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TETRODOTOXIN-RESISTANT ELECTRIC ACTIVITY IN PRESYNAPTIC TERMINALS

BY B. KATZ AND R. MILEDI

From the Department of Biophysics, University College London and the Stazione Zoologica, Naples

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SUMMARY

1. The electric properties of the giant synapse in the stellate ganglion of the squid have been further investigated.

2. During tetrodotoxin (TTX) paralysis, a local response can be elicited from the terminal parts of the presynaptic axons after intracellular injection of tetraethyl ammonium ions (TEA).

3. The response is characterized by an action potential of variable size and duration, whose fall is often preceded by a prolonged plateau. The response, especially the duration of the plateau, is subject to 'fatigue' during repetitive stimulation.

4. The TTX-resistant form of activity is localized in the region of the synaptic contacts, and shows a marked electrotonic decrement even within less than 1 mm from the synapse. It is found only on the afferent, not on the efferent, side of the synapse.

5. During the plateau of the response, the membrane resistance is greatly reduced below its resting value.

6. The response depends on presence of external calcium and increases in size and duration with the calcium concentration. Strontium and barium substitute effectively for calcium. Manganese and, to a lesser extent, magnesium, counteract calcium and reduce the response. The response also declines, and ultimately disappears, if sodium is withdrawn for long periods.

7. The relation of the local TTX-resistant response to the influx of calcium ions and to the release of the synaptic transmitter is discussed.

INTRODUCTION

It has been shown that abolition of nerve impulses by TTX does not prevent the release of the transmitter from nerve endings in response to locally applied depolarization (Katz & Miledi, 1965, 1966, 1967*a*, *b*, *e*;

Bloedel, Gage, Llinás & Quastel, 1966; Kusano, Livengood & Werman, 1967). There is increasing evidence that depolarization leads to transmitter release via inward movement of calcium ions, and that the opening up of 'calcium gates' at specific sites of release is not prevented by TTX (Katz & Miledi, 1967*c*, *e*).

Whether the local influx of calcium is strong enough to reinforce the depolarization has been a matter of conjecture. In general, after TTX dosage, the relation between applied current strength and resulting presynaptic depolarization (as well as the ensuing post-synaptic response) is continuously graded and shows no sign of a regenerative effect. But there is an interesting exception: at the frog nerve–muscle junction, addition of a few mM-TEA completely alters the character of the response (Katz & Miledi, 1967*b*). One now obtains an explosive type of release which appears abruptly above a certain current strength and results in a very large end-plate potential. In a subsequent paper it will be shown that this triggered form of release occurs even when all the external sodium has been replaced by calcium (cf. Katz & Miledi, 1967*d*).

A possible explanation of this finding is that calcium inward current in the presynaptic terminals can reinforce the applied depolarization and become regenerative, provided the counter-current of potassium has been sufficiently reduced by TEA (see Armstrong & Binstock, 1965). To examine this possibility further, similar experiments were made on the synaptic terminals in the stellate ganglion of the squid which are large enough to permit direct measurements with intracellular electrodes. Clear evidence has been obtained for a TTX-resistant local regenerative response, confined to the presynaptic terminals and observed whenever TEA was applied internally and the external calcium concentration was high. The ionic mechanism of the response remains uncertain, but there are several indications that calcium current is involved.

METHODS

The experiments were made during the summers of 1967 and 1968 on the stellate ganglion of the squid *Loligo vulgaris*. In most cases the 'distal' giant synapse (Young, 1939) formed by an axonic contact between the second-order giant fibre and the largest motor axon (in the hindmost stellar nerve) was used, though occasionally the 'accessory' presynaptic fibre, or a motor axon in one of the short stellar nerves was chosen. The preparation consisted either of a small piece of mantle muscle containing the ganglion, or of the isolated ganglion with its in- and outgoing nerves. The procedure of dissecting and mounting the preparation in a cooled chamber has been described in detail (Miledi, 1967; Katz & Miledi, 1967*e*). The temperature of the bath (approx. 20 ml.) was usually about 11° C (varying in different experiments between 7 and 14° C). Oxygenated solutions flowed continuously through the preparation chamber.

Once intracellular electrodes had been inserted in the synaptic region, solution changes were made only slowly, so as to reduce the risk of damage and dislocation of the electrodes.

In a dye dilution test it was found that, with the usual rates of flow, it took approximately 10 min for 50 %, 30 min for 75 %, 1 hour for 90 %, and 2 hours for 99 % change of the bath solution. The alternative method, of removing and reinserting electrodes each time the bath was emptied and changed, could not be used routinely because of the inherent technical difficulties and the time usually required for placing electrodes into the presynaptic terminal. It was necessary, however, to do this in some experiments in which the effects of sodium deficiency, and especially of replacing sodium by calcium, were studied.

Tetrodotoxin (Sankyo) was used in most experiments, in concentrations of 10^{-7}–$1{\cdot}1 \times 10^{-6}$. TEA was injected iontophoretically by a method previously described (Katz & Miledi, 1967*e*). During the present experiments the current pulses used to inject TEA were often of higher intensity than in the previous work. Usually, pulses of about 150 msec duration at a frequency of 5/sec were applied for periods of 10–60 min, the intensity being adjusted so as to limit the depolarization to about 20 mV. At times, the injecting pulses were strong enough to give rise to noticeable post-synaptic responses. This procedure caused the effect of TEA on the presynaptic terminals to appear much more quickly, but it was apt to produce severe deterioration in the post-synaptic response, apparently leading to a drastic, and sometimes complete, failure of transmitter release. We believe that this damaging effect arose not from the injection of TEA as such, but from the frequent application of iontophoretic (depolarizing) pulses which exceeded the intensity required for synaptic transfer. It was found that, in the presence of TEA, frequent repetition of strong pulses produced a prolonged refractoriness of the release mechanism (cf. Fig. 18 below).

As regards the exact positioning of the presynaptic 'current' and 'voltage' electrodes, the precautions described in the previous paper (Katz & Miledi, 1967*e*) were used; that is, the voltage-recording electrode was placed as close as possible to the beginning of the synaptic contact region, and the current-passing (usually TEA containing) pipette a fraction of a millimetre 'upstream'. This is important, for, as pointed out previously (Katz & Miledi, 1967*e*), reversal of this position gives spuriously low values of the presynaptic voltage change for a given post-synaptic response.

RESULTS

The effect of presynaptic TEA injection

Figures 1 and 2*A*, *B* recapitulate the results described in a previous paper (Katz & Miledi, 1967*e*; see also Kusano *et al.* 1967). The preparation had been treated with a paralytic dose of TTX. The top trace in each recording shows the depolarizing current pulse applied to the presynaptic terminal, the middle trace shows the resulting presynaptic potential change, and the bottom trace the post-synaptic response. The calcium concentration ranged between 11 mM (Fig. 1) and 5·5 mM (Fig. 2*A*, *B*). Figure 2*A* shows the normal behaviour, before application of TEA: the presynaptic membrane resistance falls during the depolarizing pulse so that it is impossible to produce a maintained voltage change of high amplitude. After intracellular injection of TEA (Figs. 1, 2*B*) the picture is changed: large presynaptic potential changes can now be maintained for the duration of the current pulse, and the phenomena of suppression of transmitter release and its postponement until the end of the pulse are shown up (Figs. 1*c*; 2*B*, lower part; cf. Katz & Miledi, 1967*e*).

A local electric response in the pre-axon

The time course of the presynaptic potentials has some unusual features which are noticeable in Fig. 2*B* and quite marked in Fig. 1, namely an inflexion on the rising phase and a more or less prolonged hump during the decline. Similar changes had already been briefly commented on in the earlier paper (Katz & Miledi, 1967*e*, p. 423). With normal or low calcium concentrations, these features were very variable in extent, but they

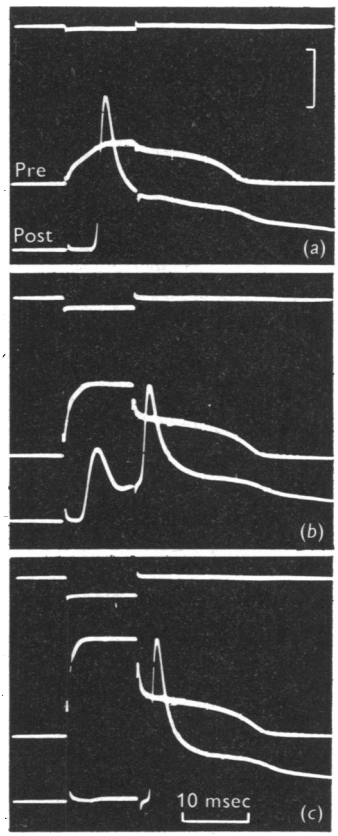

Fig. 1. Pre- and post-synaptic potentials after TEA injection. *a*, *b*, *c*: with three different current intensities applied to the pre-fibre. Calcium concentration 11 mM. In each block, top trace shows current pulse, middle trace presynaptic, bottom trace post-synaptic potential. The vertical scale represents 3·35 μA (top), 100 mV (pre), 20 mV (post). Note: 'upward swing' and delayed fall (hump) of presynaptic potential, and 'suppression' of post-synaptic potential during strong current pulse (partial suppression in *b*, nearly complete suppression in *c*). All records in this and following figures were obtained by intracellular recording from TTX-treated stellate ganglia. Position of current electrode at start of synapse; presynaptic recording electrode 0·3 mm down, within synaptic region.

