Active Calcium Responses Recorded Optically from Nerve Terminals of the Frog Neurohypophysis

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ABSTRACT Voltage-sensitive dyes were used to record by optical means membrane potential changes from nerve terminals in the isolated frog neurohypophysis. Following the block of voltage-sensitive Na^+ channels by tetrodotoxin (TTX) and K^+ channels by tetraethylammonium (TEA), direct electric field stimulation of the nerve terminals still evoked large active responses. These responses were reversibly blocked by the addition of 0.5 mM $CdCl_2$. At both normal and low $[Na^+]_o$, the regenerative response appeared to increase with increasing $[Ca^{++}]_o$ (0.1–10 mM). There was a marked decrease in the size of the response, as well as in its rate of rise, at low $[Ca^{++}]_o$ (0.2 mM) when $[Na^+]_o$ was reduced from 120 to 8 mM (replaced by sucrose), but little if any effect of this reduction of $[Na^+]_o$ at normal $[Ca^{++}]_o$. In normal $[Ca^{++}]_o$, these local responses most probably arise from an inward Ca^{++} current associated with hormone release from these nerve terminals. At low $[Ca^{++}]_o$, Na^+ appears to contribute to the TTX-insensitive inward current.

INTRODUCTION

Potentiometric dyes that function as molecular indicators of membrane potential have recently made possible optical recording of action potentials from populations of fine (0.5–1.0 μm) nerve terminals in the neurohypophysis of *Xenopus* (Salzberg et al., 1983). In that study, evidence was obtained which suggested that Ca^{++} enters the nerve terminal during the action potential and leads to a calcium-mediated increase in K^+ conductance. It was also suggested that the inward Ca^{++} current contributes to the action potential upstroke because a small regenerative potential change remained after block of the inward Na^+ current with TTX. These observations are of interest because of the central role that Ca^{++} influx plays in the secretion of neurotransmitters (Katz, 1969) and hormones (Douglas, 1978). Calcium spikes have been recorded after the block of both the voltage-

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sensitive Na^+ conductance with tetrodotoxin (TTX) and the K^+ conductance with tetraethylammonium (TEA) in the presynaptic axon of the squid giant synapse (Katz and Miledi, 1969), in growth cones of cultured neurons (Grinvald and Farber, 1981), and in rat pars intermedia cells (Douglas and Taraskevich, 1980; see also Hagiwara and Byerly, 1981). In the experiments reported here, we have used optical methods (Cohen and Salzberg, 1978; Salzberg, 1983) to examine the voltage change produced by electrical field stimulation of the nerve terminals of the neurohypophysis following block of voltage-dependent Na^+ and K^+ channels.

