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A STUDY ON THE MECHANISM OF IMPULSE TRANSMISSION ACROSS THE GIANT SYNAPSE OF THE SQUID

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The structure of the axo-axonal synapses in the stellate ganglion of the squid has been investigated by several anatomists and physiologists. In 1939, Young published the results of his gross anatomical and histological studies on the synapses in this ganglion. He showed that a large axon (about 100 μ in diameter) which has its origin in a higher ganglion divides in this ganglion into about ten smaller branches. Each of these branches ends blindly on the surface of one of the motor giant axons, making a synaptic junction. Among these synapses in the stellate ganglion, the largest one is the junction between the presynaptic axon and the so-called squid giant axon which has been used widely for electrophysiological studies of the excitable membrane. Robertson (1953) carried out electron microscope studies on this synapse and found that, in the region where the two axons make contact, there are a number of axoplasmic processes which are the extensions of the post-synaptic axon. These processes end in close apposition with the axoplasm of the presynaptic axon.

The physiological investigation of this synapse was started also by Young (1939). He found that transmission of impulses through this synapse is unidirectional and is readily blocked on repetitive stimulation of the presynaptic axon. In 1949, Bullock found that a synaptic potential, which resembles in many respects the end-plate potential of the curarized neuromuscular junction (Schäfer & Göpfert, 1937; Eccles & O'Connor, 1939; Kuffler, 1942; Fatt & Katz, 1951), can be recorded in the vicinity of this synapse. Recently, Bullock & Hagiwara (1957) examined the time course of the synaptic potential and the synaptic delay with intracellular micro-electrodes.

In the present work, an attempt is made to elucidate the mechanism of impulse transmission across this giant synapse by using various techniques

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which have been proved useful in the study of the excitable membrane of the squid giant axon. It was found possible to introduce two to three intracellular electrodes into the pre- and/or post-synaptic axons at or near the synaptic junction. By this technique, the effects of stimulating or polarizing currents upon synaptic transmission were investigated. A change in the membrane conductance associated with a synaptic potential (subthreshold) was demonstrated by the use of an impedance bridge. The mechanism of production of the synaptic potential was investigated by 'clamping' the membrane of the post-synaptic axon at various potential levels near the resting membrane potential.

The results of the present investigation were interpreted as indicating a similarity in the synaptic mechanism of the neuromuscular junction (Fatt & Katz, 1951) and this axo-axonal synapse. The outline of the present work has been presented in the spring meeting of the American Physiological Society (Tasaki & Hagiwara, 1957*b*).

METHODS

Dissection of preparations. Synapse preparations were obtained from the North Atlantic squid, *Loligo pealii*, available at the Marine Biological Laboratory in Woods Hole. The procedure of dissecting preparations out of the body of the animal was similar to that described by Bullock (1948). Both the pre- and post-synaptic axons were tied together with surrounding tissues at points about 20 mm away from the stellate ganglion. Using a pair of small scissors, the axons were freed from the underlying tissues and a preparation consisting of the presynaptic axon, the stellate ganglion and the post-synaptic giant axon was carefully dissected out of the wall of the body cavity.

The connective tissues around the synapse under study and the major portion of the small nerve fibres running together with the presynaptic axon were carefully removed under a binocular microscope while the preparation was still *in situ*. This procedure was necessary to increase visibility of the synaptic region of the preparation and to make insertion of micro-electrodes near the synapse possible. In good preparations, obtained from freshly caught specimens, stretching the preparation by way of the thread tied on the end of the axons did not bring about a failure of synaptic transmission.

Most of the experiments described in this paper were carried out at room temperature (21–22° C). On a few occasions observations were made while the preparation was exposed to cold running sea water (10–15° C).

Arrangements used for stimulation and recording. The preparation was mounted horizontally on a glass plate of approximately 34 mm width with its tied ends stretching beyond the edges of the glass plate. The synaptic region of the preparation was in the middle of the glass plate. The two tied ends were fixed to the lucite platform to which the glass plate was glued. The sea water surrounding the preparation was continuously oxygenated by circulating fine bubbles of O_2 95% plus CO_2 5%.

Intracellular potentials were recorded either with a superfine glass pipette electrode or with a metal wire electrode, or occasionally with both of them. The reference electrode (grounded) was of the Ag–AgCl–agar type and was immersed in the surrounding sea water. Glass micropipettes were filled with 0·55 M-KCl (sometimes 3 M) solution. The tip diameter of the micro-electrodes used for recording potentials was as a rule smaller than 1 μ; the micro-electrode used for applying stimulating or polarizing currents to the axons was 2–3 μ in diameter. Intracellular metal wire electrodes were made from enamelled nichrome-steel (or sometimes silver) wire of about 25 μ in

diameter, the insulating enamel of which was scraped off for a length of 1 mm (in some experiments 3 mm) near the tip.

The wire electrode was introduced into the post-synaptic axon through a hole in the axon membrane at the edge of the glass plate and was pushed along the axis of the axon until the recording tip of the electrode reached the synaptic region. Micromanipulators of the Peterfi type were used to manipulate the metal wire electrode as well as to introduce glass pipette electrodes through the surface membrane of the axons. These manipulations were done as a rule under dark field illumination.

Stimulating pulses were obtained from a stimulus isolation unit (Grass Co., Model S1U–4A). Unless otherwise stated, stimulating pulses were delivered to the presynaptic axon near the edge of the glass plate.

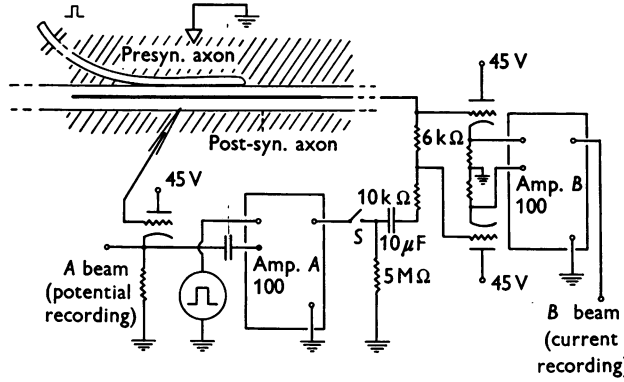

Fig. 1. Experimental arrangement used to record the 'synaptic current' by clamping the membrane potential at a constant level during synaptic activation. The thick line drawn in the post-synaptic axon represents the exposed portion of the intracellular metal wire electrode. The three cathode-followers shown in the figure were made with Z729 tubes in triode connexion. Further details in text.

Voltage clamp technique. The equipment used for voltage clamp experiments was similar to that used in a previous experiment (Tasaki & Hagiwara, 1957*a*). The internal current electrode consisted of a metal wire (25 μ) with the 3 mm long portion near the tip exposed by scraping off the insulating enamel. Since the region at which the pre- and post-synaptic axons make synaptic contact was about 1 mm long, this exposed portion of the wire covered the entire length of the synaptic region. A glass pipette micro-electrode inserted in the middle of the synaptic region of the post-synaptic axon was used to monitor the membrane potential (Fig. 1).

