

Calcium transients recorded with arsenazo III in the presynaptic terminal of the squid giant synapse

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Transient changes in free intracellular Ca^{2+} concentration were monitored in the presynaptic terminal of the giant synapse of the squid, by means of the Ca^{2+} -sensitive dye arsenazo III. Calibration experiments showed a linear relation between the amount of Ca^{2+} injected by iontophoresis into the terminal, and the peak size of the arsenazo light absorbance record. A light signal could be detected on tetanic stimulation of the presynaptic axon bathed in sea water containing 45 mM Ca^{2+} . During a 1 s tetanus the light signal rose approximately linearly, even though transmitter release declined rapidly and the light signal subsequently declined with a half-time of 2–6 s. The Ca^{2+} transient elicited by single nerve impulses was recorded by signal averaging, and showed a time course very much slower than the duration of transmitter release.

INTRODUCTION

The release of transmitter quanta following the arrival of a nerve impulse at 'chemical' synapses is triggered by an influx of calcium ions into the terminal (Katz & Miledi 1965, 1967, 1969; Llinas *et al.* 1976; Miledi 1973; Miledi & Slater 1966). Attempts have been made to record the ensuing change in intracellular calcium concentration in the presynaptic terminal of the squid giant synapse, by means of the photoprotein aequorin (Llinas *et al.* 1972; Llinas & Nicholson 1975). An increase in calcium concentration during prolonged tetanic stimulation was detected with this technique, but the calcium transient resulting from a single nerve impulse could not be observed except when the presynaptic spike was artificially prolonged by intracellular injection of tetraethylammonium (TEA) ions.

We describe here our first attempts to use the calcium indicator dye arsenazo III (Brown *et al.* 1975; Dipolo *et al.* 1976; Miledi *et al.* 1977, 1980; Parker 1979), injected iontophoretically into the nerve terminal of the squid giant synapse, to record Ca^{2+} transients during synaptic transmission.

METHODS

Preparation and electrical recording

The experiments were made during the summer of 1977 on the stellate ganglion of the squid *Loligo vulgaris*. Ganglia were isolated from small squid (mantle

lengths 9–13 cm) and mounted in a cooled chamber perfused with oxygenated natural sea water (Miledi 1967). Recordings of calcium transients were obtained during perfusion with sea water containing 55 mM Ca^{2+} , at a temperature of 12–18 °C.

Experiments were performed on the 'distal' giant synapse (Young 1939; Miledi 1967; Katz & Miledi 1969). The presynaptic axon was penetrated with two micropipettes, for arsenazo injection and electrical recording (figure 1*a*). In some experiments the KCl pipette was replaced by a pipette filled with 0.39 M CaCl_2 . Postsynaptic potentials were recorded with a microelectrode inserted within the synaptic region. The preganglionic nerve was mounted on Pt electrodes for stimulation.

Arsenazo injection

Pipettes, pulled from tubing containing a glass fibre, were back-filled with an aqueous solution of 1 mM arsenazo III (Sigma Chemical Co.; practical grade) shortly before use. Contaminating calcium in this solution was reduced by batch treatment with Chelex 100, prewashed with 0.1 M potassium acetate. The dye pipette was inserted about 0.5 mm central to the start of the synapse to ensure slow and even distribution of dye to the terminal. Arsenazo was injected by passing pipette-negative iontophoretic pulses of about 0.5 s duration at 1 Hz, over a period of 0.5–2 h. In some experiments a current of < 40 nA was sufficient to inject dye, but often larger currents were needed. To avoid excessive hyperpolarization during dye injection pulses, current was passed between the dye and KCl pipettes, by means of a circuit isolated from earth. Injection was monitored through a dissecting microscope, and was stopped when the terminal was just detectably stained. Arsenazo pipettes were found to block frequently in high-calcium sea water. Therefore the pipettes were inserted while the ganglion was perfused with natural sea water and more calcium was added to the sea water once the terminal was successfully injected.

Optical recording

Intracellular calcium transients were recorded as previously described (Miledi *et al.* 1977, 1980; Parker 1979). In most of the present experiments light transmission was recorded only at a single wavelength, to increase light collection by the photomultiplier, and thus reduce photon noise.

Figure 1*b* shows the single-wavelength optical system. Light from a 50 W quartz-halogen lamp was focused onto the ganglion by a condenser system. A field stop allowed the light to be focused as either a slit (figure 1*a*) or a spot of about 80 μm diameter. Interference filters (Balzers; half-peak bandwidth 12 nm) transmitting wavelengths of 650, 602 and 532 nm could be placed before the condenser, and light transmitted through the ganglion was collected by a photomultiplier. To avoid artefacts from ripples on the fluid surface, a hollow 'dipping cap' with a glass window of 5 mm diameter was fitted to the photomultiplier and

immersed in the fluid. The anode current of the photomultiplier was measured with a virtual earth circuit, and a sample and hold circuit allowed automatic subtraction of the baseline light level. A computer of average transients was used for signal averaging. Optical signals were capacitor-coupled to this with a time constant of 1 s.

Calcium transients were recorded at a wavelength of 650 nm, where the absorption spectrum of arsenazo III shows a large increase in the presence of calcium.

