*J. Physiol.* (1966), **184**, *pp.* 473–498
*With* 16 *text-figures*
*Printed in Great Britain*



# THE ACTION OF CALCIUM ON NEURONAL SYNAPSES IN THE SQUID

BY R. MILEDI AND C. R. SLATER

*From the Department of Biophysics, University College London; and the Marine Biological Laboratory, Woods Hole, Massachusetts, U.S.A.*

(*Received* 30 *November* 1965)

SUMMARY

1. The isolated stellate ganglion of the squid (*L. pealii*) was studied with intracellular and extracellular micro-electrodes. Three or four nerve fibres in the preganglionic nerve establish synaptic relations with the giant axon in the last stellar nerve. Accordingly, 1–3 small presynaptic spikes ($< 1$ mV) could be recorded from within the post-synaptic axon.

2. A micro-electrode was inserted in the presynaptic fibre and used to polarize and record simultaneously. In the distal (giant) synapse, hyperpolarization of the ending produced an increase in the size of the presynaptic action potential and post-synaptic potential (PSP). Depolarization had the opposite effect. These effects of polarization took more than 10 sec to develop fully, and declined with a similar time course at the end of polarization. Analogous results were obtained with two other preganglionic fibres, which make contacts in the proximal synaptic region.

3. The second of a pair of preganglionic impulses evoked a PSP larger than the first. This facilitation of PSP was sometimes accompanied by a small increase in the size of the second action potential in the presynaptic axon. At some shorter intervals, the second presynaptic action potential was reduced in amplitude, but the PSP was still increased. Hyperpolarization of the presynaptic terminal increased the size of both PSPs in a pair and abolished the facilitation. With stronger hyperpolarization the second PSP was even smaller than the first.

4. Removing or reducing the Ca in the bathing fluid reversibly abolished the post-synaptic response. The small presynaptic spikes remained practically unaffected. In these conditions a nerve impulse still invaded the ending and normal action potentials could be recorded from the presynaptic terminal. This shows that electrical coupling between pre- and post-synaptic axons is insufficient to account for synaptic transmission.

5. In low-Ca solution synaptic transmission could be restored locally by extracellular ionophoretic application of Ca to a small portion of the synapse. At sensitive spots a post-synaptic current (recorded with the

Ca pipette) and PSP could be detected earlier than 1 sec after commencing the application of Ca.

6. Ca was ineffective when injected intracellularly into the presynaptic fibre at a spot where extracellular ionophoresis of Ca restored the PSP.

7. The results indicate that synaptic transmission in the squid stellate ganglion is not electrical but due to the release of an unidentified transmitter. Release of this transmitter by the presynaptic nerve impulse requires the presence of Ca in the external medium. During the impulse Ca would combine with a 'Ca-receptor' in the membrane and initiate the reactions which lead to transmitter release. It appears that the 'Ca-receptor' is only accessible from the outside of the membrane.

## INTRODUCTION

In synapses which operate by the release of a chemical transmitter, a reduction in the concentration of calcium ions in the external medium leads to a decrease in the amount of transmitter released by the nerve impulse. Since prolonged calcium lack renders nerve fibres inexcitable, it could be thought that depression of transmitter release is a consequence of incomplete invasion of the terminal by the nervous impulse. At least in some synapses this is not so, for Katz & Miledi (1965*c*) found that at the frog's neuromuscular junction the nerve impulse continues to invade the endings even when calcium is withdrawn from the medium and the release of transmitter has been stopped. This shows that calcium ions act directly on the process that leads to transmitter release.

The experiments reported in this paper were aimed at finding out if similar results could be obtained at neuro-neuronal synapses. The giant synapse of the squid offered the obvious advantage of its size, which permits insertion of micro-electrodes in both pre- and post-synaptic elements (cf. Bullock & Hagiwara, 1957). Furthermore, such experiments, apart from giving information on the site of action of calcium ions, would also provide quantitative information on the function of the synapses in this ganglion. Some evidence for the chemical nature of transmission of impulses across the giant synapse has already been presented by Hagiwara & Tasaki (1958) and Takeuchi & Takeuchi (1962).

## METHODS

The experiments were performed at the Marine Biological Laboratory, Woods Hole, during August 1964. The stellate ganglia of the squid (*L. pealii*) were dissected with 2–3 cm of the viscero-stellate connective (preganglionic) and a similar length of the last stellar (post-ganglionic) nerve. Ligatures were placed around both pre- and post-ganglionic nerves, and the ganglion was stretched across a Perspex block which had two grooves with embedded wire electrodes for nerve stimulation or recording. The block was placed in a chamber

(volume 30 ml.) with oxygenated sea water at 10–15° C flowing at a rate of 6 ml./min. In these conditions transmission was maintained for many hours.

Fine steel hooks were used to fix and spread the ganglion and stellar nerves. Further dissection and manipulation during the experiment were done with transillumination and under a microscope. Connective tissue, muscle and blood vessels were removed from the surface of the ganglion and in some cases all fibres in the preganglionic nerve were cut except the fibre going to the giant synapse. The ganglion from the other side was kept in oxygenated sea water at 5–10° C until used several hours later.

For intracellular recording from presynaptic and post-synaptic fibres, micro-electrodes were usually filled with 3 M-KCl, and inserted into the fibres at an angle of about 45°. The local effects of calcium ions were examined using the method described by Katz & Miledi (1965*c*). The procedure was to perfuse the ganglion with artificial sea water (mM: Na 440; K 9; Mg 53·4; Cl 555·8) without calcium or with only 0·9 mM, which is about 0·1 of the normal concentration in Woods Hole sea water. Localized application of calcium was made iontophoretically from a micropipette ($< 1$–$5\ \mu$) filled with 1 M-$CaCl_2$. The efflux of calcium could be controlled by adjusting the electrical bias on the pipette. This was connected to a d.c. bias through a 100 MΩ resistance, and directly to a Grass cathode follower and condenser-coupled amplifier. In this way extracellular potentials could be recorded at the site of calcium application. The same arrangement was used to polarize and record through one micro-electrode inserted in the presynaptic axon.

## RESULTS

### *Innervation of the giant axon*

In his description of the giant nerve fibre system of the squid Young (1939) mentioned that two giant presynaptic axons enter the stellate ganglion. One of the axons, the largest in the preganglionic nerve, makes contact with the giant post-synaptic axon, forming the distal or giant synapse. The other, 'accessory' fibre ends close to the cell bodies in the giant fibre lobe in numerous synaptic contacts collectively known as the proximal synapses (see Fig. 2). Bryant (1959) has shown that both fibres are capable of generating an action potential in the last stellar giant axon.

Recording intracellularly from the giant axon near the region of the distal synapse, it is found that when the whole preganglionic nerve is stimulated with increasing strength, an intensity is reached at which suddenly an action potential appears in the post-synaptic axon (Fig. 1*B*). This action potential arises from a PSP and at higher amplification is seen to be preceded by a small spike, corresponding to the arrival of the nerve impulse at the terminal (Fig. 1*A*). If the stimulus intensity is increased further, there is usually an abrupt shortening of the latency of the potential (Fig. 1*C*). The action potential still arises from a synaptic potential, but this has a steeper rise and its transition into the spike is sometimes difficult to detect (Fig. 1*D*). The record at high amplification (Fig. 1*C*) shows two spikes preceding the main potential, indicating that another pre-fibre has been activated. The two presynaptic spikes were commonly detected by an electrode inside the post-synaptic axon in the region of the giant synapse; but occasionally the longer-latency presynaptic spike

was barely visible. The fibre which evoked the shorter-latency PSP was usually the one to the giant synapse. This was ascertained by recording simultaneously from the post-synaptic axon and from the presynaptic axon in the region of the giant synapse, which in some cases could be identified through the dissecting microscope. Besides the shift in latency there is also a difference in the form of the action potentials set up by the two presynaptic axons: the one in Fig. 1*B* is followed by a hyperpolari-