A cathode-follower and a feed-back amplifier (Amp. *A* in Fig. 1) were connected to these electrodes in such a way that the potential recorded with the micro-electrode could be maintained at a constant level during synaptic activity by an automatic control of the membrane current. The circuit used for this automatic control of the membrane current is similar in principle to that used by Hodgkin, Huxley & Katz (1952). The output of the cathode-follower (Z729 in triode connexion) was led to one of the two inputs of a differential amplifier (Tektronix, Type 122) which had a flat response between 0·1 cycle and 40 kc/s (Amp. *A*). The other input of the differential amplifier was connected to a source of a rectangular voltage pulse (Tektronix pulse generator, type 161). The output of the differential amplifier was led to the internal current electrode through a switch, a large condenser (10 μF) and resistors. To measure the current fed into the post-synaptic axon, the IR-drop across one of the resistors was led to another differential amplifier (Amp. *B* in Fig. 1) through a pair of cathode-followers.

Measurement of change in the membrane impedance associated with the synaptic potential. An alternating current Wheatstone bridge illustrated in Fig. 2 was used to investigate changes in the

membrane impedance of the post-synaptic axon associated with synaptic potentials. The internal impedance electrode was made with a metal wire similar to that used for voltage-clamp experiments. The external electrode was a long silver wire covered with a layer of agar gel. The resistors in the ratio arms of the bridge, r_1 and r_2 in the figure, were 10 Ω and 100 (or 300) Ω, respectively. The remaining arm consisting of two condensers (C and C') and a resistance (R) was used to adjust the bridge balance to eliminate the bridge output at rest.

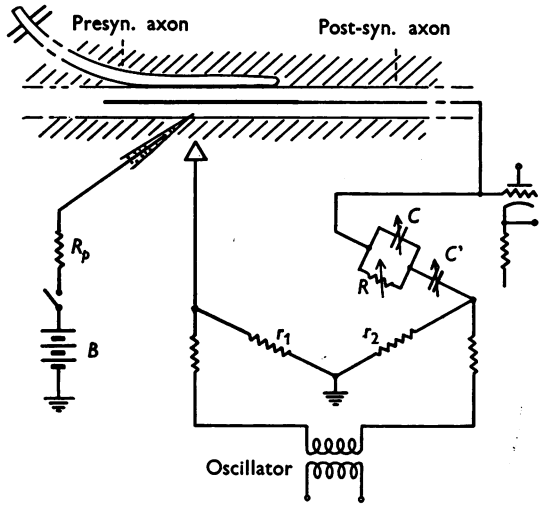

Fig. 2. An a.c. Wheatstone bridge used to detect small changes in the membrane impedance associated with synaptic potentials. Battery B and resistor R_p are used to block synaptic transmission by hypolarization of the post-synaptic axon. Further details in text.

In some experiments, condenser C' was fixed at a large value (approximately 10 μF) and R and C were varied; this parallel arrangement of C and R was used to examine the direction (increase or decrease) of the change in the membrane impedance. A series arrangement, in which C was fixed at zero and C' was variable, was used to minimize the flow of current through the internal impedance electrode during synaptic activation. Since a flow of current through the fine metal wire electrode can change the resistance of the electrode by polarization and may give rise to a false unbalance of the bridge, it was desirable to examine the membrane impedance by both parallel and series arrangements of R and C.

The bridge a.c. was 10 (or 16) kc/s in frequency and was supplied by a beat-frequency oscillator (General Radio, Type 1304-A). The bridge unbalance was amplified 100 or 1000 times with a preamplifier (Tektronix, Type 122) and then filtered by a variable electronic filter (Spencer-Kennedy Laboratory, Model 302). The unfiltered output of the bridge was taken as a measure of the potential variation in the post-synaptic axon. The filtered output of the bridge, displayed simultaneously on the dual beam oscilloscope (DuMont, Type 322), was the measure of the impedance change associated with synaptic potentials. Because of the shunting of the internal and external electrodes by the bridge, action potentials recorded by this method were slightly distorted in shape and slightly reduced in size. A micro-electrode inserted in the middle of the synaptic region of the post-synaptic axon was used to block synaptic transmission by hyperpolarization.

RESULTS

General properties of the synaptic potential

The properties of the synaptic potential as recorded with an intracellular electrode inserted into the post-synaptic axon near the synaptic region have been discussed by previous investigators (Bullock & Hagiwara, 1957). As the results of improvement of our technique of dissection and cleaning of the preparation, we were able to insert two or more micro-electrodes directly in the region of the giant synapse. By this improved method, we could confirm and extend the observation made by Bullock and his associate.

When a single electric shock was delivered to the presynaptic axon of the preparation, there was a single spike potential in the post-synaptic axon. A measurement of the latency with multiple recording micro-electrodes inserted along the post-synaptic axon revealed that the latency was shortest when the recording micro-electrode was in the region of the synapse.

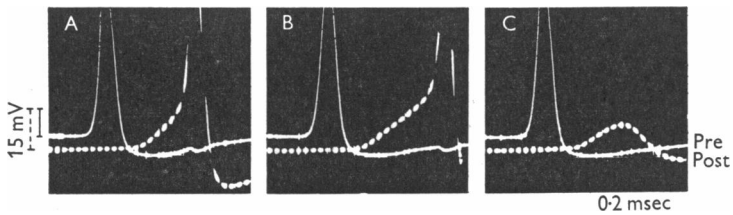

Fig. 3. Presynaptic spike potentials (continuous line) and responses of the post-synaptic axon (broken line) recorded simultaneously in the vicinity of the synapse. Records were taken when synaptic transmission started to fail as a result of a prolonged repetitive stimulation of the presynaptic axon.

The action potential recorded from the post-synaptic axon in the vicinity of the synapse was preceded by a slow, preliminary rise in the membrane potential which represented the early part of the synaptic potential. In a fresh preparation obtained from an active specimen of squid, the rise of the synaptic potential was rapid and the transition from the synaptic potential to the propagated axonal spike potential was not clear. Following repetitive stimulation of the presynaptic axon, the transition from the synaptic potential to the axonal spike became clearer. This 'fatigue' was the result of gradual decrease in size of the synaptic potential associated with a gradual prolongation of the time interval from the beginning of the synaptic potential to the start of the axonal spike potential.

The records presented in Fig. 3 illustrate this gradual change in synaptic transmission. The continuous trace in the figure represents the membrane potential recorded with a micropipette inserted into the presynaptic axon in the middle of the synaptic region. The intermittently blanked trace indicates

the potential in the post-synaptic axon recorded with a metal wire electrode (with 1 mm long exposed surface) inserted in the axis of the post-synaptic axon in the region of the synapse. The frequency of the fatiguing shocks delivered to the presynaptic axon was 40/sec. The records were taken at the time when the synaptic transmission started to fail.

The spike potential of the presynaptic axon did not change during repetitive stimulation. The beginning of the synaptic potential relative to the spike potential of the presynaptic axon did not change either when the synaptic potential underwent a progressive change. The peak value of the synaptic potential at which synaptic transmission barely failed was always between 10 and 15 mV, in most cases around 12 mV, above the resting potential. This agreed with the threshold membrane potential determined by applying a rectangular current pulse to the post-synaptic axon through an intercellular glass micro-electrode in the region of the synapse. From this fact it follows that synaptic transmission fails when the synaptic potential becomes sub-threshold for the axonal membrane around the synapse. We did not examine the decay of the synaptic potential along the post-synaptic axon very accurately. There was little doubt, however, that the decay was determined by the cable properties of the resting axon.

Within the region where the two axons make a synaptic contact, the rate of rise of the synaptic potential was found to vary slightly from spot to spot. The maximum rate of rise was encountered at around the proximal end (close to the cell body) of the 1 mm long synaptic region. The fact that the post-synaptic axon decreases its diameter as it approaches the cell body appeared to be a factor causing this difference in the rate of potential rise. But it is possible that there are some other unknown factors contributing to this slight difference.

