ROLE OF PRESYNAPTIC CALCIUM IONS AND CHANNELS IN SYNAPTIC FACILITATION AND DEPRESSION AT THE SQUID GIANT SYNAPSE

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SUMMARY

1. The roles of presynaptic calcium influx and calcium accumulation in synaptic facilitation and depression were explored at the giant synapse in the stellate ganglion of the squid.

2. Calcium currents were recorded in the presynaptic terminal, using a three-electrode voltage clamp and blocking sodium and potassium currents pharmacologically. The calcium influx was constant during pairs or trains of brief depolarizing pulses that elicited facilitating or depressing excitatory post-synaptic potentials (e.p.s.p.s).

3. The relationship between calcium influx and transmitter release during brief depolarizing pulses of varying amplitude resembled a power function with exponent of about 2.

4. Presynaptic calcium concentration transients were measured by injecting the dye arsenazo III and detecting absorbance changes microspectrophotometrically. Increments in intracellular free calcium accompanying single action potentials appeared constant for repeated action potentials that elicited facilitating e.p.s.p.s.

5. The presynaptic calcium concentration remains elevated for several seconds following action potentials.

6. Presynaptic injection of calcium ions by interbarrel ionophoresis evokes a post-synaptic depolarization, apparently reflecting a large increase in miniature e.p.s.p. frequency. Presynaptic action potentials remain unaffected by this treatment, but e.p.s.p.s triggered by them are facilitated for several seconds, and then depressed.

7. The results are consistent with the hypothesis that synaptic facilitation is due to the action of residual calcium or a calcium complex remaining in the presynaptic terminal after electrical activity. The late depression of release during calcium injection may be a result of the continual release of transmitter and consequent depletion of a presynaptic store.

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INTRODUCTION

The efficacy of chemical synaptic transmission is often a function of the pattern of previous presynaptic activity. The duration of such changes ranges from tens of milliseconds (synaptic facilitation and depression) to seconds (synaptic potentiation and fatigue) to days and weeks (competitive interactions between synapses and effects of experience). None of these processes is well understood at the molecular level. The shortest lasting changes (facilitation and depression) are the easiest to manipulate and therefore have been studied most. In this paper we explore the mechanisms underlying facilitation at the squid stellate ganglion giant synapse, and also report some observations related to synaptic depression.

The most prominent type of plasticity evident in the squid giant synapse preparation is a profound depression at high frequencies (Bullock, 1948; Horn & Wright, 1970; Kusano & Landau, 1975), leading to rapid failure of the post-synaptic action potential. This synaptic depression may be relieved by reducing calcium concentration in the external medium, unmasking an underlying synaptic facilitation whose magnitude and kinetic properties are relatively insensitive to changes in external calcium (Charlton & Bittner, 1978 a). The synaptic facilitation so evoked bears many similarities to facilitation at other chemical synapses (Charlton & Bittner, 1978 a, b) and is likely to provide a reasonable model for the phenomenon in general.

Charlton & Bittner (1978 b) considered and eliminated two hypotheses for synaptic facilitation at this synapse: (i) growth in amplitude and duration of the presynaptic action potential during repetitive activity, and (ii) enhancement of transmitter release by hyperpolarizing after-potentials. They showed that these processes together can generate at most only a small proportion of the facilitation at this synapse, and that under certain conditions normal-looking facilitation can occur in their absence. We consider here two further hypotheses: (iii) that presynaptic calcium channels themselves facilitate, such that during successive identical depolarizations the calcium influx increases, and (iv) that facilitation is a consequence of residual intracellular calcium ions or a calcium complex, which causes a presynaptic depolarization to release more transmitter than would otherwise occur (Katz & Miledi, 1965, 1968; Rahamimoff, 1968). These hypotheses are not entirely distinct, in that calcium channels may be facilitated as a consequence of residual calcium (Zucker, 1974). Nevertheless, as they are formulated above, the third hypothesis proposes only that *somehow* the calcium channels themselves facilitate, and the fourth hypothesis proposes only that *somehow* residual calcium facilitates transmitter release.

To examine the latter two hypotheses our plan was as follows. First, we investigated the properties of calcium channels to repeated depolarization, to determine whether the calcium currents themselves facilitate. We then used the calcium-sensitive dye arsenazo III to determine whether, following a single action potential or a brief train, any residual free calcium remains in the presynaptic terminal for as long as facilitation persists. Finally, we determined the effect of raising intracellular calcium on the release of transmitter by an action potential. Preliminary accounts of these results have appeared (Charlton, Zucker & Smith, 1980; Smith, Charlton & Zucker, 1980; Zucker, Charlton & Smith, 1980).

METHODS

All experiments were performed on small (10–15 cm) specimens of *Loligo pealei* collected at the Marine Biological Laboratory, Woods Hole, MA. Animals were kept in running sea water and used within 1 day of capture. The stellate ganglion was dissected as described by Charlton & Bittner (1978*a*) and continuously superfused with artificial sea water containing 466 mM-NaCl, 10 mM-KCl, 11 mM-CaCl$_2$, 54 mM-MgCl$_2$, 3 mM-NaHCO$_3$, and 10 mM-HEPES buffer pH 7·2, through which 99·5 % O$_2$:0·5 % CO$_2$ was bubbled continuously. The CO$_2$ and bicarbonate served to maintain intracellular pH buffering (Thomas, 1977). In all experiments the temperature was controlled at 15 ± 1 °C.

Electrophysiological procedures

Multiple micro-electrodes were inserted simultaneously into the presynaptic terminal and post-synaptic axon of the giant synapse. The presynaptic giant fibre was stimulated extracellularly using platinum wire electrodes.

Intracellular injection of calcium ions was accomplished by micro-ionophoresis with a constant current source passing current between two barrels of a micropipette pulled from thick-septum theta tubing (R and D Optical, New Windsor, MD). One barrel contained 3 M-KCl and the other contained 0·5 M-CaCl$_2$ plus 1·5 M-KCl added to reduce clogging. The pipettes were checked before and after use by observing the colour change at the tip when injecting calcium into a solution of arsenazo III.

Presynaptic calcium currents during depolarizations were measured using the three-electrode voltage-clamp method of Adrian, Chandler & Hodgkin (1970) at the presynaptic terminal, as described by Llinás, Steinberg & Walton (1981*a*). The first voltage-recording electrode was placed at l = about 250 μm from the end of the terminal, with the second voltage electrode about 250 μm more proximally, and the current electrode about 50 μm proximal to the latter. Two measures of membrane current were recorded. The first was total current through the current electrode, monitored as the voltage drop across a resistor in series with the current electrode and recorded through a high-voltage differential amplifier. Much of this current crossed the presynaptic membrane in the axon proximal to the terminal, where the membrane potential was poorly controlled by the clamp circuit. The local membrane current through the presynaptic terminal itself was also recorded as the difference in membrane potential between the two presynaptic voltage micro-electrodes. This potential difference was typically about 2 mV for a 50 mV voltage step, and never exceeded 10 % of the voltage step. Hence, the presynaptic potential was reasonably homogeneously controlled in the terminal region. Although voltage-clamp experiments can be performed in this preparation with only two electrodes, the three-electrode technique is probably superior because the current measured is only that flowing in the terminal region. In our hands, insertion of the third presynaptic electrode neither increased the failure rate of our experiments nor caused further membrane damage.