always became striking when the calcium level in the bath was raised to about 50 mM or more. This is shown, for instance, in Fig. 2*C* (see also Figs. 3, 5, 10). Soon after the perfusion fluid was changed to a calcium rich solution, the brief depolarizing pulse evoked a prolonged electric potential change in the presynaptic terminal, accompanied by a greatly increased post-synaptic response. This was a very regular effect, reproducible in all experiments provided (i) a sufficient dose of TEA had been

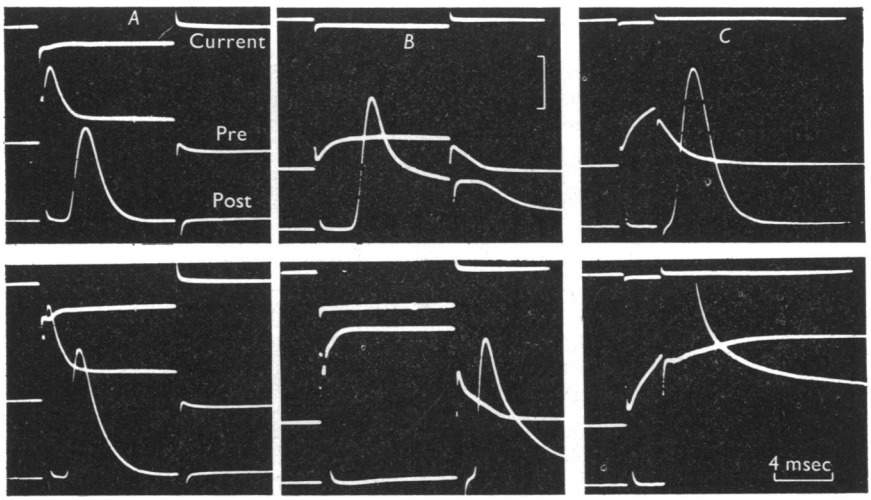

Fig. 2. Effects of TEA and of extra calcium, on pre- and post-synaptic potentials. *A*: 5·5 mM-Ca, no TEA. *B*: 5·5 mM-Ca, after some presynaptic TEA injection. *C*: Ca concentration in bath was raised towards 70 mM and more TEA injected between upper and lower blocks (same current pulse in both cases). In *A* and *B*, different current pulses were used in upper and lower blocks. In each block, the three traces show (from above downwards): current pulse, pre-, and post-synaptic potential. Vertical scale represents: 1·68 μA in *A*, 3·35 μA in *B* and *C*; presynaptic: 40 mV in *A* and *C*, 100 mV in *B*; post-synaptic: 20 mV in *A* and *B*, 10 mV in *C*. Position of presynaptic recording electrode at start of synapse; current electrode 0·28 mm upstream.

injected into the pre-axon (cf. Fig. 5), (ii) the external calcium concentration was moderately high, and (iii) the axon had not been badly damaged or subjected to excessive stimulation. Raising the TTX concentration to $1{\cdot}1 \times 10^{-6}$ g/l. had little or no effect, even after 2 hr continued perfusion. It appears therefore that under these specific conditions the terminal part of the presynaptic axon gives a local electric response which is highly TTX-resistant, at least compared to the conducted axon spike.

Other features of this presynaptic potential are illustrated in Figs. 3 and 4. In Fig. 3*A* two superimposed oscillograph traces are shown during which identical current pulses were applied. The resulting potential changes diverge, in the characteristic way of a regenerative system at

'threshold'. In Fig. 3*B*, lower trace, the duration of a presynaptic 'response' is shown on a slow time base. Incidentally, this was the longest potential obtained at normal (11 mM) calcium concentration. Figure 4*A* shows the progressive shortening of the presynaptic response during a series of pulses repeated at intervals of 2·5 sec. As with other action potentials of similar shape (e.g. in the heart or crustacean muscle), the

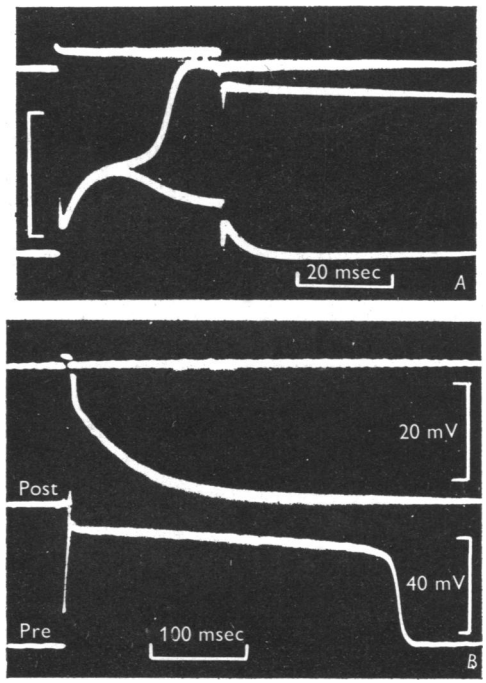

Fig. 3. *A*: 'threshold' of local response. After TEA treatment. 45 mM-Ca. Two successive superimposed traces showing current pulses (upper) and presynaptic potentials (lower trace). Vertical scale represents 1·14 μA and 50 mV. Resting potential 61 mV. *B*: from another synapse (11 mM-Ca), showing on slow time base: current pulse (top), post-synaptic (middle, transient response) and presynaptic potential (bottom, prolonged response). Resting potential (pre): 57 mV. Upper vertical scale represents also 1·83 μA.

response terminates rapidly when the potential has declined to a certain level. The plateau can be cut off much earlier by interposing a hyperpolarizing pulse as shown, for instance, in Fig. 4*B*.

For a further study of this electric response, it was important to devise some convenient way of measuring it. This presents a problem for a potential change which varies with the strength and duration of the applied current. However, with sufficient doses of internal TEA and external calcium, two features of the response become relatively independent of the applied pulse: these are (i) the duration T of the response

measured from the end of the pulse to the point of maximum rate of fall (Fig. 6), (ii) the height of the 'plateau' attained several milliseconds after the end of the pulse (i.e. the depolarization V_a, or the membrane potential V_b, Fig. 6). In any one experiment, the duration T can be made to alter by at least two orders of magnitude; it was therefore sometimes more convenient to use its logarithm (see e.g. Fig. 19 below).

In Table 1, these quantities (V_a, V_b and T) are listed for a series of experiments at different calcium concentrations. It will be seen that the

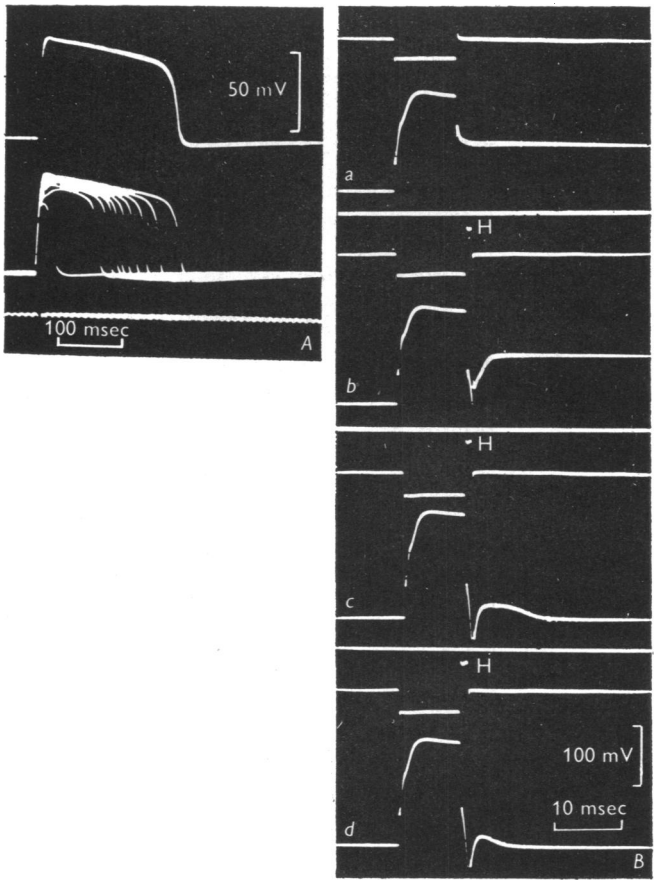

Fig. 4. *A*: effect of pulse repetition, at 2·5 sec intervals. 50 mM-Ca. Upper record shows single, 'unfatigued' presynaptic response to a brief pulse (indicated by the gap in the horizontal bottom line). Middle record shows progressive shortening of response during ten successive pulses. *B*: effect of a brief hyperpolarizing pulse in 'cutting off' presynaptic response. 70 mM-Ca. *a*: response to depolarizing pulse alone. *b* to *d*: hyperpolarizing pulse (H) added at the end of the depolarizing pulse. The duration of the short H pulse was increased from *b* to *d*. In *c* and *d* the H pulse stopped the response, in *b* it just failed to do so. Voltage scale also represents 3·35 μA.

level of the plateau rises (from −24 to +3 mV), and the duration T lengthens (on the average, from 20 to 470 msec), as the external calcium concentration is increased about tenfold (from 5·5 to 44–70 mM).