We examined, using the same arrangement, the effect of an antidromic impulse upon the potential inside the presynaptic axon. When a stimulating shock was delivered at the distal end of the post-synaptic axon, a normal action potential was observed at the synaptic region of the axon. At this moment there was no sign of a spread of electric current from the post-synaptic axon to the presynaptic axon. A small deflexion of a few mV observed with the micro-electrode in the presynaptic axon could be fully accounted for as a variation in the external field caused by the antidromic impulse. This small deflexion remained practically unchanged when the electrode in the presynaptic axon was slowly withdrawn and the recorded resting potential finally disappeared. In the next experiment we made a further attempt to determine whether or not there was a direct spread of electricity from the presynaptic axon to the post-synaptic axon.

The absence of direct spread of electricity from presynaptic axon to post-synaptic axon or in reverse direction

An experiment was designed to determine whether or not it was possible to explain the synaptic potential in the post-synaptic axon as a simple electrotonic spread of potential changes in the presynaptic axon. As shown in Fig. 4, top, two glass pipette micro-electrodes were inserted into the presynaptic axon within about 1 mm from the synapse. One of the micro-electrodes was used to deliver rectangular current pulses into the presynaptic axon; the other was used to record potential changes in the presynaptic axon. A metal wire

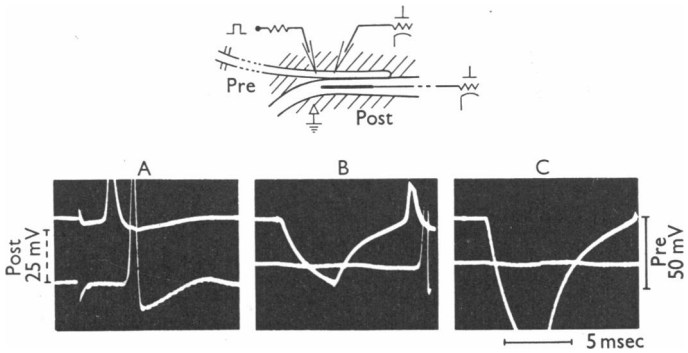

Fig. 4. Record A: Simultaneous recording the pre- and post-synaptic spike potentials. Records B and C: Demonstration of the absence of direct electrical connexion between the pre- and post-synaptic axons by hyperpolarizing the presynaptic axon. A spike potential at the end of hyperpolarization is an off-response.

electrode was inserted into the post-synaptic axon to record synaptic potentials and propagated responses. After making certain that the synapse was capable of transmitting impulses with these three intracellular electrodes in place, strong pulses of inward-directed currents were applied through one of the micro-electrodes in the presynaptic axon (records B and C in Fig. 4). The purpose of this experiment was to examine whether or not a large potential variation in the presynaptic axon is transmitted to the post-synaptic axon.

With pulses of inward membrane current, the potential variation (hyperpolarization) could be made as large as 100 mV or more. It was possible to vary the rate of potential change by varying the pulse duration and intensity. In all these cases, we could not observe any potential change in the synaptic region of the post-synaptic axon that was attributable to a direct spread of electricity from the presynaptic axon. This finding, as well as the absence of a spread of electricity from the post-synaptic axon to the presynaptic axon in antidromic excitation of the preparation, strongly indicates that there is no direct pathway (with a low electric resistance) between the axoplasm of the

presynaptic axon and that of the post-synaptic axon. This is consistent with the result of electron microscopic studies by Robertson (1953).

When pulses of outward membrane current were sent into the presynaptic axon, no potential variation was observed in the post-synaptic axon as long as the pulse was subthreshold for the presynaptic axon. When the pulse was strong enough to elicit an impulse in the presynaptic axon, there was a synaptic potential followed by a propagated impulse in the post-synaptic axon. We paid special attention to see if a 'subthreshold response' in the presynaptic axon was capable of generating any observable potential variation in the post-synaptic axon. We could not find any sign of synaptic potential related to subthreshold stimuli to the presynaptic axon.

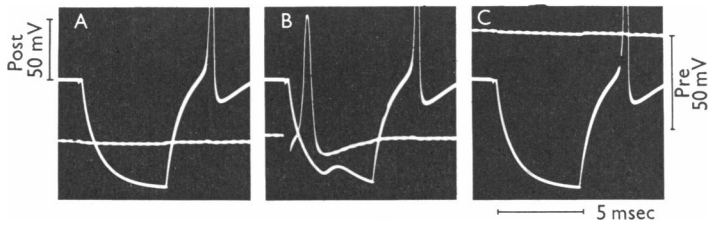

Fig. 5. Effect of hyperpolarization of the post-synaptic axon upon the membrane potential of the presynaptic axon. In record B, a stimulating shock was delivered to the presynaptic axon to show that the recording electrodes were in place. Record C was taken immediately after the disappearance of the resting potential of the presynaptic axon on gradual withdrawal of the recording electrode.

It was found recently that unidirectional transmission of impulses across the crayfish synapse is due to the existence between the pre- and post-synaptic axons of a special membrane that allows a flow of current in one direction but not in the opposite direction (Furshpan & Potter, 1957). In order to know whether or not this is the case in the squid synapse, we examined the effect of hyperpolarization of the post-synaptic axon upon the potential in the presynaptic axon (Fig. 5). Two nichrome-steel wires of 30 μ in diameter were inserted into the post-synaptic axon. The wire with 1 mm long exposed surface was connected to a source of rectangular voltage pulses of about 10–40 V through a 1·2 MΩ resistor. The other wire with a 0·5 mm long exposed surface was connected to a cathode-follower for recording the membrane potential of the post-synaptic axon. One glass pipette micro-electrode was introduced into the presynaptic axon to record the membrane potential near the synapse.

It was found that a change in the membrane potential of the post-synaptic axon caused by passing a strong inward membrane current (approximately 20 μA) did not alter the membrane potential of the presynaptic axon by any detectable amount. When a large potential variation of 50–80 mV was produced in the post-synaptic axon by the applied polarizing current, there was

a small, but detectable potential variation (1–5 mV) at the tip of the electrode in the presynaptic axon. However, this potential variation did not disappear when the tip of the electrode was withdrawn and the recorded resting potential had just disappeared (Fig. 5C). Therefore, it is evident that even this small potential variation in the presynaptic axon does not represent a spread of electricity across the synaptic membrane.

The experimental findings mentioned in this section can be summarized as follows: Neither a subthreshold depolarization nor a large hyperpolarization of the presynaptic axon brought about a detectable change in the membrane potential of the post-synaptic axon. Neither strong depolarization (associated with a propagated impulse) nor a strong hyperpolarization (caused by an applied current) of the post-synaptic axon induced any detectable potential variation across the surface membrane of the presynaptic axon. In short, electric insulation between the two axons was practically perfect.

Demonstration of a 'space' between pre- and post-synaptic axons

With a view to measuring the 'synaptic delay' of the nerve impulse in a more direct manner, we made an attempt to push a recording submicroscopic micro-electrode through the surface membrane of the post-synaptic axon in the region where the membrane of the presynaptic axon was making a synaptic contact. As has been pointed out under Methods, this synaptic region could be recognized, in a cleaned preparation under dark field illumination, as a thin shiny layer where the two axons were lying in close apposition.

