Micromolar 4-Aminopyridine Enhances Invasion of a Vertebrate Neurosecretory Terminal Arborization

Optical Recording of Action Potential Propagation Using an Ultrafast Photodiode-MOSFET Camera and a Photodiode Array

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ABSTRACT Modulation of the amount of neuropeptide released from a neurosecretory tissue may be achieved by different means. These include alterations in the quantity secreted from each active nerve terminal or in the actual number of terminals activated. From the vertebrate hypothalamus, magnocellular neurons project their axons as bundles of fibers through the median eminence and infundibular stalk to arborize extensively and terminate in the neurohypophysis, where the neurohypophysial peptides and proteins are released into the circulation by a Ca-dependent mechanism. Elevating $[Ca^{2+}]_o$ increases the magnitude of an intrinsic optical change in the neurohypophysial terminals that is intimately related to the quantity of neuropeptide released. Similarly, the addition of micromolar concentrations of 4-aminopyridine to the bathing solution enhances this change in large angle light scattering. However, we show here that, while these effects are superficially similar, they reflect different mechanisms of action. Evidence from intrinsic optical signals (light scattering) and extrinsic (potentiometric dye) absorption changes suggests that calcium increases the amount of neuropeptide released from each active terminal in the classical manner, while 4-aminopyridine exerts its secretagogue action by enhancing the invasion of action potentials into the magnocellular neuron's terminal arborization, increasing the actual number of terminals activated. Physiologically, electrical invasion of the complex terminal arborization in the neurohypophysis may represent an extremely sensitive control point for modulation of peptide secretion. This would be especially effective in a neurohaemal organ like the posterior pituitary, where, in contrast with a collection of presynaptic terminals, the precise location of release is less important than the quantity released.

INTRODUCTION

Rapid optical detection of the secretory event at vertebrate nerve terminals can provide important information about the mechanism(s) by which neurosecretion is regulated. Large and rapid intrinsic optical signals accompany and immediately follow the action potential in the peptidergic terminals of the neurohypophysis of the CD-1 mouse (Salzberg et al., 1985) and other mammals. These signals, recorded as variations in transmitted light intensity (transparency) in the image plane of a compound microscope, reflect changes in large angle light scattering, and are intimately related to the neurosecretory process (Salzberg et al., 1985; Gainer et al., 1986). An initial, transient component (E-wave) of the optical signal heralds the arrival of the action potential in the terminals, and a larger, sustained component (S-wave) monitors an early, unidentified event in the secretion of the neurohypophysial peptides. This latter change in the intrinsic optical properties of the terminals (Salzberg et al., 1985) is covariant, in sign and magnitude, with direct measures of neuropeptide secretion, e.g., radioimmunoassay of arginine vasopressin (Gainer et al., 1986) and oxytocin (Bondy et al., 1987) release, during a variety of experimental interventions. These include changes in stimulus frequency and in $[Ca^{2+}]_o$, water substitution by D_2O, and the addition of

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calcium channel blockers or micromolar concentrations of 4-aminopyridine. Of these, elevation of $[Ca^{2+}]_o$ and addition of 4-AP are classical enhancers of neurotransmitter and neuropeptide release (Katz, 1969; Douglas, 1963; Douglas and Poisner, 1964; Dreifuss et al., 1971; Heuser et al., 1979; Heuser and Reese, 1981) and are generally thought to share a common mechanism, viz., increasing intraterminal free calcium during and immediately after the arrival of the action potential. In the case of 4-AP, the effect is presumed to be mediated through a broadening of the spike resulting from blockade of one or more types of K-channels. However, at low micromolar concentrations (10–50 μM) of the drug, prolongation of the duration of the action potential requires long exposure while the effect on the light scattering signal is almost immediate. Analysis of intrinsic (light scattering) and extrinsic (potentiometric dye) signals from neurohypophysial terminals of mice (CD-1) and frogs (*Xenopus laevis*) suggests that extracellular calcium and exogenous 4-AP act in strikingly different fashions. Data presented here indicate that while raising $[Ca^{2+}]_o$ from 2 to 5 mM increases the release of neuropeptide from each active terminal, micromolar 4-AP acts instead by increasing the number of active terminals through a more complete invasion of the terminal arborization, without affecting significantly the amount of release from each depolarized terminal. This effect of 4-AP exemplifies a novel mechanism of secretagogue action, and, possibly, physiological control, viz., modulation of the spatiotemporal pattern of invasion of a highly ramified terminal arborization.

Some of this work has previously been published in abstract form (Obaid et al., 1983, 1987; Salzberg et al., 1984; Obaid and Salzberg, 1995).

METHODS

The methods that we have used for recording intrinsic optical signals from mammalian nerve terminals are essentially identical to those described in Salzberg et al. (1985) and Obaid et al. (1989). Our methods for multiple site optical recording of transmembrane voltage (MSORTV) using potentiometric probes (Cohen and Salzberg, 1978) have been described elsewhere (Salzberg et al., 1977, 1983; Grinvald et al., 1981; Hirota et al., 1985; Cohen and Lesher, 1986). For multiple site optical recording of transmembrane voltage (MSORTV) in nerve terminals of the frog (*Xenopus laevis*, 2–4 cm body length), the pituitary gland was removed 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 on its ventral aspect. Usually, 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 thick slice of transparent Sylgard (Dow Corning Corp., Midland, MI) attached to a glass cover slip. (In some instances, the infundibulum was left intact and the gland was removed together with a portion of the attached hypothalamus.) The preparation, consisting usually of pars nervosa (neurohypophysis) and pars intermedia (collectively, the neurointermediate lobe), together with the pars distalis (anterior pituitary), was immobilized by means of 50 μm tungsten wires with etch sharpened tips passing through the anterior pituitary into the Sylgard, and, in the case of 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 100-μg/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 a 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 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, Germany; run at currents between 8.5 and 9 A), was collimated, rendered quasi-monochromatic with a heat filter (KG-1; Schott Optical Glass, Inc., Duryea, PA) and an interference filter (usually 700 nm, and either 10 or 70 nm full width at half maximum [FWHM]), and focused on the preparation by means of a bright field condenser with a numerical aperture (na) matched to that of the objective. Light transmitted by the stained preparation was collected by a high numerical aperture objective (10×, 0.4 na Fluotar; Wild Heerbrug Instruments, Farmingdale, NY; 10×, 0.5 na, Nikon, or 20×, 0.75 na, Nikon, Inc., Garden City, NY) which was modified for water immersion by sealing the front element with epoxy. The objective projected a magnified real image of a region of the neurohypophysis onto an image dissector mounted atop the trinocular tube of a Zeiss compound microscope (either a UEM upright or an IM-35 inverted model; Carl Zeiss, Inc., Oberkochen, Germany). As an image dissector, we used either a 12 × 12 element silicon photodiode matrix array (MD 144-0; Centronic, Inc., Newbury Park, CA) or a differential photodiode-MOSFET camera (HR Deltaron 1700, Fuji Photo Film Co., Japan) described originally by Ichikawa et al. (1993) and recently employed by Tanifuji et al. (1994).

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 on a PDP 11/34A computer (Digital Equipment Corp., Maynard, MA) capable of acquiring a complete 124-pixel frame every 750 μs. The rise time of the light measuring system (10–90%) was 1.1 ms. This data acquisition system for MSORTV is similar to that described by Grinvald et al. (1981) and used in a variety of experiments since (e.g., Senseman et al., 1983; Salzberg et al., 1983, 1985; Konnerth et al., 1987; Obaid et al., 1985, 1989; Parsons et al., 1991, 1992; Cinelli and Salzberg, 1990, 1992).

The Fuji camera (Deltaron) is based on a sensor chip (Fuji Photo Film Co., Japan) having 16,384 photodiode pixels (128 × 128), with MOSFET gated outputs (Ichikawa et al., 1993). In this device, the light-generated electrical signal on each pixel is output to one of eight signal lines by switching a series of MOSFET gates ON using a set of vertical and horizontal shift registers. The output signals are multiplexed into a single data stream, and an

8-bit full frame buffer is used to store a reference image. During operation, the current data stream is directed to one arm of a differential amplifier, where it is compared with the synchronous analogue output obtained from digital-to-analogue conversion of the reference frame, and the difference is amplified and converted to digital form at high speed. In this manner, the camera can operate at frame rates of up to 1.7 kHz, since the difference signal is digitized at only 8-bit resolution.

Used with a 20× objective, each pixel of the photodiode array detected light transmitted by a square region of the neurohypophysis ~50 μm on a side, discounting scattered photons. The Deltaron camera had a linear spatial resolution approximately 20 times greater than this; with the same objective, each pixel had a receptive field that was roughly 2.5 μm on a side. To avoid saturation of the camera's first stage, neutral density filters were employed, together with a narrow bandwidth (10 nm FWHM) interference filter.

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. For field stimulation of the terminals (Obaid et al., 1985, 1989) short duration (300–500 μs) electric fields were applied across a population of terminals by means of platinum wires inserted into each of the suction electrodes. Maximal responses were typically obtained with shocks of 100–200 V, and were independent of stimulus polarity. For stimulation of the axons at the level of the infundibulum (Salzberg et al., 1983), the stalk was clasped between the bared regions of a pair of teflon-insulated platinum wires, and brief (500 μs) shocks were delivered to the axons. In either case, the amplitude of the extrinsic optical change was ~0.2–0.5% of the steady state light intensity at 700 ± 35 nm. The preparations were stable for hours under these conditions and neither phototoxicity of the dye, nor photolysis (bleaching) (Cohen and Salzberg, 1978; Salzberg, 1983) was a problem in this system. Evidence that the extrinsic optical signals obtained after staining with NK 2761 are dominated by membrane potential changes in the terminals themselves has been presented earlier (Salzberg et al., 1983).

