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4. The longer wave form was chosen because of the relative improvement it produced in the stability of the spectral estimates. Spectra for the shorter responses were calculated and were found to be essentially similar to those based on the longer records. The quantizing procedure limits the maximum detectable frequency to 25 hz.
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6. We thank Jeanette F. Koffler for laboratory assistance. Supported by ONR contract NONR 1858(19) (N00014-67-A-0151-0015) and by PHS grant MH 18189. Use was made of computer facilities funded in part by NSF grants NSF-GJ-34 and NSF-GU-3157.

5 January 1972; revised 21 April 1972

Calcium Transient in Presynaptic Terminal of Squid Giant Synapse: Detection with Aequorin

Abstract. *Microinjection of aequorin, a bioluminescent protein sensitive to calcium, into the presynaptic terminal of the squid giant synapse demonstrated an increase in intracellular calcium ion concentration during repetitive synaptic transmission. Although no light flashes synchronous with individual presynaptic action potentials were detected, the results are considered consistent with the hypothesis that entry of calcium into the presynaptic terminal triggers release of the synaptic transmitter substance.*

One of the prevailing hypotheses concerning the mechanism by which depolarization of a presynaptic terminal results in the release of a synaptic transmitter ("depolarization release coupling") incorporates the assumption that calcium flux into the terminal is the triggering factor for release (*1*). In the squid giant synapse, the existence of a membrane potential–dependent increase in calcium conductance at the presynaptic terminal has been demonstrated; a calcium action potential may be recorded at the presynaptic terminal after simultaneous reduction of the peak sodium and potassium currents with tetrodotoxin and tetraethylammonium ions, respectively (*2*). Under these conditions synaptic transmitter is released, as is indicated by a prolonged postsynaptic potential change in the giant axon after this calcium-dependent presynaptic spike.

The question remains whether, as a result of the action potential, calcium actually enters the axoplasm of the presynaptic terminal and is available there as a free ion, or whether it simply moves from one "membrane compartment" to another without changing the axoplasmic level. That the latter might be the case has been considered because the ionophoretic application of calcium inside the presynaptic nerve terminal is without effect on the release of transmitter (*3*). A second possibility suggested to explain these negative results is the existence of an avid intracellular calcium sequestering system that would prevent the injected calcium ions from triggering synaptic release (*3*).

We attempted to ascertain whether the intracellular calcium concentration, $[Ca^{2+}]_i$, changes during the activation of the presynaptic nerve terminal. Changes in the concentration of this ion were detected by microinjection of aequorin, a bioluminescent protein sensitive to calcium (*4*), into the presynaptic terminal. This substance emits light when exposed to very low concentrations of ionized calcium, and has been used to demonstrate calcium entry into the giant axon of the squid under various conditions (*5*). Our results demonstrate that concomitant with repetitive synaptic transmission there is an increase in $[Ca^{2+}]_i$ at the presynaptic level. The time course of this increase is consistent with the existence of a strong intracellular calcium buffering system.

Experiments were performed in the stellate ganglion of *Loligo pealii*. The stellate ganglion, together with its pre- and postsynaptic axons, was removed from the mantle and mounted in a transparent acrylic plastic chamber (*6*). The synapse was bathed with flowing, oxygenated, artificial seawater (NaCl, 466 m*M*; KCl, 10 m*M*; $CaCl_2$, 11 m*M*; $MgCl_2$, 54 m*M*; $NaHCO_3$, 3 m*M*) (*2*), and the chamber was kept at 10°C by means of a thermoelectric (Peltier effect) cooling system (Fig. 1). The presynaptic axon was activated by means of external silver–silver chloride elec-