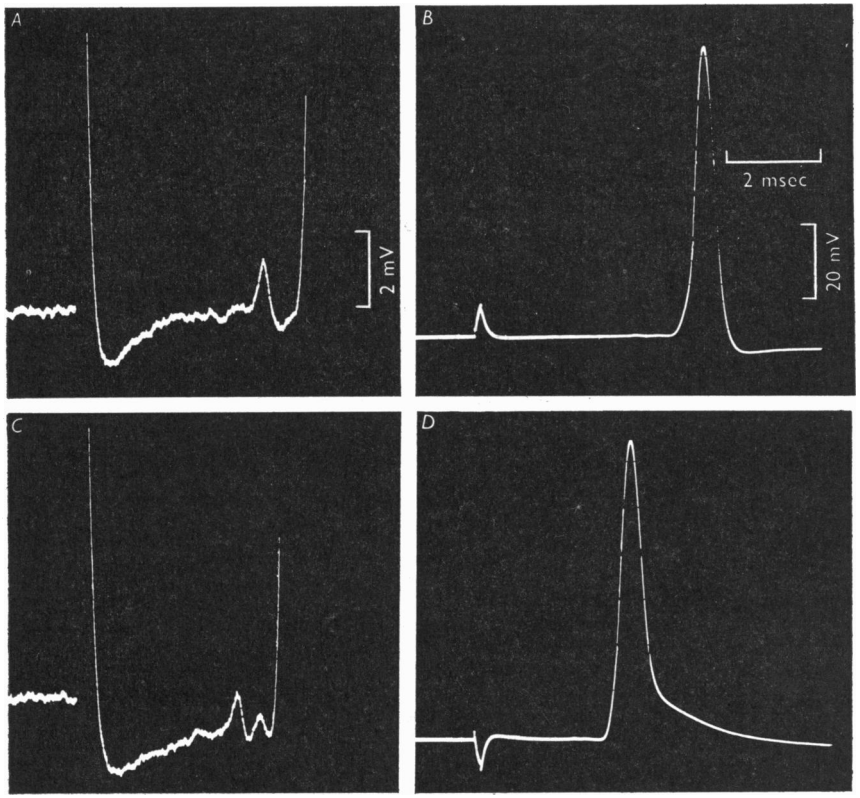


Fig. 1. Intracellular potentials evoked in the post-synaptic axon by stimulation of the preganglionic nerve. At high amplification (*A*) a small spike precedes the post-synaptic action potential. With higher stimulus intensity (*C* and *D*) two presynaptic spikes are seen (*C*). Stimulus polarity in *D* was changed inadvertently.

zation, while that in Fig. 1*D* has a depolarization instead. This residual depolarization is due to the synaptic potential produced by the slower nerve fibre which now, due to refractoriness, is incapable of eliciting an action potential in the post-synaptic axon.

In most experiments there was evidence that three or even four separate preganglionic nerve fibres made connexion with the giant axon. Accor-

dingly, in some cases, three presynaptic spikes could be detected by the electrode inside the giant axon. In the fresh preparation each of the fibres evoked an excitatory synaptic potential large enough to generate an action potential in the giant axon. In fatigued preparations or in solutions with low calcium (see below) synaptic potentials uncomplicated by action potentials were obtained and the contribution of each fibre could then be discerned more easily.

To summarize these results, it is quite clear that 3–4 and possibly more nerve fibres are in synaptic relation with the giant axon in the last stellar nerve. One of these fibres forms the large distal synapse; the exact distribution of the terminals of the other fibres is at present unknown.

### *Polarization of presynaptic endings*

*Giant synapse.* Del Castillo & Katz (1954*a*) showed that at the neuromuscular junction of the frog, anodic polarization of the nerve endings leads to an increase in the amount of transmitter released by the nerve impulse, and a concomitant increase in the amplitude of the synaptic potential. Hagiwara & Tasaki (1958) and Takeuchi & Takeuchi (1962) have found that this is also the case at the squid giant synapse, and the present results confirm their finding.

In suitable preparations the giant synapse, about 1 mm long, is visible under the dissecting microscope and a micro-electrode can be placed in the presynaptic axon in the synaptic region. The effects of local polarization can then be examined without complications introduced by changes in the propagation of the presynaptic nerve impulse. Constant repetitive stimulation of the preganglionic nerve was used to depress the synaptic potential and thus prevent the generation of action potentials in the post-synaptic axon.

It was soon noted that the effect of polarization took some time to develop fully. For instance, Fig. 2 shows that hyperpolarization of the terminal produced an increase in the amplitudes of the action potential in the presynaptic fibre and of the PSP, and that a steady level was not attained until 10 or more sec after commencing the polarization. Although we were unable to monitor the polarizing current or the membrane potential during polarization we have reason to believe that they were fairly constant throughout the period of polarization. Furthermore, a similarly slow time course was obtained for the return of the potentials to control levels after cessation of the polarizing current. Approximately the same time course was also observed during depolarization of the presynaptic axon, which decreased the amplitude of both the presynaptic action potential and the PSP.

Since the amplitude of the presynaptic action potential is modified by

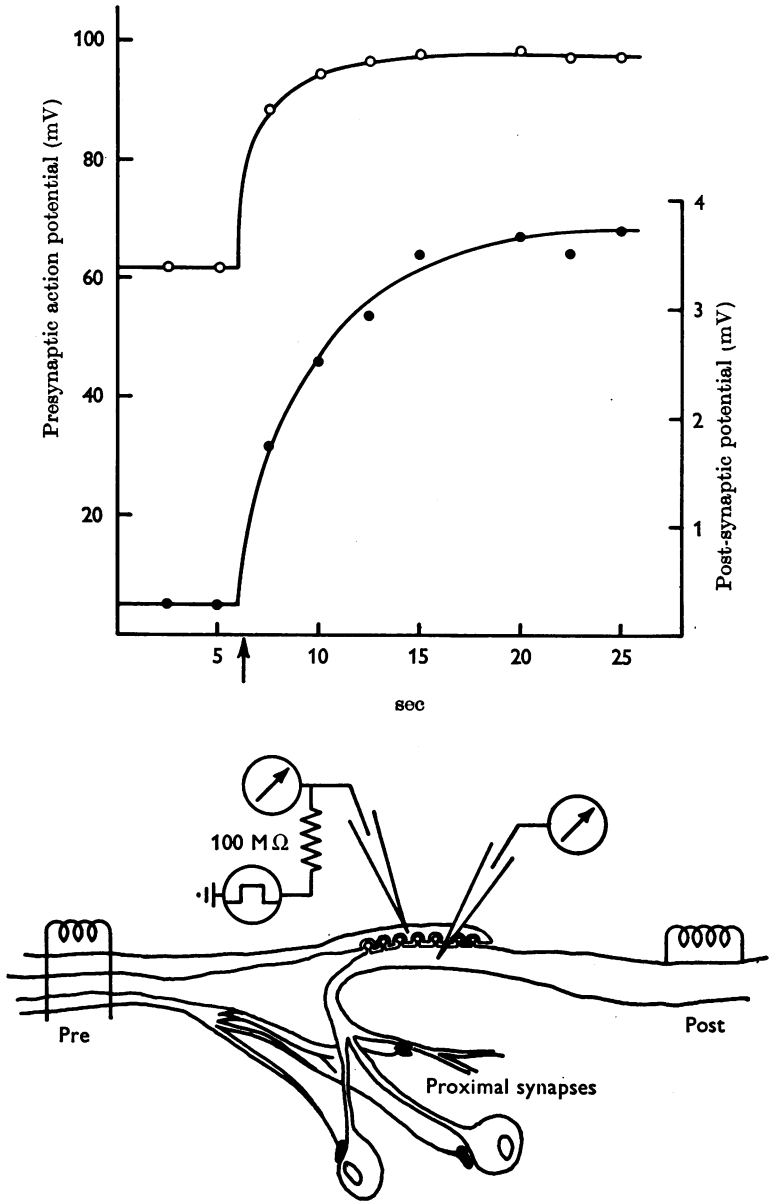


Fig. 2. Effect of maintained hyperpolarization of presynaptic axon on amplitude of presynaptic action potential (○) and PSP (●). Arrow indicates start of polarization. Lower part: diagram of experimental set-up. Only two preganglionic nerve fibres are shown. The electrode in the post-synaptic axon was sometimes located slightly beyond the distal synapse.

displacing the membrane potential, it is possible to obtain a relation between amplitude of action potential in the terminal and the PSP elicited by it in the giant axon. Figure 3 illustrates sample records from an experiment where the normal PSP had been depressed to subthreshold level by prolonged repetitive stimulation at about 2/sec. The presynaptic axon was polarized to different levels and the action potential was recorded through the same electrode; a second electrode in the giant axon