Changes in light scattering from mammalian neurohypophysial terminals were recorded in a very similar fashion, except that staining was not required (Salzberg et al., 1985). Neurointermediate lobes from CD-1 mice (Charles River Breeding Laboratories, Wilmington, MA) were fixed to the Sylgard bottom of the optical recording chamber by means of etched 50 μm tungsten pins, and a pair of teflon-covered platinum wires, with ≤500 μm exposed, were apposed 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) for 30 min before the beginning of any experiment. Supramaximal stimulation of the axons of the infundibulum was achieved by means of brief (500 μs) voltage pulses applied between the electrodes (Salzberg et al., 1985) at either 10 or 16 Hz, usually for 400 ms. This protocol was generally repeated every 10 min. The optical systems used for recording the changes in light scattering, measured as changes in transparency, were identical to those used for monitoring the extrinsic potentiometric signals in *Xenopus*, except that the interference filter had its transmission maximum at 675 nm, with a full width at half maximum of 52 nm. This choice of filter is not critical in view of the relatively weak wavelength dependence of the light scattering signals (Salzberg et al., 1985).

RESULTS

Fig. 1 *A* shows the intrinsic optical changes, measured as transmitted light intensity at 675 ± 26 nm, exhibited by the unstained neurohypophysis of a CD-1 mouse, in normal Ringer's solution, during stimulation of the infundibular stalk at 16 Hz for 400 ms. These changes in large angle light scattering (Salzberg et al., 1985), recorded by a single representative element of a 124 element photodiode matrix array from a region of the neurohypophysis 100 μm on a side, are closely correlated with the secretion of the neuropeptide arginine vasopressin (Gainer et al., 1986). The optical response to each of the six stimuli consists of at least two separable components. A rapid upstroke (an increase in large angle light scattering and a decrease in transmitted light intensity), referred to as the E-wave (Salzberg et al., 1985) marks the arrival of the propagated action potential (and action currents) in the terminals, while a large, long-lasting decrease in scattered intensity, the S-wave, monitors some step in the secretory process that ensues. The E-wave itself appears to mingle components that depend upon both current and voltage (Cohen et al., 1972*a*,*b*; Salzberg et al., 1985) and is unrelated to secretion, but provides a measure of the number of terminals that are activated (see below). The S-wave, however, is intimately related to peptide release from these terminals; the long duration change in the intrinsic optical properties of the terminals that it represents has previously been shown to exhibit prop-

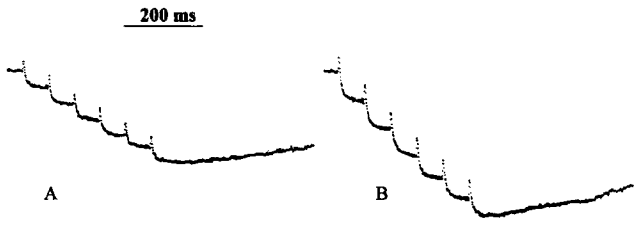

FIGURE 1. Micromolar 4-aminopyridine enhances the light scattering change associated with the secretion of neuropeptides by the mouse neurohypophysis. (*A*) Changes in transmitted light intensity at 675 ± 26 nm, recorded in normal Ringer's solution, without averaging, by a single element of a 124 element photodiode matrix array following stimulation to the axons of the infundibular stalk at 16 Hz for 400 ms. The detector element monitored light transmitted by a region of the unstained neurohypophysis 100 μm on a side. An increase in light intensity (decrease in large angle light scattering) is plotted downward. At each stimulus, the initial rapid upstroke is the E-wave and the sustained downward deflection is the S-wave. (*B*) Same as *A*, but after 35 min in 10 μM 4-AP. Objective 10×, 0.4 na. AC coupling time constant 3 s; rise time of the light measuring system (10–90%), 1.1 ms. Temperature, 23°C. Calibration bar 200 ms. MPP041.

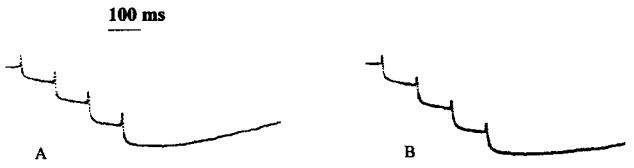

FIGURE 2. Effects of elevated $[Ca^{2+}]_o$ on the light-scattering changes recorded from the nerve terminals of the mouse neurohypophysis. (*A*) Light-scattering changes at 675 ± 26 nm accompanying stimulation at 10 Hz of an unstained mouse neurohypophysis in normal Ringer's solution (2.2 mM Ca^{2+}). (*B*) Light-scattering changes in the same preparation 40 min after exposure to a Ringer's solution containing 10 mM Ca^{2+}. Both records are the averages of 16 sweeps. Objective 10×, 0.4 na. AC coupling time constant 3 s; rise time of the light measuring system (10–90%), 1.1 ms. Temperature, 24°C. Calibration bar, 100 ms. MPP054.

erties that are typical of neurosecretory systems in general, and of the mammalian neurohypophysis in particular, viz., dependence on stimulation frequency, with facilitation and depression (Salzberg et al., 1985), dependence on $[Ca^{2+}]_o$ (Salzberg et al., 1985), sensitivity to Ca^{2+} channel blockers (Obaid et al., 1989) and to aminoglycoside antibiotics (Parsons et al., 1992), as well other interventions known to influence secretion (e.g., D_2O substitution for water) (Salzberg et al., 1985; Gainer et al., 1986).

Fig. 1 *B* shows the light scattering signals recorded by the same element of the photodiode array after the neurohypophysis had been bathed for 35 min in a Ringer's solution containing 10 μM 4-AP. The average increase in transmitted light intensity (S-wave) after exposure to 4-AP, is ~56% larger than that observed in control Ringer's solution.

Fig. 2 illustrates a very similar experiment, in which extracellular calcium is raised from its normal concentration (2.2 mM) to 10 mM. *A* shows the light scattering signals recorded by a single photodetector during 10 Hz stimulation of a mouse neurohypophysis in normal Ringer's solution. *B* demonstrates the effect of raising $[Ca^{2+}]_o$ to 10 mM. Once again, an increase in the magnitude of the individual S-waves is observed, although the effect here appears less dramatic than in the case of 4-AP. Qualitatively, however, the effects of elevated calcium ion and micromolar concentration of 4-aminopyridine seem similar. Calcium ion has other effects, however, including a direct effect on excitability (Frankenhaeuser and Hodgkin, 1957), and these need to be considered. Thus, to eliminate the effects of threshold variation, the stimulation used in the experiment illustrated in Fig. 2 was supramaximal. Examination of the optical signals still reveals significant differences between the amplitudes of the E-waves that precede each S-wave in Fig. 2, *A* and *B*. Previously, we showed (Salzberg et al., 1985) that the E-wave signals the arrival of the action potential at the terminals of the neurohypophysis, and we argued that, as a compound optical signal, the amplitude of this very early intrinsic optical change, under constant stimulation conditions, is roughly

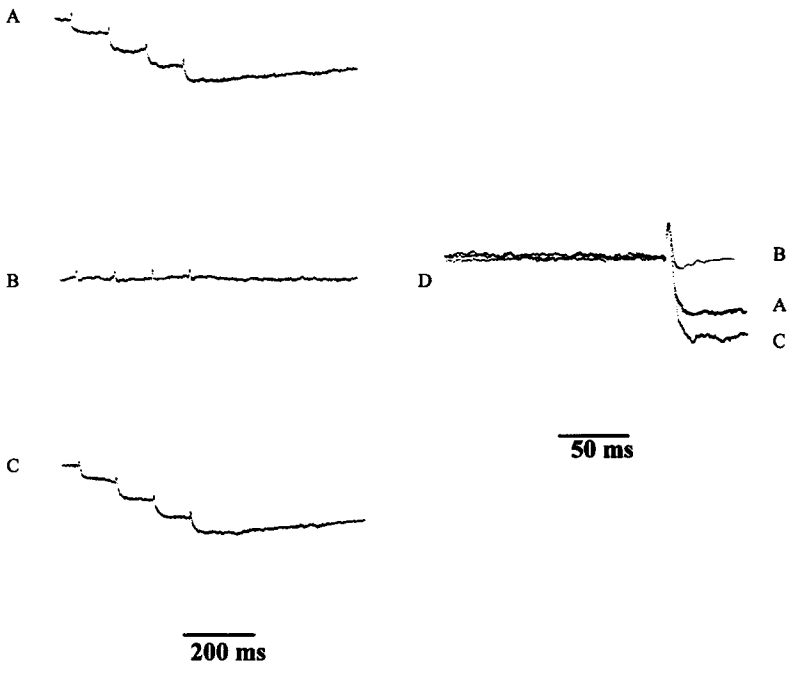

FIGURE 3. The effect of $[Ca^{2+}]_o$ on the amplitude of the S-wave of the light-scattering signal in the mouse neurohypophysis, when account is taken of the number of active terminals. (*A*) Light-scattering changes at 675 ± 26 nm accompanying stimulation at 10 Hz of an unstained mouse neurohypophysis in normal Ringer's solution (2.2 mM Ca^{2+}). (*B*) Light-scattering changes in the same preparation 50 min after a reduction in $[Ca^{2+}]_o$ to 0.1 mM by Mg^{2+} substitution. (*C*) Light-scattering changes accompanying stimulation at 10 Hz in the same preparation 30 min after an increase in $[Ca^{2+}]_o$ to 5 mM. Records *A–C* were each recorded without averaging. The magnitude of the fast component (E-wave) of the light-scattering signal remains constant during each trial, but varies inversely with $[Ca^{2+}]_o$. (*D*) Averages of 16 trials recorded under the same conditions as traces *A–C*. These records were obtained immediately after the corresponding single trains, and are shown here normalized to the height of the E-wave (see text). The normalization factor for each trace is given in parentheses: trace *A* (1.00); trace *B* (0.72); trace *C* (1.16). Objective 10×, 0.4 na. AC coupling time constant 3 s; rise time of the light measuring system (10–90%), 1.1 ms. Temperature, 24–26°C. Calibration bars 200 ms (*left*) and 50 ms (*right*). MPP057.