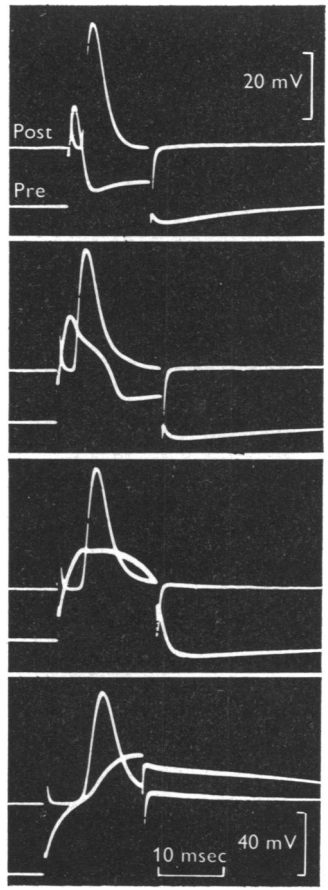

Fig. 5. Effect of progressive TEA injection into pre-axon. Each block shows post-synaptic (top trace) and presynaptic (bottom trace) potentials. 50 mM-Ca throughout. TEA was applied between successive records (from above downwards) in cumulative doses. Vertical scales refer to 'post' (top) and 'pre' (bottom) respectively. Note: appearance of local response, first only during the pulse, later outlasting the pulse.

The voltage/current relation in the presynaptic terminal

Further information can be obtained by plotting the voltage/current relation of the pre-fibre, under different experimental conditions. In the absence of TEA, the slope of the V/I curve always diminishes with increasing polarization, whether one measures the peak voltage (e.g. Katz & Miledi, 1967*e*, fig. 3; see also Fig. 20 below) or the final voltage produced

by the current pulse (Figs. 7, 9). After TEA injection the V/I relation of the pre-fibre usually shows an 'upward swing', i.e. an increasing slope over a certain range (Figs. 7, 9). This was observed even at normal or reduced calcium concentrations; for instance, in the case of Fig. 8. It may be noted that the upward swing of the V/I curve occurs in the same range of membrane potentials as the inflexions and humps (Figs. 1, 2*B*) in the time course of the potential change.

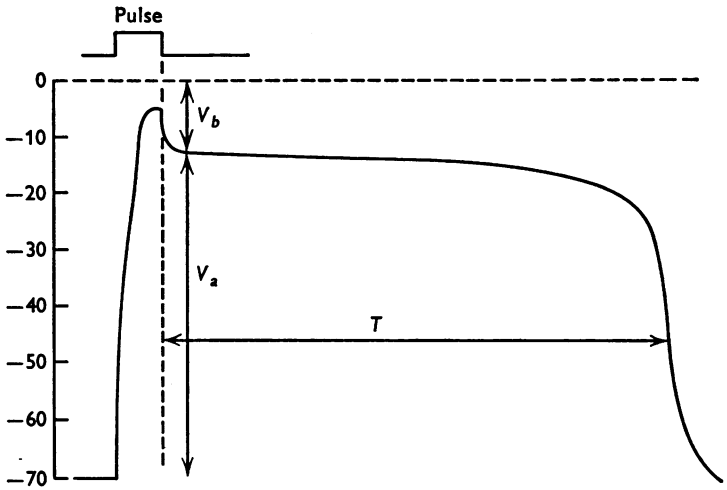

Fig. 6. Diagram illustrating measurements of V_a, V_b and T. Pulse shown at the top. Dashed horizontal line indicates zero membrane potential. Vertical scale: membrane potential, in mV.

When we first saw these changes, it was difficult to decide whether they were attributable to a regenerative inward current or to an 'anomalous' type of rectification, i.e. to a lowering of potassium conductance, and thus reduced outward current, over a certain range of depolarization. The upswing of the V/I curves, the inflexion during the rise and the hump during the fall of the depolarization could all be explained by either of these alternative mechanisms. Later, we observed the much more pronounced changes in calcium-rich solutions, which suggested that a regenerative process is involved.

Loss of membrane resistance during the plateau

What finally settled this issue was a measurement of the membrane resistance during the plateau. If the long persistence of the depolarization were due to a lowering of potassium permeability, then the membrane resistance during this period should be higher than in the resting state. If, on the contrary, the resistance during the early period of the plateau

is lower than at rest, then this explanation fails, and it becomes more probable that the response arises from inward current of external cations.

That the membrane resistance at the beginning of the plateau is low was already indicated by the high rate, and greatly reduced time constant,

TABLE 1. Effect of calcium concentration on local response. V_a = depolarization measured as indicated in Fig. 6. V_b = membrane potential corresponding to V_a. T = duration of response (see Fig. 6). *Distance*, in μ, between presynaptic recording site and start of distal synapse (zero distance means at start, or within, the synaptic region)

Calcium concentration ...			*A*: 5·5 mM		*B*: 11 mM		*B* (mean)
Distance (μ)			350		100	250	(175)
V_a (mV)			46		54·5	46	(50)
V_b (mV)			−24		−13	−11	(−12)
Calcium concentration *C*: high (44–70 mM)							
Distance (μ) ...	50	50	350	0	(80)*	550*	
V_a (mV)	63	79	68	73	56	52	
V_b (mV)	−5	+14	−2	+3	−5	−4	
							C (mean)
Distance (μ) ...	240	100	0	400	0	50	(155)
V_a (mV)	68	64	70	72	71	63	(66·5)
V_b (mV)	+8	−4	+9	+10	+10	+3	(+3)

Duration T: 5·5 mM-Ca: mean 20 msec (0–68 msec; six observations); 11 mM-Ca: mean 93 msec (25–325 msec; six observations); high Ca: mean 470 (55–1900 msec; 13 observations).

* Omitting accessory axon (bracket) and 550 μ position: mean V_a, 70 mV; mean V_b, + 4·6 mV.

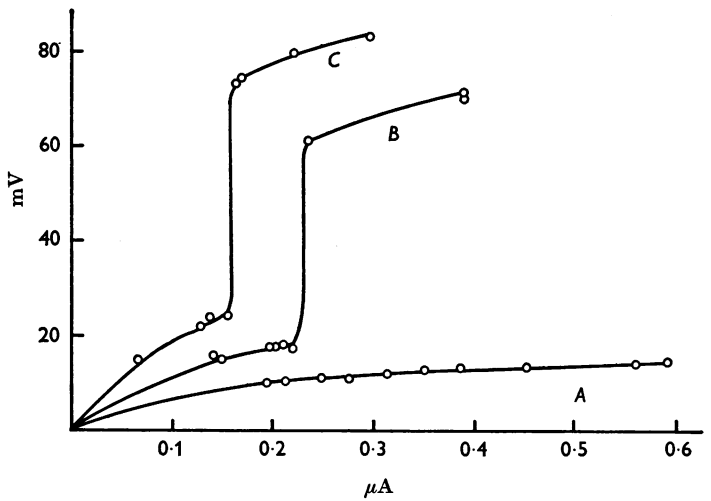

Fig. 7. Relation between steady voltage and current in presynaptic terminal. Pulse duration: 12–15 msec. Approx. 50 mM-Ca throughout. *A*, before TEA injection; *B* and *C*, after two successive doses of TEA, showing the 'jump' from 'subthreshold' to 'superthreshold' levels of depolarization. Position of recording electrode just within synaptic region; current (TEA) electrode 0·2 mm upstream.

with which the membrane potential *falls* to the plateau at the end of a strong pulse (see Figs. 1, 10). This was confirmed more directly by the method of applying repetitive test pulses throughout the period of recording. Figure 11*A* shows the long-maintained response following a brief depolarizing stimulus. In Fig. 11*B* brief hyperpolarizing test pulses are

