Calcium Channels that Are Required for Secretion from Intact Nerve Terminals of Vertebrates Are Sensitive to ω-Conotoxin and Relatively Insensitive to Dihydropyridines

Optical Studies with and without Voltage-sensitive Dyes

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ABSTRACT   Extrinsic absorption changes exhibited by potentiometric dyes have established the ionic basis of the action potential in synchronously activated populations of nerve terminals in the intact neurohypophyses of amphibia and mammals (Salzberg et al., 1983; Obaid et al., 1983, 1985b). Also, large and rapid changes in light scattering, measured as transparency, have been shown to follow membrane depolarization and to be intimately associated with the release of neuropeptides from the nerve terminals of the mouse neurohypophysis (Salzberg et al., 1985; Gainer et al., 1986). We report some experiments that help to define the pharmacological profile of the calcium channels present in intact neurosecretory terminals of vertebrates. For these, we used the peptide toxin ω-conotoxin GVIA (1–5 μM) and the dihydropyridine compounds Bay-K 8644 and nifedipine (2–5 μM), together with the after-hyperpolarization of the nerve terminal action potential. This undershoot depends upon the activation of a calcium-mediated potassium channel, as suggested by its sensitivity to $[Ca^{++}]_o$ and charybdotoxin. ω-conotoxin GVIA substantially reduced the after-hyperpolarization in neurosecretory terminals of Xenopus, while neither of the dihydropyridine compounds had any effect under conditions that mimic natural stimulation. The effects of these calcium channel modifiers on the action potential recorded optically from the terminals of the Xenopus neurohypophysis were faithfully reflected in the behavior of

the light-scattering changes observed in the neurohypophysis of the CD-1 mouse. ω-conotoxin GVIA (5 μM) reduced the size of the intrinsic optical signal associated with secretion by 50%, while the dihydropyridines had little effect. These observations suggest that the type of calcium channel that dominates the secretory behavior of intact vertebrate nerve terminals is at least partially blocked by ω-conotoxin GVIA and is insensitive, under normal conditions, to dihydropyridines.

INTRODUCTION

Optical techniques can facilitate the study of excitation-secretion coupling, especially in the small nerve terminals of vertebrates where electrical recording is generally precluded. For example, the extrinsic absorption changes (Ross et al., 1977) exhibited by plasma membrane stained with linear potentiometric dyes (Cohen and Salzberg, 1978; Salzberg, 1983; Grinvald, 1985) have been used to establish the ionic basis of the action potential in synchronously activated populations of nerve terminals in intact amphibian (Salzberg et al., 1983; Obaid et al., 1985b) and mammalian neurohypophyses (Obaid et al., 1983). Also, large and rapid changes in light scattering, measured as transparency, accompany and immediately follow membrane depolarization in the terminals of the unstained mouse neurohypophysis, and monitor an event, as yet unknown, that is intimately associated with the release of neuropeptides (Salzberg et al., 1985; Gainer et al., 1986).

The entry of extracellular calcium is the event that triggers release in many, but not all, secretory systems; calcium influx is required for the secretion of neurotransmitters (Katz, 1969) and hormones (Douglas, 1978), and, in general, entry is effected through voltage-gated calcium channels (e.g., Hagiwara and Byerly, 1981; Tsien et al., 1987). The presence of calcium channels in intact nerve terminals has been demonstrated directly by optical recording of active calcium responses in the neurohypophysis of Xenopus (Salzberg et al., 1983; Obaid et al., 1985b), and by patch-clamp measurements in isolated secretosomes from the rat neurohypophysis (Lemos and Nowycky, 1987).

Calcium channels constitute a heterogeneous class of membrane proteins, with a variety of different channel types reported in cells from many sources (for reviews see, e.g., Fox et al., 1986; Tsien, 1986), and within cells from a single source (Fox et al., 1987). These membrane-spanning proteins exhibit different activation and inactivation kinetics, single-channel conductances, voltage and ligand dependence, and sensitivity to organic and inorganic antagonists. And, while calcium channel taxonomy has advanced rapidly in the past few years, with pharmacological characterization playing a useful role, it has not previously been possible to monitor directly the effects of organic modifiers of calcium channel activity on calcium channels in the intact nerve terminals of vertebrates. More particularly, it has not been possible to demonstrate that these agents produce their effects on the calcium channels that are responsible for the release of the secretory products of vertebrate nerve terminals. Model systems, such as synaptosomes (Nachsen and Blaustein, 1979; Reynolds et al., 1986; Yeager et al., 1987) and secretosomes (Cazalis et al., 1987) provide valuable insights into the behavior of calcium channels under some conditions, but these can only approximate the physiology in situ. Some of the results reported here

have appeared in preliminary form (Obaid and Salzberg, 1985; Obaid et al., 1985a, 1986; Salzberg et al., 1987).