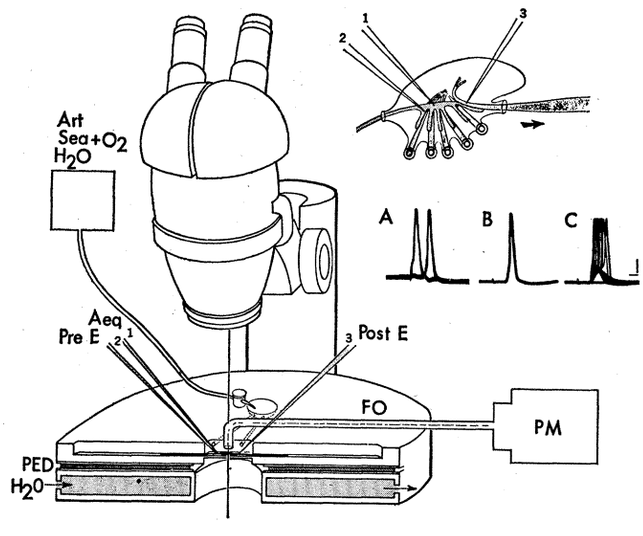

Fig. 1. Diagram of experimental arrangement and electrophysiological recordings. The chamber was kept cool by means of a Peltier effect disc (*PED*) with an underlying water-cooled heat sink. The temperature was automatically controlled from a thermistor in the chamber. The synapse was superfused with oxygenated artificial seawater throughout the experiment (*Art. sea* $H_2O + O_2$). Aequorin was injected presynaptically through a micropipette (*Aeq*) (upper right schematic diagram). The injection was performed and observed under a dissecting microscope. Electrical activity in pre- and postsynaptic fibers was recorded with electrodes *2* and *3*, respectively (details shown in upper right diagram). The presynaptic fiber was activated by means of external electrodes. *A*, simultaneous records from electrodes *2* and *3* (baselines superimposed), illustrating typical action potentials of pre- and post-synaptic fibers and synaptic delay. *B* and *C*, superimposed trains of postsynaptic responses (from electrode *3* only) evoked at 10 per second stimulation in *B* and 100 per second in *C*; those in *B* are of uniform configuration, while those in *C* show progressive diminution and failure of the EPSP. The light emission after aequorin injection was measured with a fiber-optic system (*FO*) located immediately above the synapse and in contact with the seawater. The collected light was detected with a photomultiplier (*PM*). In records *A* to *C*, voltage and time calibration are 20 mv and 1 msec, respectively.

trodes, and the pre- and postsynaptic action potentials were recorded intracellularly with micropipettes filled with 3*M* KCl, which were placed very close to the synapse. Aequorin was injected intracellularly under pressure through a third micropipette (tip diameter, approximately 1 μm) attached to an oil-filled micrometer syringe.

The aequorin was extracted from specimens of *Aequorea forskålea* (collected at Friday Harbor, Washington), and subsequently purified by a combination of gel-filtration and ion-exchange chromatography. The product was a mixture of three luminescent components of aequorin with isoelectric points of *p*H 4.42, 4.55, and 4.70 (*7*); no nonluminescent proteins could be detected on polyacrylamide-gel disc electrophoresis. The aequorin was prepared for physiological use by a technique (*8*) in which the purified protein was freed of EDTA and other salts, and lyophilized in the presence of beads of chelating resin (Chelex 100). Just before use, the lyophilized material was dissolved in 10 percent glycerol solution buffered to *p*H 7.3 with potassium phosphate (90 m*M*). The Chelex beads were filtered off with a Millipore filter in a specially constructed miniature holder, and the micropipettes were filled from the tip. The concentration of the aequorin injected was in the range of 0.1 to 0.5 m*M* (estimated from the photon yield of samples of the final solution; the quantum efficiency was assumed to be 25 percent). The amount of aequorin injected could be estimated from the position of the aequorin-oil meniscus (the oil had been treated with Sudan black). In most cases the volume injected was approximately 0.00025 μl; the injected fluid produced a distinctly visible change in the refractive index of the fiber, which indicated that aequorin entered all of the presynaptic digits visible under the microscope. In a substantial proportion of the experiments, the presynaptic terminal ceased to generate action potentials immediately after the injection, probably because of damage produced by the increase in intracellular pressure. In most cases in which presynaptic action potentials survived the injection, however, synaptic transmission was apparently normal for up to several hours after injection. The aequorin itself thus appears to have little or no deleterious effect on the presynaptic secretory system.

Luminescence was detected through a fiber-optic light guide (diameter, 2 mm; numerical aperture, 0.5; combined end and line loss, 40 percent), the tip of which was positioned directly over the presynaptic terminal with a micromanipulator. The light gathered by the fiber-optic probe passed through a shutter and was focused with a microscope objective on the photocathode of an EMI 9502S photomultiplier tube (cathode potential, −1600 volts). The anode current of the photomultiplier was converted to a voltage signal with an operational amplifier (Zeltex 133) (90 percent rise time; 100 μsec), monitored on an oscilloscope (Tektronix 565), and recorded on a Grass polygraph inkwriter. Pre- and postsynaptic membrane potentials were monitored on an oscilloscope.