A giant synapse preparation was mounted on a glass plate with a piece of solid wax fixed to its surface. By bending the synaptic region of the preparation at the corner of the piece of wax and by applying some tension to the tied ends of the axons, the preparation was arranged in such a way that the synaptic region was situated at the proximal end of the axis of the post-synaptic axon (see Fig. 6, left).

The potential difference between the grounded sea water around the preparation and the long intracellular electrode was continuously recorded during penetration of the synaptic region by the d.c. channel of a dual beam oscillograph. The potential of the same electrode was amplified with a condenser-coupled amplifier and was displayed on the other channel of the oscillograph; this channel was used to record potential variations elicited by electric shocks applied to the presynaptic axon. The d.c. potential was recorded continuously on a running film, and the responses to electric stimuli were recorded by the oscilloscope beam deflecting perpendicularly to the d.c. beam. By preliminary tetanization of the presynaptic axon, post-synaptic spike potentials were eliminated. While the micro-electrode was adavancing slowly in the axis of the post-synaptic axon, the presynaptic axon was stimulated near its tied end at the rate of one shock per second.

When the tip of the recording electrode was in the axoplasm of the post-synaptic axon, synaptic potentials of the ordinary monophasic configuration were evoked by electric shocks delivered to the presynaptic axon (Fig. 6, middle bottom). The level of the d.c. potential remained constant until the tip reached the synaptic region of the membrane as recognized by a slight

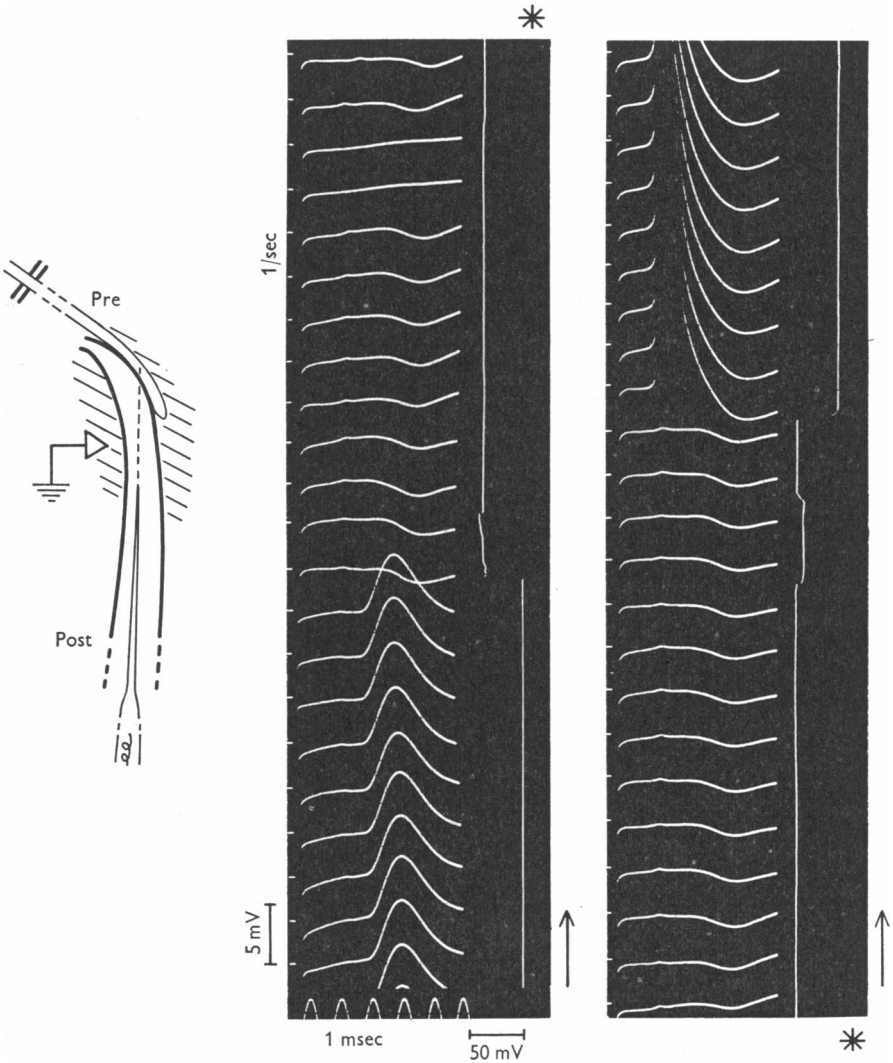

Fig. 6. Demonstration of the 'space' between the pre- and post-synaptic axon. A long recording micro-electrode was advanced slowly along the axis of the post-synaptic axon. The continuous beam displays the resting potential, and the other trace, sweeping at a rate of 1/sec, shows the responses to the shocks delivered to the presynaptic axon recorded through the same micro-electrode.

displacement of the shiny surface layer. When the electrode was slightly advanced at this stage, the d.c. potential level at the tip of the recording microelectrode was found to drop to a level close to zero. At the same moment, the intracellularly recorded monophasic synaptic potential disappeared. We could maintain the recording electrode in this position as long as we wanted.

We suspected when we made this observation that the recording electrode might have failed to penetrate the synaptic region, but instead it might have penetrated the non-synaptic surface of the post-synaptic axon. But the following observations indicated that this was not the case: In all the six cases in which the tip of the recording electrode was recognized to be pressing directly against the membrane in the shiny synaptic zone, the recording microelectrode entered the presynaptic axon on very slight advancement of the electrode in the direction of its axis. When the tip of the recording electrodes was recording no resting potential, small potential variations, which appeared to be a mirror image of the synaptic potential in the post-synaptic axon, were evoked in all-or-none manner in response to presynaptic stimulation (see Fig. 6, middle top). Since this reversed synaptic potential was too large to be attributed to a simple external pick-up of the ordinary synaptic potential, it was concluded that there is between the pre- and post-synaptic membrane actually a small 'space' where the potential is close to that of the surrounding sea water. It will be shown in the next section that the membrane current responsible for generation of the synaptic potential flows through this 'space' and generates the reversed synaptic potential.

It is seen in Fig. 6, right, that when the recording electrode penetrated the boundary between the 'space' and the presynaptic axon, there was a sudden appearance of the resting potential at the tip of the recording electrode. At the same moment, the small externally recorded action potential of the presynaptic axon changed into the large intracellularly recorded responses. The time interval between the peak of the presynaptic spike potential and the beginning of the synaptic potential as determined by this method was between 0·3 and 0·6 msec at 21–22° C.

Demonstration of membrane current responsible for production of synaptic potential

When a nerve impulse in the presynaptic axon arriving at the synapse sets up a (subthreshold) synaptic potential in the post-synaptic axon, the non-synaptic portion of the surface membrane of the post-synaptic axon is traversed undoubtedly by an outward current because of a rise in the axoplasm potential. Since the total amount of the current that leaves the post-synaptic axon has to be equal to the current that enters the axon at the same moment, there should be an inward membrane current at the synaptic region of the axon at the time when its membrane potential rises during synaptic activity.

If the membrane potential of the post-synaptic axon is 'clamped' at a constant level when a presynaptic impulse arrives at the synapse, there will be no membrane current through the non-synaptic surface of the post-synaptic axon. Now, the problem is: What is the time course of the current that flows through the surface of the post-synaptic axon when its membrane potential is held constant during synaptic activation?