METHODS

The experimental arrangement used for optical recording of potential changes in the nerve terminals of the frog (*Xenopus laevis*, 2–4 cm) neurohypophysis was similar to that previously described in detail (Salzberg et al., 1983; see also Salzberg et al., 1977) and is shown schematically in Fig. 1*A*. After decapitation, the frog's skull was opened on the dorsal surface and the brain was lifted back to expose the pituitary. The infundibular stalk was transected and the entire pituitary was removed and mounted on transparent Sylgard (Dow Corning Corp., Midland, MI) by placing a pin through the anterior pituitary while immobilizing the lateral tips of the neurohypophysis with the suction electrodes required for field stimulation (Fig. 1*B*). The isolated pituitary was vitally stained by incubating it for 25 min in a 0.1-mg/ml solution of the merocyanine-rhodanine dye NK2761 (Kamino et al., 1981; Gupta et al., 1981; Salzberg et al., 1983) in Ringer's solution (composition [mM]: 112 NaCl, 2 KCl, 2 $CaCl_2$, 33 glucose, 15 HEPES, pH adjusted to 7.35 with NaOH) and then superfused with dye-free Ringer's solution before recording. In some of the experiments, 112 mM NaCl was replaced by 200 mM sucrose. Solutions with reduced $[Ca^{++}]_o$ had Mg^{++} added to maintain the divalent cation concentration at 2 mM. All procedures were carried out at room temperature (18–22°C). Light transmitted by the preparation was collected by a water immersion objective (× 10, 0.4 N.A., Wild Heerbrug Instruments, Farmingdale, NY), which formed a real image of a region of the neurohypophysis on a silicon photodiode mounted in the trinocular tube of a Zeiss UEM microscope. The photocurrent was converted to voltage (Salzberg et al., 1977) and the AC-coupled (time constant 400 ms) output was recorded with a digital oscilloscope (TN 1500, Tracor Northern, Middleton, WI). The rise time of the light-measuring system was 1.1 ms (10–90%) and the digitized signal was stored on magnetic tape for display and subsequent analysis. Action potentials were elicited from the nerve terminals by applying brief (0.3-ms) electric fields across the population of nerve terminals by means of the suction electrodes. The amplitude of the optical spike increased with field strength, as expected, since a large number of nerve terminals were stimulated and monitored. A maximal response was obtained with stimuli of 100–200 V and was independent of stimulus polarity. The amplitude of the response was typically 0.3% of the transmitted light intensity at 700 nm. The preparation appeared to remain stable physiologically for at least 2 h, stimulated every 5 min, under normal ionic conditions. Prolonged exposure to either low $[Ca^{++}]_o$ (0.1–0.2 mM) or low $[Na^+]_o$ (8 mM, sucrose substitution) accelerated the deterioration of the preparation, as evidenced by spike broadening upon return to normal conditions. Neither phototoxicity of the dye nor photolysis (bleaching) (Cohen and Salzberg, 1978) 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 signal and only a small (20%) decrease in the magnitude of the optical signal. In order to avoid the effects of $[Ca^{++}]_o$, primarily on threshold, supramaximal

stimulation conditions were determined and the stimulus intensity required to produce a maximal active response was kept constant throughout the experiment. Evidence that these optical responses are dominated by membrane potential changes in the terminals themselves has been presented elsewhere (Salzberg et al., 1983).

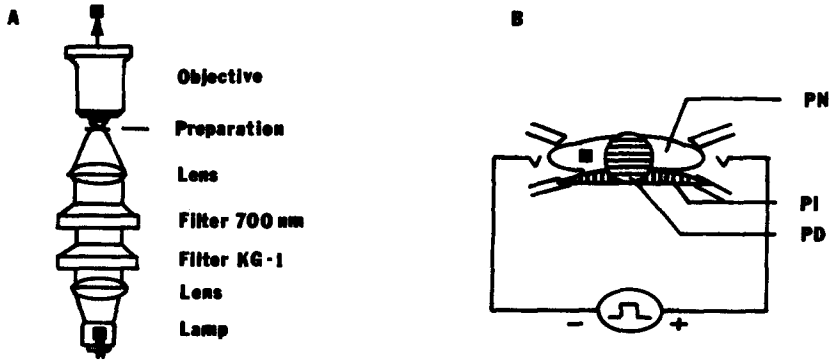

FIGURE 1. Experimental arrangement. (*A*) Schematic drawing of the optical system. Light from a tungsten-halogen lamp was collimated, rendered quasimonochromatic with a heat filter (KG-1) and an interference filter (700 nm; 70 nm full width at half-maximum), and focused on the preparation using a bright-field condenser with a numerical aperture (N.A.) matched to that of the objective. A high-numerical-aperture objective (× 10; 0.4 N.A.), modified for water immersion, projects a real image of a portion of the preparation onto the central 124 elements of a 144-element photodiode matrix array, whose photocurrent outputs are converted to voltages, AC coupled (time constant 400 ms), and amplified, multiplexed, digitized, and stored in the memory of a PDP 11/34A computer (Digital Equipment Corp., Marlboro, MA) under direct memory access (DMA). A full frame is recorded, at an effective resolution of 18 bits, every 0.8 ms. A single channel, corresponding to a region of the pars nervosa, is selected for detailed study. (*B*) Diagram of the preparation. The isolated pituitary, consisting of the anterior pituitary (pars distalis, PD), pars intermedia (PI), and posterior pituitary (pars nervosa, PN), was held between two suction electrodes. Direct field stimulation of the terminals of the pars nervosa was achieved by applying a voltage (80–150 V, 0.3 ms) between the platinum wires inserted in each of the electrodes. The black square indicates a typical region of the neurohypophysis monitored by the selected element of the photodiode array in the image plane. It contains thousands of nerve terminals.