The voltage difference $\Delta V = V_1 - V_2$, where V_2 is the voltage at the proximal voltage electrode and V_1 is the voltage at the distal voltage electrode, can be converted to a measure of the presynaptic terminal membrane current density, I_m, by a derivation similar to that of Adrian *et al.* (1970). Their equation, $I_m = a\Delta V/3R_i l^2$, where R_i is axoplasmic resistivity, must be modified for the geometry of the presynaptic terminal, which resembles a right elliptical cylinder with major radius a about three times the minor radius b (R. Joyner, personal communication). The appropriate equation for this geometry is $I_m = 2\Delta VA/3l^2 R_i c$, where A is the cross-sectional area of the terminal (πab) and c is the circumference. For a 3:1 ellipse, c is found to be 4·456a, using elliptical integrals, so $I_m = 0·1567 \, a\Delta V/R_i l^2$. We used a value of $R_i = 35 \, \Omega . \text{cm}^2$ (Hodgkin & Huxley, 1952). This treatment takes no account of tapering of the terminal, only the major axis of each terminal was measured in each specimen, R_i was not determined in these experiments, and the electrode placement (l) was not extremely precise. Therefore, this equation provides only an approximate conversion factor between ΔV and I_m.

The voltage-clamp circuit itself was fairly conventional, but used a 150 V operational amplifier. Since the major currents normally carried by sodium and potassium ions were blocked pharmacologically in these experiments, the membrane current was not excessive and series resistance errors were small and did not require compensation. A large bath electrode and small currents also reduced bath electrode polarization and eliminated the need for bath potential subtraction. Clamp performance was improved by including a series capacitor in the feed-back pathway of the control

amplifier to boost the low frequency gain (suggested by Fred Sigworth). The time required to charge the presynaptic cable was also reduced by boosting the intermediate frequency response of the clamp amplifier (suggested by Bill Huse). In some experiments, a second voltage-clamp circuit (described in Smith & Zucker, 1980) was used in the conventional two-electrode configuration to record excitatory post-synaptic current from the post-synaptic giant axon.

Most electrophysiological recordings required fast time resolution, and the analogue signals were displayed on an oscilloscope screen and photographed or averaged using a signal averaging computer. Leakage currents were removed by subtracting in the memory of the signal averager the membrane currents elicited during equal and opposite voltage-clamp steps. In experiments in which optical as well as electrical signals were recorded, all data were digitized, stored, averaged and displayed using a Processor Technology Sol 20 microcomputer system.

Intracellular calcium measurement

Presynaptic terminals from selected specimens were filled with arsenazo III by intracellular ionophoresis from a micropipette filled with 75 mM-arsenazo III (Sigma Grade I). The ionophoretic pipette was inserted about 0·75–1·0 mm from the end of the terminal, and dye was injected with cathodal current of 5–20 nA. A spot of monochromatic light was focused on the last 1 mm of the terminal, and light transmitted through the terminal was collected by a light pipe coupled to a low-noise photo-diode. The light pipe was tapered to a fine tip by pulling in a microforge and polishing the end to a rectangular wedge of dimensions $330 \times 70 \ \mu m$. The sides of the terminal-shaped tip were coated with silver paint and then with an insulating resin to prevent pick-up of stray light. The light guide was manipulated against the ventral surface of the stellate ganglion, just above the terminal, to stabilize the preparation and collect the maximum amount of light transmitted through the terminal.

The absorbance changes indicating calcium concentration changes accompanying single presyn-aptic action potentials are extremely small. To reduce extraneous noise, we identified the following sources of noise and adopted these precautions to minimize it: (i) light source fluctuations were cancelled by subtracting from the transmitted light signal another signal proportional to the incident light intensity; (ii) dust in the airborne light path was reduced by use of a dust-shielding canopy around the preparation, and dirt and tissue particles were kept to a minimum in the dissection chamber and superfusion solutions; (iii) mechanical vibration, the major source of noise, was minimized by floating the preparation on an air suspension table; (iv) photo-diode detector noise and vibration artifacts were minimized by differential multiple wave-length spectrophotometry using an air turbine spinning chopper wheel to change wave-lengths every 5 msec and a multiple wave-length spectrophotometer detector circuit to de-multiplex, equalize and ratiometrically report transmittance at four wave-lengths. In addition to records of absorbance changes at 578, 610, 660 and 690 nm, this circuit provided a signal proportional to the differential wave-length pair 660–690 nm, where 660 nm is the peak of the calcium-indicating absorbance difference spectrum of arsenazo III and 690 nm is the point on the difference spectrum nearest to 660 nm that is virtually independent of calcium concentration. Unfortunately, this apparatus provides a time resolution of only 20 msec for four wave-lengths, which is far too slow to catch the rising phase of the calcium concentration change accompanying an action potential. Therefore, to record these brief transients we abandoned the multiple wave-length multiplexing spectrophotometer and recorded responses continuously at only one wave-length at a time. The resulting signals were similar in magnitude to the noise level, and could only be observed by averaging multiple responses using a microcomputer. Further details on the procedures and apparatus for detecting arsenazo III signals are described in Smith & Zucker (1980) and Zucker (1981).

We would like to be able to quantitatively relate changes in arsenazo III absorbance to changes in average intracellular calcium concentration in the presynaptic terminal. To do this we must know the path length of light passing through the terminal, the concentration of arsenazo III in the terminal, and the relation between $\Delta A_{660-690}$ and changes in internal calcium. Unfortunately, we have accurate information on none of these, and measurements were carried out as follows:

(i) Although we can measure the width of each terminal as seen from the ventral surface, we have not histologically examined each preparation to ascertain the terminal's thickness. We selected preparations with laterally disposed terminals to maximize the average path length, which we can only estimate to be about 50 μm.

(ii) While injecting arsenazo III, we monitored the absorbance of the terminal at 578 nm, which

is the isosbestic point of arsenazo III for calcium, pH and magnesium changes. Using the above estimate of path length, the concentration of arsenazo III can be calculated from its molar extinction coefficient and the change in absorbance (Smith & Zucker, 1980). A correction was made for the fact that roughly 50 % of the light picked up by the photo-diode is scattered light that never passed through the terminal. The percentage of scattered light was estimated in most preparations by filling the terminal to darkness with arsenazo III at the end of the experiment and measuring the remaining (scattered) light directly. From this procedure, we estimate that the intracellular arsenazo III concentration ranged from 0·07 to 0·9 mM, and was typically about 0·25 mM. This concentration was reached after about 30 min of continuous injection, at which time there was a steady state between arsenazo III entry into the terminal from the micropipette and its leakage out of the terminal into the axon. To maintain this concentration of arsenazo III, we continued weak ionophoresis of dye throughout the experiment.

(iii) Finally, the relationship between absorbance change and calcium concentration change is not known accurately, because it depends on pH, magnesium concentration, ionic strength, arsenazo concentration and other factors not precisely known for the presynaptic terminal. Moreover, the stoichiometry of the arsenazo III reaction with calcium is not fully understood (Thomas, 1979; Ahmed, Kragie & Connor, 1980), and arsenazo III may not behave identically in cytoplasm and *in vitro* (Beeler, Schibeci & Martonosi, 1980). Nevertheless, using our previous calibration of arsenazo III at pH 7·3, 3 mM-magnesium, 0·5 M-ionic strength, and 0·25 mM-arsenazo III (Smith & Zucker, 1980), and assuming 50 % scattered light, we calculate that an absorbance change at 660 nm of 10^{-5} in a 50 μm-thick terminal filled with 0·25 mM-arsenazo III would correspond to a change in intracellular calcium concentration of 55 nM, averaged across the diameter of the terminal. Considering all the uncertainties in this calculation, it must be regarded as at best only an order-of-magnitude estimate.