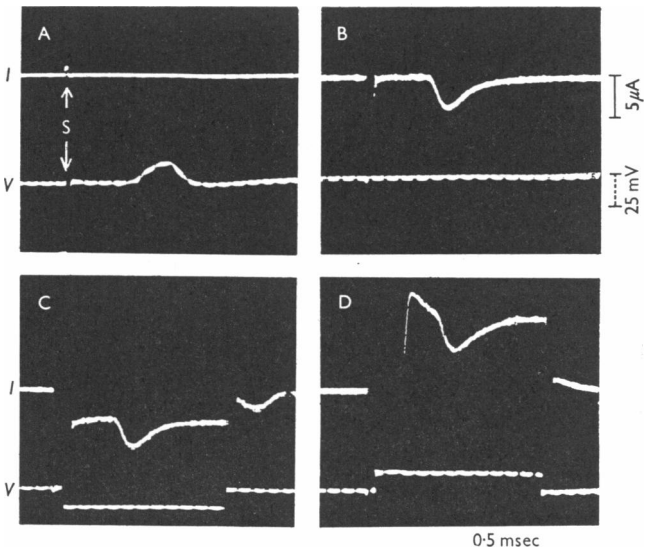

Fig. 7. Demonstration of the 'synaptic current' by clamping the membrane potential of the post-synaptic axon. The arrangement of Fig. 1 was used. Trace *V* displays the membrane potential of the post-synaptic axon; trace *I* shows the current required to control the membrane potential. S indicates the artifact caused by a stimulating shock delivered to the presynaptic axon.

To clarify this problem an experiment was designed to clamp the membrane potential along a rectangular time course when an orthodromic impulse arrives at the synapse. The arrangement of Fig. 1, described under Methods, was used for this purpose. With this arrangement, it was possible to maintain the membrane potential at a constant level by an automatic control of the membrane current. This control was not difficult in a preparation in which the synaptic transmission had been blocked by preliminary tetanization of the presynaptic axon.

In Fig. 7, the continuous trace (*I*) represents the time course of the current sent into the current-electrode in the post-synaptic axon to control the membrane potential. The blanking trace (*V*) displays the time course of the membrane potential of the post-synaptic axon recorded with a micro-electrode inserted in the region of the synapse. In record A, the current through the intracellular metal wire was kept at zero; a stimulating shock (S) given to the

presynaptic axon gave rise to a synaptic potential which was slightly distorted by the presence of a 'subthreshold response'. In record B the membrane potential of the post-synaptic axon was kept at the level of the resting membrane potential; stimulation of the presynaptic axon now gave rise to a transient *inward* current through the surface of the post-synaptic axon.

In records C and D the membrane potential of the post-synaptic axon was clamped at levels about 15 mV above and below, respectively, the resting potential when the orthodromic impulse arrived at the synapse; the intensity of the membrane current required to maintain the membrane potential at a constant level was not affected appreciably by this slight shift in the clamping level. The disappearance of the current trace in record D, following the start of the clamping pulse, is due to the shock artifact (S) followed by a large inward membrane current caused by a response of the membrane resulting from the depolarization of about 15 mV.

Let us denote the time course of the inward current recorded by the method of record B in Fig. 7 by $I(t)$. Under these voltage clamp conditions, there is obviously no membrane current in the non-synaptic surface of the post-synaptic axon. Therefore, current $I(t)$ represents the current that flows through the synaptic membrane of the post-synaptic axon on arrival of an orthodromic impulse. Since $I(t)$ is not appreciably affected by a slight change in the membrane potential (records C and D), one may infer that the same current, $I(t)$, flows through the synaptic membrane when there is no voltage clamping, i.e. when there is an ordinary synaptic potential in the post-synaptic axon. For the sake of simplicity, we call $I(t)$ the *synaptic current.*

The synaptic current flowing inward through the synaptic membrane into the post-synaptic axon tends to depolarize the post-synaptic axon. We found that the size and shape of the synaptic potential can be explained satisfactorily as the result of the synaptic current observed by the method of the voltage clamp. The argument to show that the synaptic potential is generated by the synaptic current, $I(t)$, is as follows:

The amplitude of the subthreshold synaptic current in Fig. 7 is approximately 4 μA. The effective resistance between the internal electrode and the external fluid medium is of the order of 4 kΩ (this varied between 4 and 6 kΩ among different preparations). When the synaptic current flows into the post-synaptic axon, the rise in the membrane potential will be of the order of 16 mV; actually, this is very close to what was observed in record A of Fig. 7.

The time constant of the post-synaptic membrane measured by passing a pulse of constant current was between 1 and 1·5 msec. When the delay in the rise of the membrane potential due to this time constant was taken into consideration, we were able to reconstruct the rising phase of the synaptic potential satisfactorily. A slight discrepancy in the calculated amplitude and the observed value, as well as the slight diphasicity of the observed synaptic

potential were attributed to the 'subthreshold response' in the non-synaptic surface of the post-synaptic axon.

Now, it is possible to show that the synaptic current does not pass through the surface membrane of the presynaptic axon. The presynaptic axon is smaller than the post-synaptic axon; if we assume that the synaptic current penetrates the surface membrane of the presynaptic axon, we should expect a large potential variation in the presynaptic axon at the time when there is a synaptic potential in the post-synaptic axon. Since this is actually not the case (Fig. 3), we conclude that the synaptic current flows through the 'space' between the pre- and post-synaptic axon and does not enter into the presynaptic axon.

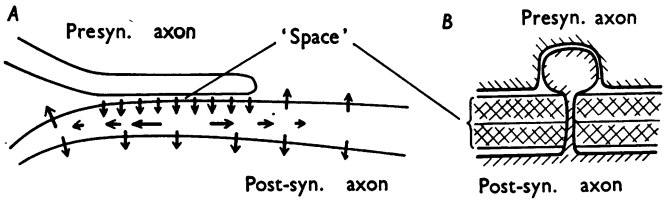

Fig. 8. *A*: distribution of electric currents in the vicinity of the synapse at the time when a synaptic potential is produced in the post-synaptic axon. *B*: simplified drawing showing the structure of the squid synapse; one axoplasmic process extending from the post-synaptic axon towards the presynaptic axon is shown. 'Space' indicates the region where the d.c. potential is close to that of the surrounding sea water.

The conclusions drawn from the electrophysiological observation mentioned above are summarized in diagram *A* in Fig. 8. It is shown in this diagram that when an orthodromic impulse arrives at the synapse, there is an inward membrane current at the synaptic region of the post-synaptic axon. This current flows outwards through the non-synaptic portion of the membrane giving rise to a synaptic potential. The pathway of the current is closed by the conducting fluid medium in the 'space' between the two axons. The diagram is highly diagrammatical and is drawn purely on the basis of our own electrophysiological and optical observations. The synaptic space is not clearly discernible as a space under our dissection microscope (60×); however, we could keep a micro-electrode of about 0·5 μ tip diameter in this space indefinitely. From this information, it seems safe to say that the thickness of this space (the distance between the axons) is not smaller than about 1 μ and not larger than about 20 μ.

We are now prepared to compare our electrophysiological findings with the results of electron microscopic studies of this synapse. Robertson (1953) showed that the 'synapsing' surfaces of the two axons are invested by two separate layers of Schwann cells and by two layers of sheath. These layers are perforated by a number of axoplasmic processes (1–10 μ in diameter). These processes are extensions of the post-synaptic axon; they attain larger diameter

and end in close apposition with the axoplasm of the presynaptic axon (diagram *B* in Fig. 8).