RESULTS

Effects of TTX, TEA, and Cd

The records shown in Fig. 2 are from one of the 24 posterior pituitaries studied in the presence of both TEA and TTX. Fig. 2*A* shows a control action potential from a population of nerve terminals. The action potential exhibits a rapid upstroke that is primarily the result of a fast inward sodium current, a rapid repolarizing phase resulting from a K^+ efflux, and an afterhyperpolarization that depends upon a calcium-mediated increase in K^+ conductance (Salzberg et al.,

1983). The action potential shown in Fig. 2*B* illustrates the effect of blocking the voltage-dependent K^+ conductance by the addition of 5×10^{-3} M TEA. The spike was prolonged and, in most experiments, a hump appeared on the falling phase, possibly because of repetitive firing of some of the terminals. The addition

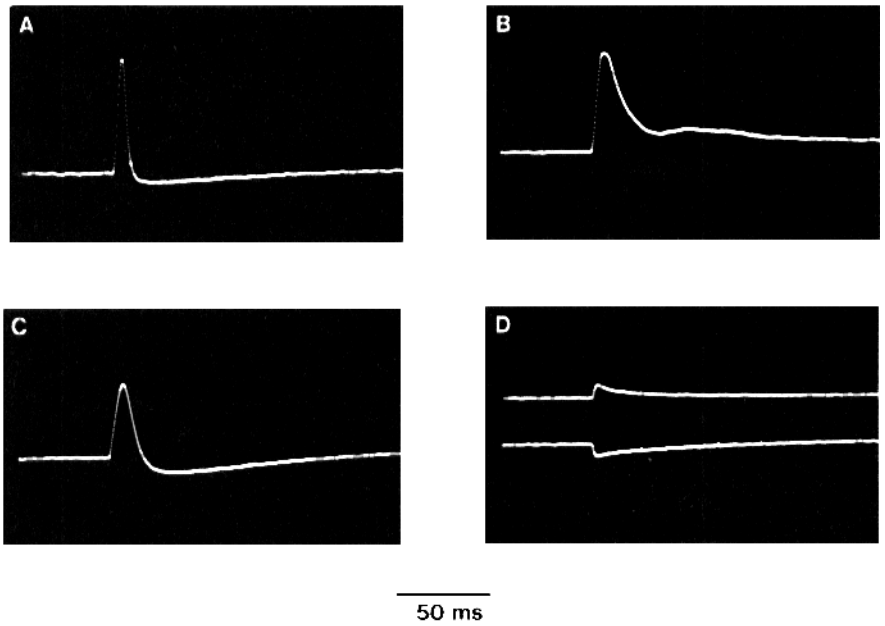

FIGURE 2. Cd sensitivity of active responses in the presence of TTX and TEA. Optical recording of action potentials in nerve terminals of the frog neurohypophysis stained with 0.1 mg/ml of NK2761 for 25 min. Records of changes in light transmission by stained nerve terminals in response to electrical field stimulation. In this and the following figure, the output of a typical single channel of the multiple-site optical recording system is shown. (*A*) Action potential recorded in normal Ringer's solution. (*B*) Action response of the nerve terminals after 17 min exposure to 5×10^{-3} M TEA. (*C*) Active response of the nerve terminals after 37 min exposure to 5×10^{-3} M TEA and 20 min exposure to 2×10^{-6} M TTX. (*D*) Passive response remaining 15 min after the addition of 5×10^{-4} M Cd^{++} to the TTX-TEA Ringer's solution bathing the preparation, upon stimulation with normal and reversed polarity. The change in transmitted light intensity at 700 nm was ~0.3% for the control record. The preparation was stimulated approximately every 5 min. The rise time of the light-measuring system was 1.1 ms (10–90%). Temperature: 18–22°C.