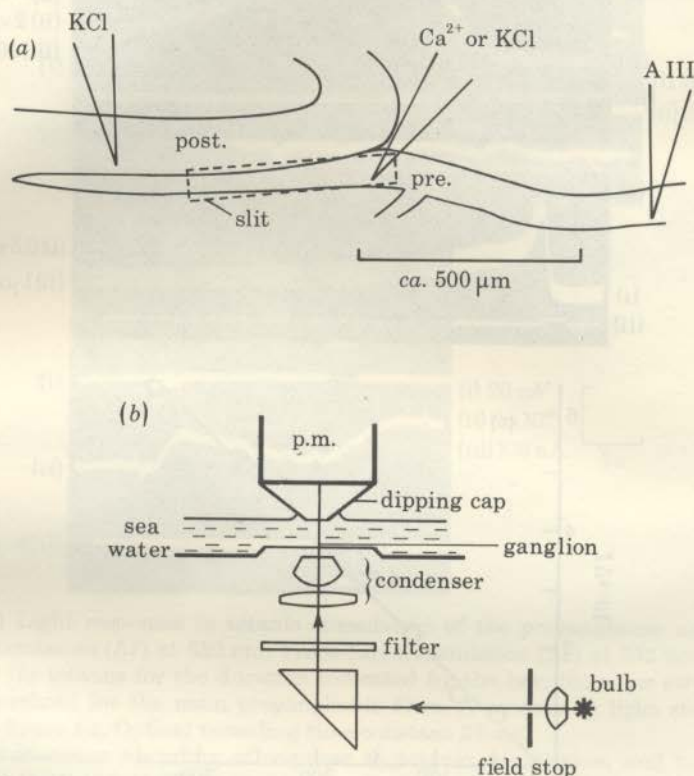


FIGURE 1. (a) Usual arrangement of electrodes in pre- and postsynaptic giant axons. The measuring light slit for optical recording is shown. The length calibration is only approximate. A III, arsenazo III. (b) Schematic diagram of the optical recording system; p.m., photomultiplier. See text for details.

This wavelength gave a slightly better signal-to-noise ratio than the other absorption peak at 600 nm. Records at 532 nm were also taken at intervals throughout an experiment. Arsenazo III shows a small decrease in absorption with calcium at this wavelength, and artefacts due to movement or transparency changes of the ganglion could be detected.

RESULTS

Injection of arsenazo III into the nerve terminal was difficult, mainly due to blocking of the pipette during injection. Bevelling the pipette tips did not seem to offer any advantage, and the tips were instead simply broken to a diameter of

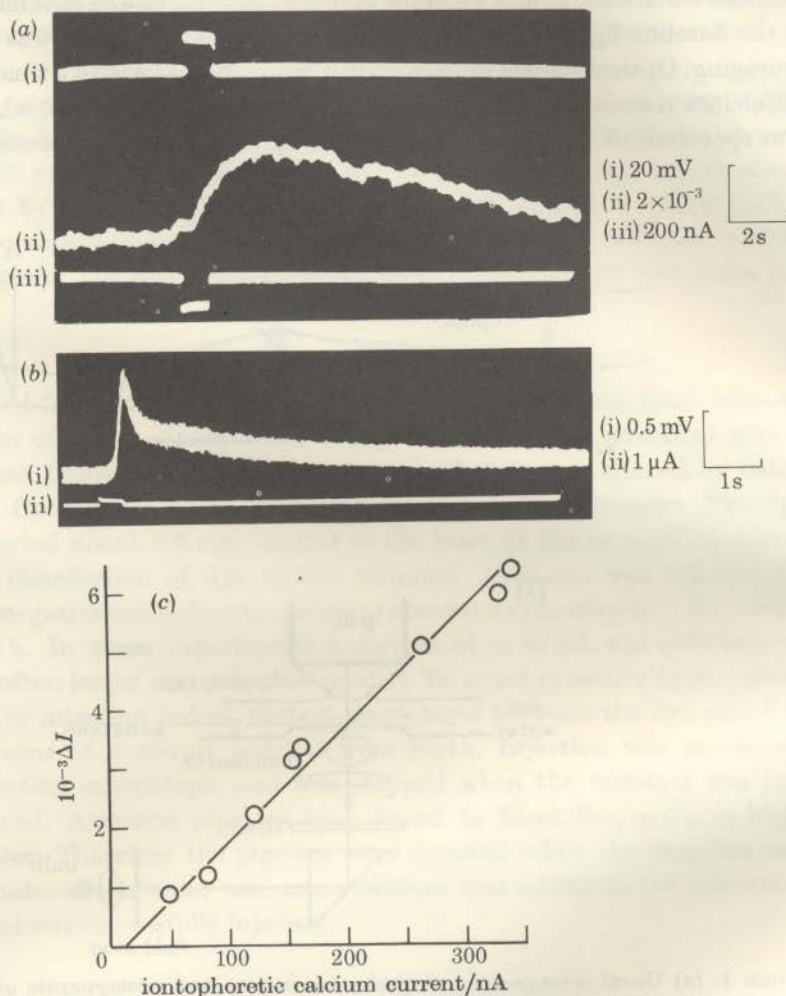


FIGURE 2. (a) Light response to an iontophoretic pulse of calcium injected midway along a presynaptic terminal loaded with arsenazo III. Trace (i) shows membrane potential in pre-terminal, trace (ii) shows light transmission at 650 nm, and trace (iii) monitors current through the Ca^{2+} pipette. The optical response was recorded through a low-pass filter of time constant 33 ms, and in this and all following figures an upward deflexion indicates a decrease in light transmission.