It is evident from what has been stated above that the 'space' between the two axons revealed by our electrophysiological study corresponds to the space occupied by loose connective tissue between the two axons and not to the narrow gap between the two axolemma at the end of the processes of the post-synaptic axons. It is this space that is traversed by the 'synaptic current' observed in the experiment of Fig. 7. We could not determine whether the synaptic current penetrates the axonal membrane only at the ends of the process, or it is distributed uniformly over the entire surface of the post-synaptic axon facing the 'space'.

The reversed synaptic potential recordable in the 'space' between the pre- and post-synaptic axon (Fig. 6) is not exactly a mirror image of the record obtained from inside the post-synaptic axon. We attribute this fact to the capacitative pick-up of the potential variation in the post-synaptic axon. When the electrode tip is in the interaxonal space, the major portion of the long (approximately 15 mm) recording electrode is still in the post-synaptic axon. The capacitative leakage of current through the wall of the micro-electrode is expected to counteract the potential variation at the tip of the electrode. Assuming that this interpretation is right and also that the conducting medium in the space between the two axons has a specific resistance of 20–30 Ω.cm, it is possible to estimate the thickness of the 'space' between the two axons from the size of the reversed synaptic potential. This estimation indicates that the 'space' should be 2–5 μ thick. (In this calculation, we assumed the length of the space to be 1 mm and the width to be 0·1 mm.) This figure is in good agreement with what has been stated by Robertson (1953).

Change in membrane conductance associated with the synaptic potential

With the a.c. Wheatstone bridge shown in Fig. 2 operated at 10 or 16 kc/s, it was possible to detect a small change in the membrane impedance of the post-synaptic axon associated with a synaptic potential. When the stimulating shock delivered to the presynaptic axon gave rise to a propagated spike potential in the post-synaptic axon, there was a large impedance loss of the membrane associated (record D in Fig. 9). When the transmission across the synapse was blocked by repetitive stimulation of the presynaptic axon, the unbalance of the Wheatstone bridge decreased to 1/20–1/50 of the original amplitude. On further repetitive stimulation, the bridge unbalance decreased further. There was a close parallelism between the amplitude of the synaptic potential and the bridge unbalance. The records obtained resembled in many respects those published by Katz (1942) showing the impedance loss of the frog sartorius muscle associated with the end-plate potential.

With an impedance bridge of the type shown in Fig. 2, a small bridge unbalance can be produced by various sources other than a change in the membrane conductance. One of the possible sources of artifact is polarization of the metal wire electrode in the post-synaptic axon. If there is a weak flow of current through the internal impedance electrode in association with a synaptic potential, a small bridge unbalance may occur as the result of a change in the resistance of the internal electrode. We think, however, that this source of artifact is excluded in our experiment. When the capacity and the resistance in the variable arm of the bridge (Fig. 2) were connected in series (i.e. when $C=0$), the bridge balance was obtained when C' was between 0·0005 and 0·0007 μF and R was between 12 and 15 kΩ (at 16 kc/s). This means that a flow of current through the internal electrode should vanish with a time constant of about 8 μsec. This time constant is too short to have any effect upon our impedance measurement.

Another source of artifact is the impedance loss of the non-synaptic membrane of the post-synaptic axon associated with its subthreshold potential variation. In so far as we regard the synaptic current as an electric stimulus for the post-synaptic axon, it is possible to make some distinction between a synaptic potential and a so-called subthreshold response. When the variation in the membrane potential is within the range where there is an approximately linear relation between the applied current and the evoked potential change, the observed potential variation can be regarded as being uncontaminated by a subthreshold response of the membrane. When the membrane of the post-synaptic axon is hyperpolarized by a constant current, the potential variation caused by stimulation of the presynaptic axon is limited within such a linear range. It should be possible therefore to determine, by hyperpolarizing the axon, whether or not the observed impedance loss is due to the subthreshold response in the non-synaptic membrane.

In record A of Fig. 9, the upper record was taken when no polarizing current was applied through the glass pipette electrode in Fig. 2. The bridge a.c. was not yet turned on, and the capacities in the variable arm of the bridge were close to zero. A shock delivered to the presynaptic axon gave rise to a propagated impulse in the post-synaptic axon preceded by a barely discernible synaptic potential. Next, the post-synaptic axon was hyperpolarized to lower the membrane potential by approximately 50 mV (lower record in Fig. 9A); a stimulus delivered to the presynaptic axon gave rise to a synaptic potential which had a long falling phase. Next, the bridge a.c. was turned on and the bridge was balanced for the impedance of the polarized membrane. A shock delivered to the presynaptic axon gave rise to a prolonged impedance loss whose time course was roughly parallel to the time course of the synaptic potential (records B and C). Finally, when the polarizing current was turned off, impulse transmission across the synapse was restored and a large bridge

unbalance associated with a propagated impulse was produced on stimulation of the presynaptic axon (record D).

From the observations described above, it seems safe to conclude that a synaptic potential (subthreshold) is accompanied by an increase in the conductance of the post-synaptic axon. The observation to be described in the following section gives further support to this conclusion.

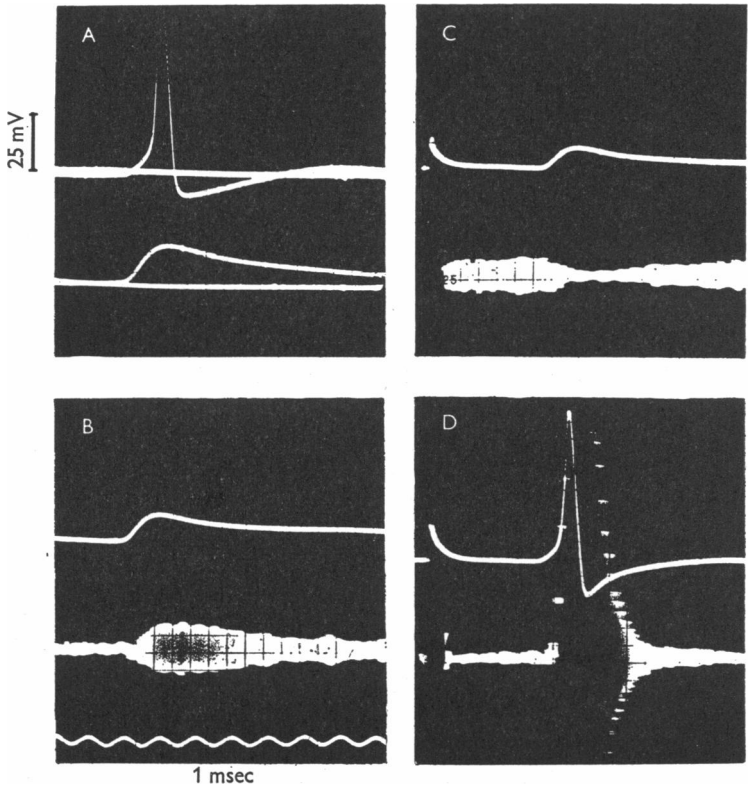

Fig. 9. Record A, top: a response of the post-synaptic axon to presynaptic stimulation, recorded in the synaptic region; bottom: response recorded during a continuous hyperpolarization (of approximately 50 mV) of the post-synaptic axon. Record B: simultaneous recording of the synaptic potential and the change in the membrane impedance. Record C: similar to record B, except that the best bridge balance was obtained at the peak of the synaptic potential. Record D: similar to record B, but taken immediately after withdrawal of the hyperpolarizing current; the time course of the action potential in record D is distorted by the impedance bridge. The arrangement of Fig. 2 was used.