METHODS

The methods that we have used for multiple site optical recording of transmembrane voltage in nerve terminals of the frog (*Xenopus laevis*, 2–4 cm) neurohypophysis have been described in detail elsewhere (Salzberg et al., 1977, 1983; Grinvald et al., 1981; Hirota et al., 1985). After decapitation, the frog's skull was opened from the dorsal surface, and the brain was transected at the level of the olfactory bulb and folded back to expose the pituitary gland on its ventral aspect. The infundibular stalk was cut, and the entire pituitary was removed and mounted in a simple Delrin chamber having a bottom consisting of a 1-mm slice of transparent Sylgard (Dow Corning Corp., Midland, MI) attached to a glass cover slip. The preparation was immobilized by means of a 50-μm tungsten wire with an etch-sharpened tip passing through the anterior pituitary into the Sylgard, and, in some instances (field stimulation, see below), a pair of suction electrodes on the lateral tips of the pars nervosa. The isolated pituitary was vitally stained by incubating it for 25 min in a 0.1-mg/ml solution of the merocyanine-rhodanine dye NK 2761 (Nippon Kankoh Shikiso Kenkyusho, Okayama, Japan) (Gupta et al., 1981; Kamino et al., 1981; Salzberg et al., 1983) in Ringer's solution (composition in millimolar: 112 NaCl, 2 KCl, 2 CaCl$_2$, 33 glucose, 15 HEPES, pH adjusted to 7.35 with NaOH), and the excess (unbound) dye was washed out of the chamber by extensive flushing with dye-free Ringer's solution before recording. All procedures were carried out at room temperature (20–24°C). Light from a 12-V 100-W tungsten-halogen lamp (64625; Halogen-Bellaphot, Osram, West Germany) was collimated, rendered quasimonochromatic with a heat filter (KG-1; Schott Optical Glass, Inc., Duryea, PA) and an interference filter (700 nm, 70 nm full width at half maximum), and focused on the preparation by means of a bright field condenser with a numerical aperture (n.a.) matched to that of the objective. Light transmitted by the preparation was collected by a high numerical aperture objective (10X, 0.4 n.a. Fluotar; Wild Heerbrug Instruments, Farmingdale, NY; 10X, 0.5 n.a., Nikon, or 20X, 0.75 n.a., Nikon Inc., Garden City, NY), modified for water immersion by sealing the front element with epoxy. The objective projected a real image of a region of the neurohypophysis onto a 12 X 12 element silicon photodiode matrix array (MD 144-0; Integrated Photomatrix Inc., Mountainside, NJ) mounted in the trinocular tube of a Zeiss UEM microscope (Carl Zeiss, Inc., Oberkochen, West Germany). Each pixel of the array detected light transmitted by a square region of the preparation 100 μm on a side (10X objective), discounting scattered photons. The photocurrents generated by the central 124 elements of the photodiode array were separately converted to voltages, AC coupled (time constant 400 ms or 3 s), and amplified as described previously (Salzberg et al., 1977; Grinvald et al., 1981; Cohen and Lesher, 1986). All of the amplifier outputs were directed to a data acquisition system based upon a PDP 11/34A computer (Digital Equipment Corp., Maynard, MA) capable of acquiring a complete 124-pixel frame every 750 μs. The rise time (10–90%) of the light measuring system was 1.1 ms. This data acquisition system for multiple site optical recording of transmembrane voltage is similar to that described by Grinvald et al. (1981) (see Cohen and Lesher, 1986) and used in a variety of experiments since (Grinvald et al., 1982; Orbach and Cohen, 1983; Senseman et al., 1983; Salzberg et al., 1983, 1985; Ross and Krauthamer, 1984; Hirota et al., 1985; Konnerth et al., 1987). Action potentials were elicited from the nerve terminals of the *Xenopus* neurohypophysis either by direct field stimulation of the terminals, or by stimulation of the axons that constitute the infundibular stalk. With the former method, short duration (300 μs) electric fields were applied directly across a population of nerve terminals by means of the platinum wires inserted in each of the suction electrodes. A maximum response was

obtained with stimuli of 100–200 V, and was independent of stimulus polarity. For infundibular stimulation, the stalk was clasped between the bared regions of a pair of teflon-coated platinum wires, and brief (500 μs) shocks were delivered to the axons of the infundibulum. In either case, the amplitude of the resulting optical signal was typically 0.2–0.3% of the transmitted light intensity at 700 nm. The preparation remained stable physiologically for at least 2 h, stimulated every 5 min, in normal Ringer's solution. Neither phototoxicity of the dye, nor photolysis (bleaching) (Cohen and Salzberg, 1978; Salzberg, 1983) was a problem in this system. Illumination for ~5 s every 5 min over a span of 2 h produced no discernible changes in the time course of the optical signals, and only a small change (20%) in their magnitude. Evidence that the optical signals obtained after staining with NK 2761 are dominated by membrane potential changes in the terminals themselves has been presented elsewhere (Salzberg et al., 1983).