<div style="text-align:center">RESULTS</div>

Calcium influx during repeated depolarization

We measured calcium current in the presynaptic giant synapse terminal after blocking sodium and potassium currents pharmacologically. A three-electrode voltage clamp measured calcium influx selectively in the well clamped portion of the terminal from which transmitter is released. Sodium current was blocked by adding tetrodotoxin (2×10^{-7} g/ml.) to the medium, and potassium current was blocked by injecting tetraethylammonium ion intracellularly into the terminal by inter-barrel ionophoresis between two of the three presynaptic micro-electrodes and by adding 2 mM-3,4-diaminopyridine to the external solution. Both these drugs are use-dependent in their blocking efficacies: tetraethylammonium enters the potassium channel and blocks it from inside on depolarization (Armstrong, 1966), while 3,4-diaminopyridine is driven out of the channel by depolarization, relieving its blocking action (Kirsch & Narahashi, 1978). Thus the best block of potassium channels is secured by use of both agents simultaneously (Llinás *et al.* 1981*a*).

Presynaptic calcium channel kinetics to single depolarizing pulses have been described by Llinás, Steinberg & Walton (1976, 1981*a*). The channels open rapidly with voltage-dependent sigmoidal kinetics and close even more rapidly and exponentially with a voltage-dependent time constant. This behaviour is similar to that described for calcium channels in gastropod neural somata (Akaike, Lee & Brown, 1978; Adams & Gage, 1979).

We examined calcium channel behaviour to paired depolarizations of duration similar to action potentials in nine preparations. The external calcium was raised to 25 mM to enhance calcium currents. Moderate-amplitude depolarizations (about 50 mV in magnitude and 0·75–1·5 msec in duration and separated by 1–3 msec) were

used to produce subthreshold excitatory post-synaptic potentials (e.p.s.p.s) which displayed synaptic facilitation. Figs. 1 and 2 illustrate the results of one typical experiment. Each depolarization was accompanied by a rapidly activating inward calcium current, which did not reach steady-state activation during the brief pulses used. The leak current in the terminal was typically very small, so the net current
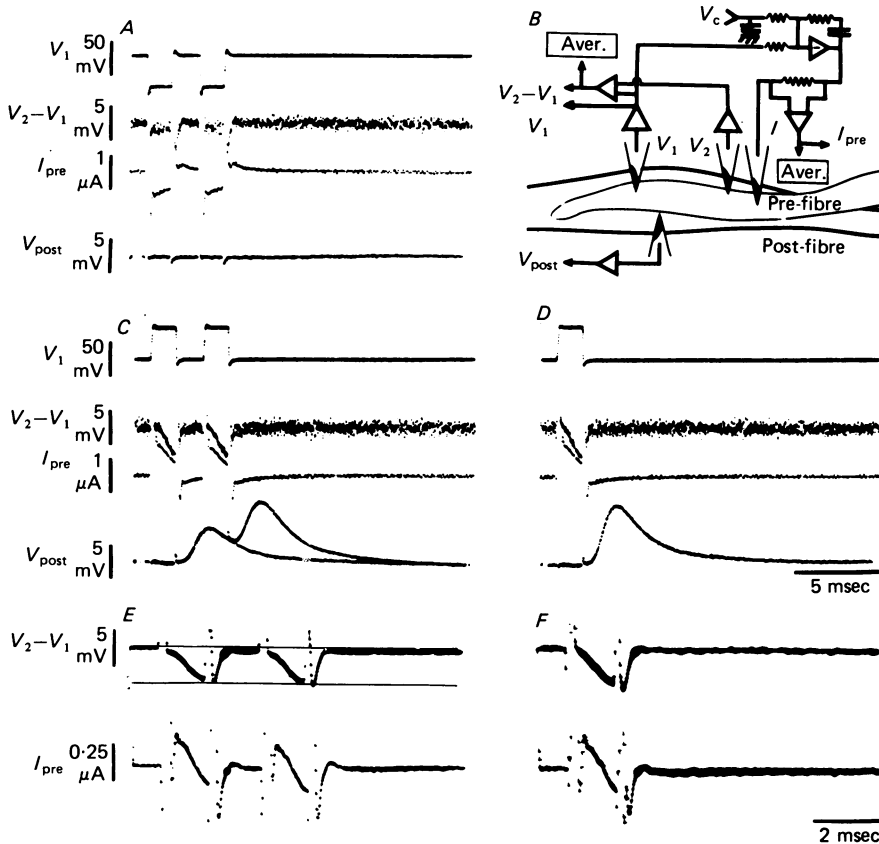

Fig. 1. Calcium currents in the presynaptic terminal. *B*, layout of experiment, showing location of two presynaptic voltage (V_1 and V_2) and current (I) electrodes and post-synaptic voltage electrode. Amplifiers for presynaptic potential (V_1), potential gradient ($V_2 - V_1$), total current (I_{pre}) and post-synaptic potential (I_{post}) signals and the voltage-clamp feed-back amplifier with command input (V_c) are also shown, as well as the two inputs to the signal averager (Aver.). *A* and *C*, responses to two hyperpolarizing or depolarizing command pulses. In *C*, the post-synaptic trace is a superposition of responses to one and two pulses, to allow the accurate measurement of the second e.p.s.p. *D*, a single depolarization was delivered with amplitude adjusted to elicit an e.p.s.p. equal in magnitude to the second e.p.s.p. in *C*. *E*, Computer-averaged records of local and total presynaptic current during eight depolarizing and eight hypoerpolarizing paired pulses. A slight downward shift arises from a slight increase in the leak current during the second pulse (See Fig. 2). *F*, current averaged from eight depolarizing and hyperpolarizing pulses adjusted to release the same amount of transmitter as the second pulse in *A*. Note that the currents in *F* are larger than the second of the currents in *E*. Holding potential: −70 mV. In this terminal, a 5 mV difference between V_2 and V_1 corresponds to a current density of 107 $\mu A/cm^2$ or a current of 72 nA in the distal half of the terminal beyond the V_2 electrode.

began near zero and was inward throughout the pulse. A rapid inward current tail, sometimes seen at the termination of the pulse, was often obscured by the capacitance current. The total current through the current electrode included a large outward leak current into the axon, on which an inward calcium current was superimposed. The records of Fig. 2 confirm that the inward going current is carried by calcium ions.
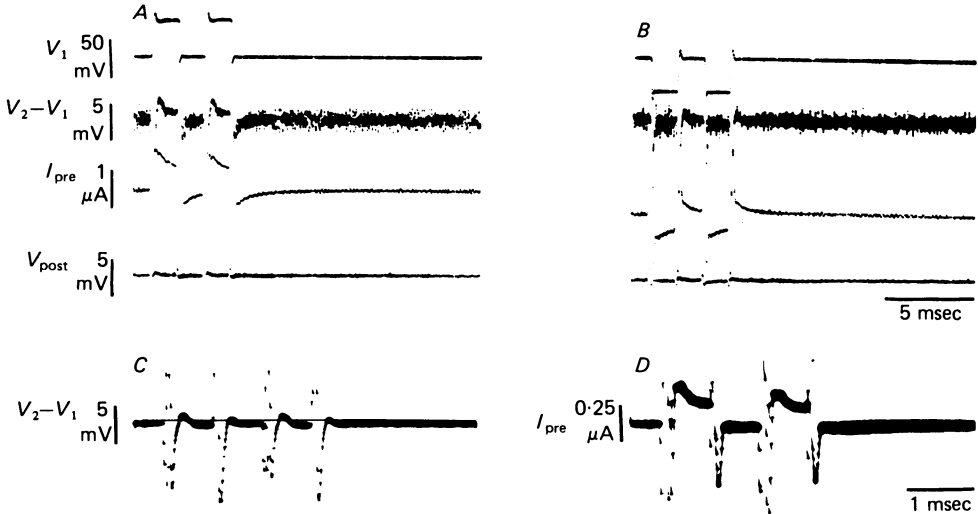

Fig. 2. Capacitance and leak currents in the same preparation as Fig. 1 after blocking calcium current with cadmium. *A* and *B* show depolarizations and hyperpolarizations; *C* and *D* are both averages of eight depolarizing and eight hyperpolarizing pulse pairs. *C* shows the local current, *D* the total current. A slight downshift in the leak current during the second pulse is evident.