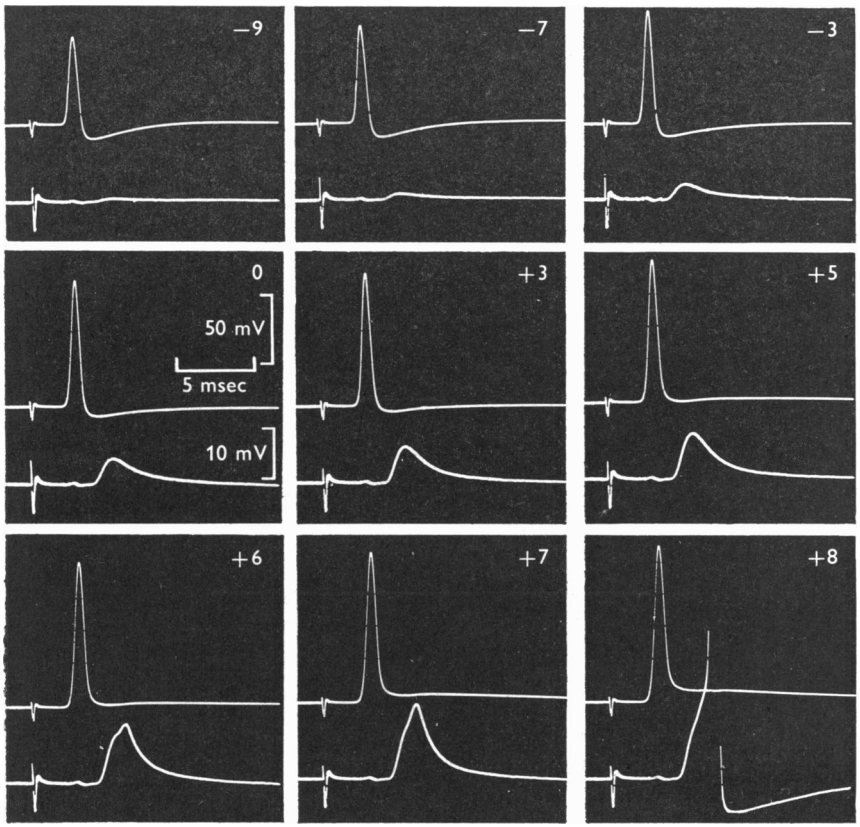


Fig. 3. Simultaneous intracellular recording from presynaptic (top traces) and post-synaptic (bottom traces) axons; during hyperpolarization (+) and depolarization (−) of presynaptic axon. Figures indicate intensity of polarization in relative units.

served to record the PSP. In order to allow for the slow changes in transmitter release described above, records were made several seconds after the onset of polarization. It can be seen that decreasing the membrane potential leads to a decreased amplitude of PSP. Increasing the membrane potential increased the PSP, which ultimately became large enough

to generate an action potential in the giant axon (Fig. 3). Hyperpolarization also produced an expected reversal of the after-hyperpolarization of the presynaptic action potential. What effect, if any, this has on the PSP produced by the impulse we do not at present know.

Figure 4*A* shows the relation between amplitude of presynaptic action potential and post-synaptic potential. The relation is very steep and is similar to the one described previously by Hagiwara & Tasaki (1958) and Takeuchi & Takeuchi (1962). In normal conditions the curve would be slightly different because transmission fatigue produced by repetitive stimulation causes a reduction of the PSP without greatly altering the size of the presynaptic action potential.

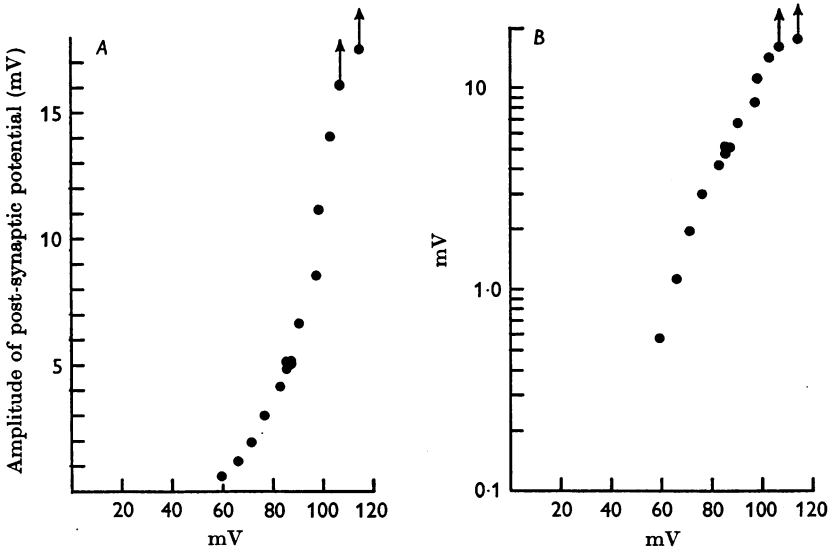


Fig. 4. Relation between amplitude of presynaptic action potential (abscissae) and PSP (ordinates). Logarithmic ordinate scale in *B*. At points marked by arrows the PSP generated action potentials. Same experiment as in Fig. 3.

In Fig. 4*B* the same results have been plotted on semilogarithmic scale, to show that there is an approximately exponential relation between the PSP and action potential amplitudes. There is a 10-fold change in amplitude of PSP for about 30 mV change in amplitude of pre-fibre action potential (the steepest relation observed was 10-fold change for 20 mV). This is approximately the same as that found by Takeuchi & Takeuchi (1962); but the relation is less steep than that postulated by Liley (1956) over a smaller range of potential in the mammalian neuromuscular junction; a difference which could be partly due to synaptic fatigue caused by the repetitive stimulation.

We found, in accord with Hagiwara & Tasaki (1958), that depolarizing pulses applied alone to the presynaptic fibre did not produce a detectable PSP until they triggered an action potential in the axon. This was the case even when the duration of the pulse was many times greater than the duration of the action potential.

*Proximal synapses.* In the experiments above, the polarizing electrode inside the presynaptic axon was placed within the actual synaptic region; but even when it was as much as 1 mm from the synapse it was still possible to affect the PSP. This made it possible to examine the effects of polarization on the much smaller synapses which other large nerve fibres establish with the giant axon and its contributing mass of cell bodies (cf. p. 475 and Fig. 2). For this purpose it was convenient to insert the microelectrode into the axons somewhere between 0·5 and 1·5 mm from the synapses. The results of these experiments were the same as with the giant synapse: large increases in the amplitude of the PSP could be produced by hyperpolarizing the axon, and depolarization had the opposite effect. In general, stronger currents were now needed to affect the PSP, as would be expected from the longer distance between polarizing electrode and synapses and the concomitant spatial decay of potential. With large hyperpolarizations there was a marked increase in the latency of the PSP clearly attributable to slowing of the impulse as it traversed the hyperpolarized region.

The effects of polarization seemed to be confined to the PSP evoked by the axon in question; but the possibility of interaction between axons needs to be more thoroughly examined. The important point to bear in mind at this stage is that the endings of at least two other axons in the ganglion react to polarization like the giant synapse.

### *Facilitation of post-synaptic potential*

At the vertebrate neuromuscular junction, if a second nerve impulse arrives at the endings shortly after a first, it produces a larger e.p.p. than that evoked by the first impulse (Eccles, Katz & Kuffler, 1941; Feng, 1941; Lundberg & Quilisch, 1953). This facilitation has been shown to be due to an increase in the amount of transmitter released by the second nerve impulse (del Castillo & Katz, 1954*b*; Katz & Miledi, 1965*c*). A similar facilitation is seen at crustacean nerve–muscle junctions (Dudel & Kuffler, 1961), cat motoneurones (Kuno, 1964), and ciliary ganglion cells of the chick (Martin & Pilar, 1964), as well as in the giant synapse of the squid (Takeuchi & Takeuchi, 1962). It has been suggested that potentiation of synaptic potentials may be due to an increase in the amplitude of the second action potential which would lead to a greater amount of transmitter being released. This can be examined in the giant synapse of the

squid because of the possibility of recording intracellularly from both pre- and post-synaptic axons.