Changes in light scattering from nerve terminals of mammalian neurohypophyses were obtained in a similar fashion, except that staining with a potentiometric dye was, of course, not required. Neural lobes from CD-1 mice (Charles River Breeding Laboratories, Wilmington, MA) were fixed in the recording chamber by means of etched tungsten pins (50 μm), and electrodes constructed from Teflon-covered platinum wires with their final 500 μm exposed were attached to the neural lobe at its juncture with the infundibulum. The preparation was equilibrated in oxygenated Ringer's solution (composition in millimolar: 154 NaCl, 5.6 KCl, 2.2 CaCl$_2$, 1 MgCl$_2$, 20 HEPES, 10 glucose, pH 7.4, adjusted with NaOH) (23–26°C) for 30 min before any stimulation occurred. Supramaximal stimulation of the axons of the infundibulum was effected by means of brief (0.5 ms) voltages applied between the electrodes (Salzberg et al., 1985) at 16 Hz for 400 ms. Although this stimulation pattern is not optimal for release of either arginine vasopressin or oxytocin, it is known to evoke significant release of both peptides. The protocol was repeated every 10 min. The optical system used for recording changes in light scattering, measured as transparency, was identical to that used to monitor extrinsic absorption changes in *Xenopus*, except that the interference filter had its transmission peak at 675 nm, with a full width at half maximum of 52 nm.

RESULTS

Fig. 2 A illustrates an optical recording of the normal action potential in a synchronously activated population of nerve terminals in the neurohypophysis of *Xenopus*. The record shown is actually the analogue output of a single representative element of the photodiode matrix array, photographed directly from the oscilloscope screen without signal averaging. The action potential exhibits several salient features. The early inward current associated with the fast upstroke is carried primarily by sodium, with a small but measurable calcium contribution (Salzberg et al., 1983; Obaid et al., 1985b). The repolarization results from the activation of a tetraethylammonium (TEA)-sensitive potassium efflux, and the prominent after-hyperpolarization reflects the contribution of a large charybdotoxin (CTX)-sensitive (Miller et al., 1985) calcium-mediated potassium conductance (Obaid and Salzberg, 1985; and see below). We have shown previously (Salzberg et al., 1983) that the time course of the compound action potential reproduces the voltage change in an individual nerve terminal with good fidelity; temporal dispersion in the population accounts for only ~0.5 ms of the full width at half maximum. In the results that follow, we display either the analogue or digitized output of a single representative photodiode in the array, or the digitized sum of several contiguous detectors. In no case is time averaging employed, and in all cases the criteria for selection of the element or elements

chosen include large signal amplitude to ensure good shape resolution, and long distance from the stimulating electrodes in the object plane to minimize contamination of the optical signal by stimulation artifacts. Once chosen, of course, the same elements were maintained throughout the experiment.

Organic modifiers of calcium channel behavior have been used by several workers to distinguish different calcium channel types 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. In the neurohypophysis, the magnitude of the after-hyperpolarization that follows the action potential depends upon $[Ca^{++}]_o$ (Salzberg et al., 1983), and, since we are unable to clamp the voltage in these terminals, this component of the action potential is our most sensitive indicator of the size of the inward calcium current, although it actually reflects a potassium conductance. Further evidence that the activation of a g_{K-Ca} plays a prominent role in shaping the nerve terminal action potential in vertebrates is provided by some experiments with CTX, a purified protein toxin from scorpion venom (Miller et al., 1985) that inhibits a calcium-activated potassium channel from rat skeletal muscle, incorporated into planar lipid bilayers.

Figure 1, *A* and *B* shows the effect of an 8-min exposure to 50 nM CTX. The top trace (*A*) is the action potential elicited in normal Ringer's solution by direct field stimulation. The bottom trace (*B*) illustrates the complete elimination of the after-hyperpolarization in the optical record of the nerve terminal spike. By itself, this experiment cannot distinguish between direct block of g_{K-Ca} and indirect inhibition mediated by a reduction in calcium entry. The experiment shown in *C-F*, however, provides evidence that CTX acts directly on the calcium-activated potassium conductance in these terminals, rather than indirectly by blocking calcium entry (Obaid and Salzberg, 1985). Again, *C* shows the action potential recorded optically in normal Ringer's solution. *D* shows the active calcium response (Obaid et al., 1985*b*) observed after blocking voltage-dependent sodium and potassium channels with 1 μM TTX and 5 mM TEA. The response also exhibits the characteristic after-hyperpolarization. *E* shows the effect of a 23-min exposure to 50 nM CTX. The afterpotential is again eliminated, while calcium entry is actually enhanced. At the same time, the loss of the outward current carried by the CTX-sensitive channel prolongs the duration of the response. Evidence that the active response is mediated by calcium is shown in *F*, where 2 min in 0.5 mM Cd^{++} serves to abolish completely the active response. (It should be noted that a small contribution to the active calcium response could come from TTX-insensitive, Cd^{++}-sensitive sodium channels (Jones, 1987). However, the fact that the active response is relatively insensitive to $[Na^+]_o$ (120 vs. 8 mM), compared with its sensitivity to $[Ca^{++}]_o$ (2 vs. 0.1 mM) (Fig. 3 of Obaid et al., 1985*b*) suggests that this is not a major contribution to the optical response recorded in TTX/TEA Ringer's solution).

