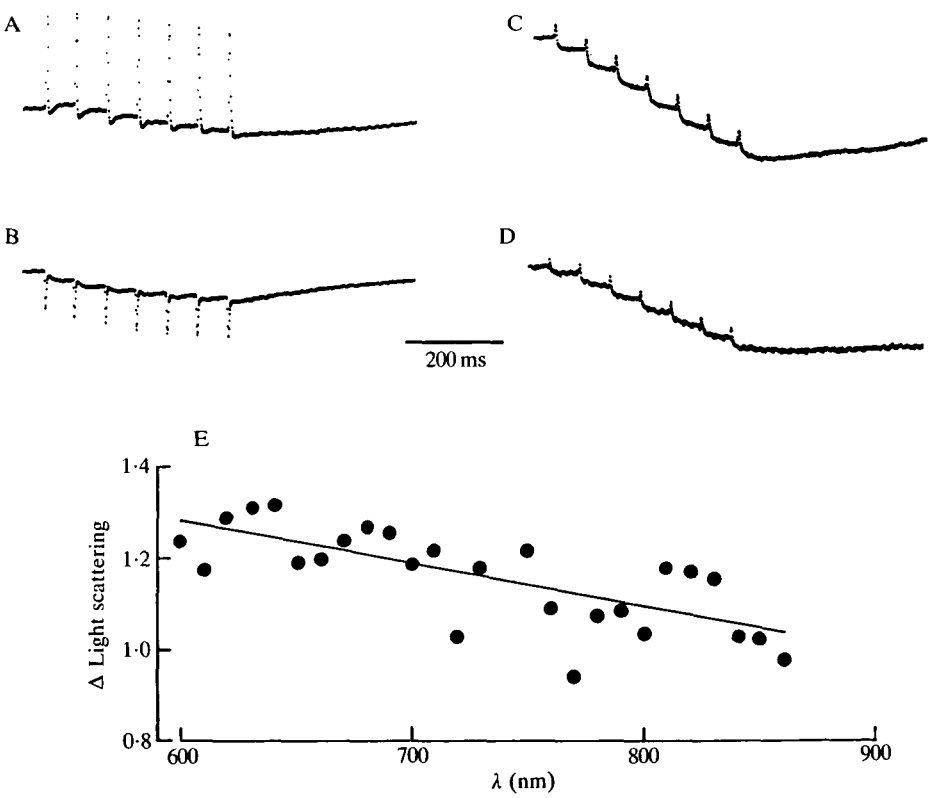

Fig. 10

$CaCl_2$, 2·2; glucose, 10; Hepes, 20, adjusted to pH 7·4 with NaOH) containing 0·1 mg ml^{-1} of the merocyanine–oxazolone dye NK 2367 (Salzberg *et al.* 1977). The method used here was virtually identical to that used to record electrical activity from the neurosecretory terminals of the frog neurohypophysis. The trace in Fig. 10A exhibits a gradual decline in baseline, followed by recovery, with the appearance of a transient hyperpolarization. The constant size of the upstroke, however, suggested to us that this apparent hyperpolarization might not, after all, represent a change in membrane potential. Fig. 10B shows a single trial recorded by the same detector element, except that the measuring wavelength was 540 nm. At this wavelength, the potential-dependent absorption change exhibited by membrane stained with NK 2367 is known to be opposite in sign to that observed at 675 nm (Senseman & Salzberg, 1980). Notice, however, that although the optical spikes are reversed in sign, the slow component of the signal is essentially unchanged. This weak dependence on wavelength of the slow component of the optical trace suggested that it might reflect a change in light scattering. Fig. 10C

Fig. 10. Extrinsic and intrinsic optical changes recorded from nerve terminals in the neurohypophysis of the mouse. (A) Changes in the extrinsic absorption at 675 nm (52 nm full width at half-maximum) of nerve terminals following staining for 25 min in 0·1 mg ml^{-1} NK 2367. A single train of 500 μs (60 V) stimuli at 16 Hz was delivered to the posterior pituitary through the axons of the infundibulum for 400 ms. The resulting changes in transmitted light intensity are shown, recorded by a single element of the photodiode matrix array. Here, a downward deflection of the trace represents an increase in transmitted light intensity. The a.c. coupling time constant for the light-measuring system was 400 ms. The fractional change in light intensity during the action potentials was approximately 0·3 %. Single sweep; single array element. (B) Same experiment as in A, except that the transmitted intensity was monitored at 540 nm (60 nm full width at half-maximum). At this wavelength, the voltage-dependent extrinsic absorption change exhibited by the merocyanine–oxazolone probe NK 2367 is known to be opposite in sign to that observed at 675 nm. Single sweep; single array element. (C) Intrinsic optical changes recorded at 675 nm (52 nm full width at half-maximum) from the nerve terminals of an unstained neurohypophysis. The preparation was stimulated as in A, and the resulting changes in transmitted light intensity recorded by a single element of the photodetector array are shown. Here again, a downward deflection of the optical trace represents an increase in transmitted light intensity, or a decrease in opacity. This is probably equivalent (see E) to a decrease in large-angle light scattering. The a.c. coupling time constant for the light measurement was 3 s. The intrinsic optical signal, resulting from the train of stimuli shown here, corresponds to a fractional change in intensity of approximately 0·2 %. Single sweep; single array element. (D) Similar experiment to C, except that the optical signal was d.c. coupled using a digital sample-and-hold circuit that eliminated the distortion introduced by capacitative coupling. Unstained preparation, 675 nm (52 nm full width at half-maximum). Single sweep; single array element. (E) Wavelength-dependence between 600 and 860 nm of the intrinsic optical changes recorded from nerve terminals of the unstained mouse neurohypophysis. The fractional intensity changes have been corrected for the efficiencies of the apparatus and for the gradual deterioration of the preparation. The stimulus trains were delivered at 3-min intervals. All trials were carried out in normal Ringer's solution. The rise time of the light measuring system (10–90 %) was 1·1 ms. (After Salzberg, Obaid & Gainer, 1985.)

illustrates an identical experiment, carried out on another mouse neurohypophysis, before staining. Here, the dye-dependent extrinsic absorption signal that depends upon membrane potential is absent, revealing a series of increases in transmitted light intensity (downward deflections of the trace), each preceded by a transient decrease in transparency (upward deflection) occurring at the time of the action potential. This experiment employed a relatively long a.c. coupling time constant (3 s) to minimize the distortion of the time course of the optical signal. Nonetheless, the tendency towards recovery following the train of stimuli is largely an artefact of the coupling time constant. A similar experiment is illustrated in Fig. 10D, in which a digital sample-and-hold circuit, consisting of 12-bit analog-to-digital and digital-to-analog converters connected in series is used instead of capacity coupling. This method avoids any a.c. distortion of the signal, which is observed to remain nearly constant for seconds following the cessation of the stimuli.

Fig. 10E illustrates the wavelength-dependence of these intrinsic optical signals between 600 and 860 nm, corrected for the spectral efficiency of the apparatus. The wavelength range was selected to exclude major absorption peaks of intrinsic pigments, e.g. haemoglobin. The fractional change in transmitted intensity exhibits a gradual decline with increasing wavelength, consistent with a change in light scattering. All components of the optical signal were eliminated when the neurohypophysis was depolarized with KCl or when the Ringer's solution contained micromolar concentrations of TTX.

It should be clear to the reader that optical techniques which employ visible light, especially those that rely upon intrinsic optical changes that require no exogenous probes, offer unique capabilities in terms of resolution in time and space, combined with a gentleness that permits the non-destructive measurement of many cellular and subcellular processes. In particular, light-scattering methods have been applied to biological systems, *in vitro* and *in vivo*, since the experiments of Tyndall (1868) and have played a role in a variety of recent efforts to study changes in neurone structure during action potential propagation and synaptic transmission (Cohen, 1973). Cohen & Landowne (1970) reported small (8×10^{-7}) changes in light scattering in the skate electric organ that appeared to be presynaptic in origin, and Shaw & Newby (1972) detected calcium-dependent movement in a thoracic ganglion of a locust (*Schistocerca gregaria*) by monitoring the power spectrum of 'twinkling' (light beating) produced by scattered laser light (see also Piddington & Sattelle, 1975; Englert & Edwards, 1977). These preliminary reports, however, have not been expanded.

In the absence of potentiometric dye staining of the terminals of the neurohypophysis, two intrinsic optical signals, illustrated in Fig. 10C, were evident following each stimulus. The rapid upward deflection of the trace (decrease in transparency), which we have termed the E-wave (Salzberg *et al.* 1985) appears to coincide with the arrival of the action potential at the terminals (cf. Fig. 10A), and the subsequent increase in transparency, the S-wave, appears to correspond to a slower and longer-lasting process occurring in the nerve terminals. Several lines of

evidence suggested that this latter intrinsic optical signal (Fig. 10C,D) is closely correlated with the secretory process. If this association is more than accidental, we would anticipate that the long-lasting changes in transparency (but not the E-wave) would exhibit certain properties that characterize neurosecretory systems in general, and the release of neuropeptides in particular; viz, dependence on stimulation frequency, with marked facilitation; dependence on $[Ca^{2+}]_o$; and sensitivity to Ca^{2+} antagonists and to pharmacological procedures known to influence secretion (Douglas & Poisner, 1964).