At the end of each experiment, the medium was changed to one containing 1 mM-calcium and 1 mM-cadmium, a potent calcium-channel blocker (Kostyuk & Krishtal, 1977). The inward current was absent in this solution, leaving only the capacitance and leak currents. An outward rectification of leak current was often evident in the total current recording.

Both the local inward currents appear identical during the two depolarizations. However, the second e.p.s.p. was typically 65 % larger than the first. In order to detect small changes in presynaptic currents, we averaged 8–16 responses to pairs of stimuli applied at 10 sec intervals. To cancel symmetrical leak currents, the average included an equal number of responses to hyperpolarizing pulses of equal duration and magnitude. In five of nine preparations, these computer-averaged local and total calcium currents were also identical during the two pulses. In three of these experiments, a small increase in peak net inward current was clearly due to a change in the leak current during depolarizing or hyperpolarizing pulses. A change in leak during a hyperpolarizing pulse was easily recognized, and a change in leak during a depolarizing pulse could be observed in the presence of cadmium (see Fig. 2*C*). In normal medium it was manifested as a downward shift in the net current during the second pulse, with no change in the amplitude or time course of the inward-going calcium current component (Fig. 1*E*). In the ninth experiment, the peak calcium currents during the two pulses were identical, but the calcium current activated

slightly faster during the second pulse. The interval between pulses in this preparation was unusually brief (only 1 msec).

If transmitter release were very sensitive to very small changes in presynaptic calcium influx, facilitation of the second e.p.s.p. could be due to such a small change in calcium current that we would fail to detect it. To test this possibility, we increased
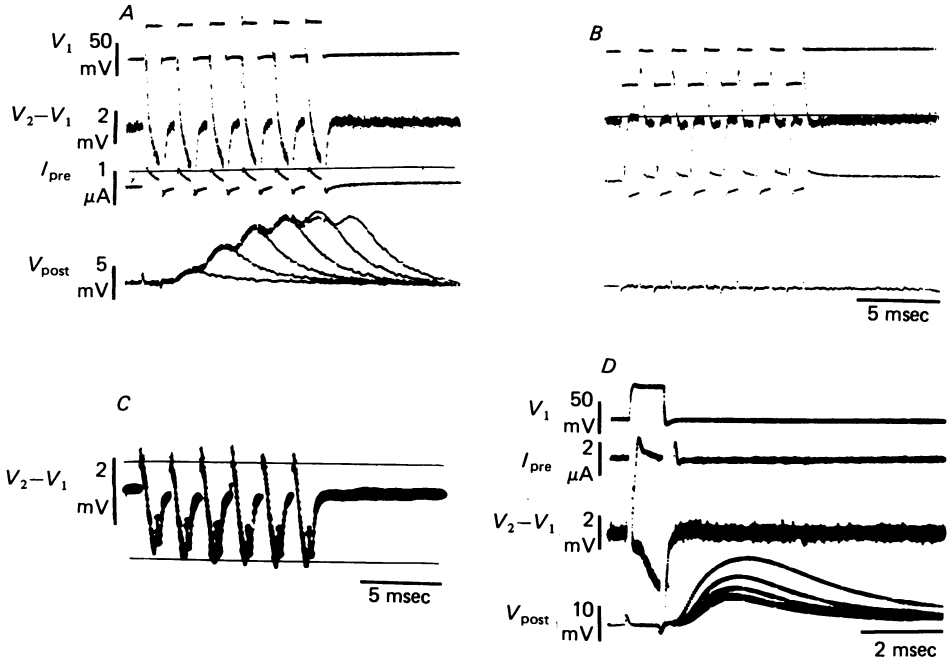

Fig. 3. Calcium currents during a train of depolarizing pulses evoking facilitating, then depressing e.p.s.p.s. A, 6 depolarizing pulses, with post-synaptic responses to 1, 2, 3, 4, 5 and 6 pulses superimposed. The inward calcium currents are constant. B, 6 hyperpolarizing pulses, showing a slight progressive increase in the leak current. C, average of 16 depolarizing and hyperpolarizing pulses. D, from a different preparation: calcium currents during a sequence of 5 single depolarizing pulses delivered at 4 Hz, eliciting e.p.s.p.s showing depression. Holding potential: -70 mV. $V_2 - V_1$ of 2 mV corresponds to 62 μA/cm^2 or 53 nA in the distal half of the terminal in A–C and to 49 μA/cm^2 or 66 nA in the distal half of the terminal in D.

the size of a single presynaptic depolarization until an e.p.s.p. equal in size to the facilitated e.p.s.p. was produced, and we recorded the calcium current eliciting this larger e.p.s.p. In every case, the single larger e.p.s.p. was accompanied by an easily detectable increase in presynaptic calcium current. In nine preparations, an e.p.s.p. adjusted to match the facilitated e.p.s.p. (on average 65 % larger than the control e.p.s.p.) was evoked by a calcium current whose peak was on average only 27 % larger than that accompanying the control e.p.s.p. In every case the increase in calcium influx was easily detectable in both single and averaged local and total presynaptic current records. We conclude that no increase in calcium current sufficient to account for facilitation occurs, and that our methods are sensitive enough to detect the increase in calcium current that would be necessary to lead to facilitation if this mechanism were operating alone.

In two of the above nine preparations we also recorded the presynaptic calcium currents accompanying a train of depolarizing pulses. Fig. 3 A–C illustrates the results of one of these experiments. The inward currents accompanying clearly facilitating e.p.s.p.s. appear identical. An apparent facilitation of the average currents is due to changes in the leak currents accompanying the train of hyper-polarizing pulses.
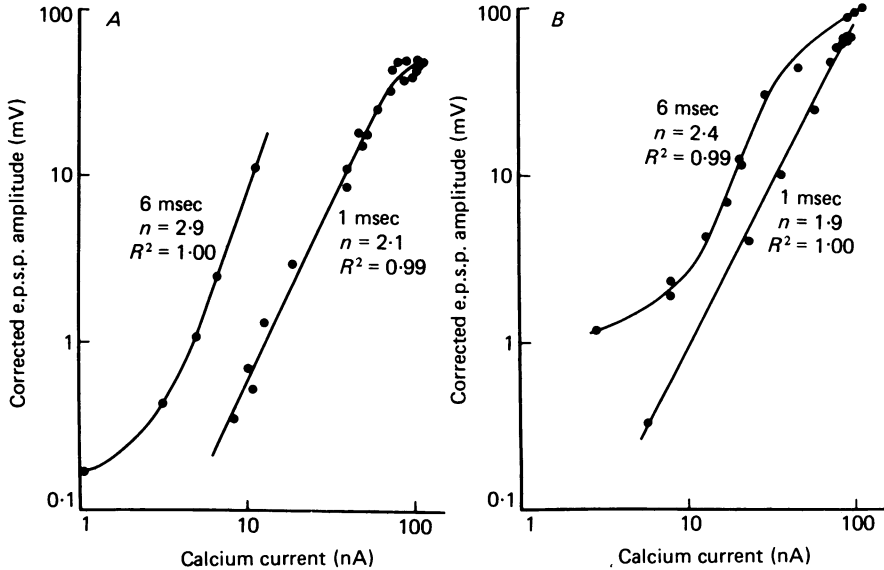

Fig. 4. Relation between transmitter release, estimated from corrected e.p.s.p. amplitude, and peak calcium current, for 1 and 6 msec pulses, plotted on logarithmic co-ordinates. R^2 is the correlation coefficient and n is the slope of the least squares line through the linear part of each curve. A and B are from different preparations.