Fig. 2 illustrates the dramatic effect on the action potential in the intact nerve terminals of Xenopus, of a 1-μM concentration of ω-conotoxin GVIA (lot No. 012169; Peninsula Laboratories, Inc., Belmont, CA). This 27–amino acid peptide (molecular weight, 3037) was originally purified from the venom of the marine snail *Conus geographus* (Olivera et al., 1985), and is now available commercially. *A* shows

an optical recording of the nerve terminal action potential in control Ringer's solution, after staining for 25 min in 0.1 mg/ml of the merocyanine-rhodanine dye NK 2761. *B* shows 1 min in 1 μM ω-conotoxin, and *C* shows the effect of 1 μM toxin after 11 min. After only 1 min, the after-hyperpolarization is reduced by 50%, and there is a small reduction in the upstroke. After 11 min, the calcium-dependent after-potential is all but gone.
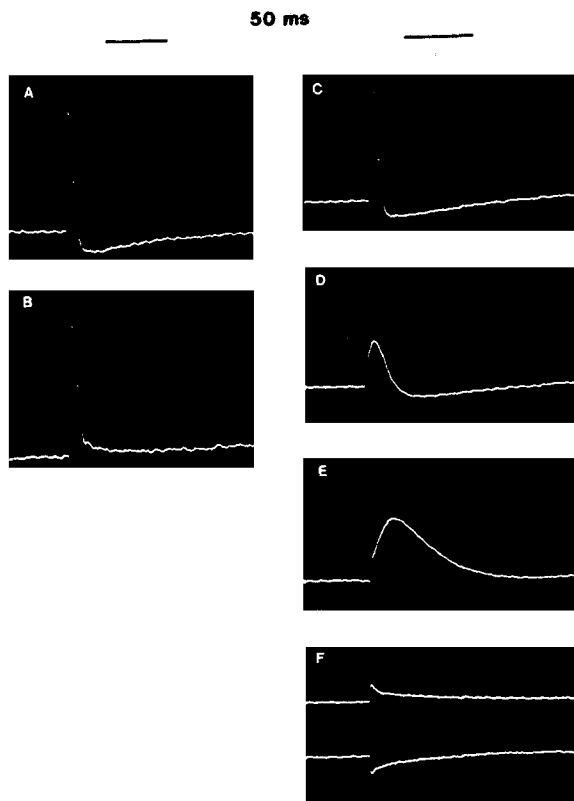
50 ms

FIGURE 1. CTX eliminates the after-hyperpolarization in the nerve terminals of the frog (*Xenopus*) neurohypophysis without blocking calcium entry. *A* shows the action potential recorded in control Ringer's solution from a neurohypophysis stained for 25 min in 0.1 mg/ml NK 2761. *B* illustrates the effect of an 8-min exposure to 50 nM CTX. The after-hyperpolarization is completely abolished. (*C*) Another experiment showing the action potential recorded in control Ringer's solution. (*D*) The active calcium response recorded 8 min after the addition of 5 mM TEA and 1 μM TTX to the Ringer's solution bathing the neurohypophysis. (*E*) The action potential recorded 23 min after the addition of 50 nM CTX to the Ringer's solution already containing 5 mM TEA and 1 μM TTX. The after-hyperpolarization of the pure calcium spike is eliminated. (*F*) The purely passive responses (both polarities) remaining after the addition of 500 μM Cd^{++} to the Ringer's solution already containing CTX, TTX, and TEA. Digitized output of a single representative element of the photodiode array: single sweep; field stimulation; 10X; 0.4 n.a.; 700 ± 35 nm, AC coupling time constant, 400 ms; response time constant, (10–90%) 1.1 ms. (*A* and *B*): Experiment PPG133, (*C–F*): experiment PPG135.

Dihydropyridines bind to high affinity sites in cardiac, smooth (see review by Miller and Freedman, 1984), and skeletal muscle (Rios and Brum, 1987), and some of them block, while at least one enhances the currents through voltage-dependent calcium channels. There is considerable disagreement in the literature, however, over the degree to which the dihydropyridines modify voltage-dependent calcium

channels in neuronal preparations (for a summary, see Rane et al., 1987). In the cell bodies of the chick dorsal root ganglion, for example, the dihydropyridines Bay-K 8644 and nifedipine increase and decrease, respectively, one component of the current carried by calcium (Nowycky et al., 1985; Fox et al., 1987; McCleskey et al., 1987), although nifedipine, at least, may require chronic membrane depolarization for binding (Cazalis et al., 1987; Rane et al., 1987; and see Discussion).