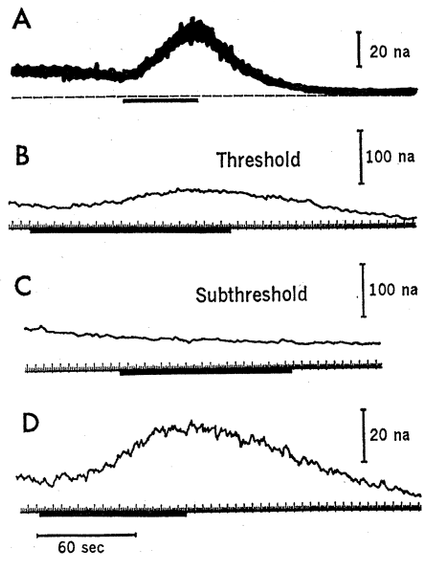

Fig. 2. Light emission during stimulation of presynaptic fiber injected with aequorin. Typical results observed after aequorin injection; in all records the background luminescence was constantly decreasing with time. (A) Repetitive stimulation (dark bar) produced a slow increase in luminescence at the presynaptic terminal to approximately three times background level. After stimulation, the luminescence slowly decreased to the dark current level (dashed line); subsequent stimulation gave no luminescent response. The synapse had been stimulated repetitively several times after the aequorin injection. This record illustrates the last effective stimulation sequence, after which the aequorin was exhausted but the synapse continued to function normally; it was selected to show a tracing in which the baseline (dark current) is not off scale. (B, C, and D) Another experiment; the time constant of the amplifier was increased to filter out high frequency photomultiplier noise. In (B) threshold repetitive activation of presynaptic fiber generated increased luminescence. In (C), after a small reduction of the stimulus intensity to subthreshold level, there was no increase in luminescence. In (D) the stimulus intensity was raised again and luminescence was produced as before (note the change in gain between records B and D). Baseline was shifted between records (B) and (C) and again between (C) and (D) to keep records on scale. The time calibration is the same for all records; luminescence was measured from photomultiplier in nanoamperes.

Baker *et al.* (*5*) have indicated that in the squid giant axon injected with aequorin and bathed in normal seawater, the light output at rest was stable for long periods and increased only slightly by repetitive stimulation. Large calcium transients during repetitive stimulation were observed only when the extracellular calcium concentration was greatly increased (*9*). On the other hand, we found that in normal seawater the level of luminescence in the presynaptic terminal decreased progressively after the injection of aequorin and, more important, that repetitive electrical activation of the presynaptic fiber resulted in a substantial increase in luminescence (Fig. 2). When the light output at rest disappeared, so did the luminescent response to stimulation, suggesting that the decline resulted from the utilization of the injected aequorin (which reacts only once) rather than from a decrease in $[Ca^{2+}]_i$ (*10*).

The results of two different experiments in which a series of trains of conducted action potentials were evoked in the presynaptic fiber are shown in Fig. 2 (100 per second for 1 second every 5 seconds in A, and 100 per second for 3 seconds every 8 seconds in B, C, and D) (*11*). The stimulation produced an increase of the luminescence only when presynaptic action potentials were generated (compare Fig. 2C with Fig. 2, B and D); under such conditions the background light intensity often more than doubled (Fig. 2A). Results similar to those in Fig. 2 were obtained in ten different trials in four preparations. In several experiments the extracellular calcium concentration was increased from 12 to 150 m*M*, and this immediately produced a large increase of light emission and consequently a very rapid depletion of the intracellular aequorin. When deterioration of the presynaptic terminal (inexcitability or failure of synaptic transmission) occurred before the injected aequorin had been depleted, it also was accompanied by a very large increase of luminescence and a rapid depletion of the remaining aequorin.

Our findings indicated that a signifi-

cant rise in the intracellular concentration of Ca^{2+} results from repetitive activity in the presynaptic terminal of the giant synapse even when bathed in normal seawater (*12*). However, the time course of that rise is probably several orders of magnitude slower than that of the events responsible for the release of transmitter. This suggests that if a rapid increase in $[Ca^{2+}]_i$ is responsible for transmitter release, it does not take place throughout the space occupied by the injected aequorin. If such a rise occurred in the bulk of the aequorin space, one might expect a rapid flash of greater intensity than the slow rise in luminescence actually recorded, since the increase in $[Ca^{2+}]_i$ which produced the slow change in luminescence did not result in or interfere with synaptic transmission. The possibility may be considered that a fast calcium transient did occur throughout the aequorin space, but that it was too rapid to be detected by the aequorin reaction, which does not precisely follow very rapid changes in calcium concentration (*13*). This seems unlikely, however, since some luminescence is detectable within the first millisecond after aequorin is exposed to calcium (*13*), and it is improbable that any calcium transient produced by the action potential would be substantially briefer than the action potential itself (Fig. 2).