Figure 5 shows the PSP produced by a nerve impulse at different intervals after a first. At intervals greater than about 4 msec the second PSP was larger than the first, while the size of the presynaptic action potential was only slightly increased and remained practically unaltered at longer intervals. At shorter intervals both PSP and presynaptic action potential were reduced. These results are different from those reported by Takeuchi & Takeuchi (1962) who found that the increase in PSP was accompanied by an increase in the amplitude of the presynaptic action

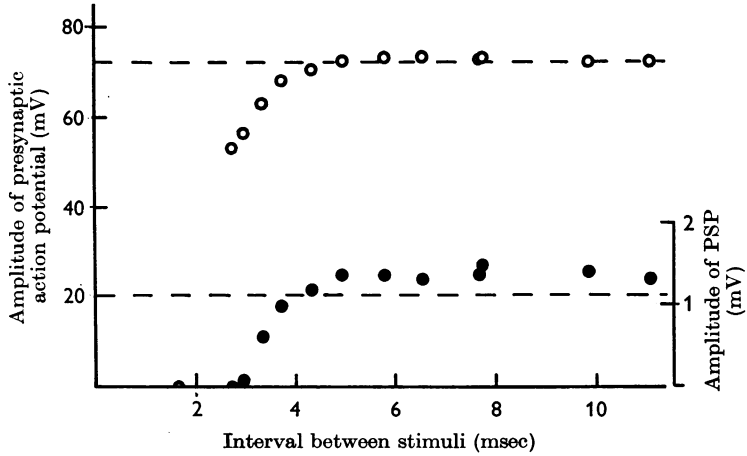


Fig. 5. Facilitation of PSP. (●): amplitude of PSP evoked by an impulse at different intervals after a first. (○): amplitude of intracellular action potential in presynaptic axon. Interrupted lines indicate mean amplitude of potentials produced by the first impulse.

potential. We do not know the reason for this discrepancy; but whatever the cause may be, the important fact is that facilitation of the PSP could be obtained without a concomitant increase in the amplitude of the presynaptic action potential, and in some cases even when the amplitude of the second action potential was slightly reduced.

Analogous observations have been made at the neuromuscular junction, where at short intervals the presynaptic action current produced by the second nerve impulse is reduced, and yet there is an increase in the number of transmitter units released by it (Hubbard & Schmidt, 1963; Katz & Miledi, 1965*c*). Similarly, Martin & Pilar (1964) find that in the 'hybrid' junctions in the ciliary ganglion of the chick (possessing electrical as well as chemical synapses) there is a facilitation of the PSP evoked by the transmitter while the electrical coupling potential and the presynaptic action potential remain unaltered.

Hyperpolarization of the presynaptic axon has a marked effect on facilitation. This is shown in Fig. 6 for two levels of hyperpolarization. There is the expected increase in amplitude of presynaptic action potential and PSP but notice that during polarization the second PSP is smaller than the first—that is, instead of facilitation there is now a depression. A similar effect has been seen at mammalian and frog neuromuscular junctions (Hubbard & Willis, 1962; R. Birks & B. Katz, unpublished observation).

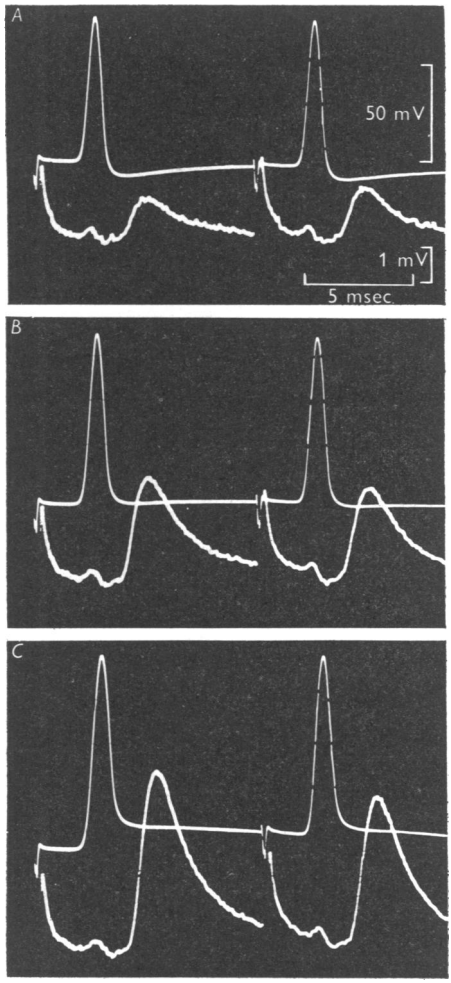


Fig. 6. Effect of hyperpolarization of the nerve terminal on synaptic facilitation. Intracellular records from presynaptic (top traces) and post-synaptic (bottom traces) axons. *A* is control without polarization; *B* with slight, and *C* during more intense, hyperpolarization. Notice that during polarization the second PSP is smaller than the first.

The effect of hyperpolarization can be seen in more detail in Fig. 7, which shows the amplitudes of presynaptic action potential and PSP at various intervals. The top part of the figure shows the results of Fig. 5 but the potentials associated with the second impulse have been plotted relative to those of the first. The lower part of the figure shows results obtained during hyperpolarization of the presynaptic axon. In the latter case it is clear that throughout the range of intervals explored a nerve

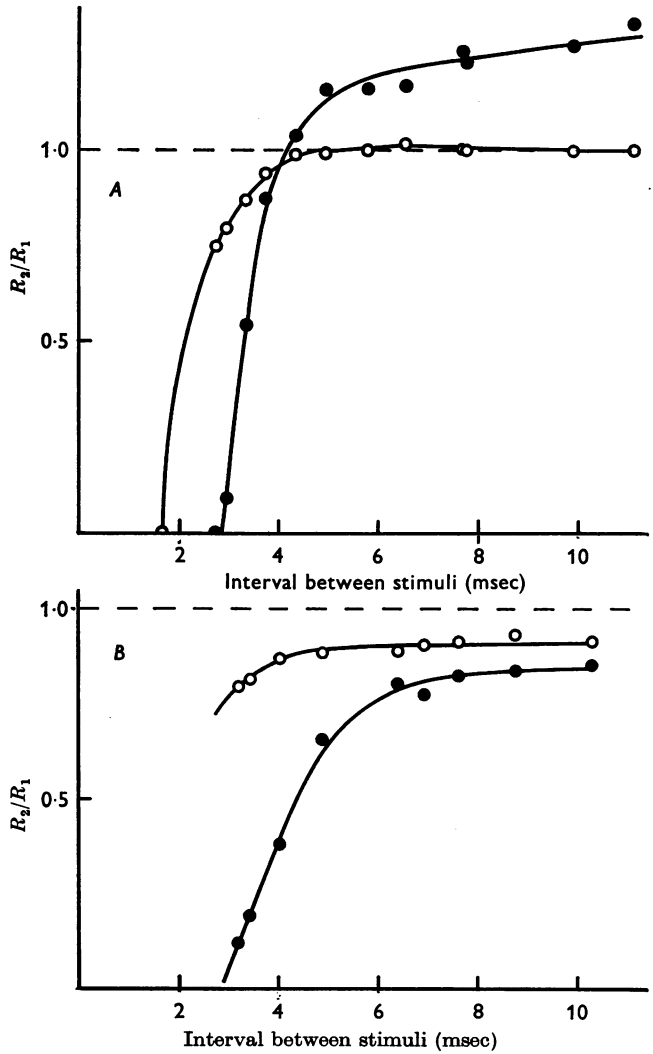


Fig. 7. Effect of hyperpolarization on presynaptic action potential (○) and PSP (●) evoked by a second nerve impulse, expressed as fractions of the mean control. Same experiment as Fig. 5. *A*: without polarization. *B*: during hyperpolarization of presynaptic axon.

impulse is followed by a period during which both presynaptic action potential and PSP are depressed. At the longest intervals used in Fig. 7*B* the second action potential is smaller than the first, but it is riding on the negative after-potential of the latter; the level reached by the peaks of both action potentials is practically the same. It may well be that in these conditions the depression of the synaptic potential is in some way related to the changes that follow the main part of the action potential.

### *Effect of calcium*

*Giant synapse.* A study of the effect of calcium on synapses is of special interest because, at all chemical synapses yet examined, the release of the transmitter substance by the nerve impulse requires the presence of calcium in the external medium. This requirement, plus the antagonistic action of magnesium, is so characteristic of chemical transmission that the effects of these ions on a synapse can be used to diagnose its mode of operation. Takeuchi & Takeuchi (1962) and Bryant (1958) found that increasing the calcium concentration enhanced the synaptic potential and improved transmission across the giant synapse, while magnesium had the opposite effect. In the present experiments the effect of reducing the calcium concentration was examined.