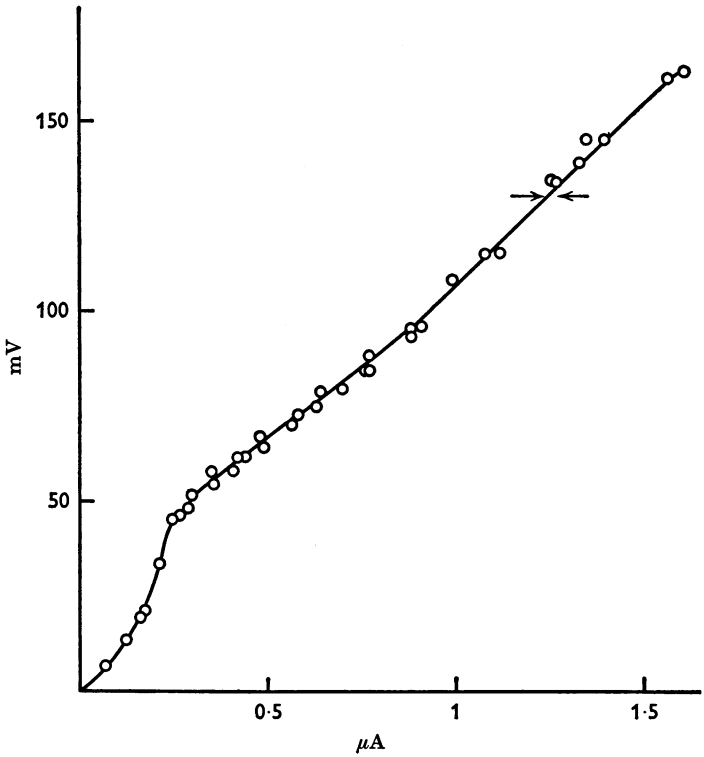

Fig. 8. Voltage/current relation, after TEA treatment in 11 mM-Ca. Pulse duration 18 msec. Arrows show the 'post-synaptic suppression' potential. The resting potential in this terminal was only 52·2 mV. Position of recording electrode 75 μ upstream from synapse; current (TEA) electrode 0·45 mm upstream.

added. From the change in amplitude and time course of the modulating potentials, it is clear that the membrane resistance drops to a low value at the beginning of the plateau and gradually recovers during its later part. Two points require comment: (i) the added test potentials, being in the hyperpolarizing direction, cause the response to be terminated earlier than in its 'unmodulated' form; (ii) the first two or three test pulses following the depolarizing stimulus are reduced in strength. This is an artifact arising from a transient resistance change in the current-passing pipette, and must be allowed for if one compares the amplitudes of the first few modulating potentials. It does not, of course, affect their time course.

In Fig. 11*C* simultaneous recordings were made at the synaptic contact region (upper) and 0·625 mm 'upstream' (lower), while the current pulses were applied at a point 1·175 mm upstream from the synapse. The differences between recordings at the two distances will be discussed in the following section.

No accurate estimates could be made from these experiments, but the change in time constant indicates that the loss of membrane resistance

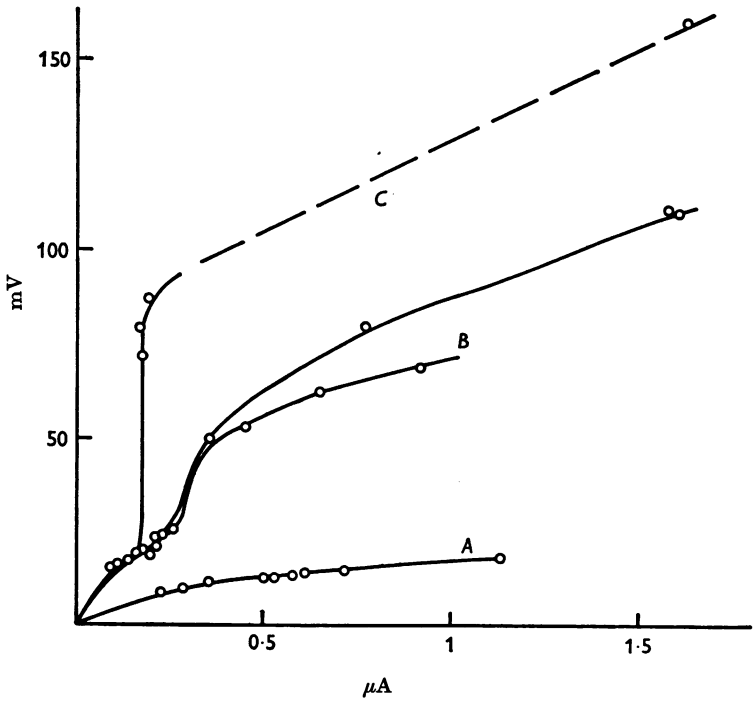

Fig. 9. Effect of calcium concentration on voltage/current relation. *A*: 11 mM-Ca, before TEA injection; *B*, 11 mM-Ca, after TEA injection. The lower curve was obtained first, the upper curve later. *C*: after raising calcium concentration to 70 mM. Ordinate: steady potential change produced by 10 msec current pulse. Abscissa: current intensity. Position of current (TEA) electrode just within synaptic region; recording electrode 0·1 mm further down.

amounted to 80 % or possibly more. This is probably made up in part by increased permeability to external cations whose influx produces the maintained depolarization, and of a residual increase of potassium conductance which was not completely obviated by the TEA injection.

Localization of the TTX-resistant responses

While the TTX-resistant local response could be obtained regularly from the terminals of presynaptic 'distal' as well as 'accessory' axons, nothing of the kind was ever observed in the post-axon. It may be argued that the larger size and lower input resistance of the post-synaptic fibre make it necessary to inject a greater amount of TEA before the response

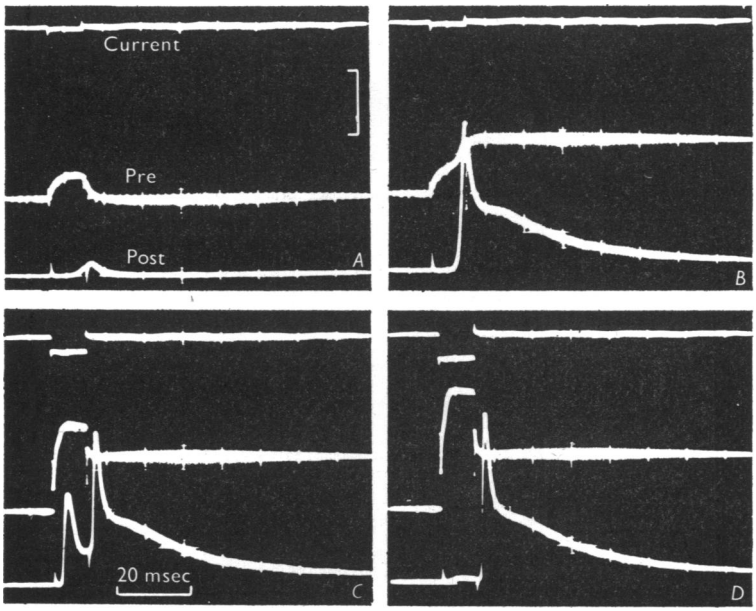

Fig. 10. Pre- and post-synaptic responses in high calcium (70 mM) and after TEA dosage. In each block: current pulse (top), presynaptic (middle), and post-synaptic (bottom) potentials. Four different pulse intensities showing presynaptic 'upswing' from threshold level (in *B*), and rapid fall to final level (in *C* and *D*); also post-synaptic response during pulse (*A*, *B*), partial (*C*) and nearly complete (*D*) suppression. Vertical scale represents 3·35 μA, 100 mV (pre) and 20 mV (post).

could become detectable. However, TEA injection periods were made long enough to show up a clear effect on the delayed rectification of the post-fibre. Also, experiments were made on some of the motor axons which run in the smaller stellar nerves (see Miledi, 1967). These axons have an input resistance comparable to that of the presynaptic terminals, yet in spite of prolonged TEA injection no trace of a TTX-resistant response was obtained.

Experiments were then made to find out whether the local response can be initiated anywhere in the pre-fibre, or whether it is confined to the region of the synaptic terminals. To examine this point, three intracellular

electrodes were placed into the presynaptic axon at different distances from the giant synapse, the TEA pipette being farthest away.

Results of such experiments are illustrated in Figs. 12–14. Figure 12 was obtained at low calcium concentration (5·5 mM). In *A* and *B*, recording was at the start of the synapse (*a*) and at 625 μ upstream (*b*), while the stimulus was applied through the TEA pipette, 1175 μ from the site of the

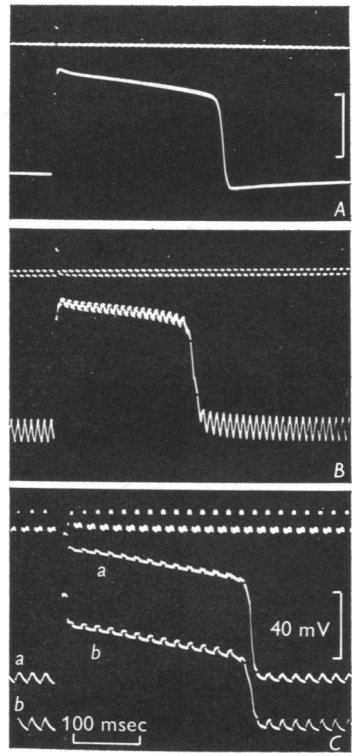

Fig. 11. Membrane resistance during plateau. *A* and *B*, 55 mM-Ca. Record *A* shows 'unmodulated' response to brief pulse (monitored in top trace). Record *B* shows response with hyperpolarizing pulse modulation. *C*: from another preparation. 45 mM-Ca. Simultaneous recording at start of synaptic contact (*a*), and 625 μ 'upstream' (*b*). The pulse pipette was farther upstream (1175 μ from *a*). The upper vertical scale applies to all records except *C* (*a*), and represents 1·76 μA and 40 mV. The lower scale (40 mV) refers to record *C* (*a*).

synapse. In *A*, the pulse was just 'below threshold'. The difference between the two records reflects mainly the ordinary electrotonic attenuation, from *b* to *a*. In *B*, a local response develops and outlasts the pulse. The response is clearly larger at *a* than at *b*, i.e. the attenuation is now in the reverse direction. In *C*, the connexions of the stimulating and the

lower recording electrode were interchanged,i.e. the upper record is obtained as before, at the synapse, while the lower record is now 1175 μ (instead of 625 μ) away. The response is attenuated much more severely, again in the upstream direction.