(b) Transmitter release induced by an iontophoretic pulse of Ca^{2+} into a presynaptic terminal. Trace (ii) shows current through the Ca^{2+} pipette (the recording polarity is inverted from (a)), and trace (i) is a high-gain recording of postsynaptic potential. Different ganglion from that in (a).

(c) Relation between iontophoretic Ca^{2+} current (1 s pulse duration) and peak change in light transmission at 650 nm. Light transmission change (ΔI) is expressed as the fractional change in resting transmission. A backing current of about 20 nA was applied to the Ca^{2+} pipette. Data are from the same experiment as in (a).

about 1 μm before insertion. A further problem was that the presence of the dye in the terminal reduced the amount of transmitter release evoked by presynaptic impulses. This did not appear to be due to damage caused by penetration of the pipettes, or by the iontophoretic current, but may have been a toxic effect of the dye. The arsenazo III used was a crude preparation, containing many impurities.

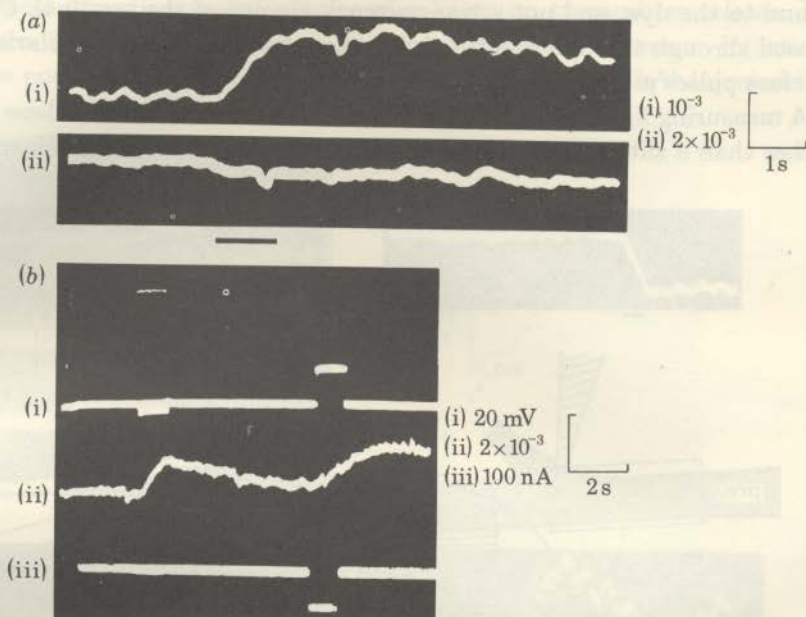


FIGURE 3. (a) Light responses to tetanic stimulation of the preganglionic nerve. Trace (i): light transmission (ΔI) at 650 nm. Trace (ii): transmission (ΔI) at 532 nm. The stimulus was a 50 Hz tetanus for the duration indicated by the bar. Stimulus strength was just above threshold for the main preganglionic fibre. A measuring light slit was used, as shown in figure 1a. Optical recording time constant 33 ms.

(b) Simultaneous recording of response to tetanic stimulation, and to a Ca^{2+} pulse injected in the terminal. Trace (i) shows pre-terminal membrane potential, trace (ii) light transmission (ΔI) at 650 nm, and trace (iii) Ca^{2+} current. Nerve stimulus was a 50 Hz tetanus for 1 s. The size of the spikes is greatly reduced by the time constant of the arsenazo III pipette that was used for recording. At the low gain used for postsynaptic recording (20 mV/cm), no depolarization was detected from the Ca^{2+} pulse. A spot of light of about 100 μm diameter was used for measurement and was centred on the tip of the Ca^{2+} pipette. Optical recording time constant 33 ms.

An alternative possibility, that the reduction in transmitter release was due to a calcium buffering action of the dye, is unlikely, as Brinley *et al.* (1977) found that arsenazo III concentrations of 2 mM had a very small buffering capacity compared to axoplasm from the postsynaptic axon. From the degree of staining of the terminal we estimate that the arsenazo III concentration was less than 0.5 mM.

Light response to Ca^{2+} injections

Iontophoretic injection of calcium pulses into arsenazo III-loaded terminals gave rise to a slow transient decrease in light transmission at wavelengths of 602 and 650 nm (figure 2*a*). Recording at 532 nm, a small increase in transmission was seen, demonstrating that the light signal records a change in the level of calcium bound to the dye, and not a transparency change of the terminal. Current pulses passed through the dye pipette giving the same membrane depolarizations as the calcium pulses produced no light response.