After inserting a micro-electrode in the post-synaptic axon and equilibrating the ganglion in artificial sea water for 1–2 hr, the solution was sucked out and replaced by a similar one, but containing little (0·9 mM) or no Ca. The former was preferred, because in 'Ca-free' solutions, pre- and post-synaptic axons sometimes showed spontaneous repetitive activity, and impulse propagation in the presynaptic axon was eventually blocked (Frankenhaeuser & Hodgkin, 1957). Test stimuli were applied to the preganglionic nerve at intervals of 1–5 min. A few minutes after depriving the preparation of external calcium the rate of rise of the PSP began to decline and the action potential arose progressively later on the synaptic potential, until eventually only the synaptic potential remained (Fig. 8). The PSP continued to fall and became undetectable after a time which varied from 0·5 to 3 hr in different preparations. The mean half-time for the fall in PSP amplitude after removal of calcium was 6 min in eleven experiments (range 3–10 min). The relatively slow time course is partly due to slow diffusion: a faster time course is seen with close iontophoretic Ca application (see below).

The effect of Ca lack was easily reversible. On restoring the calcium concentration to normal the PSP re-appeared, increased progressively in amplitude, and impulse transmission across the synapse was re-established (Figs. 8*B* and 9). The amplitude of the antidromic action potential in the post-synaptic axon was not greatly altered throughout these experi-

ments, indicating that the changes in PSP amplitude are not due to changes in the electrical characteristics of the post-synaptic axon. Another interesting feature, shown in Fig. 9, is that the presynaptic spike recorded by an electrode in the post-synaptic axon is not greatly modified by changes in calcium concentration which have a striking effect on the size of the PSP.

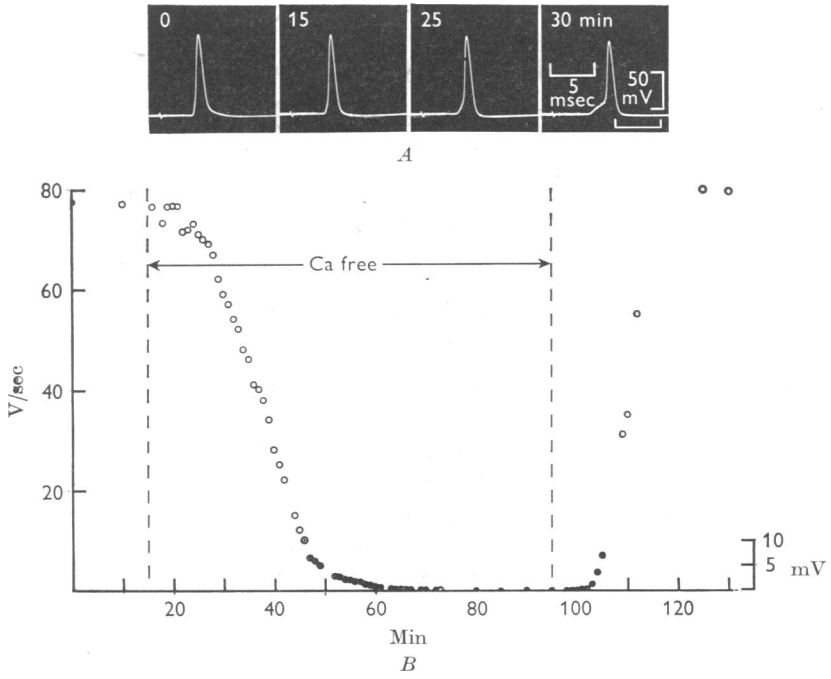


Fig. 8. Calcium action on synaptic transmission in the giant synapse. At first interrupted line the artificial sea water (containing 9 mM-Ca) was replaced by 'Ca-free' solution. Circles indicate maximal slope of post-synaptic potential, at indicated time after removal of Ca, measured from records like those in *A*, but on a faster time base and higher amplification. Dots show amplitude of PSP after the action potential in the post-synaptic axon had been abolished. At second interrupted line the perfusion fluid was switched back to artificial sea water.

*Other synapses.* In some cases, selective stimulation of two or three preganglionic nerve fibres enabled us to follow changes in the amplitude of the PSP evoked by fibres going to the proximal synaptic region. The effect of Ca lack on these synapses was the same as for the giant synapse. The PSPs evoked by stimulation of the whole preganglionic nerve vanished in low calcium. As with the giant synapse, the presynaptic spike recorded from the post-synaptic axon remained practically unaffected, while the PSP disappeared. In three cases the fibre to the giant synapse and one to the proximal synapses were tested regularly while in low-calcium solution;

the rate of fall of PSP amplitude was slower in the proximal synapse (half-time 8 min). Since the whole ganglion was immersed in the solution the unequal time course may simply reflect differences in the diffusion pathways around the synapses.

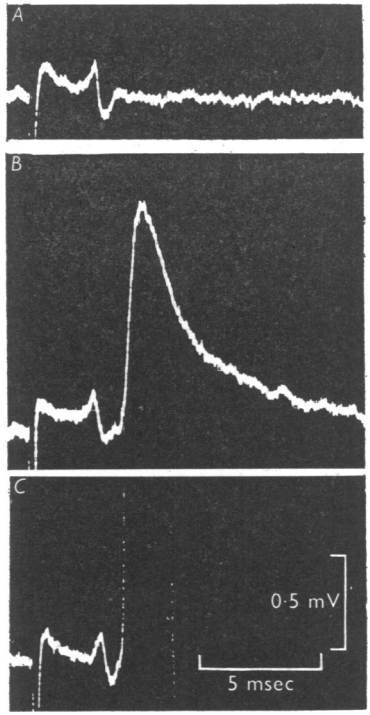


Fig. 9. Effect of calcium on synaptic transmission. *A*: intracellular record from post-synaptic axon of ganglion in 'Ca-free' solution. Stimulation of the preganglionic nerve evokes only a presynaptic spike. *B*: 8 min after starting perfusion with artificial sea water containing 9 mM-Ca. The presynaptic spike is followed by a PSP. *C*: 42 min later the post-synaptic potential is fully recovered, and generates an action potential which is off the screen at this amplification. Same experiment as Fig. 8.

*Extracellular synaptic potentials*

In experiments to be described in the next section, calcium was applied micro-ionophoretically to different parts of the giant synapse. In only a few cases was the presynaptic fibre clearly visible in the synaptic region, so electrical clues obtained by recording extracellular potentials with the calcium pipette were used to identify regions of synaptic activity. Besides helping to locate such regions, extracellular records from the synapse gave further information on the pre- and post-synaptic electrical events. Therefore, a short account of extracellular potentials at the synapse seems appropriate here.

A micro-electrode just outside the presynaptic fibre in the synaptic region records a potential which consists of both pre- and post-synaptic components. This may be seen, for instance, in Fig. 10, taken shortly after restoring calcium in a preparation where Ca lack had abolished all PSPs. The extracellular electrode records first a positive–negative deflexion which corresponds to the arrival of the presynaptic nerve impulse at the recording site; and, secondly, a slower negative deflexion corresponding to the current responsible for the PSP in the post-synaptic axon. In the blocked synapse only the presynaptic component is seen (see Fig. 15).

The extracellular potential obtained from a normally transmitting synapse is complicated further by the action potential in the post-synaptic axon. Figure 11 shows that, concurrent with the intracellular action potential, there is a positive-going deflexion superimposed on the post-synaptic current recorded by an extracellular electrode critically positioned in the synaptic region. The important feature is that in the absence of local post-synaptic current, as for instance during antidromic activation of the post-synaptic axon or when the action potential is generated by the proximal synapses, the extracellular action potential is a mainly negative-going potential. Records such as those in Fig. 11 were obtained at the neuromuscular junction by Katz & Miledi (1965*a*, *d*; cf. also Kuffler, 1942) and it seems likely that a common explanation applies: namely, that the positive-going deflexion represents a reduction in the local synaptic current as the membrane potential of the post-synaptic axon is brought near the equilibrium potential of the post-synaptic current (cf. Katz & Miledi, 1965*a*). If the potential of the post-synaptic element swings beyond the equilibrium potential, there should be a reversal of the local synaptic current. This, in fact, happens at the neuromuscular junction. At the giant synapse, in most cases the synaptic current was simply reduced to almost zero during the peak of the action potential and only in a few cases was a small reversal seen.