Effect of d.c. polarization of post-synaptic axon upon the amplitude of synaptic potential

Confirming the observation by Bullock & Hagiwara (1957), hyperpolarization of the post-synaptic axon was found to increase the amplitude of the synaptic potential. The arrangement of the electrode used in our experiment

is shown in Fig. 10, top. The potential of the post-synaptic axon was recorded with a metal wire electrode, and the polarizing current was delivered to the post-synaptic axon through a glass pipette electrode. In the example shown in Fig. 10, brief stimulating pulses were applied to the presynaptic axon approximately 1 msec after the start of the polarizing current.

With a weak polarizing current, the time interval from the start of the synaptic potential to the beginning of the spike potential was prolonged. At levels of hyperpolarization strong enough to block synaptic transmission, the amplitude of the synaptic potential (measured as the potential level attained by hyperpolarization) was found to increase with increasing shift in the membrane potential. A weak depolarization of the post-synaptic axon had an opposite effect upon the amplitude of the synaptic potential. A constant outward current through the post-synaptic axon membrane could block transmission because of the reduction in the amplitude of the synaptic potential.

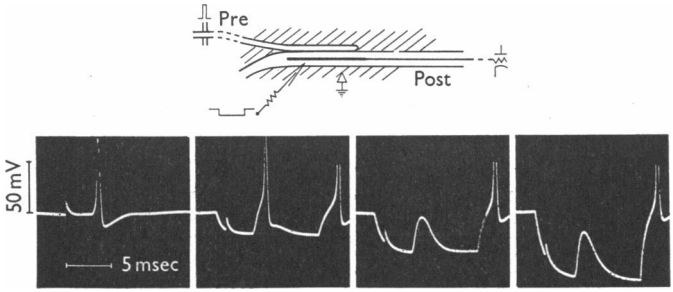

Fig. 10. Effect of hyperpolarizing current pulse upon the synaptic potential. Stimulating shocks were delivered to the presynaptic axon and the polarizing current pulses to the post-synaptic axon.

Fig. 11 shows the relationship between the change in the membrane potential brought about by the polarizing currents and the amplitude of the synaptic potential. When the frequency of stimulation of the presynaptic axon was 1/sec, as in previous experiments, a hyperpolarization of about 30 mV was required to block synaptic transmission in this preparation. As the polarizing current was increased above this blocking level, the amplitude of the synaptic potential, measured from the steady potential level of the hyperpolarized membrane, was found to increase linearly with the membrane potential (see straight line *A* in Fig. 11). When the frequency of stimulation of the pre-synaptic axon was increased, transmission block occurred at a lower intensity of polarizing current. The change in the amplitude of the synaptic potential caused by hyperpolarization was smaller under such circumstances (straight lines *B* and *C*).

When the linear relationship between the amplitude of the synaptic potential and the membrane potential was extrapolated, it was found that, at

approximately 60 mV above the resting potential, the amplitude of the synaptic potential should reach zero. Since the resting potential of the post-synaptic axon is around 60 mV below the potential of the surrounding sea water, the finding mentioned above indicates that the (hypothetical) membrane potential at which the synaptic potential vanishes is close to zero. This is in close agreement with the result of similar observations in the neuromuscular junction by Fatt & Katz (1951). They explained their experimental finding by assuming that the membrane became permeable non-specifically to all kinds of ions when the transmitter substance was released from the ending of the motor nerve fibre. This explanation of Fatt & Katz appears to be the simplest explanation of our findings on the squid synapse. It seems to us, however, that this is not the only possible explanation of the experimental results.

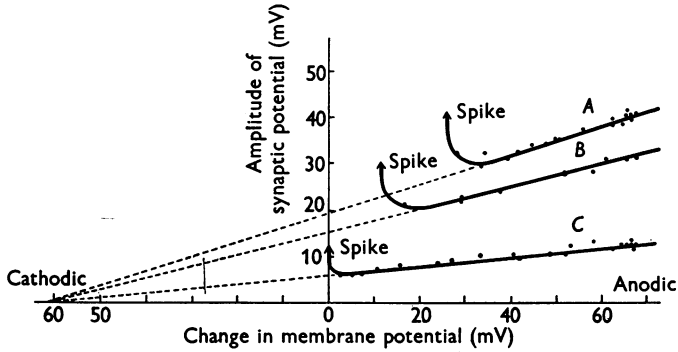

Fig. 11. Relationship between the amount of hyperpolarization of the post-synaptic axon and the amplitude of the synaptic potential. The observed points along straight line *A* were obtained when the rate of repetition of the presynaptic stimulation was 1/sec. In *B* the shocking rate was 1·5/sec; in C it was 10/sec. All the results were taken from one preparation.

Relationship between size of presynaptic spike potential and amplitude of synaptic potential

As is well known, the amplitude of the action potential recorded at the site of stimulation varies to some extent, depending upon the intensity of stimulus (see the upper trace in Fig. 12). It is also possible to reduce the spike amplitude by preliminary cathodal polarization of the axon. Anodal polarization does not alter the potential at the peak of a spike appreciably in normal axons; but in poor axons the spike amplitude can be increased to a considerable extent by anodal polarization. When the amplitude of the presynaptic axon spike was modified by these electric means, it was found that a slight change in the spike amplitude was effective in modifying the amplitude of the synaptic potential by an appreciable amount.

The records in Fig. 12 were obtained with two micro-electrodes inserted into the presynaptic axons and one metal wire electrode in the post-synaptic axon

(as in the experiment of Fig. 4). All these electrodes were within about 0·5 mm from the region of the synaptic contact of the two axons. One of the two micro-electrodes was used to deliver a pulse of constant (inward or outward) current to the presynaptic axon; the other micro-electrode was used to record potential variations in the presynaptic axon. When the stimulating shock delivered to the presynaptic axon was well above the threshold value, the synaptic potential (recorded with the metal wire electrode) was large enough to initiate a propagated impulse in the post-synaptic axon (record C). When the stimulating shock was barely above the threshold, however, the spike amplitude in the presynaptic axon was slightly smaller, and the synaptic potential induced failed to initiate a propagated impulse (record B). Small potential variations that could be regarded as subthreshold responses in the presynaptic axon never produced any measurable potential variation in the post-synaptic axon.

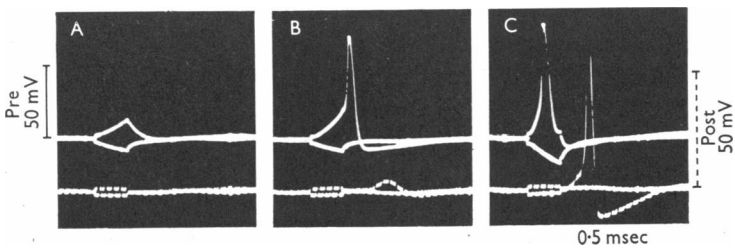

Fig. 12. The effect of direct stimulation of the presynaptic axon in the vicinity of the synapse upon the membrane potential of the post-synaptic axon. The arrangement of Fig. 4, top, was used. The lower trace showing the potential recorded from the post-synaptic axon is contaminated by stimulus artifacts.