A measuring light spot of about 100 μm diameter was used in these experiments, rather than a slit, since this gave a better signal-to-noise ratio. When the spot was

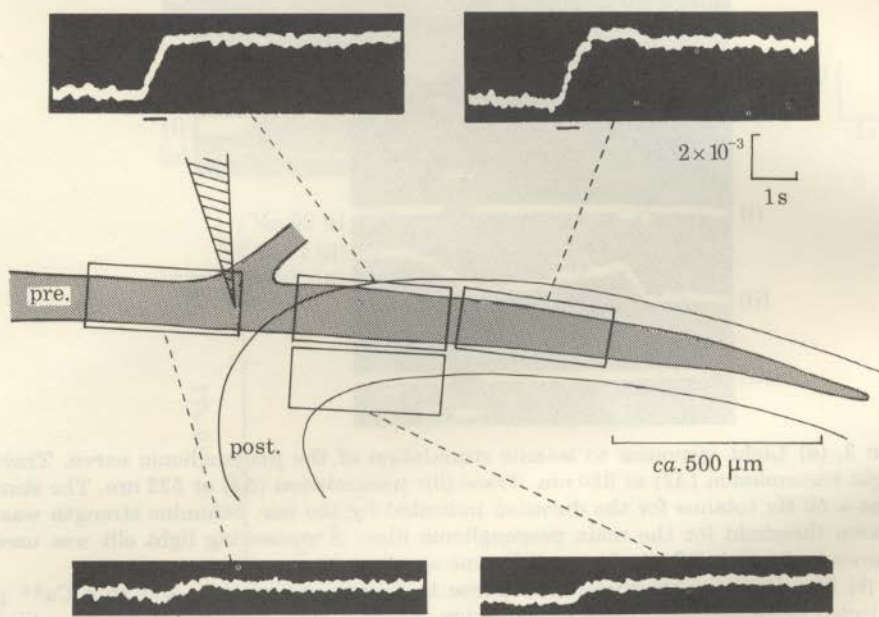


FIGURE 4. Variation in the arsenazo light response to a tetanus, recorded from different parts of the presynaptic axon. The pre-axon is shown shaded and records were obtained with the measuring light slit at the different positions indicated. The length calibration is approximate. The position of the arsenazo injection pipette is also shown. Nerve stimulus was a 100 Hz tetanus for 0.5 s, as indicated by the bars underneath the records. A small amount of TEA had been injected into the terminal, and the duration of the presynaptic spike was prolonged by two or three times. Records were obtained with a dual wavelength recording system (cf. Parker 1979) and show light transmission at 532 nm minus transmission at 650 nm. Large responses were obtained with the measuring light at two positions on the presynaptic terminal (upper traces), but only a small response was obtained from the presynaptic axon just central to the synaptic region (lower left trace). A control record with the measuring light away from the presynaptic axon (lower right trace) showed a small response, possibly resulting from stray light passing through the axon. The decay phase of the arsenazo signal was unusually prolonged in this experiment, possibly because of the low temperature used (10 °C).

moved away from the Ca²⁺ pipette so that the edge of the spot was about 20 μ m from the tip, virtually no light response could be detected. With these pulses, diffusion of Ca²⁺ through the cytoplasm must therefore have been restricted to a radius of about 20 μ m. The decline of the light response gives a measure of the rate at which Ca²⁺ is removed from the cytoplasm, and in three experiments the half-time of decline ranged between 6 and 13 s. A comparable slow decline is also seen in the effect of an intracellular pulse of calcium on transmitter release. For instance figure 2*b*, from a separate experiment (without arsenazo injection), illustrates the postsynaptic depolarization caused by a pulse of Ca²⁺ injected into