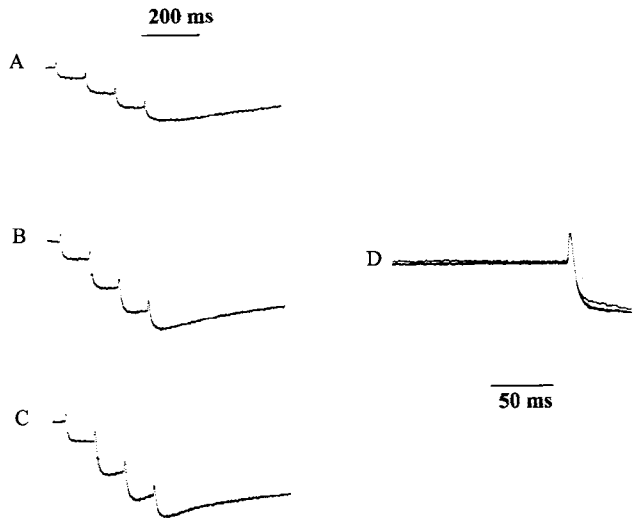

FIGURE 4. The effect of 4-AP differs from the effect of $[Ca^{2+}]_o$ when account is taken of the number of active terminals. (*A*) Light-scattering changes at 675 ± 26 nm accompanying stimulation at 10 Hz of an unstained mouse neurohypophysis in normal Ringer's solution. (*B*) Light scattering changes in the same preparation 50 min after the addition of 20 μM 4-AP to the Ringer's solution. (*C*) Light-scattering changes accompanying stimulation at 10 Hz in the same preparation 60 min after the addition of 50 μM 4-AP to the Ringer's solution. Note that the magnitude of the fast component (E-wave) of the light-scattering signal increases with 4-AP concentration. (*D*) Averages of 16 trials, recorded under the same conditions as traces *A–C*. These records were obtained immediately after the corresponding single trains, and are shown here normalized to the height of the E-wave. The traces are essentially indistinguishable. 4-AP appears to increase the slow component (S-wave) of the light-scattering signal by increasing the effective number of active terminals. The normalization factor for each trace is given in parentheses: trace *A* (1.00); trace *B* (0.78); trace *C* (0.68). Objective 10×, 0.4 na. AC coupling time constant 3 s; rise time of the light measuring system (10–90%), 1.1 ms. Temperature, 24–26°C. Calibration bars 200 ms (*left*) and 50 ms (*right*). MPP056.

proportional to the number of terminals activated at a given time. We can again use this comparative measure of the size of the active population to compensate for changes in the excitability of the tissue resulting from changes in $[Ca^{2+}]_o$, by normalizing the S-wave according to the size of the initial E-wave (c.f. Salzberg et al., 1985).

Fig. 3 illustrates the dramatic effect of extracellular calcium concentration on the light scattering signal elicited by electrical stimulation, when the records are normalized to the height of the first peak (E-wave) to compensate for differences in the extent of tissue activation. Here, single sweeps are shown (*A–C*) during stimulation at 10 Hz for 400 ms in different extracellular calcium concentrations. *A* shows the raw light scattering signal in normal Ringer's solution (2.2 mM Ca^{2+}). *B* shows the reduction in the size of the S-wave 50 min after reduction of $[Ca^{2+}]_o$ to 100 μM, with Mg^{2+} substitution, and *C* shows the optical change 30 min after an increase in $[Ca^{2+}]_o$ to 5 mM. *D* shows the averages of 16 trials recorded under the identical conditions as the traces in *A–C*. The records in *D* were obtained immediately after the corresponding single trains, and are shown here normalized to the height of the E-wave. The effect of $[Ca^{2+}]_o$ on the light scattering signal (S-wave) per active terminal, is demonstrated very clearly. There is complete block of the S-wave in very low Ca^{2+}, and enhancement of the S-wave in elevated Ca^{2+}. (Very similar effects were observed on secretion by Gainer et al., 1986.)

The identical analysis applied to the effect of micromolar concentrations of 4-aminopyridine on the light scattering signal from the neurohypophysis yields a strikingly different result, however. Fig. 4 shows an experiment in which the effects of 20 and 50 μM 4-AP are observed. Again, single sweeps are shown (*A–C*) during 10 Hz stimulation for 400 ms in normal Ringer's solution (Fig. 4 *A*) and in 20 μM (Fig. 4 *B*) and 50 μM 4-AP (Fig. 4 *C*). There is, as in Fig. 1, a dramatic increase in the size of the intrinsic optical signal with the addition of 4-AP, but, in contrast with the Ca^{2+} data in Fig. 3, the magnitude of the fast component (E-wave) of the light scattering change also increased with 4-aminopyridine concentration. In Fig. 4 *D*, we show the averages of 16 sweeps, recorded immediately after each of the individual trials in different Ringer's solutions, but these records are normalized to the number of active terminals estimated from the size of the initial E-wave. Here, we find a very different result from that seen when $[Ca^{2+}]_o$ levels are manipulated; viz., there appears to be no effect of 4-AP per active terminal, but, rather, there seems to be an increase in the number of active terminals. This is clear in Fig. 4, *B* and *C*, where the E-waves are very obviously larger than in the control (Fig. 4 *A*). It is as though 4-AP acts by an entirely different mechanism from calcium, namely an enhanced invasion of the highly ramified terminal arborization of the neurohypophysis.

If the preceding explanation of the 4-AP effect is correct, we would expect that when the entire photodiode array is used to record the light scattering changes from a large portion of the neurohypophysis, each pixel would record a larger intrinsic signal, and, the S-wave would be detected over a larger spatial domain. Fig. 5 illustrates such an experiment, in which the extent of invasion of the terminal arborization of the neurohypophysis is increased in 50 μM 4-AP. Fig. 5 *A* (*left*) shows the changes in transmitted light intensity at 675 ± 26 nm, recorded without averaging by each of the 124 elements of the photodiode array after stimulation of the axons of the infundibular stalk at 16 Hz for 400 ms.

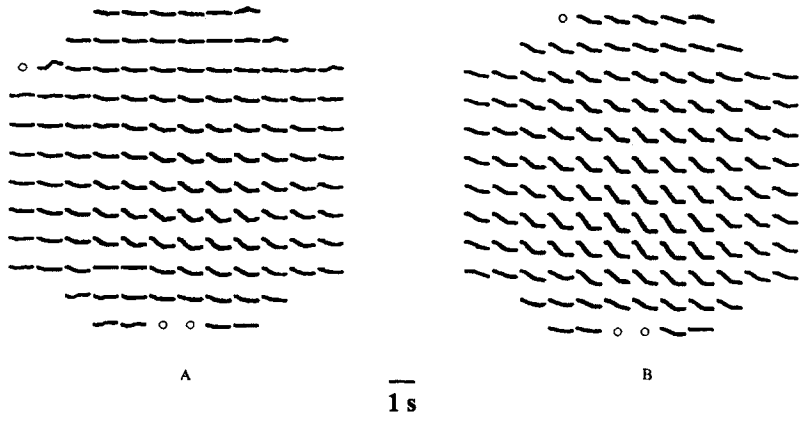

FIGURE 5. The extent of invasion of the terminal arborization of the neurohypophysis is increased in micromolar 4-aminopyridine. Changes in light-scattering at 675 ± 26 nm recorded without averaging by each of the 124 elements of the photodiode array after stimulation of the axons of the mouse infundibular stalk at 16 Hz for 400 ms. The displays represent the changes in light-scattering from many sites, plotted against time. The individual traces are each 1 s records of the intrinsic optical signal resulting from the train of stimuli delivered beginning 50 ms after the start of each trace. The small open circles mark detector elements that were defective. (*A*) Multiple site recording of changes in light-scattering in normal Ringer's solution. (*B*) Multiple site recording after 22 min in a Ringer's solution containing 50 μM 4-AP. Note that identical stimulation produces a much wider spatial spread of activation in the presence of 4-AP, as well as an enhanced light-scattering change in a given region of the mouse neurohypophysis. Objective 20×, 0.75 na. AC coupling time constant 3 s; rise time of the light measuring system (10–90%), 1.1 ms. Temperature, 23–25°C. MPP134.