Similar results, with higher calcium concentrations, are shown in Figs. 13 and 14. In every case the response measured after the end of the current pulse is largest at the synaptic contact, and reduced in size as the recording is moved upstream. Examination of the modulating potentials

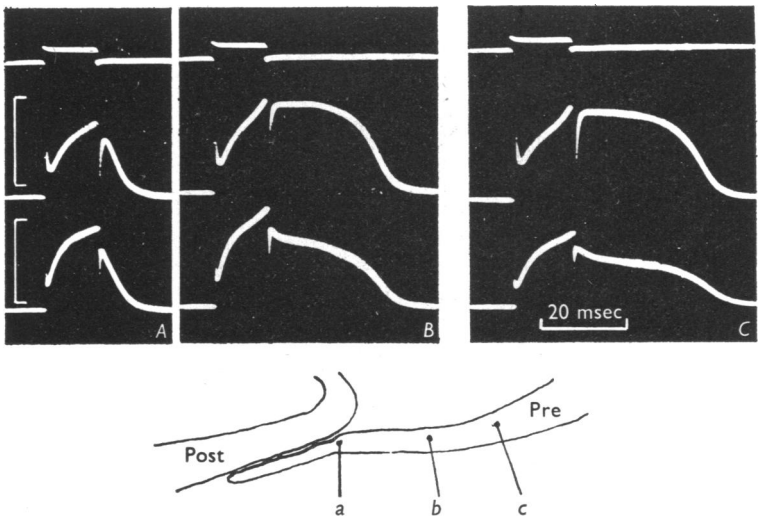

Fig. 12. Simultaneous recordings at different distances from synapse. 5·5 mM-Ca. Top trace in each block shows current pulse. Pulse in *A* is just 'subthreshold'; in *B* and *C* 'above threshold'. Middle trace: recorded at start of synapse (position *a* in diagram). Bottom trace: in *A* and *B*, at 625 μ from synapse (position *b*); in *C*, at 1175 μ from synapse (position *c*). Upper scale represents 1·83 μA (top) and 40 mV (middle trace). Lower scale: 40 mV (bottom trace).

in Fig. 11*C* shows that the reduction in membrane time constant is more severe at the synapse than at the 625 μ position upstream, in spite of the fact that the pulses were applied even further upstream. This indicates that the lowering of membrane resistance is most pronounced at the synaptic contact region.

The superimposed tracings in Fig. 14 illustrate even more clearly the electrotonic decrement of a 'subthreshold' depolarization, from *b* to *a*, and the even more severe *reverse* decrement of the 'superthreshold' response, from *a* to *b*. It seems possible that the active process may be confined entirely to the synaptic region of the terminals, and that the upstream decrement from *a* to *b* is purely passive. It would, however, be difficult to exclude the alternative possibility, namely that some active reinforcement

occurs all along the terminal branches, but with a very steep spatial gradient and a high peak of activity in the synapse itself.

Fractionation of the local response

The pre-axon divides into 8–11 terminal branches (Young, 1939), each of which forms a synaptic contact with the main motor axon of the corresponding stellar nerve. Presumably a local response is elicited at each of these synaptic endings, though the largest terminals (in the last stellar

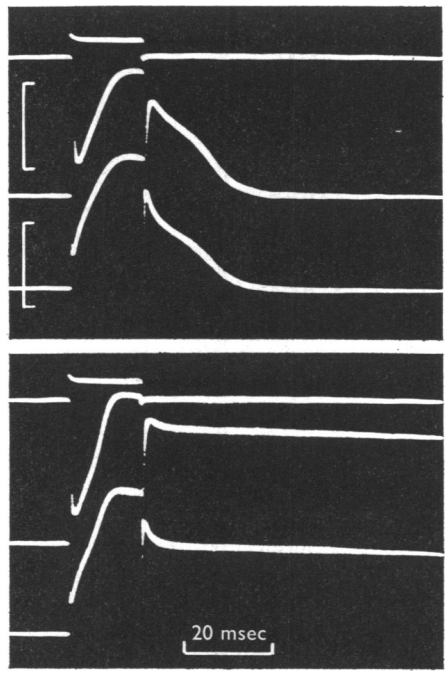

Fig. 13. Effect of calcium on TEA-treated terminal. The bath concentration was changed from 5·5 mM-Ca (top recording) towards 45 mM. The lower block was obtained several minutes after changing the perfusion fluid to higher calcium. Simultaneous recording at start of synapse (middle trace in each block) and 625 μ upstream (lower trace). Upper scale represents 1·83 μA and 40 mV (synaptic recording); lower scale 40 mV (upstream recording).

nerves) would make the chief contribution and probably act as pacemaker to the others. Normally, therefore, with the recording electrode placed near the largest synapse, one would expect to obtain a relatively simple wave form of the response dominated by the activity of the nearby 'giant terminal'. Frequently, however, there were indications of a step in the final decline of the potential, suggesting that the response was not cut off simultaneously in all the terminals. Furthermore, during deterioration the response was found in some experiments to break up into two or three

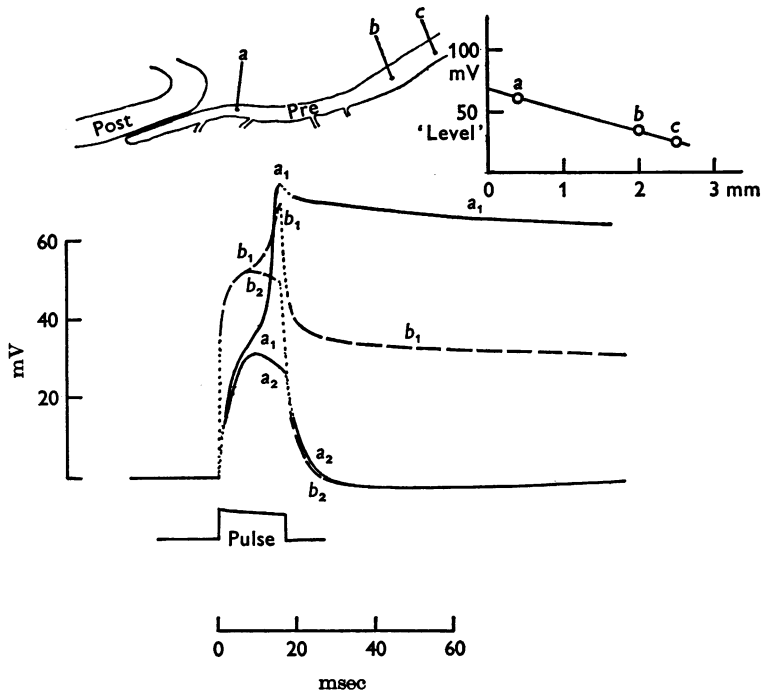

Fig. 14. Superimposed tracings of records obtained at position *a* (0·4 mm from synapse) and *b* (2 mm from synapse). Two pulses, one just below and one above 'threshold', were applied through the TEA pipette, at position *c*. The sub-threshold potentials show a decrement from *b* to *a*, the level of the response shows opposite decrement, from *a* to *b*. Diagram at top left shows positions of intracellular presynaptic electrodes. Inset diagram at top right shows the decrement of response levels, recorded at *a*, *b* and *c*.

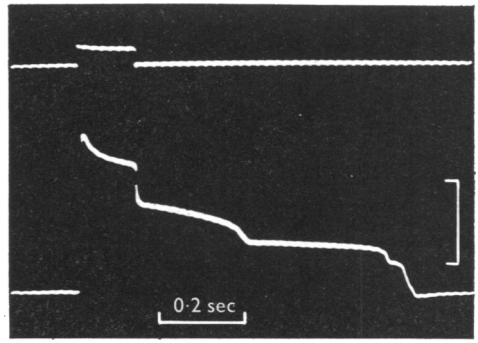

Fig. 15. Fractionation of response in deteriorating axon terminal. Local response to a large pulse is shown. It has several discrete 'steps' on its falling phase. 50 mM-Ca. Resting potential 60 mV. Vertical scale represents 0·88 μA and 40 mV.

steps of different duration and amplitude. An extreme example of this fractionation is shown in Fig. 15. The explanation is probably that the response deteriorated first at the large terminal nearest the electrode, and its duration there became shorter than at the more distant, smaller terminal branches. The fact that the response breaks into discrete fragments provides further evidence for the conclusion that the activity originates, or at least is concentrated, in discrete synaptic contact areas.