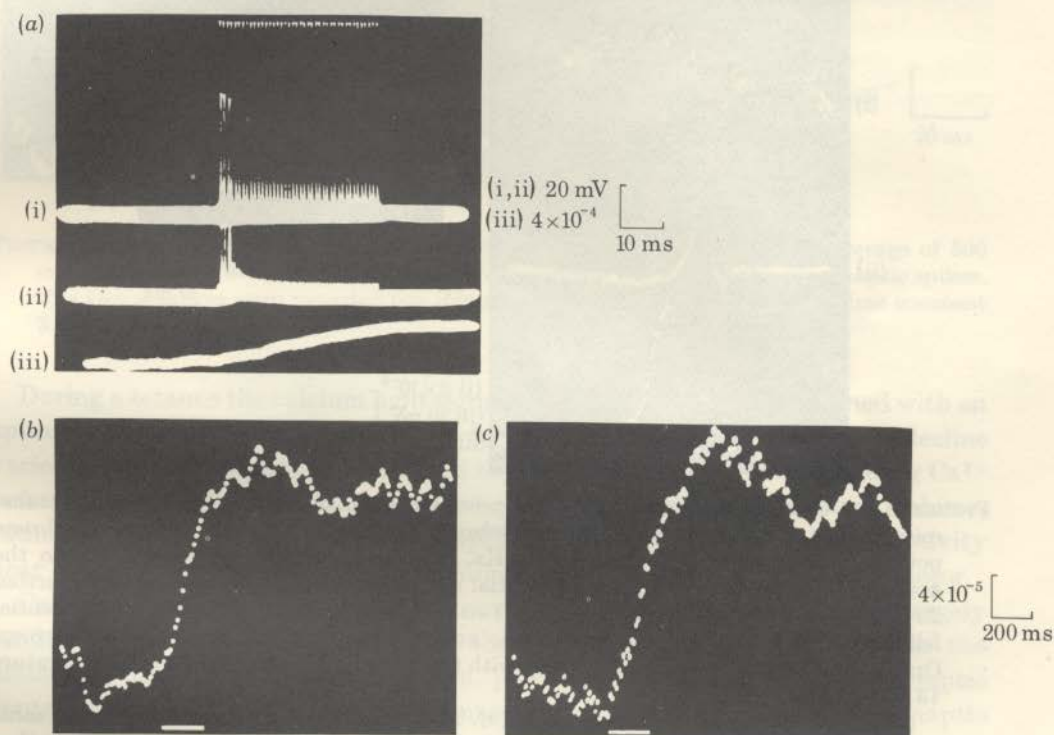


FIGURE 5. (*a*) Rising phase of Ca²⁺ response during a tetanus. Upper trace shows pre-fibre membrane potential, middle trace post-fibre membrane potential, and lower trace light transmission at 650 nm. The first three stimuli gave rise to action potentials in the post-axon, but transmission then failed rapidly, and after about the 30th impulse there was no detectable synaptic potential. The deflections remaining in the post record after this time are stimulus artefacts. Optical time constant 10 ms.

(*b*, *c*) Signal averages of five sweeps, showing the change in light transmission at 650 nm produced by 20 nerve impulses (100 Hz for 200 ms). In (*b*) trains of stimuli were repeated at intervals of 1 min, and synaptic potentials of about 3 mV were recorded at the start of each tetanus, and of about 0.5 mV at the end. In (*c*) the trains were repeated at intervals of 3 s, and five were given before starting the average to fatigue transmitter release. No synaptic potential was detectable at a gain of 1 mV/cm in any of the sweeps averaged for this record. Same experiment as figure 4*a*. Optical time constant 100 ms.

the nerve terminal. This depolarization resulted from the release of many thousands of transmitter packages (Miledi 1973), and transmitter release continued for several seconds after the end of the Ca^{2+} pulse.

A linear relation was observed between the size of the calcium pulse and the peak amplitude of the light response (figure 2c). This relation is the same as previously observed in the postsynaptic axon of the squid's giant synapse (Miledi & Parker, unpublished results) and in frog muscle fibres (Miledi *et al.* 1980), loaded with arsenazo III.

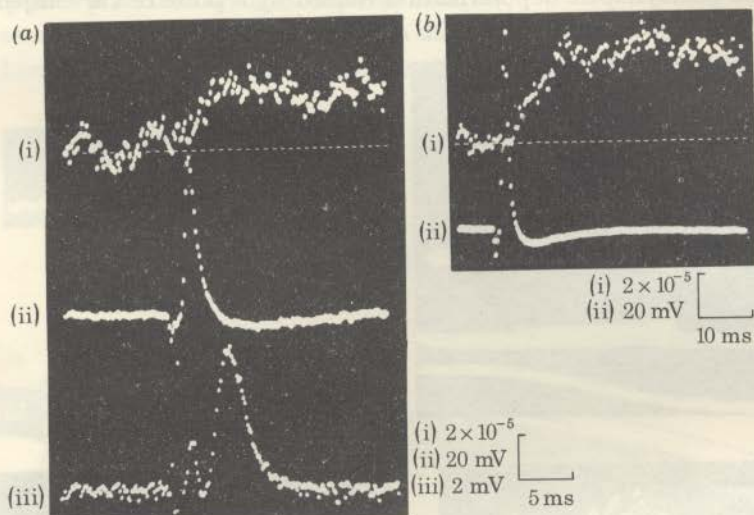


FIGURE 6. (a) Calcium response to a single presynaptic spike. Trace (i) shows light transmission (ΔI) at 650 nm, trace (ii) pre-membrane potential, and trace (iii) post-membrane potential. Average of 500 stimuli at 2 Hz. Only two channels were available on the averager, and the post membrane potential record was obtained by averaging a further 20 sweeps after photographing the other two channels. The size of the synaptic potential fell from 19 mV on the first sweep of the average, to about 1 mV on the 500th sweep. Optical recording was bandpass filtered with time constants of 1 s and 1 ms. Temperature 13 °C.

(b) Signal average of 500 sweeps as in (a). This record was obtained earlier in the same experiment, when a larger calcium response could be recorded. Calibrations and other details as for (a), except that the time calibration is 10 ms.

Light responses during tetani

With the ganglion in normal sea water we were unable to detect a light response to brief tetanic stimulation of the preganglionic nerve; but in high-calcium (55 mM) sea water, a response was obtained in all injected terminals. Figure 3a shows the response to a 100 Hz tetanus for 1 s. Figure 3b shows another experiment, where the light response to a tetanus (50 Hz, 1 s) was followed by an intracellular pulse of Ca^{2+} . In figure 3a a decrease in light intensity was seen at 650 nm, and a small increase at 532 nm. In some experiments a small increase in transparency of the ganglion was seen during stimulation. This light change was

independent of wavelength, and could be recorded anywhere on the ganglion or preganglionic nerve. This artefact could be minimized by adjusting the stimulus strength to just above threshold for the main preganglionic fibre. Reduction of stimulus strength to just below threshold abolished the calcium light response, but had little effect on any remaining 'non-specific' transparency increase. Changes in light scattering of the small nerve fibres in the preganglionic nerve, some of which are triggered before the main fibre, may be responsible for this signal (cf. Von Muralt 1975).