The display exhibits the changes in light scattering (trains of alternating E-waves and S-waves) from many sites, each plotted against time, in normal Ringer's solution. The data shown in Fig. 5 *B* (*right*) were recorded in the same fashion, after the neurohypophysis had been bathed for 22 min in a Ringer's solution containing 50 μM 4-AP. Notice that identical stimulation produces a much wider spatial spread of activation in the presence of 4-AP, as well as an enhanced light scattering change in a given region of the neurohypophysis, consistent with a mechanism that reduces branchpoint failure and increases the invasibility of this highly ramified structure.

If micromolar concentrations of 4-aminopyridine increase peptide release in a neurosecretory structure like the vertebrate neurohypophysis by causing a more complete invasion of its terminal arborization, we would predict that there would be a large increase in the magnitude of the compound action potential recorded optically from the neurohypophysial terminals in the presence of 4-AP. Optical recordings of the action potential from the terminals of the CD-1 mouse neurohypophysis, using the extrinsic absorption changes exhibited by voltage-sensitive dyes (Salzberg et al., 1985; Obaid et al., 1989), are contaminated by the intrinsic optical signals associated with excitation and secretion (E-wave and S-wave). However, in the frog, *Xenopus laevis*, there are no light scattering signals associated with activation of the terminals of the neurohypophysis, and the optical signals obtained from this tissue upon stimulation, after staining with the merocyanine-rhodanine dye NK 2761, are a pure measure of the compound action potential. That is to say, the nerve terminal optical spike from the frog neurohypophysis is a population recording of the intracellular action potential, uncontaminated by any light scattering signal, whose amplitude is proportional to the membrane area undergoing rapid depolarization and, hence, to the number of active terminals. For this reason, we examined the effect of low concentrations (10–100 μM) of 4-AP on the amplitude of the compound action potential recorded from the neurohypophysis of *Xenopus laevis* during a single brief stimulus delivered to the infundibular stalk in a preparation that had been stained for 25 min in a frog Ringer's solution containing 100 μg/ml NK 2761 (Kamino et al., 1981; Gupta et al., 1981; Salzberg et al., 1983). Fig. 6 illustrates the progressive increase in the amplitude of the population spike in the presence of

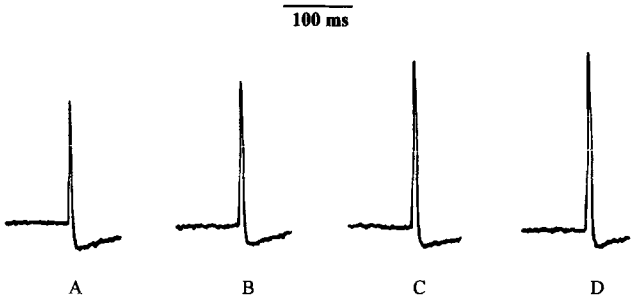

FIGURE 6. Progressive increase in the magnitude of the compound action potential in the nerve terminals of the frog (*Xenopus laevis*) neurohypophysis produced by 50 μM 4-aminopyridine. The individual signals represent changes in transmitted light intensity (extrinsic absorption) at 700 ± 35 nm detected without averaging by a single representative element of the 124-element photodiode array during field (suction electrode) stimulation. The neurohypophysis had been stained for 25 min with 100 μg/ml of the merocyanine-rhodanine dye NK 2761. (*A*) A single compound action potential recorded in normal Ringer's solution. An upward deflection of the trace represents a decrease in the transmitted light intensity. (*B–D*) Compound action potentials recorded optically after, respectively, 11, 21, and 31 min in 50 μM 4-AP. Objective 20×, 0.75 na, AC coupling time constant 400 ms; rise time of the light measuring system (10–90%), 1.1 ms. Temperature, 23°C. Calibration bar, 100 ms. PPG250.

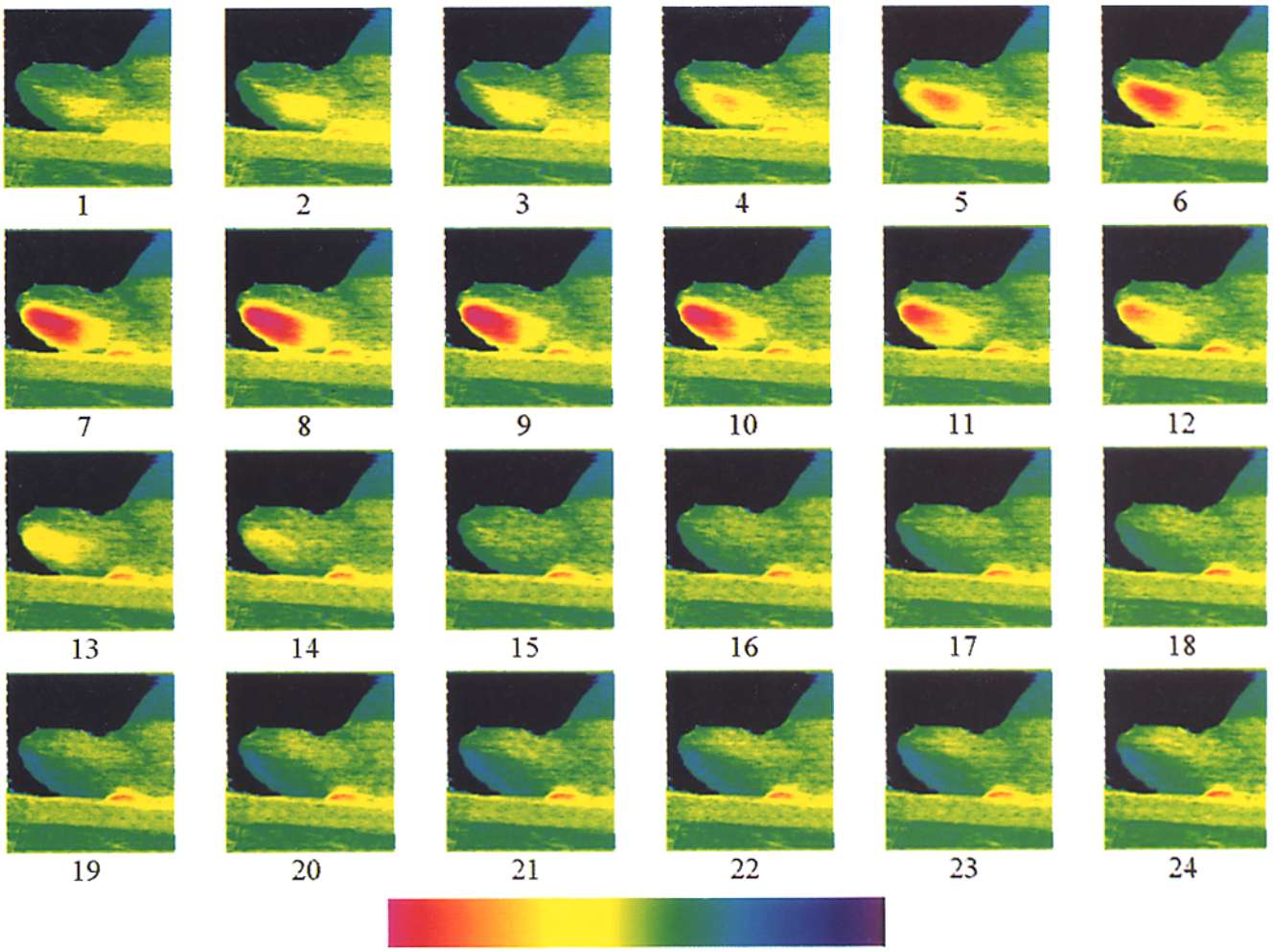

FIGURE 7. Invasion of the terminal arborization of the frog neurohypophysis by the action potential in a normal Ringer's solution containing 4 mM Ca^{2+}. The display shows 24 consecutive frames from a 512 frame sequence recorded by the HR Deltaron 1700 photodiode-MOSFET camera. A single 500-μs shock was delivered to the infundibular stalk between frames 2 and 3. The pseudocolor display of the differential image is an 8-bit linear scale, where the maximum change in transmitted light intensity is ~0.5% and a reddening of the image corresponds to a depolarization and an increase in dye absorption (decrease in transmitted light intensity). Objective 10×, 0.4 na. Camera gain = 1; amplifier gain = 400. Temperature, 22°C. Average of 16 sweeps. PPFROG21/2.

50 μM 4-AP. The action potential on the left (*A*) was recorded in normal Ringer's solution. After 11 min in 50 μM 4-AP (*B*), the upstroke of the action potential is increased by ~17%, and, by 31 min (*D*), the upstroke has increased in amplitude 45% over control. These values are inconsistent with a proportional increase in the size of the action potential in individual terminals, and most likely represent a progressive increase in the number of active terminals. At the lowest concentrations (not shown), a broadening of the spike wasn't observed for at least 15 min while the effect on the height was immediate.

The optical signals shown in Fig. 6 were recorded by a single representative detector of the 124 element photodiode array. And, while spatially resolved recordings of the population action potential obtained with the full array (not shown) provided direct evidence for enhanced invasion of the terminal branches of the frog neurohypophysis, similar to the light scattering evidence seen in the mouse, the spatial resolution of the array (50 μm in the object plane using a 20× objective) was usually not sufficient to reveal differences in the spatial pattern of invasion.