Presynaptic response and transmitter release

The findings up to this point may be summarized by saying that a regenerative reinforcement of membrane depolarization, resistant to TTX, is found to be localized in the terminal region of the presynaptic axon, i.e. in the area in which transmitter release occurs.

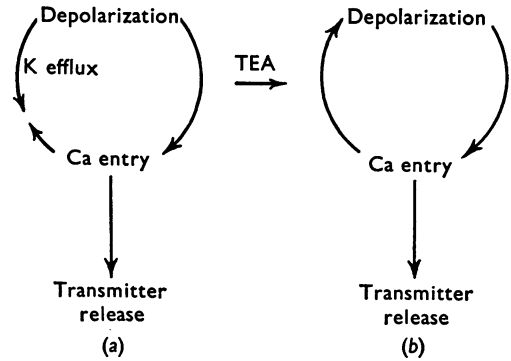

Fig. 16. Diagram illustrating 'calcium hypothesis'. This applies to the presynaptic terminal treated with TTX.

It is natural to look for a link between these two processes, and the most obvious suggestion is the one made at the outset, namely that both, electric reinforcement and transmitter release, depend on influx of calcium which is turned on by the primary depolarization.

The hypothesis, presented diagrammatically in Fig. 16, is that the regenerative effect of calcium entry is normally opposed by potassium efflux (*a*), and that TEA, by reducing potassium flux, removes this blockage (*b*). In either situation, with or without TEA, the rate of calcium entry is controlled by the membrane potential, and the calcium influx in turn controls the rate of transmitter release.

Regeneration and input/output relation. On this hypothesis the transmitter release is reinforced by the regenerative cycle, but only as a side effect, via the reinforced depolarization. Taking the scheme in its bare outline, there would be no reason why the 'input/output' relation (i.e. the relation between presynaptic potential change and post-synaptic response) should be altered by TEA and its regenerative effect.

In actual fact, however, as pointed out earlier (Katz & Miledi, 1967*b*, *e*; 1968), the transmitter output depends not only on the amplitude of the recorded presynaptic potential, but also on its time course and on the electrotonic decrement between the sites of recording and of transmitter release. Both features are altered by TEA, and it is therefore not surprising that the input/output relation becomes steeper after TEA injection (Fig. 17). This was seen even with small doses of TEA which were not sufficient to produce a clear regenerative potential change. In the case of

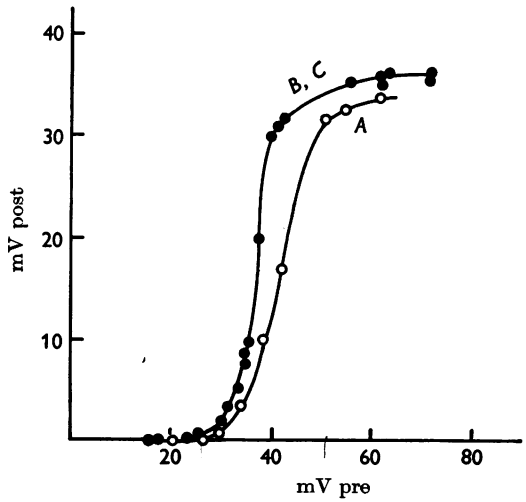

Fig. 17. Effect of TEA on 'input/output' relation of synapse. 50 mM-Ca throughout. *A*, before TEA injection; *B* and *C*, after two and three successive doses of TEA. Pulse duration 12–15 msec. Ordinate: peak amplitude of post-synaptic response. Abscissa: peak amplitude of presynaptic potential. Presynaptic recording electrode just within synaptic region.

Fig. 17, adding more TEA caused no further increase in the slope of the input/output curve, and eventually a decline in the post-synaptic response supervened.

Another important point arises from the results showing the localized nature of the response. Suppose a transient regenerative reinforcement occurs, confined to the distal part of a single terminal. This would be recorded upstream only in greatly attenuated form, but it could lead to a large increase in transmitter release and so produce a steep increment in the observed input/output curve.

In calcium-rich solutions, when a large regenerative response had developed, its relation to transmitter release showed a number of divergent as well as parallel features. In general the 'threshold depolarization' for a local regenerative response was higher than that for transmitter release,

so that post-synaptic potentials of small to moderate size could be evoked without presynaptic regeneration, or preceding the latter in time. As the preparation deteriorated, however, the situation sometimes reversed, and it then appeared that a post-synaptic potential could only be elicited when the threshold for presynaptic reinforcement was exceeded.

There was a large difference between pre- and post-synaptic responses in the rate of 'fatigue' or 'inactivation'. This shows up in two ways: (i) the post-synaptic potential usually died out long before the plateau of

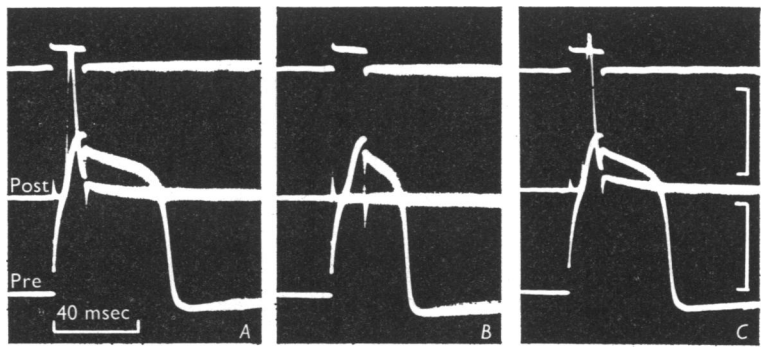

Fig. 18. 'Fatigue' of synaptic transfer. 50 mM-Ca. The three blocks, from left to right, show responses to identical pulses. *A*, rested preparation; *B*, pulse repeated 1·2 sec after *A*; *C*, after 5 min rest. In each block: current pulse (top); post-synaptic response (middle); presynaptic response (bottom). Upper vertical scale: 0·92 μA and 20 mV (post); lower scale: 40 mV (pre).

the presynaptic response came to an end; (ii) when pulses were repeated at intervals of a few seconds, the presynaptic response shortened in duration (Figs. 4, 18), but the post-synaptic potential was reduced much more drastically, and at times was practically abolished (Fig. 18). The exact site of this striking inactivation cannot at present be ascertained, but very probably it occurs on the presynaptic side and involves a failure of transmitter release. In the terms of our hypothesis, one might suggest that this inactivation is a secondary effect resulting from internal accumulation of calcium during prolonged regenerative current flow.

That the 'inactivation' of the synaptic transfer process (Fig. 18) is not simply due to *post-synaptic* changes (e.g. desensitization) is indicated by the fact that large responses can be repeated at much shorter intervals provided the presynaptic depolarizations are brief pulses (see figs. 17 and 18 in Katz & Miledi, 1967*e*) and not prolonged as in Fig. 18. On the other hand, the synaptic 'fatigue' cannot be attributed to the long-lasting presynaptic potential change *by itself*, for if one raises the potential to the suppression level (and thereby presumably prevents calcium influx) this level can be maintained for several seconds without greatly diminishing the large off-response which occurs at the end of the applied current (unpublished observations). These findings support the suggestion that the presynaptic inactivation probably results from prolonged influx and accumulation of calcium on the inside of the membrane.

Effects of ionic changes

In order to obtain more evidence on the mechanism of the TTX-resistant response, various changes in the ionic environment were made, and their effects on the level ($V_{a,b}$) and duration (T) of the regenerative potential were determined.

Calcium. The dependence of the presynaptic reinforcement on the external calcium concentration has already been described and is summarized in Table 1. No great accuracy can be claimed for the values of the levels V_a and V_b, as these are affected to some extent by attenuation (over the small distances stated in Table 1) and by other variables such as the internal TEA concentration. Nevertheless, in a qualitative way, these results support the calcium hypothesis. It should be noted that the values of V_b do not represent calcium equilibrium potentials, but levels at which calcium (or other cation) influx is balanced by potassium efflux plus, possibly, chloride influx (see Discussion). The calcium equilibrium level is at a much higher, inside-positive, potential, and on our hypothesis could be estimated by determining the potential at which transmitter release is completely suppressed (see Katz & Miledi, 1967*e*).

Strontium and barium. Both strontium ions and barium ions were effective substitutes for calcium ions and enabled large and prolonged regenerative responses to be produced in the presynaptic terminal. When 50 mM-Sr was added to a calcium-free solution, a response of 0·7 sec duration was obtained; in another experiment, with 80 mM-Sr, the value of T reached 2 sec. With 50 mM-Ba an even larger response, of 6·5 sec duration, was recorded. Strontium and barium are also able, though much less effectively, to substitute for calcium in the release of the transmitter. This aspect will be dealt with in a later paper.

Magnesium and manganese. Both magnesium ions and manganese ions were found to act as calcium antagonists and caused the response to be shortened appreciably. The effect of magnesium was comparatively weak, that of manganese quite strong. The concentration of magnesium in the normal bath was 54 mM; raising it to 151 mM, in the presence of 11 mM-Ca, caused T to drop from 124 to 4 msec. In this experiment the recovery (on returning to 54 mM-Mg) was very slow and incomplete: after 75 min T had increased to only 7 msec; perfusion was then switched to a magnesium-free solution which caused T to rise to 100 msec. In another experiment, at half the normal calcium (5·5 mM) and normal magnesium (54 mM), T was 3·3 msec, and increased to 9·6 msec when a magnesium-free solution was substituted.