The last e.p.s.p. in the train of Fig. 3 A–C is actually smaller than the previous one, indicating depression of synaptic transmission. Depression may be studied in isolation by stimulating at low frequencies. Then facilitation will decay completely between stimuli, and only longer-lasting depression will accumulate (Kusano & Landau, 1975). Fig. 3 D shows presynaptic calcium currents accompanying five successive e.p.s.p.s at 4 Hz showing a marked depression. Again, the calcium currents are constant.

When single depolarizing pulses were increased to elicit an e.p.s.p. that was on average 65 % larger, the calcium current (I_{Ca}) increased only an average of 27 %. This suggests a highly non-linear relationship between calcium influx and transmitter release. In fact, the ratio log (e.p.s.p.$_1$/e.p.s.p.$_2$)/log (I_{Ca-1}/I_{Ca-2})was 2·23 ($\pm$0·42 s.d., $n = 9$), suggesting a 2–3 power law relation between e.p.s.p. and I_{Ca}. This relation was examined more carefully in three experiments by stimulating the presynaptic terminal with depolarizing pulses of different amplitudes and plotting the e.p.s.p. amplitude corrected according to Martin (1955) *vs.* the peak calcium current. The results of two experiments, using 1 and 6 msec long pulses, are shown in Fig. 4. There appears to be a sigmoid relationship between log e.p.s.p. and log I_{Ca}, with a maximum slope of about 2, and some suggestion of both a threshold level of calcium influx for

transmitter release and saturation of release at large calcium currents. A similar relationship is obtained on plotting e.p.s.p. area *vs.* I_{Ca} area.

Presynaptic calcium concentration transients following electrical activity

The above results indicate that calcium influx did not facilitate to repeated depolarization. We used arsenazo III to measure the persistence of internal calcium
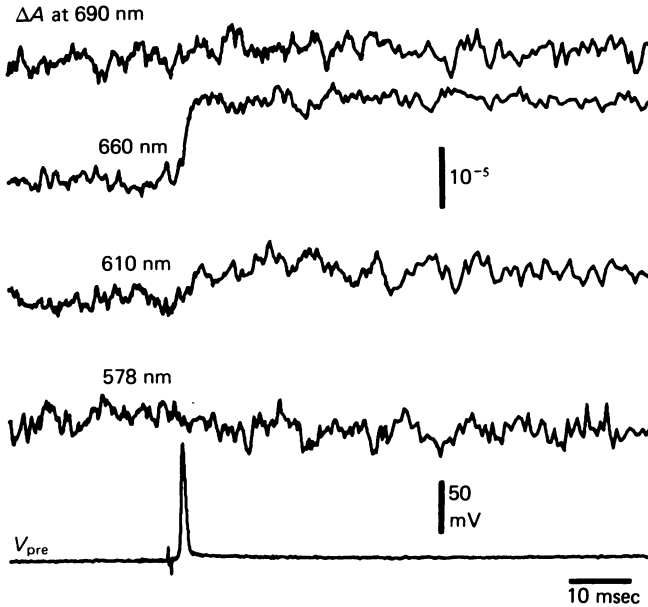

Fig. 5. Absorbance changes (ΔA) at four wave-lengths in a presynaptic terminal filled with arsenazo III. The presynaptic axon was stimulated orthodromically every 25 sec, and sixteen absorbance signals were averaged at each wave-length, recording one wave-length at a time. The presynaptic potential (V_{pre}) was recorded with the dye-injecting electrode. The post-synaptic response in this experiment was over 20 mV, and elicited a post-synaptic action potential.

transients following its influx, and to investigate whether there is any facilitation of intracellular calcium concentration changes.

Fig. 5 illustrates the effect of a single action potential on presynaptic calcium concentration, from one of five similar experiments. There was a marked increase in absorbance at 660 nm, a smaller change at 610 nm, and no response at all at 578 nm and 690 nm. This spectrum indicates a change in dye state due to an increase in calcium concentration (DiPolo, Requena, Brinley, Mullins, Scarpa & Tiffert, 1976; Brown, Brown & Pinto, 1977; Kendrick, Ratzlaff & Blaustein, 1977; Gorman & Thomas, 1978). The average internal calcium concentration rises in about 1 msec, during the falling phase of the action potential, to a peak about 75 nm above the ambient level. The calcium transient is quite long-lived, not returning to base line for about 10 sec.

In three experiments we observed the arsenazo III signals during paired presynaptic action potentials (Fig. 6*A*). The absorbance changes accompanying the two spikes were identical. The results were clearest in normal calcium medium, when the calcium

influx is large. Transmission then is suprathreshold and facilitation is not manifest. Even when the post-synaptic current is recorded under voltage clamp, a predominant depression in normal calcium masks facilitation (Kusano & Landau, 1975). In one experiment, we were able to record arsenazo III signals in reduced calcium while the post-synaptic current facilitated (Fig. 7). The response to the second action potential
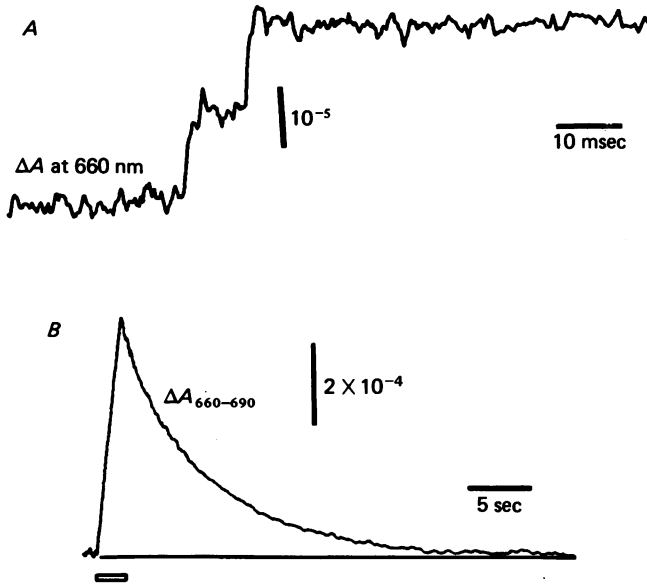

Fig. 6. Absorbance changes (ΔA) to repeated action potentials in two terminals filled with arsenazo III. *A*, the presynaptic axon was stimulated twice with an interval of 12 msec, and the absorbance change at 660 nm recorded; sixteen paired responses were averaged for this record. The calcium increments during the two action potentials were identical. *B*, the presynaptic axon was stimulated at 33 Hz for 2 sec (indicated by open bar), and the differential absorbance at the wave-length pair 660–690 nm was recorded using the interference filter light chopper and multiplexing spectrophotometer. Trains were repeated every 45 sec; sixteen responses were averaged for this record. Calcium concentration, 11 mM.

still appeared identical to that accompanying the first. The slow rising phase of the arsenazo III signals shown in this Figure suggests that the light pipe may have detected a calcium concentration change not immediately adjacent to the major site of calcium influx.