The frequency-dependence of the transparency changes (intrinsic optical signals), which we have referred to as 'light scattering' (Salzberg *et al.* 1985), is illustrated in Fig. 11. In each case, the stimuli were delivered to the neurohypophysis for 400 ms, but at different frequencies. Fig. 11A–D shows, not surprisingly, a monotonic increase in the total size of the light-scattering change with the number of stimuli. The size of the fractional change in light scattering per stimulus ($\Delta_{50-460\,ms}/N$), however, varied with frequency, reaching a broad maximum at between 8 and 16 Hz. Fig. 11D demonstrates the tendency of the total optical change to saturate at very high stimulation rates. In each instance, a marked facilitation of the optical response is evident between the first and second stimulus. Fig. 11E was obtained under identical conditions (16 Hz) to Fig. 11C, except that 1 mmol l^{-1} $CdCl_2$ was added to the bath for 15 min before the single trial recorded here. In Fig. 11E, Cd^{2+} is seen to block more than 90 % of the S-wave (downward deflection of the trace; decrease in opacity) of the light-scattering change without significantly affecting the rapid upstroke (E-wave) that occurs coincident with the action potential. The plot in Fig. 11F summarizes the dependence of the magnitude of the light-scattering change on the rate and number of stimuli and also includes the effect of barium ions added to the normal calcium concentration (see below).

Effects of extracellular calcium

Calcium profoundly influences neurosecretory activity (Dodge & Rahamimoff, 1967; Katz, 1969; Douglas, 1978) and the extracellular concentration of this ion would be expected to modulate the size of any intrinsic optical signal related to secretion from the terminals of the neurohypophysis. Fig. 12 shows the effect of $[Ca^{2+}]_o$ on the light-scattering signal evoked by stimulation of the infundibulum at 10 Hz for 400 ms. Traces A–F, which are each the average of 16 sweeps, were obtained in alphabetical order. The effect of calcium concentration is apparent, and the blockade of the optical signal by 0·5 mmol l^{-1} Cd^{2+} in Fig. 12F is dramatic. Calcium, of course, has other effects, including a direct effect on excitability (Frankenhaeuser & Hodgkin, 1957) and these must be considered. To eliminate the effects of threshold variation, the stimuli were supramaximal in each instance. Examination of the records in Fig. 12 (compare, for example, B and D) reveals significant differences in the amplitudes of the E-waves that precede each S-wave in the light-scattering signal and coincide in time with the terminal action

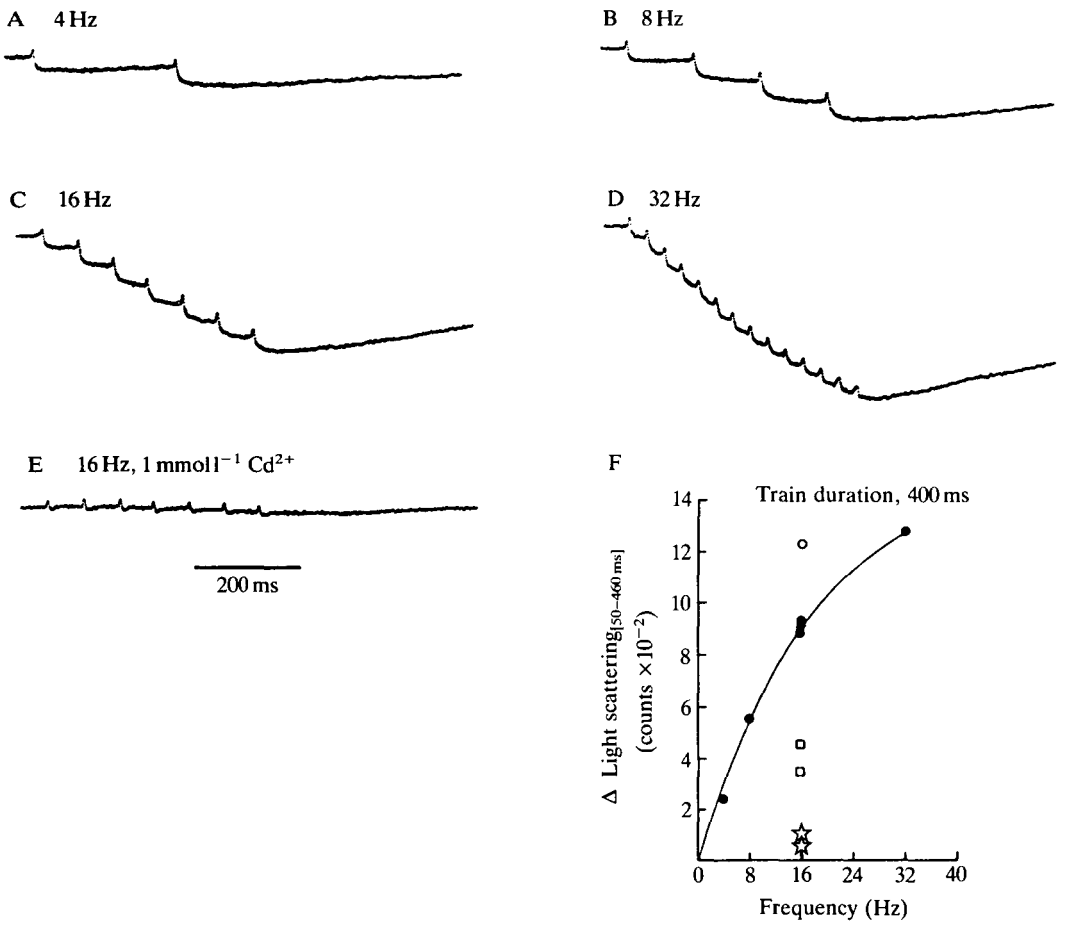

Fig. 11. Frequency-dependence and cadmium block of the light-scattering change in nerve terminals. A, B, C and D show the intrinsic optical signals at 675 nm resulting from stimulation of the terminals of the mouse pars nervosa at 4 Hz, 8 Hz, 16 Hz and 32 Hz, respectively, for 400 ms, in normal Ringer's solution. (E) Identical experiment to C, except that the intrinsic optical signal was recorded 15 min after the addition of 1 mmol l^{-1} Cd^{2+} to the Ringer's solution bathing the neurohypophysis. (F) Fractional change in transparency between 50 and 460 ms, as a function of stimulation frequency, during stimulation for 400 ms. The data are not corrected for the absolute number of stimuli. At 16 Hz, data are also plotted from two trials from the same experiment, in which 1 mmol l^{-1} Cd^{2+} was added to the bath (☆), and 2·2 mmol l^{-1} Ba^{2+} (○) was added to the normal Ringer's solution. A–E were single sweeps, recorded by a single array element. ●, Control; □, 2 mmol l^{-1} Ba^{2+}, 0 mmol l^{-1} Ca^{2+}. a.c. coupling time constant 3 s; rise time of the light-measuring system (10–90 %) 1·1 ms; temperature 23 °C. (After Salzberg, Obaid & Gainer, 1985.)

potential. The E-wave evidently has a different origin from the large S-wave that is blocked by cadmium. We have suggested (Salzberg *et al.* 1985) that this very early intrinsic optical change (E-wave) reflects the arrival of the action potential at the