To examine more closely the effect of micromolar concentrations of 4-AP on the pattern of invasion of the terminal arborization of frog and mouse neurohypophyses, we employed a novel ultrafast photodiode-MOSFET camera, the HR Deltaron 1700 (Fuji Photo Co., Inc.) having square pixels 70 μm on a side, corresponding to receptive fields in the object plane 2.5 μm on a side, when employed with a 20 × objective. Figs. 7 and 8 illustrate the effect that a 20-min exposure to 50 μM 4-AP has on the spatiotemporal pattern of excitation in the neurohypophysis of *Xenopus*. Fig. 7 shows a

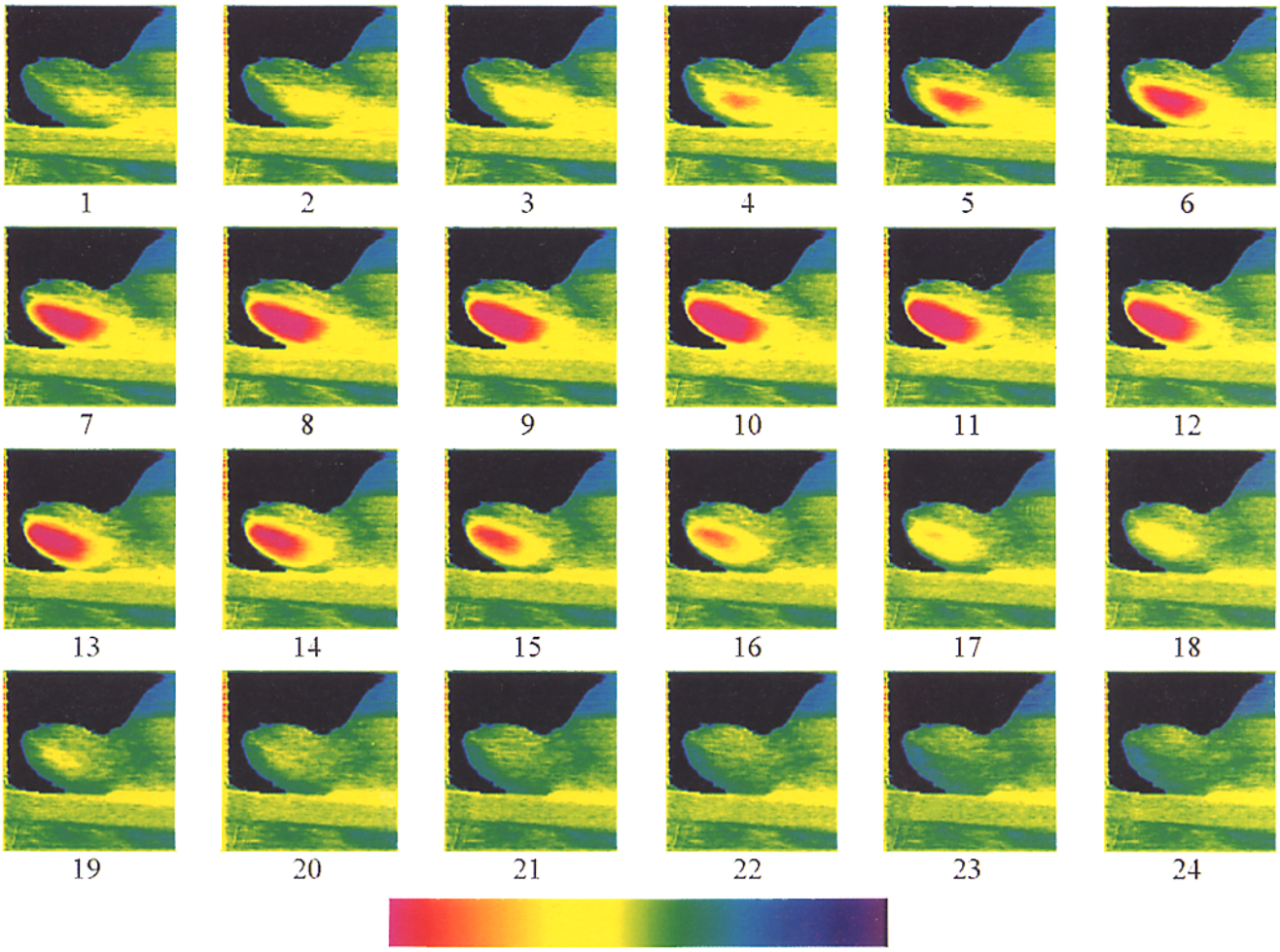

FIGURE 8. Invasion of the terminal arborization of the frog neurohypophysis by the action potential after 20 min exposure to a Ringer's solution containing 4 mM Ca^{2+} and 50 μM 4-aminopyridine. The display shows 24 consecutive frames from a 512 frame sequence recorded by the HR Deltaron 1700 photodiode-MOSFET camera. A single 500-μs shock was delivered to the infundibular stalk between frames 2 and 3. The pseudocolor display of the differential image is the same 8-bit linear scale as that in Fig. 7, where the maximum change in transmitted light intensity is ~0.5% and a reddening of the image corresponds to a depolarization and an increase in dye absorption (decrease in transmitted light intensity). Objective 10×, 0.4 na. Camera gain = 1; amplifier gain = 400. Temperature, 22°C. Average of 16 sweeps. PPFROG21/5.

24 frame sequence, recorded during a control experiment in which the infundibular stalk was stimulated by a brief shock (500 μs duration) delivered by a pair of platinum wire electrodes. A 10×, 0.4 na objective (Wild Heerbrug Instruments) was used to form the image. The upper electrode is clearly visible at the bottom of each frame, and the left "wing" of the neurointermediate lobe (pars nervosa and pars intermedia) is seen just to the left of center, with the anterior pituitary (pars distalis) on the right. The frames depicted were acquired with a separation of 588 μs, and the entire sequence was averaged sixteen times. In this experiment, the preparation had been bathed in a Ringer's solution in which $[Ca^{2+}]_o$ was raised to 4 mM, 13 min before the sequence was recorded. (Raising $[Ca^{2+}]_o$ from 2 to 4 mM has little or no effect on the invasion of the terminal arborization; see below). The action potential is first visible in frame 3, as a red "bloom," and has reached its maximum spatial extent by frame 7, ~4.7 ms later. Note that the optical signal monitoring action potential invasion is limited to the region of the neurohypophysis (pars nervosa), and does not invade the intermediate lobe (pars intermedia) at the top of the "wing." By frame 15, the excitation of the neurohypophysial terminals is no longer visible. Fig. 8 shows an identical experiment, recorded from the same preparation 64 min later, after a 20-minute exposure to a Ringer's solution containing 50 μM 4-AP. Here, the enhancement of invasion is clear (compare frames 7–11) and the prolongation of excitation is very evident. (Compare, e.g., frames 14–17). Fig. 9 shows the actual extrinsic optical signals (at 588 μs per point) recorded by 9 of the

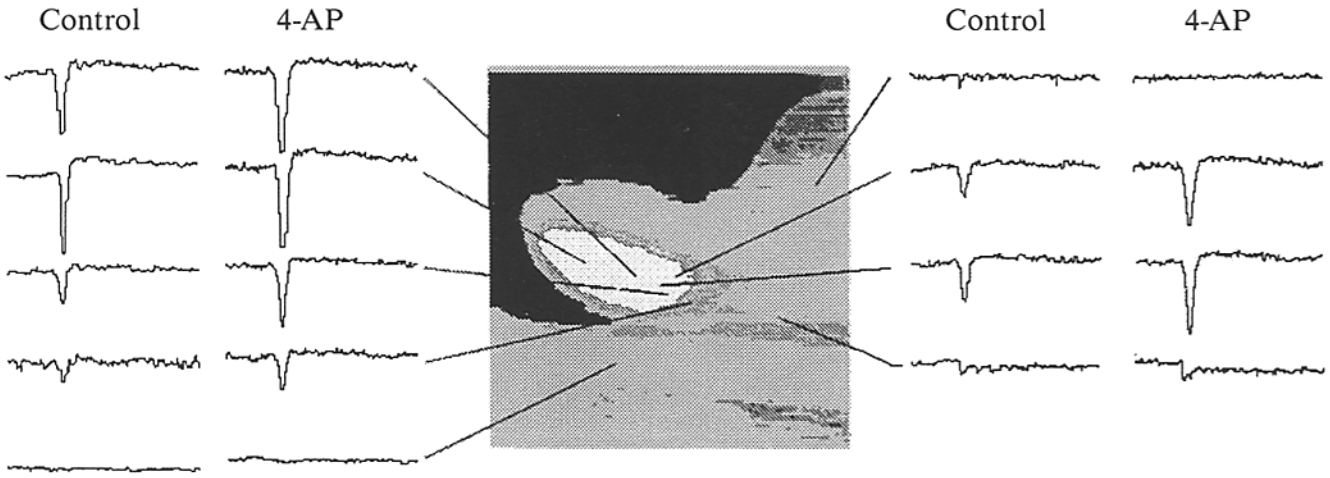

FIGURE 9. The effect of 50 μM 4-AP on the extrinsic optical signals recorded by 9 of the 16,384 pixels of the camera at 588 μs per point (frame). Pointers indicate the locations of the corresponding pixels. For each pair of traces, the one on the left was recorded in 4 mM Ca^{2+} Ringer's solution (represented by the frame sequence in Fig. 7) and the one on the right was recorded 64 min later, after 20 min exposure to 50 μM 4-AP (corresponding to the frame sequence in Fig. 8). For each trace, transmitted light intensity is plotted upward. Thus, with the dye NK 2761, the action potential is seen inverted and the after-hyperpolarization appears as an overshoot. Wire stimulation (500 μs) of the infundibular stalk. Objective 10×, 0.4 na. Camera gain = 1; amplifier gain = 400. Temperature, 22°C. Average of 16 sweeps. PPFROG21/2,5.