In four experiments we also recorded the change in calcium concentration accompanying trains of 7–74 spikes at 30–40 Hz (Fig. 6*B*). In every case, the arsenazo III signal consisted of a linearly rising absorbance at 660 nm during the train, followed by a gradual decline with a half-time of from 4·5 to 15 sec. The peak absorbance change was similar to that expected from multiplying the response to one action potential by the number of spikes in the train. These results, along with the responses to paired action potentials, indicate that no facilitation or depression of calcium-concentration increments accompanies multiple action potentials that elicit facilitating post-synaptic responses.

Arsenazo III absorbance declined most rapidly in cells filled with the least dye,

suggesting that high dye concentrations may bind a significant fraction of the intracellular calcium and retard its removal and diffusion. This effect of arsenazo III is further suggested by records of post-synaptic current under voltage clamp made before and after injecting arsenazo III into different terminals. When a terminal was injected with a high concentration of dye (0·54 mM-arsenazo III), the post-synaptic
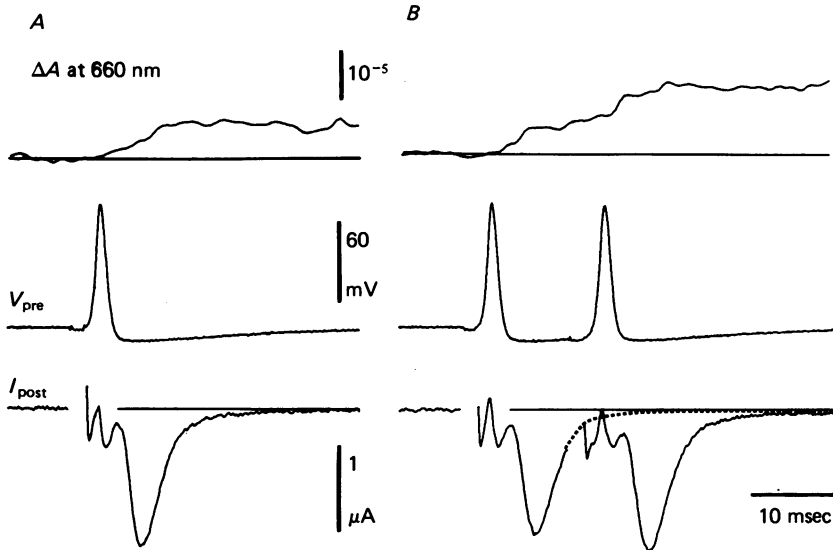

Fig. 7. Absorbance changes (ΔA) at 660 nm to single and paired action potentials in a terminal filled with arsenazo III. Presynaptic potential was recorded with the dye-injecting electrode, and post-synaptic current (I_{post}) was recorded from a post-synaptic axon under voltage clamp. Absorbance records are averages of responses to 160 stimuli repeated every 45 sec; and post-synaptic currents are averages of sixteen responses. The dotted line in B shows the post-synaptic current tail traced from A: the second post-synaptic response is 11 % larger than the first. The sea water contained 7·4 mM-calcium and 2·5 mM-manganese, reducing transmission to a level exhibiting facilitation.

current was reduced by 50 %. When only 0·07 mM-dye was injected, synaptic transmission was reduced only 22 %, which is similar to the normal deterioration that often occurs during a long experiment. Thus we feel that the faster decay of the arsenazo III signal seen in cells with little dye (Fig. 6B) is closer to the situation that prevails in the absence of arsenazo III. In any case, these results show that there *is* an elevation in internal calcium, or a 'residual' calcium, for several seconds following one or more presynaptic action potentials.

Facilitation of spike-evoked transmitter release by elevated presynaptic calcium

    We next turned to the question of whether an increase in intracellular calcium in the terminal can alter the amount of transmitter released by an action potential. In fourteen experiments we injected calcium ionophoretically (ejection current of 20–500 nA from a retaining current of −2 nA) into the presynaptic terminal. Synaptic transmission was reduced to a facilitating level by dropping external calcium concentration to 2·4–5·5 mM and adding 5–7 mM-manganese. In all experiments, we observed a slight post-synaptic depolarization which sometimes declined

after a few seconds of calcium injection. The peak post-synaptic depolarization ranged from 0·2 to 1·0 mV (average 0·39 mV), and was accompanied by a slight increase in post-synaptic noise, apparently due to an increase in miniature e.p.s.p. frequency. These results are similar to those reported by Miledi (1973), except that in our experiments no injection current was passed across the presynaptic membrane, and there was no presynaptic depolarization.

In eleven of these preparations the spike-evoked e.p.s.p. also increased for about 10 sec after the calcium injection began. Two experiments are illustrated in Figs. 8 B and 10 A. The amount of facilitation was variable and often small, ranging from 4 to 50 % (average 16 %). In seven of these eleven preparations, the period of facilitating e.p.s.p.s was followed by a small and gradual depression of transmission that lasted until calcium injection was stopped; full recovery sometimes took several minutes. Such a depression was observed without facilitation in two of the remaining three preparations. The e.p.s.p. was unaffected by calcium injection in the fourteenth experiment.

We suspected that the small effects of calcium injection on evoked release might be due to a limited intracellular diffusion of injected calcium, with the calcium concentration becoming high only in the immediate region of the injection electrode, and therefore influencing only a small portion of the giant synapse, which may extend a full millimeter (Young, 1973). Such a circumscribed increase in calcium following injection has been observed in epithelial cells (Rose & Loewenstein, 1975). We expected to be able to increase the effectiveness of calcium injection by restricting calcium influx during an action potential to the same part of the terminal into which the calcium was injected. We accomplished this in three experiments by blocking calcium influx with a medium containing only 1 mM-calcium plus 7 mM-manganese and restoring transmission locally with an extracellular ionophoretic calcium pipette (Miledi & Slater, 1966) positioned adjacent to the intracellular ionophoretic calcium pipette (see Fig. 8 A). Calcium was ejected from the extracellular pipette with current of 400–1000 nA. Pre- and post-synaptic potentials were recorded as usual with separate 3 M-KCl micro-electrodes.

Figs. 8 B and C and 10 A and B illustrate two experiments, comparing in each the two experimental procedures described above: calcium injection with evoked release throughout the synapse, and with evoked release restricted to the region of injection. In each preparation, the effects of calcium injection – on both the initial e.p.s.p. facilitation and the subsequent depression – were more marked using the second procedure. Fig. 10 C shows results from the third experiment, using restricted release. In these three experiments, the average peak facilitation during calcium injection was 26 %, compared to 16 % using the first procedure.

In two experiments, we used paired stimulation of the presynaptic fibre to elicit facilitating e.p.s.p.s. In both cases presynaptic injection of calcium enhanced both e.p.s.p.s about equally, so that facilitation was still present and little affected by elevating presynaptic calcium (see Fig. 10 B).