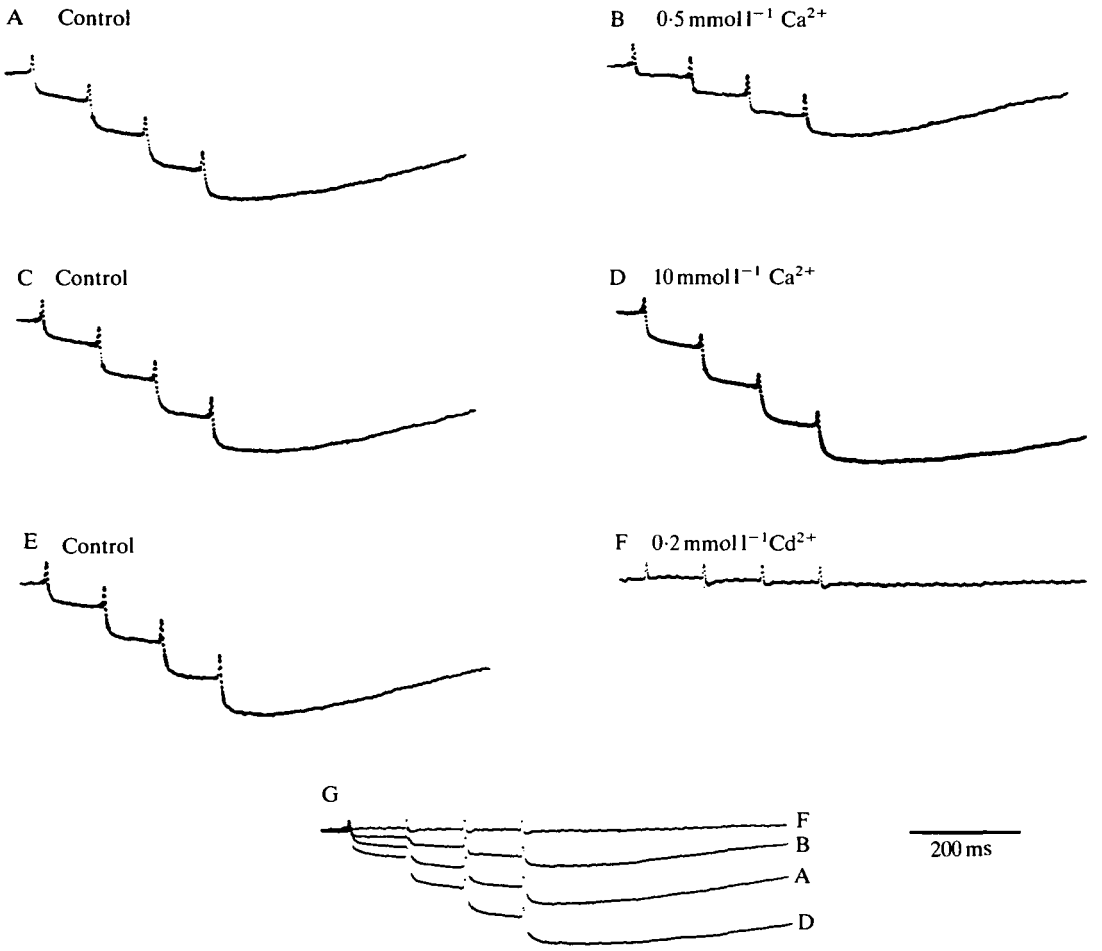

Fig. 12. Effects of extracellular calcium on the light-scattering changes recorded from the nerve terminals of the mouse neurohypophysis. (A,C,E) Light-scattering changes accompanying stimulation at 10 Hz of an unstained mouse neurohypophysis in normal Ringer's solution (2·2 mmol l^{-1} Ca^{2+}). (B) Light-scattering changes in the same preparation 20 min following reduction of the extracellular calcium concentration to 0·5 mmol l^{-1} by Mg^{2+} substitution. (D) Light-scattering changes in the same preparation 40 min following exposure to a Ringer's solution containing 10 mmol l^{-1} Ca^{2+}. (F) Light-scattering changes in the same preparation 10 min following the addition of 0·2 mmol l^{-1} Cd^{2+} to the bathing solution. Records A–F are each the average of 16 sweeps, recorded by a single representative array element, and were recorded in the order shown. (G) Traces A, B, D and F are shown superimposed, after normalization to the height of the first E-wave (see text). a.c. coupling time constant 3 s; rise time of the light-measuring system (10–90 %) 1·1 ms; temperature 24 °C. (After Salzberg, Obaid & Gainer, 1985.)

terminals of the neurohypophysis, and we assumed that, as a compound optical signal, its size is roughly proportional to the number of terminals activated synchronously under a given set of conditions. On this assumption, we have

compensated for changes in the invasibility of the tissue, resulting somehow from changes in extracellular calcium concentration, by normalizing the S-wave according to the size of the initial E-wave. This procedure has two potential difficulties. The first has to do with the interpretation of the E-wave. The peak of the E-wave occurs slightly before the peak of the voltage change in the terminals, as determined by the response of voltage-sensitive dyes. And, although the extrinsic absorption signals could be distorted if the dye response exhibited a slow component, the merocyanine–rhodanine and merocyanine–oxazolone dyes, at the concentrations used in these experiments, respond in less than 2 μs to step changes in voltage (B. M. Salzberg, A. L. Obaid & F. Bezanilla, unpublished observations) and their overall response is dominated by this fast component. If, instead, the E-wave depended on the inward current, it might be expected to reach a maximum at the time of the maximum rate of rise of the extrinsic absorption (voltage) signal. In fact, the time-to-peak of this early component of the opacity change falls somewhere between the time-to-peak of the voltage change and the time-to-peak of the time derivative of the absorption (voltage) change. This result suggests that the ultimate interpretation of the E-wave will be complicated and that, although this portion of the intrinsic optical signal is related to excitation, it may exhibit both current- and potential-dependence (Cohen, Keynes & Landowne, 1972*a*,*b*).

A second possible difficulty involves our assumption that the size of the E-wave, whether related primarily to current or to voltage, is proportional to the number of active terminals. This can only be approximately correct, since it will be affected by changes in the magnitude of the upstroke of the terminal action potential, particularly those resulting from changes in the resting membrane potential. It will also be sensitive to changes in the temporal dispersion of excitation of the terminals, and small changes in the size of the factor used for normalizing the optical signals might seriously distort the quantitative analysis of the optical signals. On balance, however, under constant stimulation conditions, the height of the E-wave should provide a rough comparative estimate of the degree of invasion of the tissue and thereby offer a convenient normalization for the larger light-scattering changes that appear to be related to secretion (S-wave). In any event, we have taken care that none of the qualitative conclusions that we have drawn (Salzberg *et al.* 1985) are affected by this procedure. Accordingly, Fig. 12G shows records obtained in normal Ringer's solution (A), together with records obtained in low $[Ca^{2+}]_o$ (B), high $[Ca^{2+}]_o$ (D), and normal Ringer's solution to which 0·2 mmol l^{-1} Cd^{2+} had been added (F), all normalized so as to equalize the sizes of the initial E-waves. In this way, the effect of $[Ca^{2+}]_o$ on the light-scattering signal per active terminal is demonstrated most convincingly.

Deuterium oxide decreases the size of the light-scattering response to stimulation

Heavy water (deuterium oxide) is known to depress excitation–contraction coupling in different muscle types (Kaminer, 1960; Svensmark, 1961; Bezanilla &

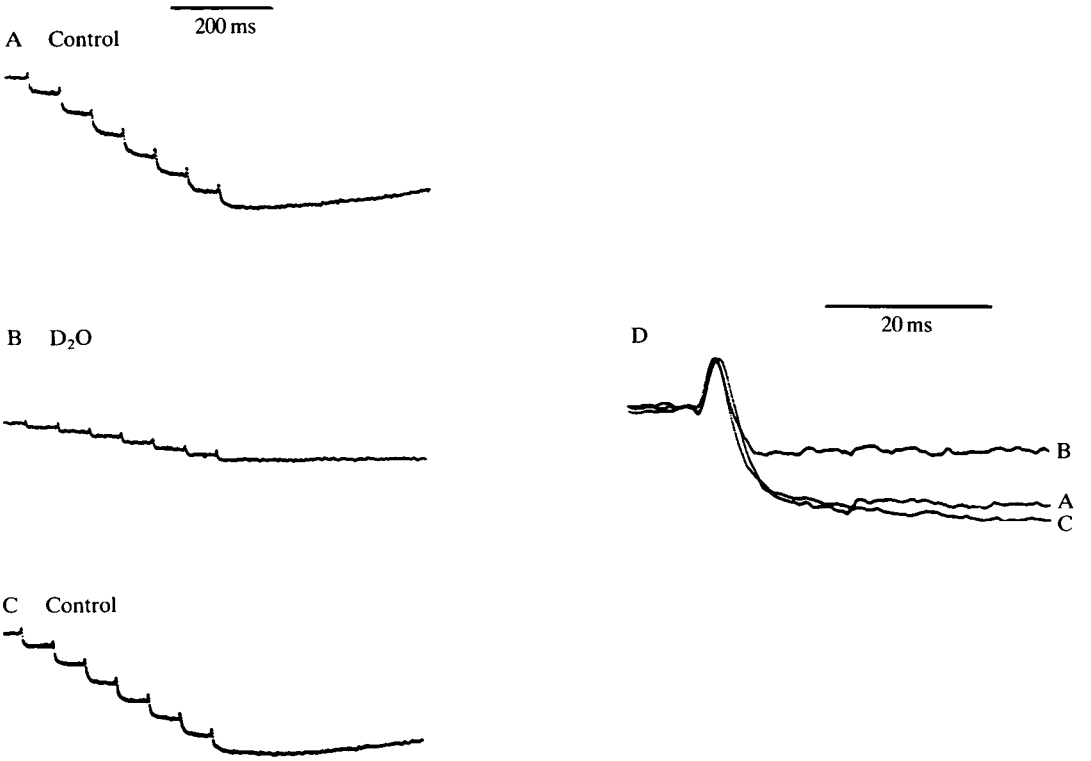

Fig. 13. Depression of the light-scattering changes by heavy water. (A) Light-scattering changes at 675 nm following a single train of stimuli delivered to the neurohypophysis at 16 Hz for 400 ms, in control Ringer's solution. Single sweep; single array element. (B) Light-scattering changes at 675 nm in the same preparation 31 min following the substitution of more than 98 % of the H_2O in the Ringer's solution by D_2O. Single sweep; single array element. (C) Same as A, 41 min following return to normal Ringer's solution. Single sweep; single array element. (D) Light-scattering responses to single stimuli, obtained immediately following the corresponding trains. The records are shown superimposed following normalization to the height of the first E-wave. Averages of 16 sweeps, stimulated every 5 s. a.c. coupling time constant 3 s; rise time of the light measuring system (10–90 %) 1·1 ms; temperature 23°C. (After Salzberg, Obaid & Gainer, 1985.)