It would have been interesting to determine the potential at which the synaptic current reverses, but unfortunately the post-electrode was not inserted directly under the extracellular electrode. This was important because antidromic action potentials were found to decrease markedly in amplitude as they entered the relatively narrow proximal portion of the post-synaptic axon. In view of this complication the null point for the post-synaptic current could not be determined accurately, but the experiments indicate that it is close to zero membrane potential.

In order to test whether the nerve impulse actively propagated along the synaptic region of the presynaptic axon, extracellular records were obtained simultaneously from two points on the synapse. As shown in

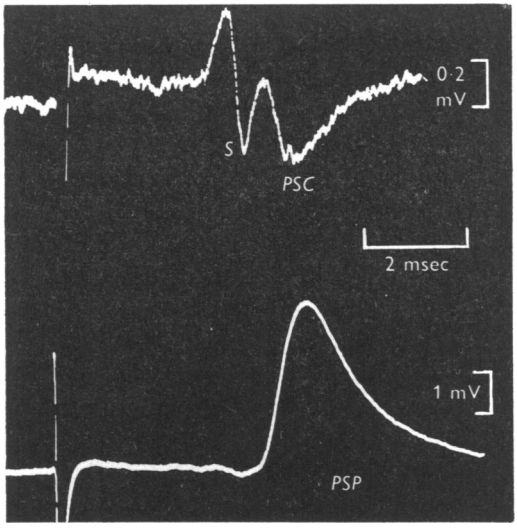


**Fig. 10.** Potentials recorded simultaneously with an extracellular electrode on the giant synapse (top trace) and an intracellular electrode in the post-synaptic axon (bottom trace). *S*: presynaptic spike. *PSC*: post-synaptic current. Transmission had been depressed by Ca lack.

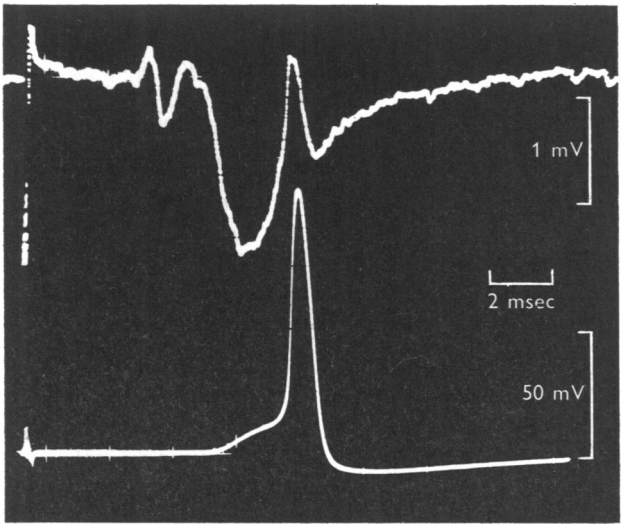


**Fig. 11.** Synaptic potentials in a transmitting giant synapse. Top trace: extracellular record from synaptic region. Bottom trace: intracellular record from post-synaptic axon.

Fig. 12 the presynaptic spike arrives earlier at the proximal recording site than at the distal. From the time difference, the conduction velocity along the intermediate stretch could be calculated; in two experiments it was approximately 3 m/sec. A further point, not illustrated in the figure, is that the shape of the presynaptic spike alters as the impulse travels towards the end of the axon. It is mainly negative at the beginning of the

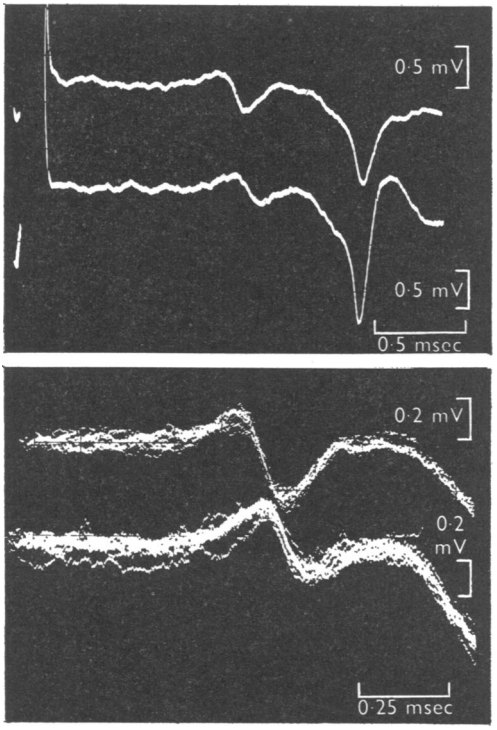


Fig. 12. Simultaneous extracellular recording from two spots 380 $\mu$ apart along the presynaptic nerve terminal in the giant synapse. Lower part shows the presynaptic nerve spikes alone on a faster time base. Total length of synapse was about 1·1 mm. The proximal electrode was approximately 0·1 mm distal to the start of synapse. Preganglionic nerve reduced to one fibre. Temp. 15° C.

synapse and becomes mainly positive near its end. This change in configuration is similar to that seen in the nerve terminals at the neuromuscular junction and is probably due to the distribution of current at the closed end of the axon (cf. Katz & Miledi, 1965*a*).

Thus, at least in the frog's nerve–muscle junction and at the axo–axonal squid synapse, it seems that an impulse is normally propagated along presynaptic nerve terminals. It is possible that this is the case in all terminals, but this can only be regarded as a tentative generalization until other synapses have been examined.

Similar changes in the latency and configuration of the extracellular spike of the presynaptic axon were seen in ganglia where synaptic transmission was blocked by Ca lack. This shows that the impulse still propagates along the synaptic portion of the presynaptic axon even in these conditions when it does not evoke a PSP in the post-synaptic axon. That it is still capable of doing so if calcium is available may be shown by applying calcium locally to small regions of the synapse.

*Ionophoretic application of calcium*

The method for localized application of calcium consisted essentially in depriving the preparation of calcium and in using the same calcium-filled pipette both for ionophoretic release of calcium and for external focal recording. The method has been useful at the nerve–muscle junction (Katz & Miledi, 1965*b*, *c*), an important difference being that the extracellular recording of spontaneous miniature end-plate potentials provided a valuable clue in positioning the Ca pipette on synaptic spots. Since nothing comparable to miniature potentials is observed at the squid giant synapse (Takeuchi & Takeuchi, 1962) we had to rely solely on the extracellular potential of the presynaptic axon.

The effect of calcium was tested at different spots along the synapse; at some points releasing calcium by reducing or inverting the negative bias on the pipette partially restored the PSP in the post-synaptic axon (Fig. 13). At the same time the extracellular Ca pipette recorded the synaptic current associated with the PSP. The positioning of the pipette was very critical, and displacements of only a few microns caused both synaptic current and PSP to disappear, even though a presynaptic spike could still be recorded by the pipette. The amplitude of the presynaptic spike was not directly related to the Ca sensitivity of a spot. At the most sensitive spots so far encountered, the release of only $10^{-14}$–$10^{-13}$ moles in about 1 sec was sufficient to produce a PSP in a blocked preparation, but no special effort was made to determine the maximum sensitivity attainable.

This method of selective activation of a minute portion of a synapse can be used to determine the spatial extent of the synaptic contact. Calcium had an effect when released at points along the stretch of presynaptic axon which under the microscope appears to lie adjacent to the post-synaptic axon. Young (1939) mentions that in this region the post-synaptic axon gives off numerous fine collaterals which run through the connective-tissue sheath enclosing both nerve fibres and end in close contact with the presynaptic axon. These contacts show some of the characteristics found at synapses elsewhere (Robertson, 1953; Hama, 1962), and it is conceivable that the nerve impulse releases the trans-

mitter at these spots. Certainly, the very sharp localization of calcium action does not contradict this supposition; but whether this is so and whether transmitter release occurs only at these regions are questions that require a finer approach.