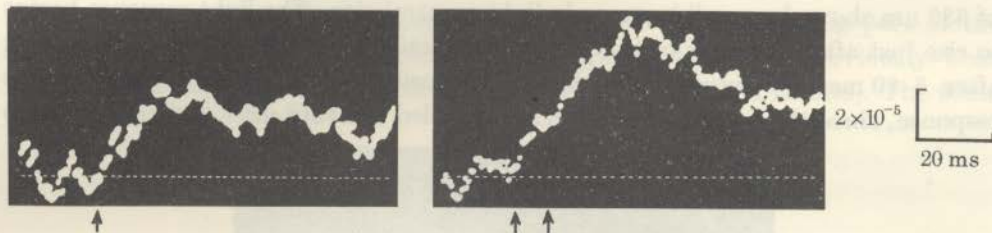


FIGURE 7. Light responses to single and paired impulses. Each record is an average of 500 sweeps, obtained at intervals of 0.5 s. Arrows indicate the time of the presynaptic spikes. The two averages were recorded one immediately after the other. Optical time constant 3.3 ms. Temperature 13 °C.

During a tetanus the calcium light signal rose steadily, and then declined with an approximately exponential time course. In four experiments the half-time of decline varied between 2 and 6 s. This is about twice as fast as the decline following Ca²⁺ pulses, probably because the entering Ca²⁺ is distributed over a large volume, rather than being localized to a small spot; but higher Ca²⁺-sequestering activity near the synaptic contacts (Martin & Miledi 1978) may be a contributing factor.

Ca²⁺ influx during preganglionic nerve stimulation appeared to be largely confined to the synaptic terminal of the presynaptic axon. Records with the measuring light positioned on the axon, just central to the start of the synaptic contact, showed a much smaller arsenazo signal than those from the synaptic region (figure 4) even though the amount of dye in the two regions was approximately the same.

Fatigue of transmitter release

The amount of transmitter released by each impulse decreased rapidly during tetanic stimulation of the pre-axon. However, it is interesting that the Ca²⁺ entry per impulse did not appear to be greatly decreased.

The light response recorded from a terminal stimulated at 50 Hz for 1 s is shown in figure 5*a*. There was a steady increase in the light response during stimulation, although synaptic transmission in this synapse failed after the first three impulses, and there was practically no detectable synaptic potential after about thirty impulses. Moreover, averaged records of light responses to short

tetani obtained when transmitter release was present (figure 5*b*) or very much depressed by rapid stimulation (figure 5*c*) were not significantly different.

Calcium transients from single impulses

In these first experiments we were just able to detect a light response from four stimuli given at 10 ms intervals, and it was therefore necessary to use signal averaging to examine the time course of the Ca^{2+} transient following a single nerve impulse. Figure 6 gives two examples of averaged responses to 500 stimuli, showing the transient fall in light transmission at 650 nm. Control averages at a wavelength of 532 nm showed a small increase in light transmission. The light response begins to rise just after the peak of the presynaptic action potential, and reaches a peak after 5–10 ms. We were unable to record accurately the falling phase of the response, since the signals were capacitor-coupled to avoid drift, but from records

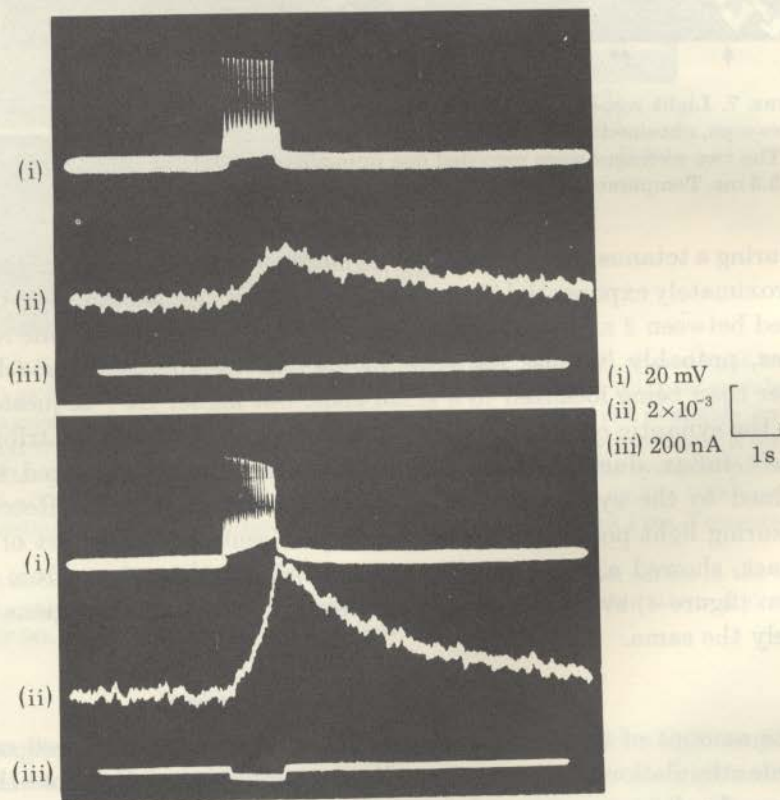


FIGURE 8. Light recordings from an arsenazo III-loaded nerve cell soma. The cell was penetrated by two micropipettes for recording and arsenazo injection, and trains of action potentials were elicited by passing depolarizing current pulses through the arsenazo pipette. The two records show responses to different intensities of depolarizing current. Traces in each record show: top, membrane potential; middle, light transmission (ΔI) at 532 nm minus that at 602 nm; lower, current through the arsenazo pipette. The optical record was filtered with a time constant of 33 ms. Temperature 22 °C; 55 mM Ca^{2+} sea water.

taken at slower sweep speeds it was apparent that the Ca²⁺ level did not return to the baseline for at least several hundred milliseconds.