Horowicz, 1975) and excitation–secretion coupling in a variety of systems including pancreatic beta cells (Lacy, Walker & Fink, 1972). In the neurohypophysis of the mouse, complete (>98 %) replacement of the water in the Ringer's solution by D_2O resulted in a reduction in the size of the light-scattering signal by about 60 %. Fig. 13 illustrates the effect of D_2O substitution on the optical response to stimulation of the terminals of the mouse neurohypophysis. Fig. 13A–C shows light-scattering changes at 675 nm obtained during single trials in normal Ringer's solution (A), 30 min in D_2O-substituted Ringer's solution (B), and 40 min following the return to normal Ringer's solution (C). The depression produced by deuterium oxide seems to reflect primarily a decrease in the light

scattering from each active terminal, consistent with earlier observations of the effects of D_2O (Kaminer, 1960; Svensmark, 1961; Lacy *et al.* 1972; Bezanilla & Horowicz, 1975), rather than a decrease in the number of active terminals, as judged by the very small variation in the size of the E-wave. This is illustrated in Fig. 12D, where the averages of 16 trials are shown, each record obtained immediately following the corresponding single trial. These records have been normalized to the height of the first peak, to compensate for small differences in the number of active terminals. It should be noted that replacement of water with D_2O produces a shift in the true pD, compared to the value measured with a glass pH electrode (Glascoe & Long, 1960) of approximately 0·4 pD units in the 'alkaline' direction. To control for the possibility that the depression of the light-scattering change shown in Fig. 13 resulted from a pD shift, we repeated these experiments in normal Ringer's solution with its pH adjusted to 8·0. This procedure had no effect on the normal light-scattering signals.

The effect of increasing the extracellular volume fraction with hypertonic medium

Rendering the Ringer's solution bathing the neurohypophysis hypertonic is expected to increase the volume of the extracellular space by shrinking the terminals. We might be able to detect this effect qualitatively in several different ways. Perhaps simplest would be to note the effect of hypertonicity on the appearance of extracellular potassium accumulation (Frankenhaeuser & Hodgkin, 1956). Fig. 14 illustrates this behaviour indirectly in a neurohypophysis that had been stained with the merocyanine–oxazolone dye NK 2367 (Salzberg *et al.* 1977). Fig. 14A shows the sum of the extrinsic absorption change, representing membrane potential, and the light-scattering signal, during stimulation at 16 Hz for 400 ms in normal Ringer's solution. The decrease in the overall size of the action potential (20 % between the first and last spike in the train) suggests that, in this preparation, sufficient potassium is accumulating extracellularly to affect the undershoots of the action potentials, but that this effect is masked by the downward deflection produced by the S-wave of the light-scattering signal. When the solution bathing the preparation is made 20 % hypertonic by the addition of sucrose, the reduction in spike amplitude is less pronounced (8 %), as though the effective extracellular volume was decreased by the shrinking of the terminals. The light-scattering signal, however, is not appreciably reduced.

Connection of the large intrinsic optical signal to secretory events at the nerve terminals

The intrinsic optical signals described above are closely correlated with the secretory activity of the magnocellular neurone terminals located in the neurohypophysis of the mouse. A mechanical artefact associated with contraction of vascular smooth muscle cannot be ruled out unequivocally and, indeed, such an effect would be expected to exhibit many of the same properties as an intrinsic

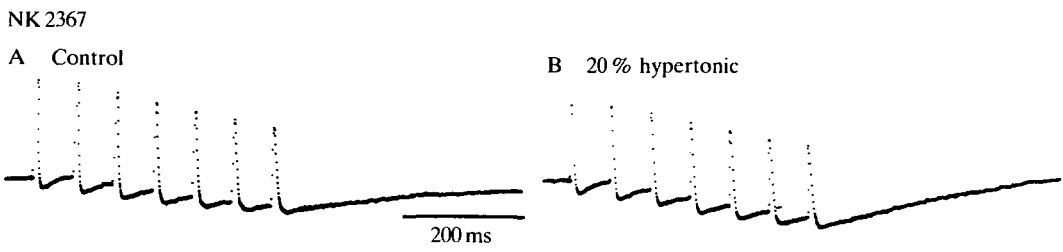

Fig. 14. Effects on intrinsic and extrinsic optical signals of increasing the extracellular volume fraction with hypertonic saline. (A) Extrinsic and intrinsic optical signals at 675 nm recorded by a single array element in a single sweep during stimulation at 16 Hz of a mouse neurohypophysis that had been stained with 25 μg ml^{-1} NK 2367 for 25 min in control Ringer's solution. The progressive decline in the height of the action potentials probably reflects the accumulation of K^+ in the extracellular space. The downward light-scattering signal masks the Frankenhaeuser–Hodgkin effect on the envelope of the undershoots. (B) Same experiment as A, following 10 min exposure to a Ringer's solution made 20 % hypertonic by the addition of sucrose. Extracellular accumulation of K^+ is less evident here (nearly constant peak heights and reduced Frankenhaeuser–Hodgkin effect), although the light-scattering component of the signal is not decreased. Single sweep; a.c. coupling time constant 400 ms; rise time of the light-measuring system (10–90 %) 1·1 ms; temperature 19 °C. (After Salzberg, Obaid & Gainer, 1985.)

signal related to secretion. However, the time course of the S-wave, the component of the light-scattering change that we associate with secretion, would seem to be too fast by at least an order of magnitude to have its origin in the contraction of smooth muscle. The S-wave reaches its half-maximal value within 7–8 ms of the application of the stimulus pulse to the region of the pars nervosa where the infundibular stalk enters (and 3–4 ms following the peak of the extrinsic signal). Even neglecting any conduction latency, this is considerably faster than any known mechanical response of vascular smooth muscle. Similarly, we feel justified in ruling out changes related to the terminal action potential alone, in accounting for the major component of the intrinsic optical signal. For example, extracellular accumulation of potassium ion might give rise to a change in light scattering, mediated by a transport number effect (Girardier, Reuben, Brandt & Grundfest, 1963; Barry & Hope, 1969; Cohen *et al.* 1972*b*) resulting from an increased salt concentration in the restricted space immediately outside the terminals. At least two kinds of evidence argue against such a mechanism. First, the addition of 2·2 mmol l^{-1} barium to the normal complement of extracellular calcium increases the size of the S-wave, despite the fact that barium is unlikely to replace calcium in activating a potassium conductance (Standen & Stanfield, 1978; Vergara & Latorre, 1983) and barium is likely, in fact, to reduce the voltage-dependent potassium conductance (Eaton & Brodwick, 1980; Armstrong, Swenson & Taylor, 1982; Armstrong & Taylor, 1980). Thus, the accumulation of potassium is expected to be severely decreased under these conditions. However, extracellular barium does replace calcium in some secretory systems, (Dicker,