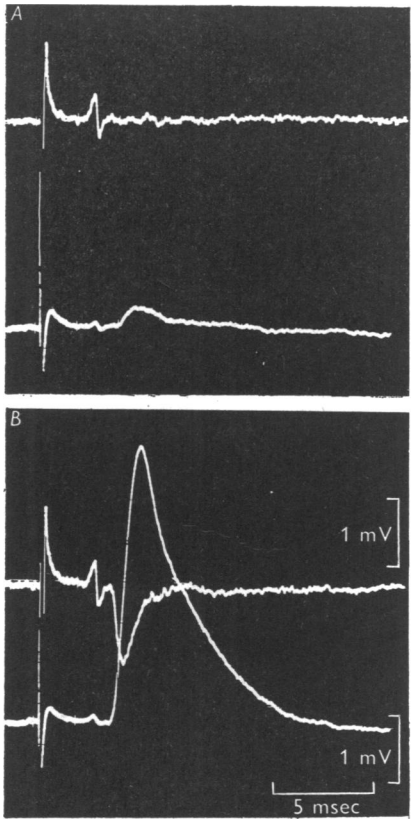


Fig. 13. Localized action of calcium at the giant synapse. Top traces: extracellular records from synaptic region. Bottom traces: intracellular records from post-synaptic axon. In *A*, the synapse was almost fully blocked in low-Ca solution. In *B*, ionophoretic release of calcium restores the post-synaptic current and potential.

The effect of calcium applied in this manner is quite rapid. An appreciable PSP can already be seen in less than 1 sec after the onset of the Ca-releasing current. If the pulse of calcium is prolonged the PSP continues to grow for many seconds (Fig. 14). At the same time the post-synaptic current recorded by the focal pipette also grows slowly, but attains a maximum before the intracellular PSP. A similar phenomenon has been observed at the neuromuscular junction (Katz & Miledi, 1965*c*) and it seems likely that the explanation of this difference is that during

prolonged application the calcium diffuses in effective amounts to progressively increasing distances and acts at points outside the recording range of the extracellular pipette. By contrast, the spatially less discriminating intracellular electrode is able to record the contribution of the more distant spots.

*Synaptic delay.* It is important to know if there is a delay between the arrival of the nerve impulse at the terminal and the beginning of the post-synaptic potential. The experiments above allow us to measure this delay without the complications introduced by conduction time in the terminal. This is so because the extracellular electrode records both the arrival of the presynaptic nerve impulse and the post-synaptic current from the same spot. Furthermore, the active part of the synapse is also restricted to the

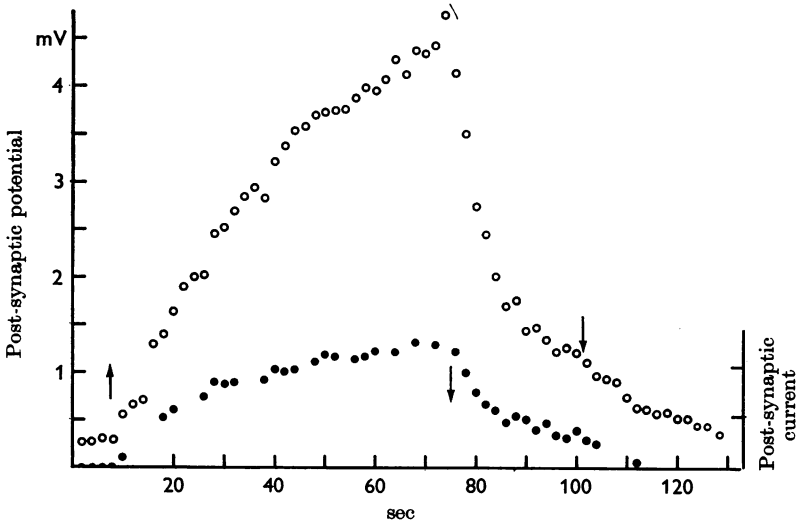


Fig. 14. Time course of calcium action at the giant synapse. (○) PSP recorded intracellularly from post-synaptic axon. (●) post-synaptic current recorded extracellularly with Ca pipette. Ionophoretic release of Ca, at first arrow. At second arrow the Ca-releasing current was stopped, and at third the biasing voltage was re-applied to prevent outward diffusion of Ca from pipette. Same experiment as Fig. 13.

immediate vicinity of the pipette, where alone the calcium concentration is sufficient for transmitter release to occur.

Figure 15 shows a superposition of two extracellular focal records obtained from a synaptic spot. The ganglion was in low-Ca solution and transmission across the synapse was almost completely blocked. One of the traces shows, after the initial stimulus artifact, a presynaptic current spike which signals the arrival of the nerve impulse, and practically nothing afterwards. A moment later, a small amount of calcium was

released from the pipette and the second record obtained. Besides the presynaptic current, this record also shows the post-synaptic current, and a measure may be obtained of the synaptic delay, defined as the time interval between the peak inward current of the presynaptic spike and the beginning of the post-synaptic current (Katz & Miledi, 1965*b*). The synaptic delay thus measured was 0·5–0·8 msec at 10–15° C. This is somewhat shorter, but not importantly so, than the synaptic delay of the frog's neuromuscular junction at the same temperature (Katz & Miledi, 1965*d*).

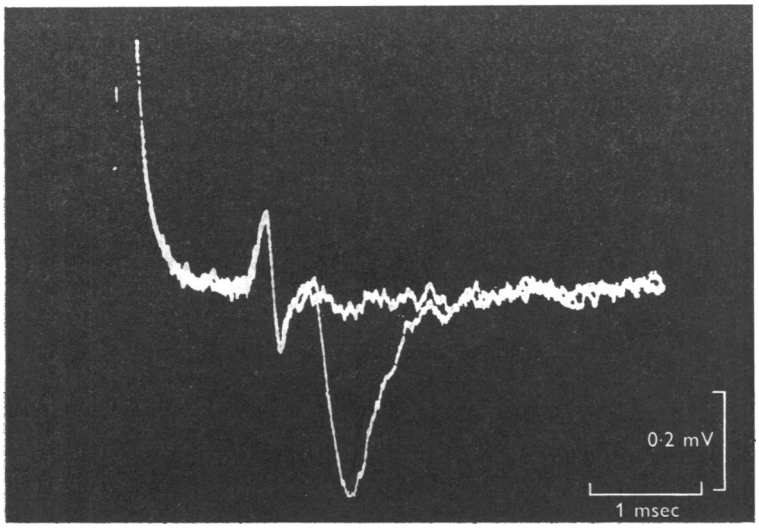


Fig. 15. Synaptic delay at giant synapse. Superimposed photographs of potentials recorded at a synaptic spot. The ganglion was in low-Ca sea water and in the first trace the extracellular recording electrode registers only a spike due to arrival of the presynaptic nerve impulse. The second trace was obtained during ionophoretic release of Ca from the recording (Ca-filled) electrode. A local post-synaptic current (large negative deflexion) is now recorded.

*Intracellular injection of calcium.* It has been seen above that in squid synapses, calcium is necessary for the production of the PSP. In all likelihood here, as at the neuromuscular junction, the presynaptic nerve impulse requires calcium in order to release a transmitter substance. The next question raised is whether the site of Ca action is on the membrane surface or whether calcium crosses the membrane and causes transmitter release by acting inside the presynaptic nerve terminal.

To test these possibilities calcium was injected ionophoretically into the presynaptic terminal in synapses where transmission had been blocked by Ca lack. A calcium-filled pipette was positioned outside the presynaptic axon on a spot where a PSP could be restored by reducing the negative bias on the pipette, thus allowing calcium to diffuse out in small amounts

(Fig. 16*A*). The release of calcium was then stopped and with a slight downward movement the Ca pipette was inserted into the presynaptic axon. With the Ca pipette inside the terminal a similar reduction of the bias was without effect (Fig. 16*B*). The nerve impulse did not evoke a PSP, even when a positive voltage was applied to the inside of the pipette to release more calcium. It should be pointed out that in this case the current flowing from the pipette and out through the membrane depolarized the nerve fibre and greatly reduced the amplitude of the action

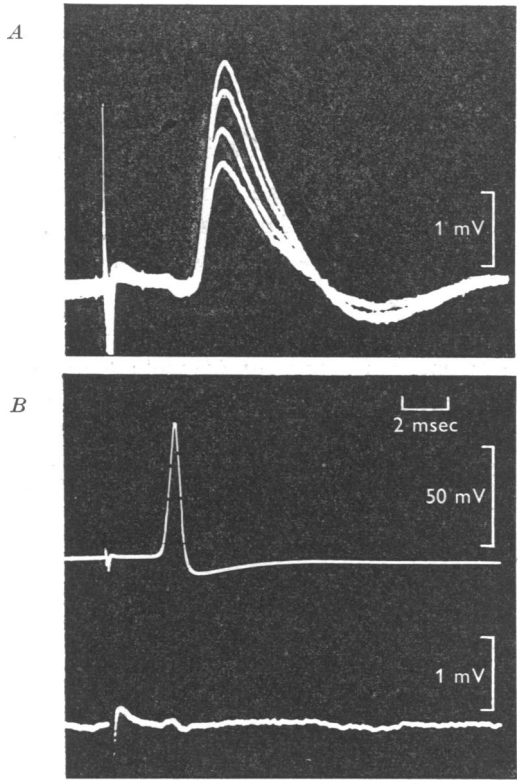


Fig. 16. Intracellular injection of calcium into the presynaptic terminal. Transmission was blocked in low Ca and no PSP could be recorded by the electrode inside the post-synaptic axon.