Although we found no direct indication of rapid calcium transients synchronous with individual synaptic events, we believe that our results are consistent with the hypothesis that a transient increase in calcium concentration somewhere in the axoplasm of the presynaptic terminal is a significant step in the release phenomenon. This belief is based on the fact that our experiments did demonstrate a rise in $[Ca^{2+}]_i$ associated with repetitive activation of the presynaptic terminal, suggesting the flux of free calcium into the axoplasm. Our inability to detect rapid calcium transients associated with the individual action potentials might well result from the restriction of these transients to a small portion of the axoplasm of the presynaptic terminal. At least two likely mechanisms could produce such a restriction. First, the axoplasm might contain a calcium buffering system capable of confining large calcium transients to a very narrow zone of axoplasm in the immediate vicinity of the plasma membrane. (Such a system would ensure that the calcium transient generated by an action potential was terminated rapidly.) Mitochondria bind calcium in large amounts and might represent at least one component of such an intracellular calcium sequestering system (*5, 14*). In the presence of such a system the calcium concentration detected by the bulk of the intracellular aequorin would be that in equilibrium with the buffering system, and changes in that concentration would reflect changes in the degree of saturation of the buffer capacity. In a well-buffered system these changes would be slow and relatively slight except in the immediate vicinity of the site of calcium entry.

A second possible factor that might contribute to the difficulty in detecting rapid calcium transients is the small diameter of the terminal digits relative to their length (considerably smaller than indicated in the schematic diagram of Fig. 1). If rapid calcium transients were more pronounced in the digits than elsewhere (as they might well be, if for no other reason than the greater surface to volume ratio), the aequorin in the digits might be discharged relatively early. Unspent aequorin would diffuse down the long narrow digits relatively slowly, with the result that the rapid transients would go undetected. Further experiments are required to test these possibilities.

R. LLINÁS
J. R. BLINKS
C. NICHOLSON

Division of Neurobiology, Department of Physiology and Biophysics, University of Iowa, Iowa City 52240, and *Department of Pharmacology, Mayo Medical School, Rochester, Minnesota 55901*

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9. In an ancillary study in the squid giant axon, we found this to be true also under our experimental conditions, which differed from those of Baker *et al.* (*5*) primarily in that a different species of squid was used, and the concentrations of active aequorin that we injected were two orders of magnitude higher.
10. The much more rapid consumption of aequorin in the presynaptic terminal is presumptive evidence that the resting $[Ca^{2+}]_i$ is considerably higher than in the giant axon. This conclusion requires the assumption that other conditions influencing the rate of the luminescent reaction (of which $[Mg^{2+}]$ is the most important one known) are similar in the two fibers.
11. When stimulation at 100 per second was maintained for much over 3 seconds, there was in most cases a large reduction and on occasions an apparent blockade of the excitatory postsynaptic potentials (EPSP's) (Fig. 2C), whether or not aequorin had been injected. The repetitive stimulation was interrupted for the periods described above in order to have effective EPSP's recorded throughout the period of stimulation.
12. The very unlikely possibility that the increase in luminescence is due to liberation of aequorin into the extracellular medium by the synaptic release process is ruled out by the slow decay of the luminescence change after the end of stimulation.
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28 January 1972; revised 28 February 1972

Ascent of Sap in Trees

Abstract. *Experimental results concerning the ascent of sap in the xylem are usually interpreted in terms of gradients of hydrostatic pressure in the xylem conduits. In this report an alternative model is proposed that is equally consistent with the experimental results: under static conditions the water column is supported by a gradient in the chemical activity of the water, and the hydrostatic pressure is constant throughout. Observations that support the new model are cited, and experiments are suggested that would permit a choice between the two models.*

Many definitive experiments concerning the ascent of water through the xylem have been performed. Colloidal metal particles and large organic dye molecules (*1*, p. 182) are carried by water ascending the column, which shows that the water does not pass through semipermeable membranes. Samples of xylem water removed from the xylem conduits during the growing period have very low concentrations of inorganic and organic solutes although