1966; Rubin, 1974), although it is not as effective, and this is consistent with our observation that the increase in the size of the light-scattering signal with barium is smaller than that contributed by the addition of a similar amount of calcium. In this context, it would be helpful to demonstrate directly, using ion-selective electrodes, that potassium accumulation is, in fact, reduced in the presence of barium, but this has not yet been done. The experiment using sucrose (Fig. 14B) to increase the extracellular volume fraction also suggests that the light-scattering signal does not depend primarily on an alteration of extracellular space, since the effect of repetitive stimulation should be less under conditions in which the terminals are shrunk by the hypertonicity of the bath, but the light-scattering change was, in fact, not diminished. Indeed, the S-wave transparency changes that seem to reflect rapid alterations in light scattering are dramatically affected by many of the same interventions that are known to alter the release of neuropeptides from these same terminals. The dependence on stimulation frequency (Fig. 11) is in good accord with evidence obtained in several laboratories suggesting that neuropeptide secretion increases with frequency of stimulation (Dreifuss *et al.* 1971; Nordmann & Dreifuss, 1972; Poulain & Wakerley, 1982), and the facilitation of the intrinsic optical response seen in virtually all of the records seems particularly significant. Our own experiments, carried out in collaboration with Seth Wolfe and Hal Gainer (Gainer, Wolfe, Obaid & Salzberg, 1986) have used radioimmunoassays (RIA) for arginine vasopressin (AVP) to demonstrate the dependence upon stimulation frequency of vasopressin release in the CD-1 mouse. Fig. 15 shows the RIA measurements of arginine vasopressin release from mouse neural lobes, measured during two complete cycles of 600 stimuli delivered at 1 Hz and at 20 Hz. The data were highly reproducible (Gainer *et al.* 1986), and exhibited an enhancement of approximately 20-fold between 20 Hz and 1 Hz. Release at 1 Hz was not distinguishable from basal levels, so that it was not possible to calculate an accurate ratio of specific AVP released per pulse at 20 Hz *versus* 1 Hz. However, it was apparent that the AVP released per pulse in the mouse neurohypophysis is highly frequency-dependent, as is the case in the rat neurohypophysis (Dreifuss *et al.* 1971; Dutton & Dyball, 1979; Nordmann & Dreifuss, 1972). Further, the amount of AVP released during a second cycle of electrical stimulation was virtually identical to that released during the first cycle, suggesting that the stimulation paradigm was not deleterious to the release mechanism, and did not deplete the terminals to any significant degree.

The effect of extracellular calcium concentration on the magnitude of the S-wave of the intrinsic optical signal (Fig. 12) is also in remarkably good agreement with the classical behaviour of secretory systems (Douglas, 1978). The effect of replacing all the water in the Ringer's solution with D_2O, however, had not been reported in the mammalian neurohypophysis before our experiments (Salzberg *et al.* 1985). Under these conditions, we found (Fig. 13) a depression of approximately 60 % in the S-wave of the light-scattering signal. Radioimmunoassay measurements of secreted vasopressin (Gainer *et al.* 1986), revealed a nearly identical decrease (Table 1) in vasopressin release from the mouse neural lobes

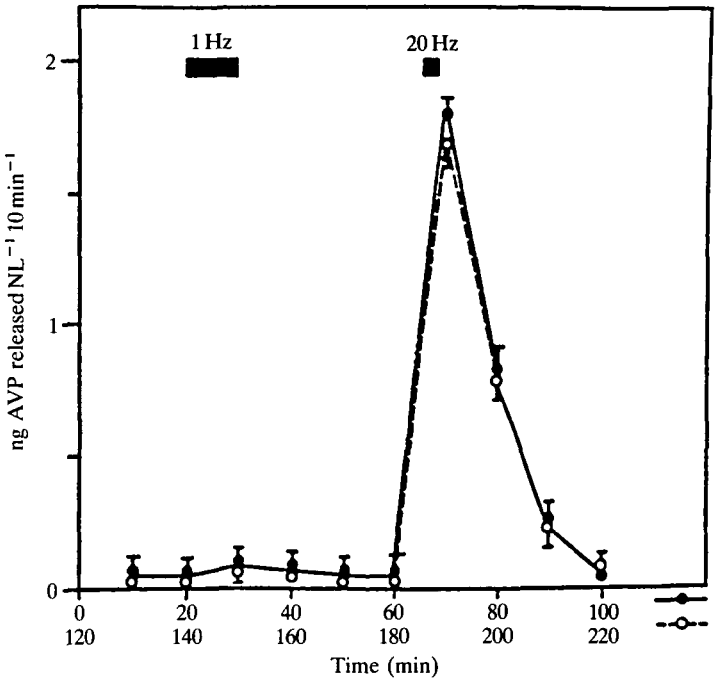

Fig. 15. Radioimmunoassay measurements of arginine vasopressin (AVP) release from mouse neural lobes (NL) stimulated electrically *in vitro*. Each point on the graph represents the average (±S.E.M.) amount of AVP released in six independent experiments. Electrical stimulation consisted of 600 pulses (0·5 ms duration) at either 1 Hz (600 s) or 20 Hz (30 s). The neural lobes were stimulated in two cycles of 1 and 20 Hz. The first cycle is shown by filled circles (0–100 min), followed by wash-out (data not shown) and a repeat second cycle shown by open circles (120–220 min). Calcium concentration in the medium was 1 mmol l^{-1} in these experiments. Following the experiments, the total residual AVP in the lobes was determined, and AVP release at 20 Hz was found to be 0·4 % of total lobe content. (After Gainer, Wolfe, Obaid & Salzberg, 1986.)

when D_2O was substituted for H_2O under comparable stimulation conditions. These experiments also provided direct corroboration of our observations on the effects of $[Ca^{2+}]_o$ on AVP release, estimated from the variation in the size of the S-wave, and reproduced the saturation that we found at concentrations above 5 mmol l^{-1} (Table 1).

Another series of experiments, carried out in our laboratory together with T. D. Parsons, at the suggestion of David Quastel, has shown that neomycin and other aminoglycoside antibiotics depress the S-wave of the light-scattering signal (Parsons, Obaid & Salzberg, 1985). This finding is significant because, at low concentration, these drugs act presynaptically at the snake neuromuscular junction and depress evoked transmitter release by competing with $[Ca^{2+}]_o$ (Fiekers, 1983). Our observation that, for example, neomycin at 220 μmol l^{-1} in normal Ringer's solution depressed the magnitude of the S-wave by 60 % after

Table 1. *Effects of calcium ions and selected deuterium oxide on the secretion of arginine vasopressin (AVP) from mouse neural lobes (NL)**

Experimental treatment	*N*	ng AVP released NL^{-1} †	*P* ‡
Effect of external calcium			
0·1 mmol l^{-1} Ca^{2+}	6	0·2 ± 0·1	$P<0·01$
1·0 mmol l^{-1} Ca^{2+}	6	2·4 ± 0·3	$P<0·01$
2·0 mmol l^{-1} Ca^{2+}	6	3·7 ± 0·5	$P<0·01$
5·0 mmol l^{-1} Ca^{2+}	6	6·7 ± 0·8	
10·0 mmol l^{-1} Ca^{2+}	6	6·2 ± 1·2	$P>0·5$ (NS)
Effect of D_2O§			
Control	6	1·0 ± 0·2	
D_2O	6	0·4 ± 0·1	$0·05>P>0·01$

* This table is abstracted from Gainer, Wolfe, Obaid & Salzberg (1986).

† Radioimmunoassay measurement of AVP released (after subtracting 30 min basal AVP release) over a 30-min sampling period, following 600 stimulation pulses at 20 Hz. Data expressed as average values ± S.E.M. for *N* samples.

‡ *P*-values determined using the Student's *t*-test. NS, not significantly different. Each measurement was independently compared with the maximum release at 5 mmol l^{-1} Ca^{2+} when the effects of external calcium were examined.

Experiments were performed as shown in Fig. 15, except that the first 20-Hz stimulation was the control, and the second 20-Hz stimulation was performed under the experimental condition, i.e. where the H_2O in the medium was completely replaced by D_2O.

11 min provides additional evidence that these agents act presynaptically, since there are no postsynaptic elements in the neurohypophysis, and that they act as competitive antagonists of $[Ca^{2+}]_o$ (Fiekers, 1983; Parsons *et al.* 1986). Further, the compatibility of the aminoglycoside effects on the light scattering from the neurohypophysis, with voltage-clamp data reported from the neuromuscular junction (Fiekers, 1983) reinforces our interpretation (Salzberg *et al.* 1985) that the light-scattering signals reflect processes closely related to neuropeptide release.