The results of calcium injection might be due to uncontrolled effects of our procedures. For example, hyperpolarization of the terminals would facilitate transmitter release (Charlton & Bittner, 1978b). Although no change in presynaptic membrane potential was evident, a small presynaptic hyperpolarization might go
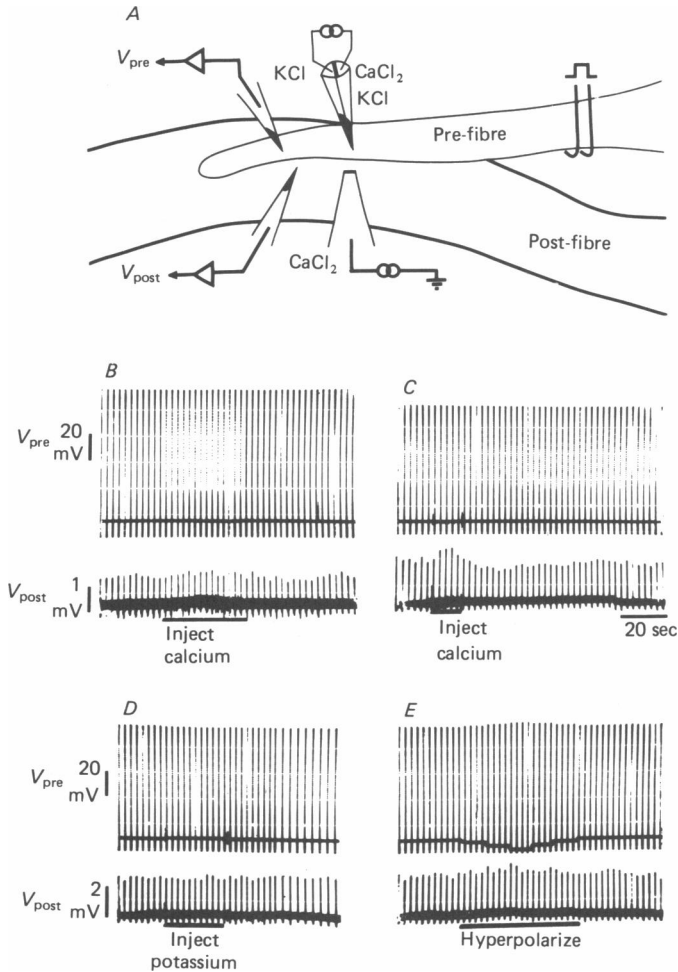
Fig. 8. Effect of elevated presynaptic calcium on spike-evoked transmitter release. *A*, arrangement of electrodes for recording pre- and post-synaptic potentials (V_{pre} and V_{post}) and for injecting calcium presynaptically and raising calcium locally extracellularly. *B*, ionophoretic injection of calcium with a current of 100 nA during the period indicated by the horizontal line without external calcium ionophoresis, in sea water containing 2·4 mM-calcium and 6 mM-manganese *C*, injection of calcium into the same terminal after changing to a solution containing 1 mM-calcium and 7 mM-manganese and restoring transmission locally by ejecting calcium from a pipette containing 1 M-CaCl₂ with a current of 800 nA. *D*, injection of potassium with an interbarrel current of 100 nA, under the same conditions as in *C*, *E*, effect of hyperpolarization of the presynaptic terminal on spike-evoked transmitter release and presynaptic action potential amplitude.

undetected and result in enhanced transmission; this was particularly worrisome because the resistance of the intracellular calcium pipette often rose to several hundred megohms, and leakage pathways from the constant current source to ground would be expected to hyperpolarize the terminal. To control for this problem, we checked to see how much hyperpolarization of the presynaptic terminal, caused deliberately by passing current between the potassium barrel and ground, would be
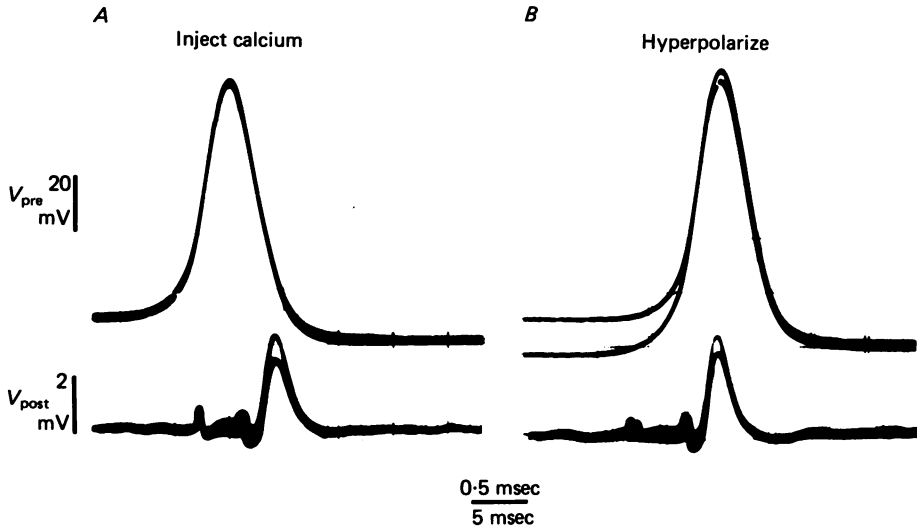
Fig. 9. Effects of presynaptic calcium injection (*A*) and hyperpolarization (*B*) on the presynaptic action potential and the post-synaptic potential, from the same experiment as Fig. 8.
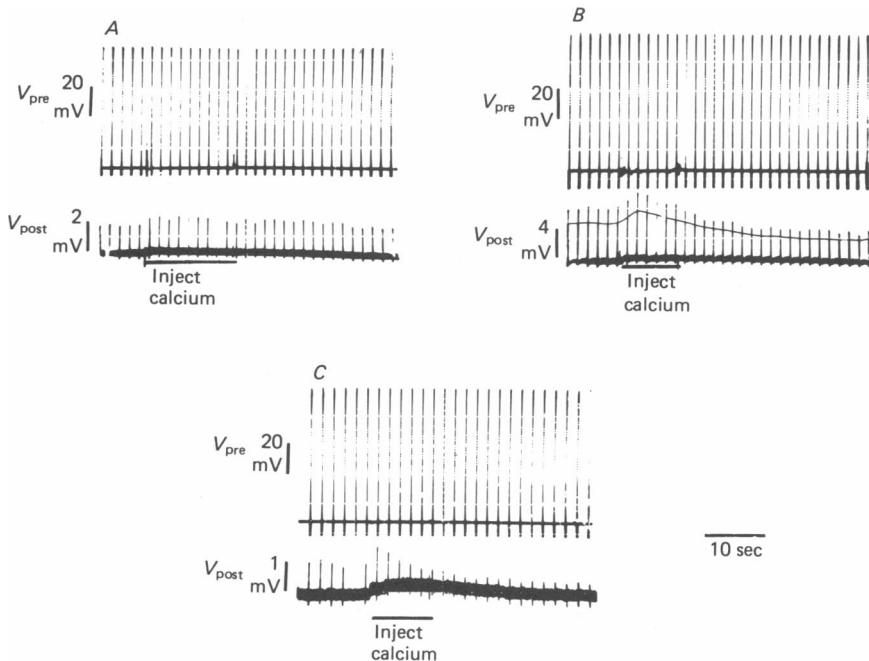

Fig. 10. Effect of presynaptic calcium injection on synaptic transmission in two additional experiments. In *A*, calcium was injected (60 nA current) in sea water containing 3·75 mM-calcium and 5 mM-manganese; in *B*, calcium was 1 mM, manganese was 7 mM, and calcium was applied locally from an external pipette with 1 μA current. Paired stimuli separated by 9 msec were delivered in *B*, and the fine line is drawn through the peaks of the first e.p.s.p. *C*, in this preparation, calcium was injected using 20 nA in 1 mM-calcium and 7 mM-manganese with transmission restored locally using a 400 nA current through the extracellular calcium pipette.

necessary to mimic the effect of calcium injection. In five experiments we found that if the terminal were hyperpolarized about 10 mV, the presynaptic action potential was increased slightly and transmission was facilitated by about the same amount as by calcium injection (Figs. 8E and 9). It was clear that no hyperpolarization even one tenth this magnitude occurred during calcium injection (cf. Figs. 8B, C, 9A and 10A–C). Reversing the interbarrel current to inject potassium ions was also always without effect (Fig. 8D). We conclude that the effects of calcium injection are due to the intracellular action of this ion.