Under "natural" (i.e., unperturbed resting potential) stimulation conditions, in which a normal action potential is elicited, neither of these agents appear to have any effect upon calcium entry into intact nerve terminals. In Fig. 3, trace *A* shows an optical recording of the control action potential in the neurohypophysial terminals of *Xenopus*, and trace *B* shows that 5 μM Bay-K 8644 (Miles Laboratories, Westport,
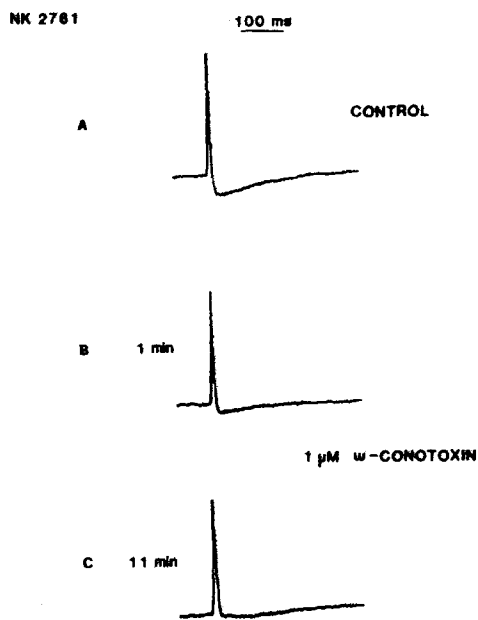

FIGURE 2. ω-Conotoxin GVIA reduces the calcium-dependent components of the nerve terminal action potential in the neurohypophysis of *Xenopus*. (A) Optical recording of the action potential in control Ringer's solution, after staining with NK 2761. (B) The effect of a 1-min exposure to 1 μM ω-conotoxin GVIA. (C) 11 min in 1 μM ω-conotoxin GVIA. Analogue output of a single representative element of the photodiode array; single sweep; infundibular stimulation; 10X; 0.4 n.a.; 700 ± 35 nm; AC coupling time constant 400 ms; response time constant (10–90%) 1.1 ms. Experiment PPG233.

CT), which increases the size of calcium currents in membranes having dihydropyridine-sensitive calcium channels (Nowycky et al., 1985) has little effect on the calcium components of the action potential after 32 min. Indeed, the decrease in the height of the upstroke, in this case 19%, was often observed in control experiments using the same 0.25% ethanol as was used to dissolve the dihydropyridines. Similarly, Fig. 3, *C* and *D* illustrates our observation that 5 μM nifedipine (#N-7634 Sigma Chemical Co., St. Louis, MO) has essentially no effect on the action potential. Fig. 3 (*E–G*) also demonstrates that in the same population of nerve terminals in which there was no effect of nifedipine (2 μM) there is a dramatic reduction in calcium entry produced by 3.3 μM ω-conotoxin GVIA. *E* shows the control action