With these results in mind, it is perhaps worthwhile returning to our earlier discussion of the pharmacological profile of the calcium channels that are present in the nerve terminals of vertebrate neurohypophyses. The evident correlation between the intrinsic optical changes in the terminals, on the one hand, and the release of neuropeptides, on the other, allows us to pose the question of whether the calcium channels in the terminals which are sensitive to ω-conotoxin and insensitive to dihydropyridines are the same calcium channels that are required for normal secretion. Apparently, they are. Fig. 16 shows an experiment in which we attempted to observe the effects of dihydropyridine calcium channel modifiers on the light-scattering signal. Fig. 16A shows the intrinsic optical change recorded during a single stimulus train applied in normal Ringer's solution. Fig. 16B shows the result of a stimulus train following 22 min in 5 μmol l^{-1} nifedipine. No effect observed, and this result is consistent with the absence of any effect of nifedipine

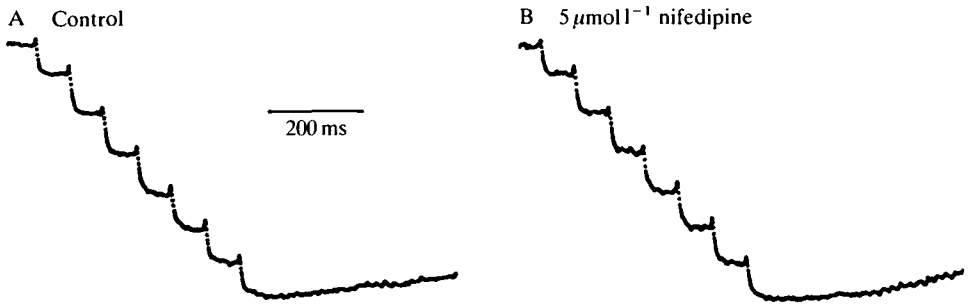

Fig. 16. Nifedipine has no effect on the light-scattering change associated with the release of hormones from the nerve terminals of the mouse neurohypophysis. (A) Light-scattering signal resulting from stimulation of the infundibulum of an unstained mouse (CD-1) neurohypophysis at 16 Hz for 400 ms in control Ringer's solution. (B) Light-scattering signal following a 22-min exposure to a Ringer's solution containing 5 μmol l^{-1} nifedipine (in 0·05 % ethanol). Spatial average of the digitized outputs of four contiguous elements of the photodiode array; single sweep; ×10; 0·4 numerical aperture; 675 ± 26 nm; infundibular stimulation; a.c. coupling time constant 3 s; response time constant (10–90 %) 1·1 ms. (After A. L. Obaid, R. Flores & B. M. Salzberg, in preparation.)

on the action potential in the neurohypophyseal nerve terminals of the frog (Fig. 7). Similarly, Bay-K 8644, at 5 μmol l^{-1} for at least 40 min (not shown) had no effect on the intrinsic optical signal exhibited by the nerve terminals of the CD-1 mouse. The calcium channels required to produce the normal S-wave and, by extension, secretion of neuropeptides, are blocked by ω-conotoxin, however. This is illustrated in Fig. 17, where the inhibition of the S-wave of the light-scattering signal in the presence of 5 μmol l^{-1} ω-conotoxin GVIA is shown.

We have, as yet, no reason to implicate any particular step among the sequence of events that couples excitation to secretion in the generation of these large intrinsic optical signals, and the identity of their physiological basis remains unclear. The fusion of secretory vesicles during exocytosis should result in the loss of relatively high refractive index particles, and should thereby reduce the refractive index gradients in the terminal. However, the very early onset and fast rise of the S-wave suggest that the optical signal might arise as a result of some calcium-dependent process prior to the fusion of secretory vesicles and the release of their contents. For example, it is possible that the light-scattering changes described here reflect a swelling of secretory vesicles, or they may reflect, instead, changes in the cytoskeleton, or rapid alterations in the state of intracellular calcium stores following calcium entry but prior to secretion (Neering & McBurney, 1984). In the latter case, these intrinsic optical changes may be related to the transparency changes that precede tension development which have been observed in cut skeletal muscle fibres (Kovacs & Schneider, 1977; Rios, Melzer & Schneider, 1983). Identification of the physiological origin of the intrinsic optical changes must await precise measurement of light scattering *per se*. Quasi-elastic

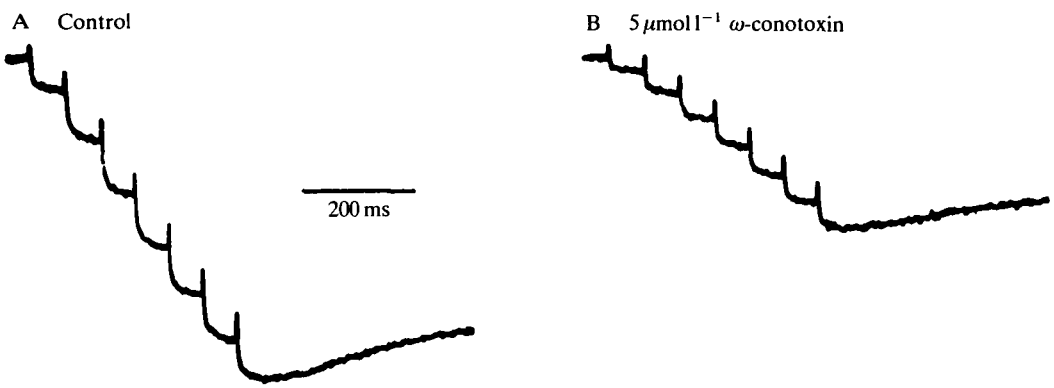

Fig. 17. ω-Conotoxin GVIA (5 μmol l^{-1}) blocks calcium channels required for neuropeptide release in the nerve terminals of the mouse neurohypophysis. (A) Light-scattering signal resulting from stimulation of the infundibulum of an unstained mouse (CD-1) neurohypophysis at 16 Hz for 400 ms in control Ringer's solution. A downward deflection of the trace represents an increase in the transparency of the tissue. (B) The effect of an 18-min exposure to a Ringer's solution containing 5 μmol l^{-1} ω-conotoxin GVIA. Spatial average of the digitized outputs of 12 contiguous elements of the photodiode array; single sweep; ×10; 0·5 numerical aperture; 675 ± 26 nm; infundibular stimulation; a.c. coupling time constant 3 s; response time constant (10–90 %) 1·1 ms. (After A. L. Obaid, R. Flores & B. M. Salzberg, in preparation.)

light-scattering measurements, particularly on simplified model systems such as secretosomes or vesicle suspensions (see Sattelle, 1988; this volume) may assist in the interpretation of these very interesting signals.

In any case, the weak dependence on wavelength of the intrinsic signal related to secretion, contrasted with the strong wavelength-dependence of the extrinsic absorption signals provided by linear potentiometric probes (e.g. merocyanines) (Ross, Salzberg, Cohen & Davila, 1974; Ross *et al.* 1977; Cohen & Salzberg, 1978; Salzberg, 1983) should permit one to monitor simultaneously, in a stained preparation, the voltage changes in the nerve terminals and the time course of events intimately associated with the release of secretory products. The inherently fast responses of the optical measurements may then improve our ability to resolve early events in the coupling of excitation to secretion.

We have chosen the vertebrate neurohypophysis to illustrate the technique of optical recording of membrane potential for the study of excitation at nerve terminals. These experiments led to the use of light-scattering methods for the investigation of excitation–secretion coupling, and it seems very likely that intracellular indicators for the measurement of $[Ca^{2+}]_i$-transients will also find applications in the optical study of the secretory event in this system.

We are grateful to Dr H. Gainer for introducing us to the neurohypophysis as an experimental system and for his participation in many of the experiments described here, to Drs C. M. Armstrong, L. B. Cohen, M. Morad, R. K. Orkand, D. B. Sattelle and R. W. Tsien for valuable discussions, and to Drs Orkand,

Parsons, Sattelle and D. M. Senseman for their participation in some of the experiments discussed. We are also grateful to Dr Chris Miller for his generous gift of charybdotoxin, and to L. B. Cohen and S. Lesher for providing the software used for data acquisition and display. Supported by USPHS grant NS 16824.

References

ARMSTRONG, C. M., SWENSON, R. P. & TAYLOR, S. R. (1982). Block of squid axon K channels by internally and externally applied barium ions. *J. gen. Physiol.* **80**, 663–682.

ARMSTRONG, C. M. & TAYLOR, S. R. (1980). Interaction of barium ions with potassium channels in squid giant axons. *Biophys. J.* **30**, 473–488.

BARRETT, J. N., MAGLEBY, K. L. & PALOTTA, B. S. (1982). Properties of single calcium activated potassium channels in cultured rat muscle. *J. Physiol., Lond.* **331**, 211–230.

BARRY, P. H. & HOPE, A. B. (1969). Electroosmosis in membranes: effects of unstirred layers and transport numbers. *Biophys. J.* **9**, 700–728.

BEZANILLA, F. & HOROWICZ, P. (1975). Fluorescence intensity changes associated with contractile activation in frog muscle stained with Nile Blue A. *J. Physiol., Lond.* **246**, 709–735.

BLAUSTEIN, M. P. & GOLDRING, J. M. (1975). Membrane potentials in pinched-off presynaptic nerve terminals monitored with a fluorescence probe: Evidence that synaptosomes have potassium diffusion potentials. *J. Physiol., Lond.* **247**, 589–615.

BRANDT, B. L., HAGIWARA, S., KIDOKORO, Y. & MIYAZAKI, S. (1976). Action potentials in the rat chromaffin cell and effects of acetylcholine. *J. Physiol., Lond.* **263**, 417–439.

COHEN, L. B. (1973). Changes in neuron structure during action potential propagation and synaptic transmission. *Physiol. Rev.* **53**, 373–418.