of a high concentration of TTX (2×10^{-6} M), sufficient to block voltage-sensitive Na^+ channels, abolished the hump (Fig. 2*C*) and revealed an afterhyperpolarization that presumably resulted from the activation of a Ca-mediated K^+ conductance. The further addition of 5×10^{-4} M Cd^{++} to the bath (Fig. 2*D*) completely blocked the remaining active response. Low levels of Cd^{++} do not block the action potentials in the nerve terminals of the neurohypophysis in the

absence of TTX (Salzberg et al., 1983). The residual optical signals shown in Fig. 2*D* represent the passive electrotonus, as demonstrated by their symmetric response to stimulus polarity. The combined effects of TEA and Cd^{++} could generally be reversed, but it was never possible to reverse completely the effect of TTX.

Ca^{++} and Na^{+} Dependence

The relative contributions of Ca^{++} and Na^{+} to the active response were determined qualitatively by recording optical signals at different $[Ca^{++}]_o$ at high ($n = 7$) and low $[Na^{+}]_o$ ($n = 2$), and at different $[Na^{+}]_o$ at high ($n = 4$) and low $[Ca^{++}]_o$ ($n = 2$). These experiments were all carried out in the presence of 10^{-6} M TTX and 5×10^{-3} M TEA. The results are illustrated in Fig. 3. Panel *A* shows that at normal $[Na^{+}]_o$, both the amplitude and rate of rise of the upstroke, as well as the amplitude of the afterhyperpolarization, increased as $[Ca^{++}]_o$ increased. This is consistent with the idea that Ca^{++} carries inward current in the presence of both TTX and TEA.

Fig. 3*B* shows that at reduced extracellular concentrations of Na^{+}, a decrease in $[Ca^{++}]_o$ from normal 2 (trace *a*) to 0.2 mM (trace *b*) eliminated virtually all of the active response. A comparison of trace *b* in Fig. 3*B* (low Ca, low Na) with trace *c* in Fig. 3*A* (low Ca, normal Na) suggests that at low $[Ca^{++}]_o$, Na^{+} might contribute to the inward current conducted by TTX-insensitive channels (Kostyuk et al., 1983; Hess and Tsien, 1984).

Fig. 3*C* illustrates the optical responses in the presence of 1 μM TTX and 5 mM TEA, in low Ca^{++} (0.2 mM, Mg^{++} substitution) at the following Na^{+} concentrations: (*a*) 120 mM (normal) and (*b*) 8 mM (sucrose substitution). These records demonstrate that at low $[Ca^{++}]_o$, the active phase of the response was markedly Na^{+} sensitive. On the other hand, at normal $[Ca^{++}]_o$ (Fig. 3*D*), varying $[Na^{+}]_o$ from 120 (trace *a*) to 8 mM (trace *b*) had little effect on the rate of rise of the optical signal, as if Na ions were relatively unsuccessful in competing with normal concentrations of Ca^{++}. One cannot ignore, however, the overall decrease in the size of the active response in Fig. 3*D* when Na^{+} is replaced by sucrose. Thus, it appears that, particularly in low $[Ca^{++}]_o$, Na ions can carry depolarizing current through these Cd-sensitive (TTX- and TEA-insensitive) channels. Fig. 3*D* raises another issue, however: traces *a* and *b* cross during the repolarizing phase, which indicates that at normal $[Ca^{++}]_o$, the afterhyperpolarization was bigger at normal $[Na^{+}]_o$ than at low $[Na^{+}]_o$. This finding was surprising, since one would expect a larger undershoot when more Ca^{++} is available to activate the Ca-dependent K^{+} conductance, and this situation should prevail when there are fewer Na ions to compete with Ca^{++} for the same channels. This decrease in $[Na^{+}]_o$ was accompanied by an important decrease in ionic strength, which may explain the result. (Attempts to replace Na^{+} by other cations [TMA, Tris] in an effort to keep ionic strength constant resulted in an inexcitable preparation. Thus, we do not have independent evidence of the effect of changing ionic strength per se. Furthermore, our methods [see Discussion] do not permit us to record changes in resting potential that may result from changes in the ionic composition of the solutions and could alter the action potential shape.)