In *A*, a Ca pipette was placed on a synaptic spot just outside the presynaptic axon. Calcium was allowed to diffuse out of the pipette and the presynaptic impulses regained their ability to produce PSP. The four records in *A* illustrate the still increasing PSPs at 16, 18, 20, and 22 sec after beginning the Ca application.

Between *A* and *B*, the Ca pipette was lowered slightly so that it penetrated the presynaptic axon. In *B*, during the release of a similar amount of calcium to that in *A*, the top trace shows the intracellular presynaptic action potential and the bottom trace records the intracellular potential from the post-synaptic axon, as in *A*. During intracellular application of Ca no PSP is observed.

potential during the time of Ca injection. However, no PSP was seen immediately after the injection period when the amplitude of the presynaptic action potential had recovered its initial amplitude. It should be remembered that, during extracellular ionophoresis of calcium, the effect took many seconds to wear off (see Fig. 14).

It appears, therefore, that to exert its action on transmitter release, calcium acts through a membrane reaction and that the reactive sites are available only from the outside. Unfortunately, for the present this statement remains tentative because time was too short to test its validity fully. Up to now, technical limitations prevented proper impalement of the nerve terminal, and frequently the action potential amplitude fell rapidly after insertion of the pipette. Usually we could not test more than one 'intracellular spot', and it is clear that many spots must be tested in view of the sharp localization required for effective ionophoretic application of calcium from the outside. With large doses of calcium one might conceivably overcome this difficulty, but large injections of Ca seemed to have a deleterious effect on the axon.

## DISCUSSION

The present results confirm in many respects those of Hagiwara & Tasaki (1958) and Takeuchi & Takeuchi (1962), and support their conclusion that transmission across the giant synapse is due to release of a transmitter substance. They show further that a similar mechanism probably operates at the proximal synapses, and that calcium is directly involved in the process of transmitter release.

A striking example which shows that electrical coupling between pre- and post-synaptic axons is not sufficient to play an important role in synaptic transmission across these synapses is that in low calcium the nerve impulse still reaches the ending but does not evoke a PSP. In these conditions, the action potential in the nerve endings produces in the post-synaptic axon only a brief small spike ($< 1$ mV) which is not much larger than the potential recorded just outside the post-synaptic axon.

One of the most interesting aspects of our experiments concerns the lack of action of calcium when injected inside the presynaptic terminal. Several explanations come to mind in this connexion. In the first place, it could be that damage produced by insertion of the Ca pipette blocked transmitter release through that part of the synapse where calcium was applied. A second possibility is that the calcium released inside the axon is immediately 'bound' by intracellular structures other than those concerned with transmitter release. This explanation, however, loses some of its attraction in view of the fact (R. Miledi & R. Thies, unpublished) that extracellular ionophoresis of calcium can restore transmitter release by a

nerve impulse in frog neuromuscular junctions even in the presence of a strong calcium-chelating agent (ethylene glycol bis( $\beta$-amino ethyl ether) -N,N-tetracetic acid) in the external fluid.

A third, and perhaps more attractive, explanation would be that the reactions which lead to transmitter release require the combination of calcium with a membrane component, and that this 'Ca receptor' is accessible only from the outside of the membrane.

We wish to express our gratitude to The Grass Foundation for giving us the means to visit Woods Hole, and lending us equipment. We would also like to thank Professors H. Grundfest, I. Tasaki, and S. W. Kuffler for the loan of equipment.

## REFERENCES

BRYANT, S. H. (1958). Transmission in squid giant synapses. The importance of oxygen supply and the effects of drugs. *J. gen. Physiol.* **41**, 473–484.

BRYANT, S. H. (1959). The function of the proximal synapses of the squid stellate ganglion. *J. gen. Physiol.* **42**, 609–616.

BULLOCK, T. H. & HAGIWARA, S. (1957). Intracellular recording from the giant synapse of the squid. *J. gen. Physiol.* **40**, 565–577.

DEL CASTILLO, J. & KATZ, B. (1954*a*). Changes in end-plate activity produced by presynaptic polarization. *J. Physiol.* **124**, 586–604.

DEL CASTILLO, J. & KATZ, B. (1954*b*). Statistical factors involved in neuromuscular facilitation and depression. *J. Physiol.* **124**, 574–585.

DUDEL, J. & KUFFLER, S. W. (1961). Mechanism of facilitation at the crayfish neuromuscular junction. *J. Physiol.* **155**, 530–542.

ECCLES, J. C., KATZ, B. & KUFFLER, S. W. (1941). Nature of the 'end-plate potential' in curarized muscle. *J. Neurophysiol.* **4**, 362–387.

FENG, T. P. (1941). The local activity around skeletal N–M junctions produced by nerve impulses. *Biol. Symp.* **3**, 121–152.

FRANKENHAEUSER, B. & HODGKIN, A. L. (1957). The action of calcium on the electrical properties of squid axons. *J. Physiol.* **137**, 218–244.

HAGIWARA, S. & TASAKI, I. (1958). A study on the mechanism of impulse transmission across the giant synapse of the squid. *J. Physiol.* **143**, 114–137.

HAMA, K. (1962). Some observations on the fine structure of the giant synapses in the stellate ganglion of the squid, *Doryteuphis bleekeri*. *Z. Zellforsch. mikrosk. Anat.* **56**, 437–444.

HUBBARD, J. I. & SCHMIDT, R. F. (1963). An electrophysiological investigation of mammalian motor nerve terminals. *J. Physiol.* **166**, 145–167.

HUBBARD, J. I. & WILLIS, W. D. (1962). Hyperpolarization of mammalian motor nerve terminals. *J. Physiol.* **163**, 115–137.

KATZ, B. & MILEDI, R. (1965*a*). Propagation of electric activity in motor nerve terminals. *Proc. R. Soc.* B, **161**, 453–482.

KATZ, B. & MILEDI, R. (1965*b*). The measurement of synaptic delay, and the time course of acetylcholine release at the neuromuscular junction. *Proc. R. Soc.* B, **161**, 483–495.

KATZ, B. & MILEDI, R. (1965*c*). The effect of calcium on acetylcholine release from motor nerve terminals. *Proc. R. Soc.* B, **161**, 496–503.

KATZ, B. & MILEDI, R. (1965*d*). The effect of temperature on the synaptic delay at the neuromuscular junction. *J. Physiol.* **181**, 656–670.

KUFFLER, S. W. (1942). Electric potential changes at an isolated nerve–muscle junction. *J. Neurophysiol.* **5**, 18–26.

KUNO, M. (1964). Mechanism of facilitation and depression of the excitatory synaptic potential in spinal motoneurones. *J. Physiol.* **175**, 100–112.

LILEY, A. W. (1956). The effects of presynaptic polarization on the spontaneous activity of the mammalian neuromuscular junction. *J. Physiol.* **134**, 427–443.

LUNDBERG, A. & QUILISCH, H. (1953). On the effect of calcium on presynaptic potentiation and depression at the neuromuscular junction. *Acta physiol. scand.* **30**, suppl. 111, 121–129.

MARTIN, A. R. & PILAR, G. (1964). Presynaptic and post-synaptic events during post-tetanic potentiation and facilitation in the avian ciliary ganglion. *J. Physiol.* **175**, 17–30.

ROBERTSON, J. D. (1953). Ultrastructure of two invertebrate synapses. *Proc. Soc. exp. Biol. Med.* **82**, 219–223.

TAKEUCHI, A. & TAKEUCHI, N. (1962). Electrical changes in pre- and post-synaptic axons of the giant synapse of *Loligo*. *J. gen. Physiol.* **45**, 1181–1193.

YOUNG, J. Z. (1939). Fused neurons and synaptic contacts in the giant nerve fibres of cephalopods. *Phil. Trans. R. Soc.* B, **229**, 465–503.