COHEN, L. B. & LANDOWNE, D. (1970). Changes in light scattering during synaptic activity in the electric organ of the skate, *Raia erinacea*. *Biophys. J.* **10**, 16a.

COHEN, L. B., KEYNES, R. D. & LANDOWNE, D. (1972*a*). Changes in light scattering that accompany the action potential in squid giant axons: potential-dependent components. *J. Physiol., Lond.* **224**, 701–725.

COHEN, L. B., KEYNES, R. D. & LANDOWNE, D. (1972*b*). Changes in light scattering that accompany the action potential in squid giant axons: current-dependent components. *J. Physiol., Lond.* **224**, 727–752.

COHEN, L. B. & LESHER, S. (1986). Optical monitoring of membrane potential: Methods of multisite optical measurement. In *Optical Methods in Cell Physiology* (ed. P. De Weer & B. M. Salzberg), pp. 71–99. New York: John Wiley & Sons.

COHEN, L. B. & SALZBERG, B. M. (1978). Optical measurement of membrane potential. *Rev. Physiol. Biochem. Pharmac.* **83**, 33–88.

COHEN, L. B., SALZBERG, B. M., DAVILA, H. V., ROSS, W. N., LANDOWNE, D., WAGGONER, A. S. & WANG, C.-H. (1974). Changes in axon fluorescence during activity: molecular probes of membrane potential. *J. Membr. Biol.* **19**, 1–36.

DELLMAN, H.-D. (1973). Degeneration and regeneration of neurosecretory systems. *Int. Rev. Cytol.* **36**, 215–315.

DICKER, S. E. (1966). Release of vasopressin and oxytocin from isolated pituitary glands of adult and new-born rats. *J. Physiol., Lond.* **185**, 429–444.

DODGE, F. & RAHAMIMOFF, R. (1967). Cooperative action of calcium ions in transmitter release at the neuromuscular junction. *J. Physiol., Lond.* **193**, 419–432.

DOUGLAS, W. W. (1963). A possible mechanism of neurosecretion – release of vasopressin by depolarization and its dependence on calcium. *Nature, Lond.* **197**, 81–82.

DOUGLAS, W. W. (1978). Stimulus secretion coupling: variations on the theme of calcium activated exocytosis involving cellular and extracellular sources of calcium. *Ciba Fdn Symp.* **54**, 61–90.

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

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

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

EATON, D. C. & BRODWICK, M. S. (1980). Effects of barium on the potassium conductance of squid axon. *J. gen. Physiol.* **75**, 727–750.

ENGLERT, D. & EDWARDS, C. (1977). Effect of increased potassium concentration on particle motion within a neurosecretory structure. *Proc. natn. Acad. Sci. U.S.A.* **74**, 5759–5763.

FERNANDEZ, J. M., NEHER, E. & GOMPERTS, B. D. (1984). Capacitance measurements reveal stepwise fusion events in degranulating mast cells. *Nature, Lond.* **312**, 453–455.

FIEKERS, J. F. (1983). Effects of the aminoglycoside antibiotics, streptomycin and neomycin, on neuromuscular transmission. I. Presynaptic considerations. *J. Pharmac. exp. Ther.* **225**, 487–495.

FOX, A. P., NOWYCKY, M. C. & TSIEN, R. W. (1987). Kinetic and pharmacological properties distinguishing three types of calcium currents in chick sensory neurons. *J. Physiol., Lond.* (in press).

FRANKENHAEUSER, B. & HODGKIN, A. L. (1956). The after-effects of impulses in giant nerve fibres of *Loligo*. *J. Physiol., Lond.* **131**, 341–376.

FRANKENHAEUSER, B. & HODGKIN, A. L. (1957). The action of calcium on the electrical properties of squid axons. *J. Physiol., Lond.* **137**, 218–244.

FREEDMAN, J. C. & LARIS, P. C. (1981). Electrophysiology of cells and organelles: Studies with optical potentiometric indicators. *Int. Rev. Cytol.* Suppl. **12**, 177–246.

GAINER, H., WOLFE, S. A., JR, OBAID, A. L. & SALZBERG, B. M. (1986). Action potentials and frequency-dependent secretion in the mouse neurohypophysis. *Neuroendocr.* **43**, 557–563.

GERSCHENFELD, H. M., TRAMEZZANI, J. H. & DE ROBERTIS, E. (1960). Ultrastructure and function in the neurohypophysis of the toad. *Endocrinology* **66**, 741–762.

GIRARDIER, L., REUBEN, J. P., BRANDT, P. W. & GRUNDFEST, H. (1963). Evidence for anion-permselective membrane in crayfish muscle fibers and its possible role in excitation–contraction coupling. *J. gen. Physiol.* **47**, 189–214.

GLASCOE, P. K. & LONG, F. A. (1960). Use of glass electrodes to measure acidities in deuterium oxide. *J. phys. Chem.* **64**, 188–190.

GRINVALD, A. (1985). Real-time optical mapping of neuronal activity: from single growth cones to the intact mammalian brain. *A. Rev. Neurosci.* **8**, 263–305.

GRINVALD, A., COHEN, L. B., LESHER, S. & BOYLE, M. B. (1981). Simultaneous optical monitoring of activity of many neurons in invertebrate ganglia, using a 124 element photodiode array. *J. Neurophysiol.* **45**, 829–840.

GRINVALD, A. & FARBER, I. C. (1981). Optical recording of Ca^{2+} action potentials from growth cones of cultured neurons using a laser microbeam. *Science* **212**, 1164–1169.

GRINVALD, A., MANKER, A. & SEGAL, M. (1982). Visualization of the spread of electrical activity in rat hippocampal slices by voltage-sensitive optical probes. *J. Physiol., Lond.* **333**, 269–291.

GUPTA, R. K., SALZBERG, B. M., GRINVALD, A., COHEN, L. B., KAMINO, K., LESHER, S., BOYLE, M. B., WAGGONER, A. S. & WANG, C.-H. (1981). Improvements in optical methods for measuring rapid changes in membrane potential. *J. Membr. Biol.* **58**, 123–137.

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

HEUSER, J. E. & REESE, T. S. (1981). Structural changes after transmitter release at the frog neuromuscular junction. *J. Cell Biol.* **88**, 564–580.

HEUSER, J. E., REESE, T. S., DENNIS, M. J., JAN, Y., JAN, L. & EVANS, L. (1979). Synaptic vesicle exocytosis captured by quick freezing and correlated with quantal transmitter release. *J. Cell Biol.* **81**, 275–300.

HODGKIN, A. L. (1938). The sub-threshold potentials in a crustacean nerve fibre. *Proc. R. Soc. Ser.* B **126**, 87–121.

HOLZ, G. G., DUNLAP, K. & KREAM, R. M. (1988). Characterization of the electrically-evoked release of substance P from dorsal root ganglion neurons: Methods and dihydropyridine sensitivity. *J. Neurosci.* (in press).

KAMINER, B. (1960). Effect of heavy water on different types of muscle and on glycerol-extracted psoas fibres. *Nature, Lond.* **185**, 172–173.

KAMINO, K., HIROTA, A. & FUJII, S. (1981). Localization of pacemaking activity in early embryonic heart monitored using a voltage sensitive dye. *Nature, Lond.* **290**, 595–597.

KAMINO, K. & INOUYE, A. (1978). Evidence for membrane potential changes in isolated synaptic membrane ghosts monitored with a merocyanine dye. *Jap. J. Physiol.* **28**, 225–237.

KATZ, B. (1969). *The Release of Neural Transmitter Substances.* Springfield, IL.: Thomas. 193pp.

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

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

KAUFMANN, R. & FLECKENSTEIN, A. (1965). Ca^{++}-kompetitive electromechanische Entkoppelung durch Ni^{++} – und Co^{++} – Ionen am Warmblutermyokard. *Pflügers Arch. ges. Physiol.* **282**, 290–297.

KOSTYUK, P. G. & KRISHTAL, O. A. (1977). Separation of sodium and calcium currents in the somatic membrane of mollusc neurons. *J. Physiol., Lond.* **270**, 545–568.

KOVACS, L. & SCHNEIDER, M. F. (1977). Increased optical transparency associated with excitation–contraction coupling in voltage-clamped cut skeletal muscle fibres. *Nature, Lond.* **265**, 556–560.

LACY, P. E., WALKER, M. M. & FINK, C. J. (1972). Perfusion of isolated rat islets *in vitro*. Participation of the microtubular system in the biphasic release of insulin. *Diabetes* **21**, 987–998.

LEMOS, J. R. & NOWYCKY, M. C. (1987). One type of calcium channel in nerve terminals of the rat neurohypophysis is sensitive to dihydropyridines. *Neurosci. Abstr.* **13**, 793.

LINDAU, M. & FERNANDEZ, J. M. (1986). IgE-mediated degranulation of mast cells does not require opening of ion channels. *Nature, Lond.* **319**, 150–153.