Paired stimuli, given at an interval of 10 ms, produced a light response that was approximately double that obtained with a single impulse (figure 7). Therefore the calcium entry with each action potential appears to be little affected by an earlier impulse and, as already mentioned, during trains of stimuli the size of the light response rises approximately linearly with the number of impulses (cf. figure 5a).

Arsenazo records from nerve cell somas

Recordings were made from superficial nerve cell bodies forming part of the small axon system of the ganglion (Miledi 1972), which had previously been found to show calcium action potentials (Miledi, unpublished results). The soma

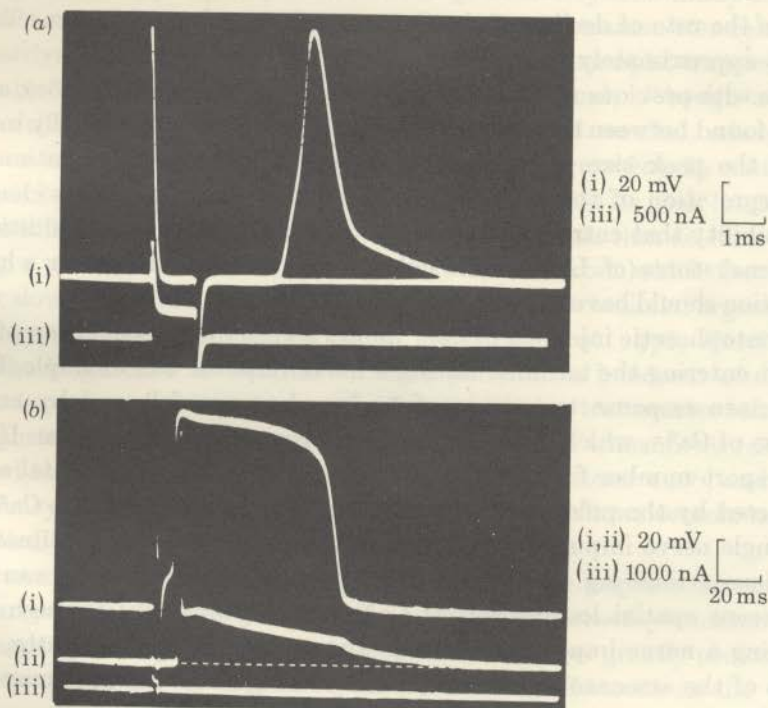


FIGURE 9. Postsynaptic potentials evoked by presynaptic action potentials. In both records the terminal was stimulated by a brief depolarizing pulse from an intracellular micro-electrode; the traces show (from bottom to top) current through the stimulating electrode, postsynaptic potential, and (in *b*) presynaptic potential.

(a) Record from a normal synapse in which an action potential was initiated in the nerve terminal. The transmitter released depolarized the postsynaptic axon sufficiently to trigger an action potential, but the falling phase of the postsynaptic potential can be seen following this.

(b) Record from the same synapse after TEA had been injected into the terminal to prolong the presynaptic action potential. Transmitter release continued during the action potential, as shown by the prolonged postsynaptic depolarization following the initial action potential and declined slowly after the terminal repolarized.

Isolated stellate ganglion preparation in natural sea water. Temperature 13 °C.

was penetrated with a recording electrode and an arsenazo pipette. Dye was injected by passing an iontophoretic current of about 20 nA for a few minutes, until the cell was stained red, with the nucleus appearing darker. From these cells bathed in high-calcium sea water, large arsenazo light records could be obtained in response to trains of action potentials elicited by depolarizing pulses (figure 8).

DISCUSSION

The present results lend further support to the view that a rise in ionized calcium within the nerve terminal is the triggering factor for transmitter release, because a rise in Ca^{2+} was detected at about the same time as the onset of transmitter release by a nerve impulse. With repetitive nerve stimulation, the increase in intracellular Ca^{2+} caused by each nerve impulse is approximately constant, but, since the rate of decline of the Ca^{2+} transient is slow, the intracellular Ca^{2+} level rises approximately linearly during a brief train of impulses.

As with previous results in frog muscle fibres (Miledi *et al.* 1980), a linear relation was found between the amount of Ca^{2+} injected iontophoretically into the terminal and the peak size of the arsenazo response. This linear relation simplifies the interpretation of the arsenazo responses and also provides evidence against the possibility that entry of external Ca^{2+} triggers the release of additional Ca^{2+} from internal stores (cf. Llinas & Nicholson 1975), for, if that were so, a highly nonlinear relation should have been obtained.