100 ms

A

CONTROL

B

5 μM BAY-K 8644

100 ms

C

CONTROL

D

5 μM NIFEDIPINE

100 ms

E

CONTROL

F

2 μM NIFEDIPINE

G

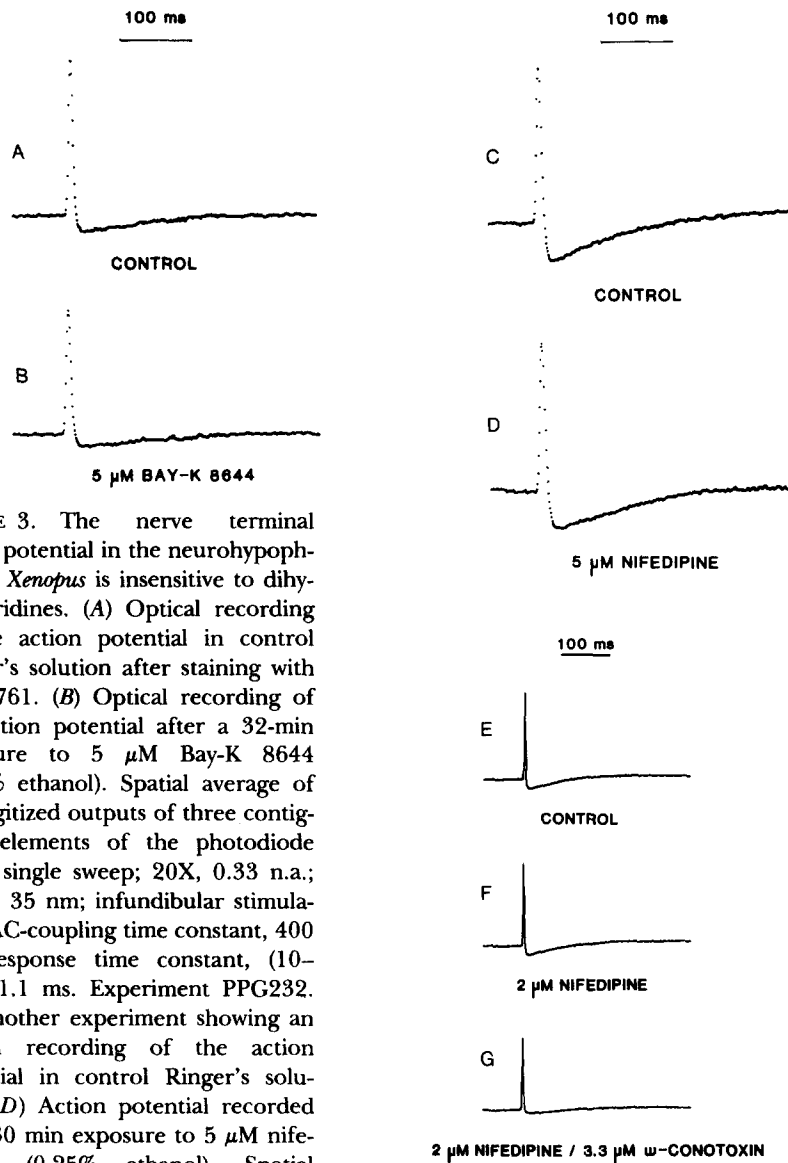**2 μM NIFEDIPINE / 3.3 μM ω-CONOTOXIN**

FIGURE 3. The nerve terminal action potential in the neurohypophysis of *Xenopus* is insensitive to dihydropyridines. (A) Optical recording of the action potential in control Ringer's solution after staining with NK 2761. (B) Optical recording of the action potential after a 32-min exposure to 5 μM Bay-K 8644 (0.25% ethanol). Spatial average of the digitized outputs of three contiguous elements of the photodiode array: single sweep; 20X, 0.33 n.a.; 700 ± 35 nm; infundibular stimulation, AC-coupling time constant, 400 ms; response time constant, (10–90%) 1.1 ms. Experiment PPG232. (C) Another experiment showing an optical recording of the action potential in control Ringer's solution. (D) Action potential recorded after 30 min exposure to 5 μM nifedipine (0.25% ethanol). Spatial average of the digitized outputs of four contiguous elements of the photodiode array: single sweep; 20X; 0.33 n.a.; 700 ± 35 nm; infundibular stimulation; AC-coupling time constant, 400 ms; response time constant, (10–90%) 1.1 ms. Experiment PPG231. (E) Another experiment showing an optical recording of the action potential in control Ringer's solution. (F) Optical recording of the action potential after 30 min exposure to 2 μM nifedipine (0.1% ethanol). (G) Optical recording of the action potential 10 min after the addition of 3.3 μM ω-conotoxin to the 2 μM nifedipine Ringer's solution. Analogue output of a single representative element of the 124-element photodiode array: single sweep; 10X; 0.4 n.a.; 700 ± 35 nm; infundibular stimulation; AC-coupling time constant, 400 ms; response time constant, (10–90%) 1.1 ms. Experiment PPG235.

potential, F shows that after 30 min in 2 μM nifedipine, the action potential in the terminals is unchanged, and G shows that just 10 min after exposure to 3.3 μM ω-conotoxin and 2 μM nifedipine, the after-hyperpolarization is reduced by at least 60%. That the snail venom is acting directly on the calcium conductance, and not on the calcium-activated potassium conductance, is demonstrated by the decrease (not shown) in the amplitude of the active calcium response elicited in the presence of 5 mM TEA/1 μM TTX.

Thus, ω-conotoxin dramatically reduced the component of the nerve terminal action potential that is most sensitive to calcium entry, while dihydropyridines such as nifedipine have no effect under normal (i.e., action potential) conditions of stimulation. A very interesting question remains, viz., whether the calcium channels that are affected by ω-conotoxin are those required for normal secretion from these terminals.

Optical changes exhibited by the neurohypophysis of the mouse allow us to answer this question. Excitation of the magnocellular neuron terminals located in the neurohypophyses of some mammals is accompanied by large and rapid changes in light scattering, measured as transparency (Salzberg et al., 1985), and these intrinsic optical signals are closely correlated with the secretion of the neuropeptide arginine vasopressin in for example, the posterior pituitary of the CD-1 mouse (Gainer et al., 1986). Fig. 4 A illustrates a typical light-scattering signal during stimulation of an unstained mouse neurohypophysis at 16 Hz for 400 ms. The optical response to an individual stimulus consists of at least two separable components. A rapid upstroke (increase in large angle light scattering and decrease in transmitted light intensity), termed the E-wave (Salzberg et al., 1985), signals the arrival of excitation in the terminals, while a large, long-lasting decrease in scattered intensity, the S-wave, reflects some aspect of the secretory event itself. The E-wave appears to include components that depend upon both current and voltage (Cohen et al., 1972a, b; Salzberg et al., 1985) and is unrelated to secretion.

The S-wave is intimately associated with the release of neuropeptides from these terminals. This long duration change in the intrinsic optical properties of vertebrate nerve terminals has already been shown (Salzberg et al., 1985) to exhibit properties that are characteristic of neurosecretory systems in general, and the release of neurohypophysial peptides in particular, viz., dependence on stimulation frequency (with marked facilitation), dependence on $[Ca^{++}]_o$, and sensitivity to Ca^{++} antagonists and to various interventions (e.g., D_2O substitution for water) known to influence secretion.