LLINAS, R. & SUGIMORI, M. (1980*a*). Electrophysiological properties of *in vitro* Purkinje cell somata in mammalian cerebellar slices. *J. Physiol., Lond.* **305**, 171–195.

LLINAS, R. & SUGIMORI, M. (1980*b*). Electrophysiological properties of *in vitro* Purkinje cell dendrites in mammalian cerebellar slices. *J. Physiol., Lond.* **305**, 197–213.

MCCLESKEY, E. W., FOX, A. P., FELDMAN, D. H., CRUZ, L. J., OLIVERA, B. M., TSIEN, R. W. & YOSHIKAMI, D. (1987). ω-Conotoxin: Direct and persistent block of specific types of calcium channels in neurons but not muscle. *Proc. natn. Acad. Sci. U.S.A.* (in press).

MASON, W. T. & DYBALL, R. E. (1986). Single ion channel activity in peptidergic nerve terminals of the isolated rat neurohypophysis related to stimulation of neural stalk axons. *Brain Res.* **383**, 279–286.

MEECH, R. W. & STRUMWASSER, F. (1970). Intracellular calcium injection activates potassium conductance in *Aplysia* nerve cells. *Fedn Proc. Fedn Am. Socs exp. Biol.* **29**, 834.

MILLER, C., MOCZYDLOWSKI, E., LATORRE, R. & PHILLIPS, M. (1985). Charybdotoxin, a protein inhibitor of single Ca^{2+}-activated K^+ channels from mammalian skeletal muscle. *Nature, Lond.* **313**, 316–318.

MILLER, R. J. (1987). Multiple calcium channels and neuronal function. *Science* **235**, 46–52.

NEERING, I. & MCBURNEY, R. N. (1984). Role of microsomal Ca storage in mammalian neurones? *Nature, Lond.* **309**, 158–160.

NORDMANN, J. J. (1977). Ultrastructural morphometry of the rat neurohypophysis. *J. Anat.* **123**, 213–218.

NORDMANN, J. J. & DREIFUSS, J. J. (1972). Hormone release evoked by electrical stimulation of rat neurohypophyses in the absence of action potentials. *Brain Res.* **45**, 604–607.

OBAID, A. L., ORKAND, R. K., GAINER, H. & SALZBERG, B. M. (1985). Active calcium responses recorded optically from nerve terminals of the frog neurohypophysis. *J. gen. Physiol.* **85**, 481–489.

OBAID, A. L. & SALZBERG, B. M. (1985). Selective block of the calcium-mediated potassium conductance [$g_{K(Ca)}$] in vertebrate nerve terminals by charybdotoxin (CTX): An optical demonstration using the neurohypophysis of *Xenopus*. *J. gen. Physiol.* **86**, 19a.

OLIVERA, B. M., GRAY, W. R., ZEIKUS, R., MCINTOSH, J. M., VARGA, J., RIVIER, J., DE SANTOS, V. & CRUZ, L. J. (1985). Peptide neurotoxins from fish-hunting cone snails. *Science* **230**, 1338–1343.

PARSONS, T. D., OBAID, A. L. & SALZBERG, B. M. (1985). Light scattering changes associated with secretion from nerve terminals of the mammalian neurohypophysis are depressed by aminoglycoside antibiotics. *Biophys. J.* **47**, 447a.

PARSONS, T. D., OBAID, A. L. & SALZBERG, B. M. (1986). Aminoglycoside antibiotics block calcium currents in nerve terminals of the frog neurohypophysis: An optical demonstration using potentiometric probes. *Biol. Bull. mar. biol. Lab., Woods Hole* **171**, 497.

PIDDINGTON, R. W. & SATTELLE, D. B. (1975). Motion in nerve ganglia detected by light-beating spectroscopy. *Proc. R. Soc. Ser.* B **190**, 415–420.

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

RANE, S. G., HOLZ, G. G. & DUNLAP, K. (1987). Dihydropyridine inhibition of neuronal calcium current and substance P release. *Pflügers Arch. ges. Physiol.* **409**, 361–366.

RIOS, E., MELZER, W. & SCHNEIDER, M. F. (1983). An intrinsic optical signal is related to the calcium transient of frog skeletal muscle. *Biophys. J.* **41**, 396a.

RODRIGUEZ, E. M. & DELLMAN, H.-D. (1970). Hormonal content and the ultrastructure of the disconnected neural lobe of the grass frog (*Rana pipiens*). *Gen. comp. Endocr.* **15**, 272–288.

ROSS, W. N., SALZBERG, B. M., COHEN, L. B. & DAVILA, H. V. (1974). A large change in dye absorption during the action potential. *Biophys. J.* **14**, 983–986.

ROSS, W. N., SALZBERG, B. M., COHEN, L. B., GRINVALD, A., DAVILA, H. V., WAGGONER, A. S. & WANG, C.-H. (1977). Changes in absorption, fluorescence, dichroism, and birefringence in stained giant axons: optical measurement of membrane potential. *J. Membr. Biol.* **33**, 141–183.

RUBIN, R. P. (1974). *Calcium and the Secretory Process.* New York: Plenum Press.

SALZBERG, B. M. (1983). Optical recording of electrical activity in neurons using molecular probes. In *Current Methods in Cellular Neurobiology*, vol. 3, *Electrophysiological Techniques* (ed. J. L. Barker & J. E. McKelvey), pp. 139–187. New York: John Wiley & Sons, Inc.

SALZBERG, B. M. & BEZANILLA, F. (1983). An optical determination of the series resistance of *Loligo*. *J. gen. Physiol.* **82**, 807–818.

SALZBERG, B. M., GRINVALD, A., COHEN, L. B., DAVILA, H. V. & ROSS, W. N. (1977). Optical recording of neuronal activity in an invertebrate central nervous system: Simultaneous monitoring of several neurons. *J. Neurophysiol.* **40**, 1281–1291.

SALZBERG, B. M., OBAID, A. L. & FLORES, R. (1987). Calcium channels required for neuropeptide release in the intact nerve terminals of vertebrate neurohypophyses are sensitive to omega-conotoxin and insensitive to dihydropyridines: Optical studies with and without voltage-sensitive dyes. *Biol. Bull. mar. biol. Lab., Woods Hole* **173**, 446.

SALZBERG, B. M., OBAID, A. L. & GAINER, H. (1985). Large and rapid changes in light scattering accompany secretion by nerve terminals in the mammalian neurohypophysis. *J. gen. Physiol.* **86**, 395–411.

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

SATTELLE, D. B. (1988). Light-scattering studies on nerve terminals associated with secretion. *J. exp. Biol.* **139**, 233–252.

SENSEMAN, D. M. & SALZBERG, B. M. (1980). Electrical activity in an exocrine gland: Optical recording using a potentiometric dye. *Science* **208**, 1269–1271.

SENSEMAN, D. M., SHIMIZU, H., HORWITZ, I. S. & SALZBERG, B. M. (1983). Multiple site optical recording of membrane potential from a salivary gland: Interaction of synaptic and electrotonic excitation. *J. gen. Physiol.* **81**, 887–908.

SHAW, T. I. & NEWBY, B. J. (1972). Movement in a ganglion. *Biochim. biophys. Acta* **255**, 411–412.

STÄMPFLI, R. & HILLE, B. (1976). Electrophysiology of the peripheral myelinated nerve. In *Frog Neurobiology* (ed. R. Llinas & W. Precht), pp. 3–32. Berlin: Springer-Verlag.

STANDEN, N. B. (1981). Ca channel inactivation by intracellular Ca injection into *Helix* neurones. *Nature, Lond.* **293**, 158–159.

STANDEN, N. B. & STANFIELD, P. R. (1978). A potential- and time-dependent blockade of inward rectification in frog skeletal muscle fibres by barium and strontium ions. *J. Physiol., Lond.* **280**, 169–191.

SVENSMARK, O. (1961). The effect of deuterium oxide on the mechanical properties of muscle. *Acta physiol. scand.* **53**, 75–84.

TSIEN, R. W. (1986). Modulation of calcium currents in heart cells and neurons. In *Neuromodulation* (ed. I. B. Levitan & L. K. Kaczmarek), pp. 206–242. Oxford: Oxford University Press.

TYNDALL, J. (1868). The optical deportment of the atmosphere in relation to the phenomena of putrefaction and infection. *Phil. Trans. R. Soc. Ser.* B **166**, 27–74.

VERGARA, C. & LATORRE, R. (1983). Kinetics of Ca^{2+}-activated K^+ channels from rabbit muscle incorporated into planar bilayers. Evidence for Ca^{2+} and Ba^{2+} blockade. *J. gen. Physiol.* **82**, 543–568.

WAGGONER, A. S. (1979). Dye indicators of membrane potential. *A. Rev. Biophys. Bioeng.* **8**, 47–68.