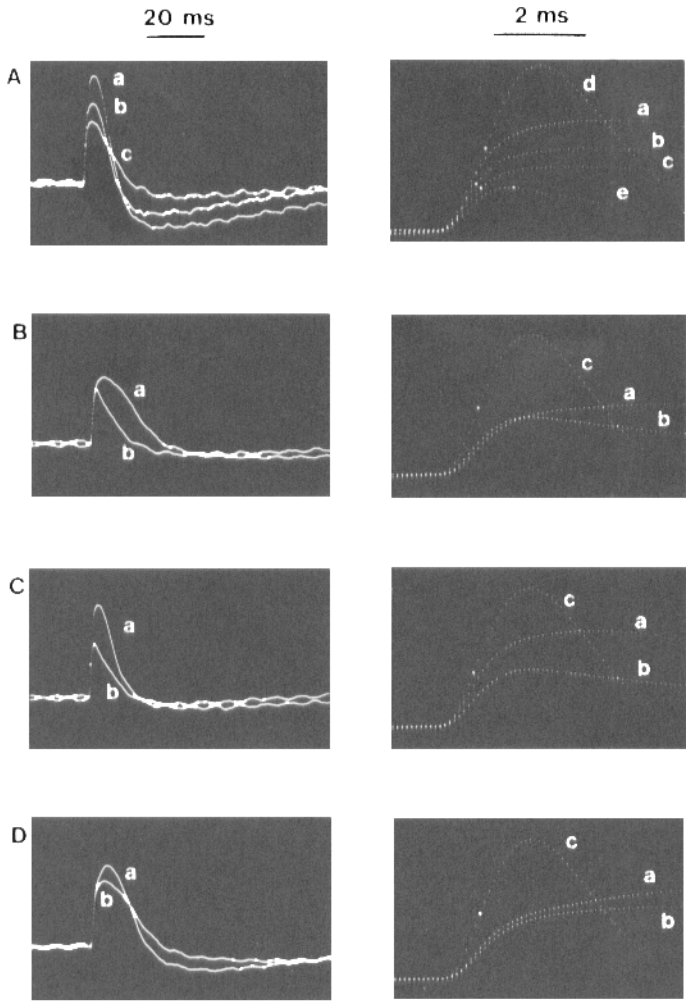

FIGURE 3. Ca and Na dependence of the active response after blocking the voltage-sensitive Na and K channels with TTX and TEA. (*A*) Left side: optical responses in the presence of 10^{-6} M TTX and 5×10^{-3} M TEA at normal Na concentration (120 mM) and the following Ca concentrations (for the times indicated): (*a*) 10 mM (19 min); (*b*) 2 mM (20 min); (*c*) 0.1 mM (1.9 mM Mg substitution, 20 min). Right side: traces *a*, *b*, and *c* are the same records as those shown on the left side, after expanding the time axis. An initial control response (trace *d*), obtained in normal Ringer's solution, and the passive response (trace *e*), obtained 10 min after the addition of 0.2 mM Cd^{++} to the bathing solution containing 10^{-6} M TTX and 5×10^{-3} M TEA, are shown for comparison. (*B*) Left side: optical responses in the presence of 10^{-6} M TTX and 5×10^{-3} M TEA, at low Na concentration (8 mM, sucrose substitution) and the following Ca concentrations (for the times indicated): (*a*) 2 mM (16 min); (*b*) 0.2 mM (1.8 mM Mg^{++} substitution, 21 min). Right side: traces *a* and *b* are the same records as those shown on the left side, after expanding the time axis. An initial control action potential (trace *c*) obtained in normal Ringer's

DISCUSSION

A molecular probe of membrane potential has been used to monitor the shape of the action potential from populations of synchronously activated neurosecretory terminals of the isolated frog neurohypophysis. The results reported here demonstrate that when voltage-sensitive sodium and potassium channels are blocked with TTX and TEA, direct field stimulation of the nerve terminals evokes regenerative calcium responses that are smaller and slower than the normal action potential. These responses are sensitive to extracellular calcium concentration and are reversibly eliminated by the addition of 0.5 mM Cd^{++}. Their dependence on $[Ca^{++}]_o$ and sensitivity to Cd^{++} block indicate that they are likely to result from a voltage-sensitive calcium influx into the terminals (Hagiwara and Byerly, 1981; Kostyuk and Krishtal, 1977), which probably mediates hormone release. The behavior of the active component of the responses, when the sodium concentration was varied in the presence of low $[Ca^{++}]_o$, suggests that Na ions may move through these calcium channels under special conditions.