In Fig. 13 is shown the relationship between the amplitude of the presynaptic spike potential and the amplitude of the synaptic potential. The arrangement of the electrodes used was similar to that used in the experiment of Fig. 4, top, except that the stimulating shock was delivered to the presynaptic axon near its tied end. By preliminary tetanization, the synaptic potential was reduced to a subthreshold level. Then the amplitude of the presynaptic axon spike was varied by changing the time interval between the start of the polarizing current of 20 msec in duration and the arrival of the impulse in the presynaptic axon, as well as the intensity and the polarity of the polarizing current.

It is seen in Fig. 13 that a slight change in the amplitude of the presynaptic spike brings about a large change in the amplitude of the synaptic potential. In this particular preparation, a 30% reduction of the presynaptic spike by preliminary cathodal polarization almost completely eliminated the synaptic potential. The significance of this finding will be considered under Discussion.

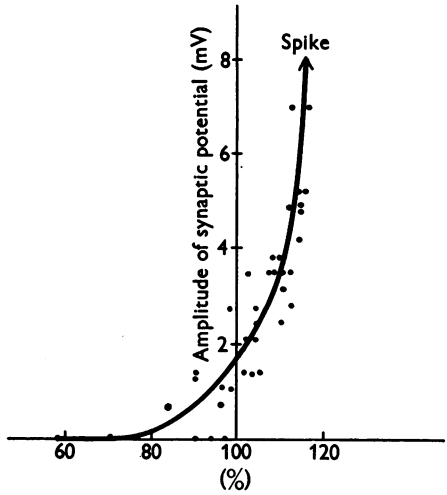

Fig. 13. The relationship between the amplitude of the presynaptic spike potential and the size of the induced synaptic potential. The presynaptic spike was altered by preliminary electric polarization.

DISCUSSION

One of our primary objectives in the present investigation on the squid giant synapse was to find out whether there is any possibility of explaining the mechanism of impulse transmission across the squid giant synapse in terms of the electrical theory. After we finished the major portion of the present studies in the summer of 1956, we were informed of the result of a recent investigation on a crayfish synapse by Furshpan & Potter (1957). According to these investigators, the membrane between the pre- and post-synaptic axon is capable of passing electric current more readily in one direction than in the reversed direction. Because of this strong rectifying action of the membrane between the two axons, the action potential of the presynaptic axon can stimulate the post-synaptic axon electrically but a response of the post-synaptic axon cannot stimulate the presynaptic axon. This explains the mechanism of uni-directional transmission across the crayfish synapse on the basis of the electrical theory.

The electrophysiological properties of the squid synapse appear to be very different from those of the crayfish synapse. It appears to us impossible to explain our results in terms of the straight-forward electrical theory.

Recently Robertson (1953) compared the ultrastructure of the squid synapse with that of the crayfish synapse (between the median giant fibres and the third motor giant fibre of the abdominal segmental ganglia). According to this electron microscopic study, the structural difference between the crayfish synapse and the squid synapse appears to be very small. This situation leads

us to a question: Why do these two structurally similar synapses behave functionally differently? At present, we do not have the answer to this question.

The difficulty of explaining our experimental results in terms of the electrical theory can be summarized in the following manner: If the property of unidirectional transmission across the squid synapse be explained on the electrical basis, the relationship between the synaptic potential and the amplitude of the presynaptic spike potential (presented in Fig. 13) would have to be interpreted as representing the rectifying property of the synaptic membrane. Then, a strong hyperpolarization of the post-synaptic axon should bring about a detectable potential variation in the presynaptic axon; but this has not been observed (Fig. 5). The reversed synaptic potential observed in the 'space' between the pre- and post-synaptic axons (Fig. 6) cannot be explained as a spread of current from the presynaptic axon. Finally, the synaptic delay, which is approximately 0·4 msec or slightly longer at 21° C (Fig. 6), is prolonged by cooling the preparation (1–2 msec at 9° C); it is somewhat difficult, if not impossible, to explain this fact in terms of the simplest version of the electrical theory.

It may be pointed out in this connexion that it is possible to modify (and to complicate) the electrical theory and to make it reconcilable with our experimental findings. We may assume, for instance, that the synaptic potential is produced by the response localized at the enlarged surface membrane at the top of the protoplasmic processes (Fig. 8*B*) and also this localized response is caused by a small amount of current spreading electronically from the presynaptic axon. If we make an additional assumption that the necks of the processes, which are 1–10 μ in diameter and 2–3 μ long (Robertson, personal communication), bring about an appreciable attenuation of the potential along its course, then it may not be impossible to reconcile our observation with the electrical theory. It appears to us, however, that the assumption of a strong attenuation along the neck of these short processes is somewhat unlikely (see also Fig. 14 in Young's article); in addition, our observation on the effect of polarization of the post-synaptic axon upon the amplitude of the synaptic potential (Fig. 10) conflicts with this assumption.

On the basis of the chemical theory of synaptic transmission, the dependence of the amplitude of the synaptic potential upon the magnitude of the presynaptic spike potential (Fig. 13) should be interpreted as indicating the dependence of the amount of the secreted transmitter substance upon the magnitude of depolarization of the presynaptic axon. A similar dependence of the synaptic activity upon the presynaptic membrane potential has been demonstrated by del Castillo & Katz (1954) and Liley (1956) for the vertebrate neuromuscular junction. We are not certain at present whether the action of the 'transmitter substance' is localized at the top of the protoplasmic processes (Fig. 8*B*) or the substance has to difluse through the 'space' between the two axons. It is interesting to note in this connexion that the observed synaptic delay (as well as the time course of the synaptic potential) can be accounted for as the time required for diffusion of the 'transmitter' through the

'space' between the two axons. This does not prove, however, the validity of the assumption made in solving the equation of diffusion for this problem.

SUMMARY

1. Electrophysiological properties of the axo-axonal synapse in the stellate ganglion of the squid were investigated by using the multiple intracellular electrode technique, the impedance bridge method and the voltage-clamp technique.

2. By delivering strong pulses of in- or outward membrane currents to the pre- or post-synaptic axon, it was shown that there was no detectable spread of electric current across the synapse. It was therefore impossible to interpret the synaptic potential as a result of direct electrotonic spread of the pre-synaptic potential across the synapse.

3. By piercing the synapse with a recording micro-electrode, a 'space' was demonstrated in which there was no resting potential. Synaptic potentials of a reversed sign were observed in this space. This space was identified as the layer of loose connective tissue sheath through which processes of post-synaptic axoplasm extended toward the surface of the presynaptic axon.

4. By clamping the membrane potential of the post-synaptic axon at a constant level during synaptic activation, an inward membrane current was demonstrated in the synaptic region of the post-synaptic axon, which was responsible for generation of the synaptic potential. It was possible to reconstruct the synaptic potential from the time course of this synaptic current.

5. With a metal wire electrode inserted in the post-synaptic axon, it was possible to detect a small decrease in the membrane impedance associated with production of a synaptic potential.

6. An investigation of the effect of d.c. polarization of the post-synaptic axon upon the amplitude of the synaptic potential supports the view that the 'transmitter substance' tends to bring the inside potential to the level of the surrounding medium.

7. A slight variation in the amplitude of the presynaptic spike potential was found to alter appreciably the amplitude of the synaptic potential. Possible interpretations of this finding were discussed.

We wish to express our gratitude to Dr M. Fuortes and Professor B. Katz for their valuable advice and criticism concerning the interpretation of the results presented in this paper. We are also indebted to Dr J. D. Robertson who gave us important information as to the structure of the squid synapse. The work presented in this article was carried out at the Marine Biological Laboratory, Woods Hole, Massachusetts.

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