In two experiments an equimolar concentration of manganese ions was added to a calcium-rich solution (both at 40 or 50 mM). This caused the

duration of the regenerative response to drop, reversibly, to a small fraction (from 430 to 19 msec in one, and from 110 to 2·3 msec in the other experiment). Manganese also caused a marked reduction in the synaptic output which will be described elsewhere.

To summarize, the effects of the divalent ions resemble the actions which have been reported for other calcium-dependent processes, in particular the action potential of crustacean muscle (Fatt & Ginsborg, 1958; Hagiwara & Nakajima, 1966) in which strontium and barium are strong synergists, manganese is a strong antagonist, and magnesium has been described as 'inert'.

Sodium withdrawal. The calcium hypothesis (Fig. 16) would be greatly strengthened if it could be shown that no other external cation is required, and that the regenerative response still occurs after complete withdrawal of external sodium. A number of experiments were made in which sodium was replaced by either sucrose, or choline or calcium ions.

In one experiment, illustrated in Fig. 19, the isolated ganglion was immersed in a solution whose sodium and magnesium contents had been entirely replaced by calcium. Intracellular electrodes were then inserted. Following the injection of TEA a large local response, of 75 msec duration, was obtained as late as 90 min after the change of the bath, but thereafter the presynaptic response failed. The post-synaptic response had disappeared earlier, within about 1 hr.

In another experiment the preparation was kept in a solution 95% of whose sodium content had been replaced by choline. In this case a local response of about 10 msec duration could still be recorded after $2\frac{1}{2}$ hours, but then it gradually declined.

In other experiments the intracellular electrodes were introduced in the normal ionic medium, and the effects of changing the perfusion fluid to a sodium-deficient solution were followed. Again, the presynaptic response persisted or even increased in size during the first hour, but it did not survive indefinitely and disappeared after 1–2 hours exposure to the sodium-free solution (see e.g. Fig. 20). The effect always came on slowly, and reversibility after return to high sodium was also slow and usually incomplete. In one case the calcium concentration was raised from 2 to 40 mM, 65 min after the bath had been perfused with a solution containing choline instead of sodium. As a result, a local response began to appear in the pre-fibre within 30 min and built up to over 150 msec in duration during the next 40 min, but declined thereafter.

The interpretation of these results is made difficult in view of the slow clearance of extracellular spaces in the stellate ganglion (see Miledi & Slater, 1966; Katz & Miledi, 1967*e*). Nevertheless, the delayed abolition and the delayed recovery cannot *both* be attributed to slow extracellular

equilibration: for if the delayed extinction were due simply to the long time it takes for the interstitial sodium to fall below a certain level, then this level would be exceeded very quickly and recovery should be rapid on readmission of the high-sodium bath. It is more likely that sodium deprivation has a slow protracted action on the nerve terminal, possibly

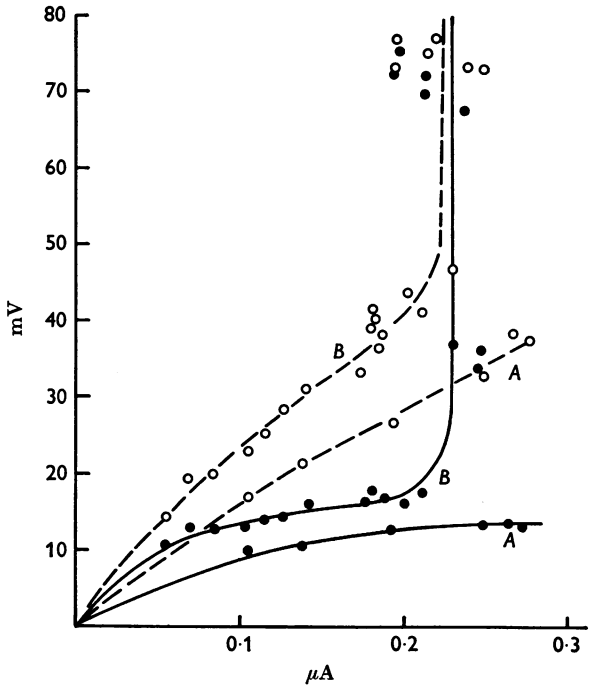

Fig. 19. Voltage/current relation, after 90 min in sodium-free isotonic calcium solution. Filled circles and continuous lines: final potentials (pulse durations 17 msec in *A*, 32 msec in *B*). Open circles and dashed lines: peak potentials. *A*, before TEA; *B*, after TEA injection. Ordinate: presynaptic potential change, mV. Abscissa: current intensity.

(see Blaustein & Hodgkin, 1968) by blocking calcium extrusion and allowing gradual accumulation of calcium to occur inside the terminals.

To summarize, while presynaptic responses could be obtained for a certain length of time in sodium-free solutions, the present experiments on sodium withdrawal complicate the issue, and make the acceptance of the calcium hypothesis more difficult. In view of recent work on the interaction between sodium and calcium fluxes across the axon membrane (Baker, Blaustein, Hodgkin & Steinhardt, 1967; Blaustein & Hodgkin, 1968), one may envisage ways of resolving the difficulties, but further evidence will be needed to test these possibilities.

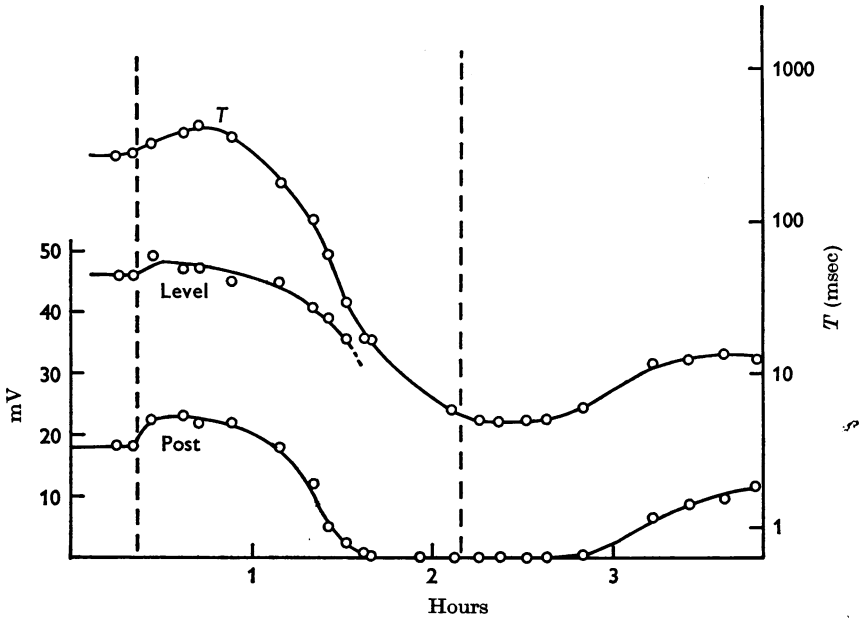

Fig. 20. Effect of sodium withdrawal on duration T (top), level V_a (middle) and post-synaptic response (bottom curve). Ordinate: left scale, mV; right scale, msec (log. scale). Abscissa: time in hours. 11 mM-Ca. TEA injected. At first dashed vertical line, perfusion was switched from sodium to choline. At second dashed line, perfusion returned to sodium.

DISCUSSION

The observations described in this paper raise a number of interesting problems.

A local electric response has been found which is apparently restricted to the terminal parts of the presynaptic axon, under conditions of complete TTX paralysis of the main parts of the afferent and efferent neurones. The response may, of course, not be totally resistant to TTX, but the doses used were 100 times larger than what is needed to block axonic propagation. Apart from TTX-resistance, the effects and interactions of various divalent cations (Ca, Sr, Ba, Mg, Mn) suggest that the situation is analogous to the electric activity of crustacean muscle fibres in which calcium (or Sr, Ba) inward current appears to be the dominant factor (Fatt & Ginsborg, 1958; Hagiwara & Nakajima, 1966). The situation differs from crustacean muscle in that presence of sodium is needed, at least for long-term maintenance of the presynaptic response.

In the squid axon, Hodgkin & Keynes (1957) observed a small influx of calcium (2–3 % of that of sodium) during periods of impulse activity, and it has been suggested that this may be the basis of the 'calcium action

potential' which Watanabe, Tasaki, Singer & Lerman (1967) obtained from the giant axon (after internal perfusion with caesium fluoride and exposure to a 200 mM-$CaCl_2$ solution). This response differs from, and is probably not related to, the one described in the present paper, for it is readily blocked by TTX (Watanabe *et al.* 1967), besides the fact that it occurs in the post-synaptic axon.