<div align="center">DISCUSSION</div>

Calcium currents are stable during facilitation and depression

Our first finding is that the presynaptic calcium current at the squid giant synapse does not change appreciably to spike-like depolarizations repeated at frequencies that elicit significant facilitation or depression of post-synaptic potentials. This result is in accord with the finding that calcium channels in molluscan central neurones also appear not to facilitate (Akaike *et al.* 1978; Tillotson & Horn, 1978; Ahmed & Connor, 1979; Connor, 1979; Smith & Zucker, 1980; Gorman & Thomas, 1980).

In one experiment, we did see a hint of calcium channel facilitation, in that the calcium current activated slightly more rapidly in the second of two depolarizations separated by 1 msec. When the interval between pulses was increased to 3 msec in this preparation, the calcium currents were identical in the two pulses, although the second pulse released more transmitter than the first. Since 1 msec is less than the refractory period for the presynaptic neurone, this minor facilitation of calcium channels at very brief intervals cannot play a role in synaptic facilitation under physiological conditions. Nevertheless, this preliminary result is interesting and should be explored further.

Calcium concentration transients are constant and prolonged

Our second major finding is that no facilitation of calcium concentration increments accompanies multiple action potentials that elicit facilitating post-synaptic responses. This suggests that under conditions leading to facilitation of transmitter release, no augmentation of calcium concentration increments occurs, due for example to saturation of internal calcium buffering or removal systems. There is also no evidence of a declining rate of internal calcium accumulation during brief trains, due for example to a decline in calcium influx or a priming of calcium buffering or removal systems.

Our third finding is that there is an increase in presynaptic calcium concentration that lasts for several seconds after even a single action potential. Previous studies using aequorin also reported a persistent change in internal calcium following presynaptic electrical activity, but in these studies it was necessary to use either long trains of action potentials (Llinás, Blinks & Nicholson, 1972) or action potentials greatly prolonged by tetraethylammonium injection (Llinás & Nicholson, 1975) to detect an intracellular calcium transient.

It might appear that calcium persists for *too* long after an action potential to be involved in synaptic facilitation. The arsenazo III signal has a half-decay time of

about 5 sec, while facilitation has a half-decay time of only about 10 msec. This is not a real discrepancy, however, because arsenazo III detects free calcium throughout the cytoplasm of the terminal, while transmitter release should be sensitive only to the submembrane calcium concentration at release sites. The decline of the arsenazo III signal reflects active processes removing calcium from cytoplasm, while the decline of submembrane free calcium is much faster if it is governed mainly by diffusion of calcium away from release sites toward the cell interior. Using the model described in Smith & Zucker (1980), we have made some preliminary calculations of calcium diffusion inside the terminal following an influx of calcium for 1 msec. The calcium concentration within 10 nm of the membrane decays with a half-time of 1 msec or less, accounting for the rapid decline of transmitter release folowing an action potential. A slower component of the decay of submembrane calcium lasts over 10 msec, and could provide the residual calcium needed to facilitate release.

Elevated presynaptic calcium facilitates transmitter release

Our fourth finding is that elevated presynaptic calcium facilitates transmitter release to action potentials for several seconds, and this facilitation is followed by a later phase of depressed release. This suggests that residual calcium following electrical activity may indeed act to facilitate synaptic transmission. Other evidence in support of this hypothesis includes the enhancement of evoked transmitter release by metabolic inhibitors that should increase intracellular calcium (Alnaes & Rahamimoff, 1975) and by fusion of calcium-containing liposomes with presynaptic terminals (Rahamimoff, Meiri, Erulkar and Barenholz, 1978), and reduction in post-tetanic increase in miniature e.p.s.p. frequency in low-calcium medium (Erulkar & Rahamimoff, 1978). These earlier results are limited by the absence of any control for effects of these treatments on presynaptic action potentials.

We can imagine at least two ways that calcium-evoked facilitation might come about. The first mechanism was proposed by Katz & Miledi (1968), and Rahamimoff (1968) and relies on a non-linear power-law type relation between transmitter release and intracellular calcium. The idea is that a given increment of calcium concentration will be much more effective when added to a small residual calcium, although the residual calcium itself will be nearly ineffective in releasing transmitter, due to the non-linear relation between release and calcium.

We have demonstrated such a relation between release and calcium influx. Moreover, the linearly rising arsenazo III signal during a train of action potentials indicates a linear relation between constant increments of influx and increments of calcium concentration change. Thus our results suggest that there is a power-law type relation between intracellular calcium and the amount of transmitter released by a spike or depolarizaton. This relation is expected because transmitter release in the squid varies with the extracellular calcium concentration to approximately the power of 2·5 (Katz & Miledi, 1970; Lester, 1970).

Results similar to ours have recently been reported by Llinás, Steinberg & Walton (1981*b*).

One aspect of our results appears to be in quantitative disagreement with a prediction of this formulation of the residual calcium hypothesis: facilitation of evoked release was obtained only by injection of enough calcium to elicit a consider-

able level of continuous release, while normally synaptic facilitation occurred in the absence of any observable increase in tonic release at this synapse. However, the very different geometries of calcium injection from one point and its entry across the plasma membrane must lead to substantial differences between the intracellular pro- files of calcium concentration in the two cases. The facilitation we observed on injecting calcium was similar in magnitude to that seen normally a few msec following an action potential. However, facilitation caused by calcium injection must be generated by a sharp gradient of calcium concentration near the ionophoretic electrode tip, and the local peak level of calcium is undoubtably higher than the residual calcium following influx across the membrane surface. Thus the higher rate of continuous transmitter release from this part of the synapse is not surprising.

A second mechanism for calcium-dependent facilitation was proposed by Balnave & Gage (1974). They suggested that calcium entering during an action potential forms two complexes, one to release transmitter immediately and another to facilitate subsequent release. This mechanism does not rely on any special form of the relation between transmitter release and calcium. We cannot distinguish these or other possible mechanisms from our results.

Our results indicate that there is residual calcium in these nerve terminals following an action potential, and that when residual calcium is mimicked by calcium injection, transmitter release is facilitated. To our knowledge this is the first direct intracellular test of the residual calcium hypothesis. Although our results strongly support this hypothesis, the relationship between residual calcium and facilitation has not been demonstrated quantitatively. To do so would require accurate measurement of calcium concentration change at transmitter release sites following an action potential, and knowledge of the exact relation between calcium concentration and enhancement of spike-evoked release.

Synaptic depression during calcium injection

Following several seconds of calcium injection, e.p.s.ps were depressed by variable amounts in most preparations. This may be a specific pathological effect of calcium, or, as the following calculation suggests, it may merely reflect the usual processes operative in synaptic depression. Using a transport number of 0·1 for calcium injection from pipettes containing one-third as much $CaCl_2$ as KCl (Gorman & Hermann, 1979), we calculate that 50 nA ionophoretic current corresponds to an injection of $1·6 \times 10^{10}$ ions/sec. If $1·7 \times 10^8$ calcium ions enter the terminal during an action potential (Llinás, 1977), we are typically injecting calcium at a rate equivalent to 93 spikes/sec, but the calcium injection and transmitter release are both confined to a small part of the terminal. No wonder this leads to depression.

The above calculation may be misleading, in that calcium injected at one point may be far less effective in releasing transmitter than calcium entering through the membrane near transmitter release sites. This is suggested by the observation that the typical post-synaptic response to calcium injection was 0·4 mV. Since a quantum is about 10 μV and declines with a 2 msec time constant (Miledi, 1973), the post-synaptic depolarization corresponds to 20,000 quanta/sec (Katz & Miledi, 1970). An action potential releases about 5000 quanta from the entire terminal, so this corresponds to a frequency of 4 spikes/sec. Even this frequency normally leads to

strong synaptic depression (Fig. 3 *D*), presumably by depleting a presynaptic store of transmitter.

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