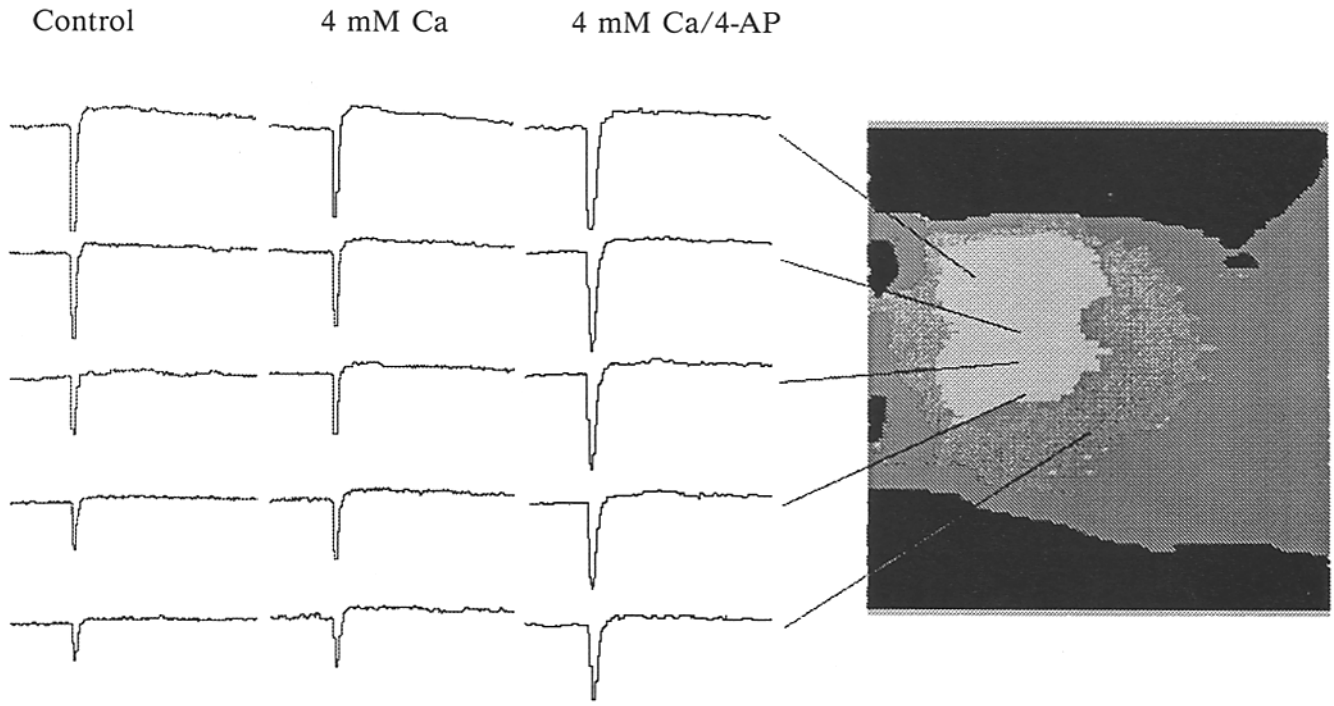

FIGURE 10. Effect of elevated Ca^{2+} and 50 μM 4-AP on the extrinsic optical signals recorded from five locations within the neurohypophysis in response to suction electrode stimulation. Each trace is the output of a single pixel (of 16,384), recorded at 588 μs per point (frame). For each location, the left trace was recorded in normal (2 mM Ca^{2+}) Ringer's solution, the middle trace was recorded after 20 min in a Ringer's solution containing 4 mM Ca^{2+}, and the right trace was recorded after 23 min in a Ringer's solution containing 4 mM Ca^{2+} together with 50 μM 4-AP. Again, for each trace, transmitted light intensity is plotted upward and the action potential is seen inverted, with the after-hyperpolarization appearing as an overshoot. Direct (suction electrode) stimulation (500 μs) of the neurohypophysis. Objective 20×, 0.75 na. Camera gain = 1; amplifier gain = 160. Temperature, 22°C. Average of 16 sweeps. PPFROG19/0,3; PPFROG20/1.

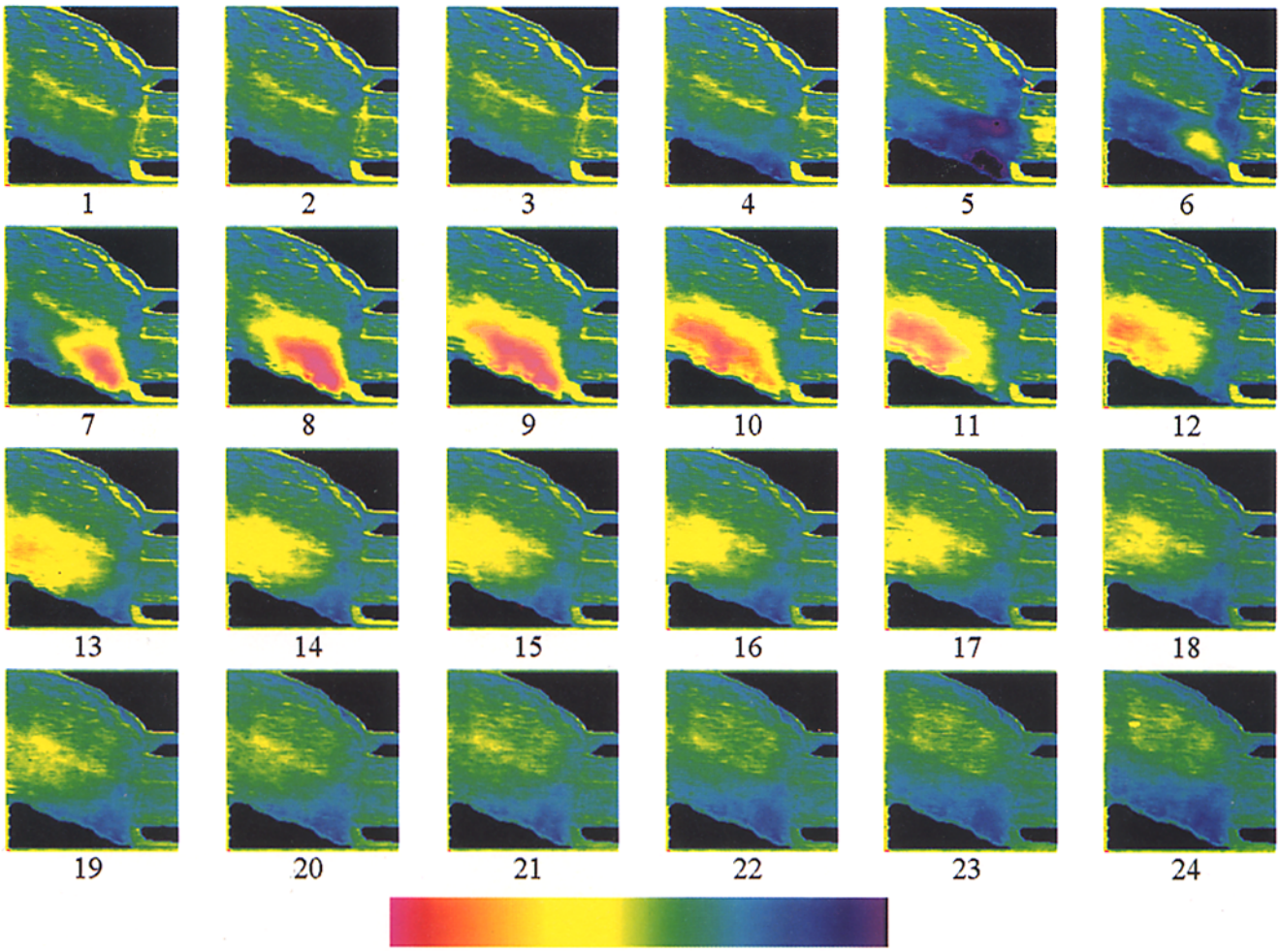

FIGURE 11. Action potential invasion of the frog neurohypophysis after direct field stimulation in a normal Ringer's solution containing 2 mM Ca^{2+}. One of a pair of suction electrodes is seen at the lower right. The display shows 24 consecutive frames from a 512-frame sequence recorded by the HR Deltaron 1700 photodiode-MOSFET camera. A single 500 μs shock was delivered to the neurohypophysis by the suction electrodes between frames 4 and 5. The pseudocolor display of the differential image is, again, an 8-bit linear scale, where the maximum change in transmitted light intensity is ~0.5% and a reddening of the image corresponds to a depolarization and an increase in dye absorption (decrease in transmitted light intensity). Objective 20×, 0.75 na. Camera gain = 1; amplifier gain = 400. Temperature, 22°C. Average of 16 sweeps. PPFROG18/1.