The record in Fig. 4 B was obtained after 18 min exposure to a Ringer's solution containing 5 μM ω-conotoxin GVIA. After seven stimuli, the reduction in the size of the cumulative S-wave was ~50%. C (inset) shows that this steady state value is, in fact, reached after only 8 min, since there is no further reduction at 18 min (traces $b(2)$, see figure legend), despite the slow onset of binding reported in other preparations (Abe and Saisu, 1987; Hirning et al., 1988). This strongly suggests that ω-conotoxin blocks calcium channels that are required for neuropeptide release in the intact nerve terminals of at least this vertebrate neurohypophysis.

Figure 4, D and E shows one of the experiments in which we attempted to observe the effects of dihydropyridine calcium channel modifiers on the light-scattering sig-

nal derived from the neurosecretory terminals of the mouse. *D* shows the intrinsic optical change recorded during a single stimulus train applied in normal Ringer's solution. *E* shows the result of a stimulus train after 22 min in 5 μM nifedipine. No effect is observed, and this result is consistent with the absence of any effect of 5 μM nifedipine on the action potential in the neurohypophysial nerve terminals of the frog. Similarly, Bay-K 8644, at 5 μM concentration 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.
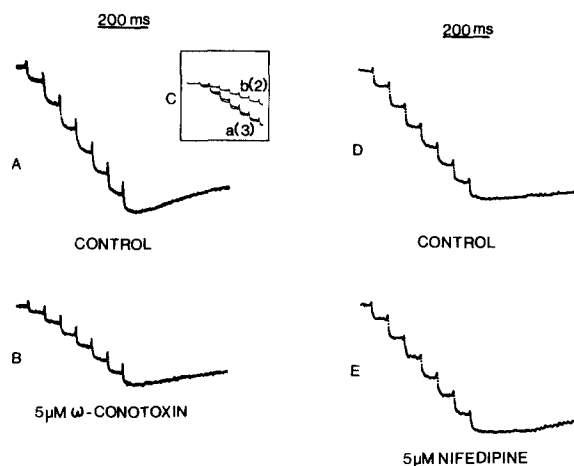

FIGURE 4. Intrinsic optical signals that accompany secretion from intact nerve terminals in the mouse neurohypophysis are sensitive to ω-conotoxin GVIA (5 μM), and relatively insensitive to nifedipine. (*A*) Light-scattering signal resulting from stimulation of the infundibulum of an unstained mouse (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 μM ω-conotoxin GVIA. ω-conotoxin GVIA blocks some calcium channels required for secretion from these terminals. (*Ca*) The three (*3*) superimposed traces represent three consecutive controls, recorded at 10-min intervals; (*b*) the two (*2*) superimposed traces indicate the effect of 8 and 18-min exposures, respectively, to 5 μM ω-conotoxin GVIA. Spatial average of the digitized outputs of 12 contiguous elements of the photodiode array: single sweep; 10X; 0.5 n.a.; 675 ± 26 nm; infundibular stimulation; AC-coupling time constant, 3 s; response time constant, (10–90%) 1.1 ms. Experiment MPP133. (*D*) Another experiment showing the 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. (*E*) Light-scattering signal after a 22-min exposure to a Ringer's solution containing 5 μM nifedipine (0.05% ethanol). Spatial average of the digitized outputs of four contiguous elements of the photodiode array: single sweep; 10X; 0.4 n.a.; 675 ± 26 nm; infundibular stimulation; AC coupling time constant, 3 s; response time constant, (10–90%) 1.1 ms. Experiment MPP154.

Because our optical recording techniques require highly synchronous activation, achievable only by electrical stimulation of the neurosecretory terminals, we are unable to duplicate the extreme depolarizations used by others. Nonetheless, we attempted to examine whether chronic depolarization results in dihydropyridine block of Ca^{++} channels in this preparation. Although fast potentiometric dyes (e.g., NK 2761) provide no information about resting membrane potential (Cohen and Salzberg, 1978), and the records shown here are all AC-coupled, we believe that we

were able to achieve a steady-state depolarization of ~25 mV by increasing $[K^+]_o$ from 5.6 to 15 mM in a series of light-scattering experiments using the mouse neurohypophysis. Increased extracellular potassium (15 mM), per se, strongly enhanced the light-scattering signals, particularly the first S-wave in a train. If calcium channels in these terminals are dihydropyridine sensitive, nifedipine should decrease the potassium effect, and Bay-K should increase it. Because the various mechanisms for potassium homeostasis might be expected to reduce gradually the magnitude of the induced depolarization, we decided to examine the effects of nifedipine and Bay-K 8644 application both before, and after elevation of $[K^+]_o$. In the first case, the dihydropyridine would be present at the membrane, and available for binding, from the onset of depolarization. The largest effect was, indeed, obtained when potassium depolarization was induced after equilibration of the neurohypophysis with nifedipine (30 min). Nifedipine's effect was to reduce the magnitude of the potassium enhancement by no more than 15%. Because the potassium effect alone was large and variable, the effect of nifedipine in the depolarized preparation was difficult to quantify, and was frequently insignificant. In one experiment, in which $[K^+]_o$ was increased after 30 min of equilibration in 5 μM Bay-K 8644, the effect of this dihydropyridine was negligible. Of course, we cannot rule out larger effects under more extreme depolarizations, but these conditions are incompatible with excitability.