An optical measurement of membrane potential is equivalent, at least in a limited sense, to a transmembrane electrode measurement. Indeed, under special conditions (e.g., Salzberg and Bezanilla, 1983), the optical measurement may be preferred. The limitations on that equivalence depend, of course, on the preparation and must be considered here. First, the optical measurement, in general, lacks an absolute voltage calibration (Cohen and Salzberg, 1978; Salzberg, 1983). In a multicellular preparation, this difficulty is compounded when the number of active elements may vary. In order to minimize the effects of this variation (e.g., from changes in threshold), tissue stimulation must be kept supramaximal. Second, optical measurements are most frequently AC coupled, in order to subtract electronically the large background light levels. As a consequence, knowledge of DC potential levels is lost, even if an absolute calibration is available. Both of these limitations are significant in the present report and restrict the scope of our conclusions. We are forced to assume that the number of terminals activated under our field stimulation conditions (see Methods) is constant and that resting potentials do not change significantly during the course of an experiment (for which there may be independent evidence), and we can attach

solution is shown for comparison. (*C*) Left side: optical responses in the presence of 10^{-6} M TTX and 5×10^{-3} M TEA, at low Ca concentration (0.2 mM, 1.8 mM Mg^{++}) and the following Na concentrations (for the times indicated): (*a*) 120 mM (normal, 21 min); (*b*) 8 mM (sucrose substitution, 21 min). Right side: traces *a* and *b* are the same records as those shown on the left side, after expanding the time axis. An initial control action potential (trace *c*), obtained in normal Ringer's solution, is shown for comparison. (*D*) Left side: optical responses in the presence of 10^{-6} M TTX and 5×10^{-3} M TEA, at normal Ca concentration (2 mM) and the following Na concentrations (for the times indicated): (*a*) 120 mM (normal, 16 min); (*b*) 8 mM (sucrose substitution, 21 min). Right side: traces *a* and *b* are the same records as those shown on the left side, after expanding the time axis. An initial control action potential (trace *c*), obtained in normal Ringer's solution, is shown for comparison. Rise time of the light-measuring system: 1.1 ms (10–90%). Temperature: 18–22°C.

no absolute magnitude to the voltage changes recorded optically. On the other hand, the time course of the optical change faithfully reproduces the time course of the voltage change across the membrane and, in the absence of significant bleaching, successive optical signals from the same loci can be compared ratiometrically. Indeed, under constant conditions, these signals are remarkably stable.

In the experiments reported here, the silicon photodiode in the image plane of the objective is actually one of 124 elements of a photodiode matrix array forming the detector portion of a multiple-site optical recording of transmembrane voltage (MSORTV) system (Grinvald et al., 1981; Salzberg et al., 1983). The major portion of the neurohypophysis is imaged onto this array and, at the beginning of an experiment, a single representative detector element is selected and this locus is retained throughout the subsequent trials. Elements close to the location of the stimulating electrode are never selected, as these may exhibit artifacts that are unrelated to membrane voltage. The passive optical responses, however, recorded in the presence of TTX, TEA, and Cd^{++} by detector elements well removed from the image of the stimulating electrodes, represent true electrotonic potential changes and exhibit the same wavelength dependence as that of the optical signal representing the normal action potential. The calcium-mediated active responses described here appear to have properties in common with the local subthreshold action potentials described by Hodgkin (1938) in unmyelinated crab nerve. Thus, with TTX blocking any propagated action potential in the axons of the infundibulum (Salzberg et al., 1983), the small active calcium currents cannot excite a sufficient length of nerve to produce a propagated action potential. These results may be viewed as providing additional evidence for the existence of a higher density of calcium channels in the specialized membrane of nerve terminals (Katz and Miledi, 1969).

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