Iontophoretic injection of Ca^{2+} allows a very rough estimation of the amount of Ca^{2+} entering the terminal during a nerve impulse. For example, in figure 3*b* the arsenazo response to a train of 50 impulses was followed by an iontophoretic pulse of Ca^{2+} , which evoked a slightly larger arsenazo response. If one assumes a transport number for Ca^{2+} of 0.1 (Kusano *et al.* 1975), the total amount of Ca^{2+} injected by the pulse would be about 3×10^{-14} mol; hence the Ca^{2+} entry during a single nerve impulse would be about 4×10^{-16} mol. This estimate is subject to various errors, e.g. the transport number for Ca^{2+} pipettes can vary, and the different spatial localization of Ca^{2+} entering diffusely through the membrane during a nerve impulse, or focally when released from a pipette, may affect the size of the arsenazo signal. Preliminary though this estimate may be, it is nevertheless interesting to compare it with measurements of the amount of Ca^{2+} entering the postsynaptic axon during an action potential (Hodgkin & Keynes 1957). It seems likely that Ca^{2+} enters the terminal mainly through specialized membrane in the regions of synaptic contacts, but, if for the sake of simplicity we assume that Ca^{2+} enters uniformly over the entire membrane surface, the Ca^{2+} entry, for a terminal with a diameter of 100 μm and a measuring light spot with a diameter of 100 μm , is estimated to be 10^{-12} mol cm^{-2} per impulse. This influx of Ca^{2+} is nearly 50 times greater than that found in the postsynaptic axon (Hodgkin & Keynes 1957) and is in accord with the finding that the comparatively large influx of Ca^{2+} in the nerve terminals is capable of eliciting in them a regenerative

Ca²⁺ action potential which is not obtained in the rest of the axon (Katz & Miledi 1967, 1969). Arsenazo recordings from different parts of the presynaptic axon (figure 4) also confirm that the Ca²⁺ influx is much greater in the nerve terminal, and further improvements in technique may allow us to determine if this influx occurs only across the synaptic membrane, or through the entire membrane surface of the terminal.

It is also clear that the number of Ca²⁺ ions entering the nerve terminal during a nerve impulse greatly exceeds the number of transmitter quanta released (cf. Katz & Miledi 1967). In figure 3*b* the amount of Ca²⁺ that entered the whole terminal is estimated to be about 4×10^{-15} mol (2.4×10^{-9} molecules), while the number of transmitter quanta released by a nerve impulse would have been about five orders of magnitude smaller (Miledi 1967).

An interesting feature of the present results concerns the very slow decay of the intracellular Ca²⁺ transient, compared with the duration of transmitter release, caused by the arrival of a nerve impulse at the terminal. For example, transmitter release is very intense immediately after a nerve impulse, and, although a few quanta can be released 10–100 ms later (Miledi, unpublished results), release is essentially over a few milliseconds after the nerve impulse (figure 9*a*). In contrast the intraterminal Ca²⁺ transient, recorded with arsenazo, reaches its peak later and persists for several hundred milliseconds (figures 6, 7). It appears that calcium ions going in during a nerve impulse diffuse into the cytoplasm from which they are removed rather slowly by soluble Ca²⁺-binding proteins and intracellular organelles (Alemà *et al.* 1973; Baker & Schlaepfer 1975; Blaustein *et al.* 1978; Martin & Miledi 1978). The heightened level of Ca²⁺, which persists for hundreds of milliseconds after a nerve impulse, is not very effective in eliciting transmitter release, but may be important in phenomena such as the facilitation of transmitter release seen with paired stimulation (Katz & Miledi 1968; Miledi & Slater 1966) and the increased frequency of discharge of transmitter quanta that follows tetanic nerve stimulation (Miledi & Thies 1971; Cooke & Quastel 1973).

Normally, transmitter release ceases rapidly after a nerve impulse. However, when the K⁺ current is blocked by intracellular injection of tetraethylammonium, both the presynaptic action potential and transmitter release are greatly prolonged (figure 9*b*), because the initial depolarization opens Ca²⁺ channels in the presynaptic membrane and the influx of Ca²⁺ itself leads to further depolarization (cf. Katz & Miledi 1967, 1969). During prolonged depolarization the increased calcium conductance shows little if any inactivation (Katz & Miledi 1971) and the Ca²⁺ going in causes the level of ionized calcium to go on increasing (cf. Llinas & Nicholson 1975) as it does during a tetanus or during an intracellular pulse of Ca²⁺ (figures 3–6). In spite of this, transmitter release diminished progressively (figure 9*b*), presumably because one or more steps in the release process become partially 'exhausted'. Following repolarization of the presynaptic membrane, transmitter release decreases rapidly at first, but is then followed by a phase of low-level release lasting several seconds. This slow phase resembles the prolonged

release caused by a large intracellular pulse of Ca^{2+} and, likewise, may be a reflection of a long-lasting increase in free calcium within the terminal.

The cause of the rapid decrease in transmitter release following a nerve impulse, at a time when the overall level of intra-terminal Ca^{2+} is still increasing (figures 6, 7) is not clear. Because of the short delay between membrane depolarization and onset of transmitter release (Miledi & Slater 1966; Llinas *et al.* 1976), calcium ions must be acting very close to their site of entry. Perhaps the sites to which Ca^{2+} binds to release transmitter are located at the inner mouth of the channel. A high concentration of Ca^{2+} builds up at these sites while the channel is open, but the concentration of Ca^{2+} falls rapidly when the channel is closed because the calcium available is removed by binding to receptor sites and by diffusion further into the terminal. A contributing factor may be increased Ca^{2+} -buffering systems in the nerve terminal close to the synaptic membrane (Martin & Miledi 1978).

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