DISCUSSION

The results presented here demonstrate directly the effect of the peptide toxin ω-conotoxin GVIA on the calcium channels involved in the release of neuropeptides from the intact nerve terminals of two vertebrates. This component of the venom of the marine snail *Conus geographus* blocks calcium channels that normally open during the nerve terminal action potential, and, as a consequence, reduces the size of a light-scattering change that reflects the release of arginine vasopressin and oxytocin. Although this is the first direct evidence from measurements on intact nerve terminals, it is consistent with the findings of others using patch clamp of neuronal preparations (Fox et al., 1987; McCleskey et al., 1987) and model systems such as synaptosomes (Reynolds et al., 1986; Abe and Saisu, 1987; Yeager et al., 1987).

The reader may question the seemingly strange juxtapositions of the two vertebrate preparations used in the experiments reported here. Several considerations dictated our choice of species. First, the optical changes associated with transmitter release are not observed in *Xenopus*. For this reason, the extrinsic absorption signal in the amphibian is uncontaminated by any intrinsic optical changes. In the mouse, on the other hand, there are three difficulties in reliably measuring pharmacological effects on the nerve terminal action potential. (a) The light-scattering signal occurs at all visible wavelengths, and is, itself, affected by the agents applied. It is not possible to separate, for example, effects on the after-hyperpolarization from effects on the S-wave of the intrinsic signal. (b) The geometry of the mouse neurohypophysis, in contrast to that of the frog, does not permit direct field stimulation of the terminals, and so, active calcium responses cannot be observed. (c) The requirement, in the mammalian preparation, for nearly continuous oxygenation results in substan-

tial phototoxicity, which is not found in the frog. It should be noted, however, that whenever any intervention modifies a calcium-dependent component of the action potential in *Xenopus*, we observe a corresponding modification of the S-wave of the light-scattering signal in the mouse. Also, the observation that the intrinsic optical signal in the mouse is extremely sensitive to the extracellular calcium concentration (Salzberg et al., 1985), and that ω-conotoxin reduces the amplitude of the very component of the light-scattering change that requires calcium entry (S-wave), are strong though indirect evidence for the presence of ω-conotoxin–sensitive calcium channels in this system (not necessarily identical to those in *Xenopus*). The incomplete block by ω-conotoxin that we observe, in steady state, may be explained either by the presence of more than one type of calcium channel, or by differences in the conditions of application, penetration, concentration, nonspecific binding, and affinity (McCleskey et al., 1987).

The relative 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). In isolated secretory nerve endings from the rat neurohypophysis, however, Cazalis et al., (1987) have shown that while nitrendipine and nicardipine inhibited the potassium stimulated release of arginine vasopressin and oxytocin, and Bay-K 8644 produced a strong augmentation of arginine vasopressin release, nifedipine had no effect. The results of Cazalis et al. (1987) were obtained under conditions of chronic high potassium depolarization, and are therefore not entirely comparable with our results if dihydropyridine binding is both voltage and time dependent, as suggested by Rane et al. (1987). Indeed, these authors concluded that chronic depolarization is required to achieve nifedipine block of calcium channels in neurons of the chick dorsal root ganglion, but is not required for Bay-K 8644 action (Holz et al., 1988). Our results with nifedipine and Bay-K 8644 suggest that neurohypophysial calcium channels are either insensitive to these agents, or that dihydropyridine-sensitive calcium channels in these nerve terminals, if they exist, are a minority and play a relatively insignificant role in normal secretion of arginine vasopressin and oxytocin.

It may be argued that the lack of any sensitivity to the class of dihydropyridine compounds in our hands could be attributed to the effects of photo-inactivation during the high intensity illumination required for optical recording. However, the extrinsic absorption changes used to monitor the nerve terminal action potential in the frog were recorded at 700 ± 35 nm, far outside the absorption peak for nifedipine (λ_{max} = 350 nm), and even the light-scattering changes were observed at 675 ± 26 nm. It is very unlikely, therefore, that there was significant photolysis of the active compound (Morad et al., 1983).

The experiments we have described constitute the first direct observation of the effect of organic modifiers of calcium channels on the action potential in the intact nerve terminals of vertebrates. Because both the electrical events in the terminals, and an intrinsic optical signal that is intimately related to neuropeptide release, can be monitored with millisecond time resolution, it should be possible to use these optical techniques to better understand the mechanisms of excitation-secretion coupling.

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