A phenomenon somewhat similar to that described here has been observed at the frog nerve–muscle junction (Katz & Miledi, 1967*b*, p. 35, and unpublished results). After TTX-paralysis, treatment with TEA enables one to elicit an 'all-or-none' type of post-synaptic response to a brief pulse applied to the terminal part of the motor axon. This response still occurs after the preparation has been kept for some hours in a sodium-free, isotonic $CaCl_2$ solution. Eventually, however, the response declines irreversibly. There is no doubt that in this case the response survives long beyond the time needed for extracellular equilibration and sodium clearance. The ultimate failure must therefore be attributed to some other change, possibly gradual accumulation of calcium inside the terminals. We have suggested that the refractoriness of transmitter release which follows a large presynaptic response (Fig. 18) may be due to intracellular calcium accumulation, and that recovery depends on elimination and extrusion of calcium from the terminal. There is evidence (Blaustein & Hodgkin, 1968) that calcium extrusion is coupled to sodium influx and becomes greatly reduced when the external sodium concentration is lowered. It is conceivable therefore that the delayed inactivation of the presynaptic response which occurs in a sodium-deficient medium is the result of slow, progressive accumulation of calcium inside the terminal.

An alternative explanation for a somewhat similar case has recently been proposed by Banks, Biggins, Bishop, Christian & Currie (1969). Their argument is based on the fact that the rate of calcium influx through the axon membrane depends on the *intracellular* sodium concentration (Baker *et al.* 1967). In a sodium-free medium there would be a gradual loss of sodium from the cell, which might in turn lead to delayed inactivation of a process which depends on calcium entry.

Thus, while the results can be said to remain compatible with the calcium hypothesis, the description of the roles which sodium and calcium play in the production of the presynaptic response remains uncertain. We shall continue the discussion on the assumption that calcium carries the inward current, alone or as a principal partner, while sodium is required for longer-term maintenance of the process.

The next question to consider is the action of TEA. Without application of TEA no trace of a local response was seen in the TTX paralysed axon; all that was seen was the ordinary 'delayed rectification'. This does not

mean that calcium inward current is completely absent, but merely that the effect of calcium influx on the membrane potential is overwhelmed by efflux of potassium. It has been suggested (Beaulieu & Frank, 1967*a*, *b*) that, at the vertebrate nerve–muscle junction, TEA has a specific action facilitating movement of calcium ions through the membrane. This is indeed a possibility which might be used to explain many of our findings, but it does not seem to be a necessary assumption, for the well-established action of TEA in blocking potassium current appears to be sufficient to account for our results.

To take some simplified quantities, let us suppose the level V_b during the local response (Fig. 6) is at zero, and equidistant from the equilibrium potentials E_K and E_{Ca} (approximately, say, $-0{\cdot}1$ V and $+0{\cdot}1$ V respectively). Further, take the potassium conductance g_K of the TEA-treated membrane as 1 mmho/cm^2. Then, g_{Ca} during the response is also 1 mmho/cm^2, and the inward current of calcium I_{Ca} is approximately 0·1 mA/cm^2, balancing a similar outward current of potassium. It will be appreciated that this is small compared to the sodium inward current during the normal action potential, or to the potassium outward current in the absence of TEA. It follows, therefore, that the same intensity of calcium current would have little effect on the shape of the action potential (in the absence of both TEA and TTX); moreover, at the normal calcium concentration (11 mM), I_{Ca} would be lower.

In Fig. 21, an attempt has been made to reconstruct schematically the V/I (steady voltage/current) relation for the TEA-treated axon terminal. The underlying assumptions are shown in the inset. They include some obvious oversimplifications, namely (i) the calcium channel is switched on and off in all-or-none manner; (ii) the resistances in the two parallel channels are fixed, and there is no residual rectification; (iii) arbitrary quantities have been chosen for E_K, E_{Ca} and the corresponding resistances. A single 'resting' and two 'active states', for different calcium concentrations have been drawn. Points of interest are (*a*) the intercepts between 'active' slopes and vertical axis which correspond to the levels V attained after the end of the applied pulse, (*b*) the intersections between 'resting' and 'active' slopes which correspond to E_{Ca}.

E_{Ca} should, *ex hypothesi*, be estimated from the 'suppression potential' (see p. 479, also Katz & Miledi, 1967*e*). This varied between approximately +70 and +140 mV in different experiments. Attempts were made to measure this level during changes of the calcium concentration, but the results were disappointing, partly because the suppression potential is a difficult 'end-point' to determine and mainly because it was not easy to maintain sufficient stability during the long experiment. In two out of four experiments, there was a significant increase in the suppression potential with increased calcium concentration; in the others no clear change was observed.

The question now arises whether the calcium current, of 10^{-4} A/cm^2, which is presumed to underly the regenerative response, can be equated to the influx of calcium at the actual transmitter release sites. We may use the information available at the frog nerve-muscle junction to help us in forming a rough estimate. A single, large terminal arborization in frog muscle has a presynaptic surface of approximately $2{\cdot}5 \times 10^{-5}$ cm^2 from which about 250 quantal packets of transmitter are released, after arrival

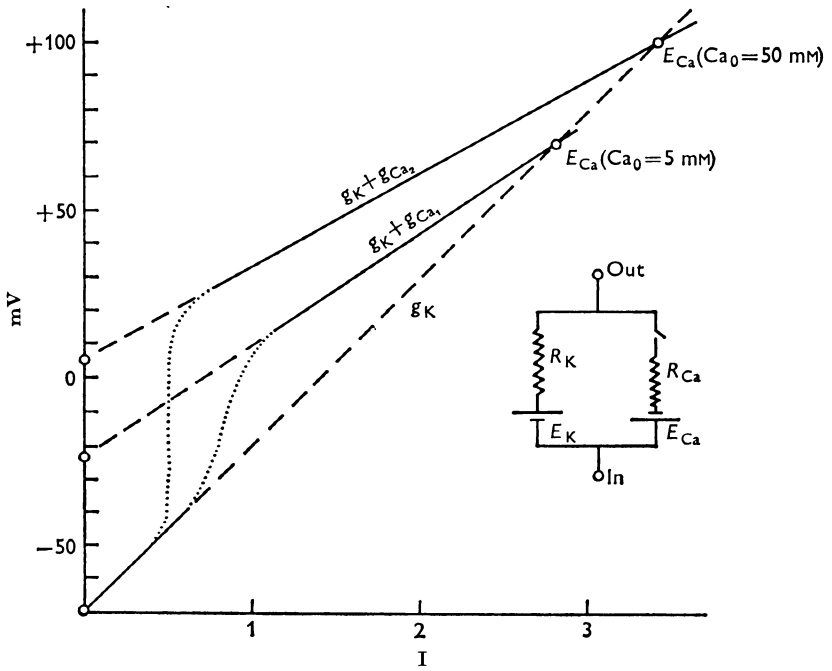

Fig. 21. Theoretical reconstruction of voltage/current relation, making assumptions illustrated in inset diagram. The three slopes represent, successively from below: resting state (g_K); active state with 5 mM-Ca ($g_K+g_{Ca_1}$); active state with 50 mM-Ca ($g_K+g_{Ca_2}$). $g_K = 1/R_K$; $g_{Ca} = 1/R_{Ca}$. The significance of the various intersections (marked by circles) is discussed in the text. Ordinate: steady presynaptic membrane potential in mV. Absciss: current intensity in relative units. The following values have been chosen: $E_K = -70$ mV, $E_{Ca} = +70$ mV (5 mM-Ca); $+100$ mV (50 mM-Ca). $R_{Ca_1} = 2R_K$ (5 mM-Ca); $R_{Ca_2} = 1{\cdot}25\ R_K$ (50 mM-Ca).

of a nerve impulse, within a period of approximately 1 msec. This gives a surface density of transmitter release of about 10^7 packets/cm^2, which may be assumed to apply also to the squid giant synapse (cf. Miledi, 1967, p. 403). If each quantal event requires the simultaneous action of four calcium ions (Dodge & Rahamimoff, 1967), then in our present example 1000 calcium ions can be assumed to have passed through $2{\cdot}5 \times 10^{-5}$ cm^2 of presynaptic membrane during 1 msec. This is equivalent to an inward

current of only $1{\cdot}3 \times 10^{-8}$A/cm^2, i.e. 0·01 % of the intensity required for the regenerative presynaptic potential.

It appears that the membrane current associated with the entry of those calcium ions which are *directly* involved in the process of release is immeasurably small. Nevertheless, this very small current may be part of the same process which leads to the reinforcement of the presynaptic potential change. The above calculation merely suggests that, for any one calcium ion which enters the terminal, the probability of it contributing to transmitter release is very low. There are many reasons which could account for this: one possibility arises from the suggestion that simultaneous action of several calcium ions is needed for transmitter release at any point (Dodge & Rahamimoff, 1967; Hubbard, Jones & Landau, 1968). Suppose, in order to be effective, four ions must penetrate a single calcium channel almost synchronously, within a period which is, say, one-tenth of the mean interval between *single* random penetrations. In this case, the expected ratio between single ion entries (which contribute to the electric reinforcement, but not to release) and quadruple entries (which are effective in transmitter release) would be about 10,000:1. Another possibility is that only a very small fraction of the membrane area which is available for calcium entry has the special properties required for the subsequent steps in the release mechanism.

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