16,384 pixels of the camera. The image displayed corresponds to frame 8 in Fig. 8, and serves only to define the spatial origins of the individual traces. (The gray-scale, here, is arbitrary and does not correspond exactly to the pseudocolor scale employed in Fig. 8.) For each location, two optical signals are shown. Each shows transmitted light intensity plotted upward, so that, with the dye NK 2761, the action potential is seen inverted, and the after-hyperpolarization that results from the activation of a prominent $g_{K(Ca)}$ appears as an overshoot. The signal on the left was recorded in 4 mM Ca^{2+} Ringer's solution, corresponding to the intensities in the frame sequence in Fig. 7 (control), while the signal on the right represents the action potential recorded in 50 μM 4-AP, corresponding to the frame sequence in Fig. 8. Note that at each location, the amplitude of the population spike is larger in 4-AP, and this is particularly dramatic in the pairs of optical signals that have their origins in the lower right region of the neurohypophysis (third row of each column). (Note that two of the extrinsic signals recorded in 4-AP are slightly truncated in this experiment.)

In some experiments, we employed direct electric field stimulation of the terminal arborization (Obaid et al., 1985, 1989) by means of a pair of suction electrodes fitted to the tips of the pars nervosa. We were particularly interested in knowing whether, under these conditions, the degree of active invasion of the highly ramified axonal tree would still be modulated by 4-aminopyridine. We also wanted to determine whether or not raising Ca^{2+} from 2 mM to 4 mM would affect our results. Fig. 10 shows such an experiment. In this experiment, we used a 20×, 0.75 na objective (Fluotar, Nikon, Inc.) and, again, the neurohypophysis was stimu-

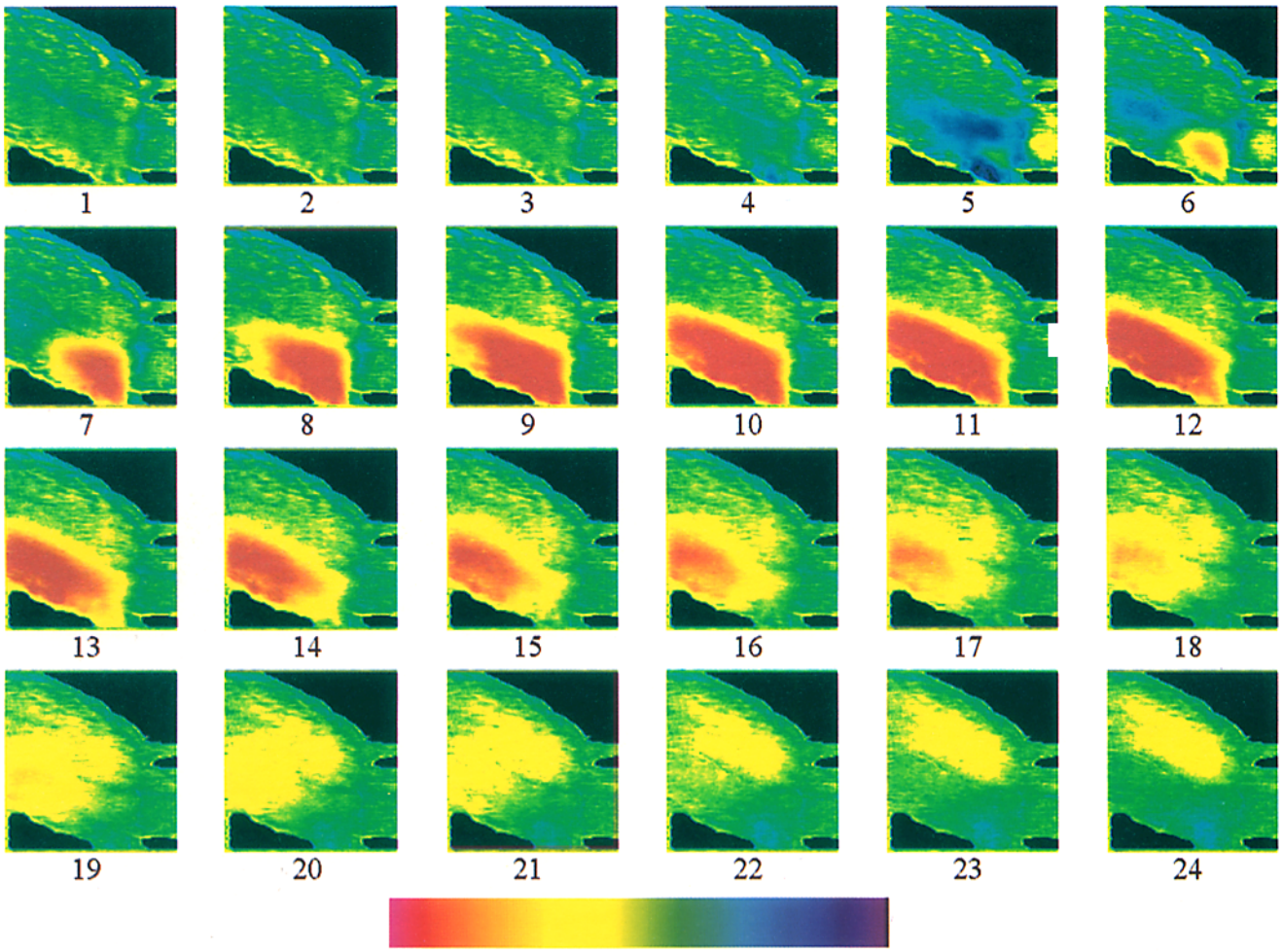

FIGURE 12. Action potential invasion of the frog neurohypophysis following direct field stimulation after a 21-min exposure to a Ringer's solution containing 2 mM Ca^{2+} and 10 μM 4-aminopyridine. One of a pair of suction electrodes is seen at the lower right. The display shows 24 consecutive frames from a 512-frame sequence recorded by the HR Deltaron 1700 photodiode-MOSFET camera. A single 500-μs shock was delivered to the neurohypophysis by the suction electrodes between frames 4 and 5. The pseudocolor display of the differential image is the same 8-bit linear scale seen in Fig. 11, where the maximum change in transmitted light intensity is ~0.5% and a reddening of the image corresponds to a depolarization and an increase in dye absorption (decrease in transmitted light intensity). Objective 20×, 0.75 na. Camera gain = 1; amplifier gain = 400. Temperature, 22°C. Average of 16 sweeps. PPFROG18/2.

lated with 500 μs shocks delivered to the suction electrodes by fine platinum wires. The suction electrode, fitted onto the tip of the pars nervosa is seen at the far left, and the expansion of the tissue at the right marks the beginning of the anterior pituitary (pars distalis). Extrinsic optical signals (inverted population spikes) are exhibited, to the left, from the five indicated locations within the neurohypophysis. In each row, the first action potential was recorded in a normal Ringer's solution containing 2 mM Ca^{2+}, the second was recorded following 20 min in a Ringer's solution containing 4 mM Ca^{2+}, and the third extrinsic optical signal was recorded 1 h later, after 23 min in a Ringer's solution containing 4 mM Ca^{2+} and 50 μM 4-AP. There is little or no systematic effect of elevating $[Ca^{2+}]_o$, on the optical signal, but, everywhere, 50 μM 4-AP results in a larger amplitude for the population signal, and in a significantly broader spike.

Figs. 11 and 12 illustrate a similar experiment, in which we monitored the effect of just 10 μM 4-AP on the invasion of the neurosecretory terminal arborization, when the neurohypophysis was excited using anode break stimulation. Fig. 11 shows a 24-frame sequence recorded at 1.7 kHz in normal Ringer's solution. At frame 5, the hyperpolarizing anode break stimulus (500 μs duration) is seen, as a blue-shift in the pseudocolor display, followed, beginning in frame 6, by the action potential represented as a reddening of the image, that persists until approximately frame 15. In Fig. 12, an identical anode break stimulus is applied to the preparation after it was bathed for 21 min in a Ringer's solution containing 10 μM 4-AP. In this exper-

iment, the enhancement in the spatial extent of the action potential's invasion of the tissue, and in the duration of the excitation are dramatic.

DISCUSSION

In the mouse neurohypophysis, elevation of extracellular calcium concentration and addition of micromolar concentrations of 4-aminopyridine each increase the amplitude of the component of a fast intrinsic optical signal (the S-wave of the large angle light scattering change) that is intimately related to secretion of neurohypophysial peptides. However, when the number of activated terminals is factored into the measurement, by normalizing the signals to a local measure (the amplitude of the E-wave) of the extent of invasion, 4-AP is seen to act in a manner very different from that of calcium ion. Calcium appears to enhance that step in the secretory pathway that is reflected by the S-wave of the light scattering signal (Salzberg et al., 1985; see also Fan and Terakawa, 1989) referred to here as secretion, in the classical fashion, i.e., by augmenting secretion from each neurosecretory terminal into which the action potential spreads. A low concentration of 4-aminopyridine, on the other hand, appears to have no effect on the amount of secretion from each active terminal, but produces its secretagogue action, instead, by improving the success with which the action potential excites the highly ramified terminal arborization, by recruiting additional boutons and secretory swellings. This is seen very clearly in Figs. 1 and 4, where the amplitudes of the E-wave and the S-wave of the light scattering signal, from a single region of the CD-1 mouse neurohypophysis, increase in parallel. At the same time, recordings that make use of the full 124-element photodiode array, e.g., those shown in Fig. 5, provide direct evidence that exposure of the mouse neurohypophysis to 50 μM 4-AP increases not only the amplitude of the light scattering signals, but also the spatial extent of the activated region. Other evidence consistent with this interpretation was obtained from experiments such as those shown in Figs. 6–12, in which voltage-sensitive dyes were used to monitor the compound transmembrane action potential in intact populations of neurohypophysial terminals in *Xenopus*. In these experiments, we could observe the augmentation, produced by micromolar 4-AP, in the amplitude of the compound action potential monitored optically by single photodetectors, either in the 124-element photodiode array (Fig. 6) or by individual pixels in the 16,384-pixel photodiode-MOSFET camera (Figs. 9–10). Since the individual photodiodes in the array monitor a much larger region of the frog neurohypophysis, the increase in the size of the extrinsic absorption signal is more dramatic and more robust than that seen in the Deltaron recordings. Nevertheless, the population spikes recorded from regions of the pars nervosa 5×5 μm^2 (at $10\times$) by single representative pixels of the Deltaron camera, in Fig. 9, exhibit considerable increases in amplitude. In this context, it is noteworthy that other blockers of potassium channels such as tetraethylammonium (TEA) and Ba^{2+} do not increase the magnitude of the compound action potential (data not shown). (TEA does, however, produce a very large increase in the size of the S-wave in the mouse neurohypophysis, but this effect, in contrast to that of 4-AP, reflects a large change in light scattering per active terminal. Indeed, the effects of TEA and 4-AP on the S-wave of the scattering signal seemed to be multiplicative, rather than additive, consistent with the notion that these two agents act independently to increase release from each terminal, and to extend the number of invaded terminals (Obaid et al., 1987).) Finally, the frame sequences ("movies") recorded by the photodiode-MOSFET camera, at 128×128 pixel and 1.7 kHz resolution show very clearly the enlarged domain of invasion in the frog neurohypophysis, whether stimulation was by means of platinum wire electrodes placed on the axons passing through the infundibular stalk (Figs. 7 and 8) or by suction electrodes on the tips of the pars nervosa (Figs. 11 and 12).

How are these phenomena related to the neurosecretory behavior of the neurohypophysis in situ? Physiologically, the relative degree of invasion of the complex terminal arborization in this tissue may represent an extremely sensitive control point for modulation of neuropeptide secretion. This would be especially effective in a neurohaemal organ like the pituitary. There, the precise location of release is far less important than it is likely to be in a collection of presynaptic terminals and their boutons. It may be supposed that under the conditions of our experiments, enhanced invasion of the neurohypophysial arborization is effected by a reduction, however slight, in branchpoint or propagation failure, resulting either from a decrease in a potassium conductance at critical bifurcations that is especially sensitive to 4-aminopyridine, or simply from the geometry of particularly large secretory swellings. It is interesting to speculate concerning the complex interplay between spike broadening in this tissue (Gainer et al., 1986), frequency and temporal pattern sensitivity of secretion (Poulain and Wakerley, 1982; Bicknell and Leng, 1981; Bicknell et al., 1982), and the physiological effects of propagation through regions of low safety factor, where there must be a delicate accommodation between a relatively stable, but complex geometry, and membrane properties that are dynamic and subject to modulation.

The terminal arbor of the posterior pituitary has a highly complex geometry in which secretory swellings appear as axonal varicosities along the distal portions

of the axons of the magnocellular neurons of the hypothalamus. In the rat, Nordmann (1977) has shown that the 18,000 magnocellular neurons give rise to some 40,000,000 secretory swellings and boutons. Thus, the axonal projection in the neurohypophysis of a single hypothalamic neuron includes ~2,000 secretory swellings and terminals. Studies of the geometry of the terminal arborization using dye injection techniques indicate that the axons within the posterior pituitary contain many en passant swellings with relatively few branches (Tweedle et al., 1989; Bourque, 1990; Jackson, 1993). These axons are fine caliber, on the order of 0.4 μm (Jackson, 1993), and the swellings and terminal boutons have average diameters of 2.7 and 1.6 μm, respectively (Nordmann, 1977). There are, however, occasional swellings of up to 20 μm in diameter referred to as Herring bodies (Mason and Bern, 1977), and, while extremely rare, a few of these have diameters as large as 32 μm (Cannata and Tramezzani, 1977). Axonal enlargements of these sizes present a dramatic impedance mismatch to the advancing action potential and might compromise impulse propagation. Several theoretical analyses have examined the effects of en passant varicosities and terminal arborization on the spatial pattern of invasion of the action potential (Khodorov et al., 1969; Goldstein and Rall, 1974; Segev, 1990; Lüscher and Shiner, 1990), and some of these have revealed the extent to which successful propagation through regions of low safety factor depends critically upon local conductances and specific membrane properties. Ultimately, channel properties and even local ionic conditions that may reflect the recent history of electrical activity (Chung et al., 1970), interact with geometrical factors to vary the activation pattern of neurosecretory or presynaptic terminals, and participate in the processing of information in the nervous system.

Recently, patterned growth of cardiac myocytes in culture, coupled with MSORTV (Rohr and Salzberg, 1994*a*,*b*), has permitted the experimental exploration, with high spatial and temporal resolution, of the effect of geometry per se, on action potential propagation in an electrical syncitium in which there is an abrupt expansion from a narrow cell strand to a large cell area. Also, Brock and Cunnane (1987) have examined action potential propagation through the relatively small varicosities in the sympathetic postganglionic axons that innervate prostate muscle. However, the question of action potential propagation in the terminal arbor of the neurohypophysis received little attention until the careful theoretical studies of Jackson and Zhang (1995). These authors were primarily interested in the question of whether depolarizing and shunting $GABA_A$ responses (Zhang and Jackson, 1995) would block action potential propagation through neurohypophysial secretory swellings. In the course of their analysis (Jackson and Zhang, 1995), based upon a very detailed and specific model of the posterior pituitary terminal arborization, they concluded that, in the absence of a significant degree of inhibition or depression, action potentials normally invade the entire posterior pituitary arbor. In their model, swellings could block propagation, but only when their sizes reached a critical value of 26 μm which exceeds that normally observed in the neurohypophysis. Adding axonal branches reduced the critical size only slightly. The results of their modeling appear to conflict with our observations that micromolar concentrations of 4-aminopyridine can enhance the invasion of the neurohypophysial terminal arborization. There are, of course, many possible explanations for this disagreement. (*a*) Specie difference is possible, but unlikely, in view of our consistent observations in mouse and frog. (*b*) Uncertainties in some of the model parameters employed by Jackson and Zhang (1995), particularly the steady state activation and inactivation of the nerve terminal sodium channels (Jackson and Zhang, 1995), quantities that are particularly difficult to assess in the posterior pituitary terminals, could lead to our different results. Or, the simplifying assumption of uniform channel density in the model may be inappropriate, with lower sodium channel, or higher potasium channel density in the swellings. (*c*) The geometric assumptions of the model may not adequately reflect the true complexity of the tissue, in that multiple swellings and branches may be clustered, in such a fashion that their combination depresses the safety factor below the prediction of the model. Or, there may be unusually large swellings, distributed infrequently along each axon, that increase the probability of block. Or, the value of 0.4 μm for the axon diameters, particularly as they approach the swellings, may be an overestimate. The model of Jackson and Zhang yielded a critical swelling size of 26 μm for propagation failure, and, with a varicosity diameter of 20 μm, the model system was close to critical. Thus, it would not require a very large correction to the model, or a very significant adjustment to its geometric parameters, for the model to generate propagation failures.

Active propagation is a threshold phenomenon and theory (Jackson and Zhang, 1995) has shown that the neurohypophysis is close to threshold and very sensitive to intrinsic membrane and geometrical properties. In the end, the question is an experimental one, and reminds us of the need for further direct experimental studies of propagation in complex terminal arbors. Another, very interesting possibility, and one that is susceptible of test, is that the stimulation employed in the optical measurements of action potential propagation resulted in the concomitant stimulation of the clusters of GABA-positive terminals that have been described in the posterior pituitary (Buijs et al., 1987). In the model

elaborated by Jackson and Zhang (1995), they were able to simulate action potential block by widespread G_{Cl} activation by GABA, thus reproducing the experimental finding that bath application of GABA blocked stimulus evoked secretion (Dyball and Shaw, 1978; Saridaki et al., 1989) and reduced the size of the optical population spike (Obaid, A.L., and B.M. Salzberg, unpublished observations). However, as Jackson and Zhang (1995) point out, "activation of only a few GABAergic fibers would have little impact on evoked hormone release from peptidergic neurons." We do not believe that the observed modulation by 4-AP of the extent of neurohypophysial terminal invasion depends upon a GABA-dependent depression of a normally complete activation of the terminal arbor.

Finally, it should be noted that, in the case of the mouse (but not the frog) experiments, brief trains of six or seven action potentials were employed, and the repetitive stimulation could have caused some propagation failure which was then ameliorated by the 4-AP. While this argument could not be used for the frog experiments, we have already described (Obaid and Salzberg, 1991) significant specie differences between frog and mouse neurohypophysial terminals (e.g., the behavior and role of Ca-activated K channels), and these could result in differences in safety factor. Future experiments will help to resolve the apparent conflict between our results and the model proposed by Jackson and Zhang (1995), but our demonstration of enhanced invasion of a neurosecretory terminal arbor mediated by 4-aminopyridine must be accommodated by any complete model.

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