The effect of repetitive stimulation on the passive electrical properties of the presynaptic terminal of the squid giant synapse

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(Communicated by E. J. Denton, F.R.S. – Received 9 November 1978 – Revised 4 June 1979)

The resting electrical properties of the presynaptic terminal of the squid giant synapse have been determined by using constant current pulses. After short periods of repetitive stimulation, the terminal resistance, time constant and capacitance are found to be increased. These changes are absent in terminals bathed in artificial sea water containing no calcium, and sea water containing 5 mM cobalt. It seems likely that these changes are associated with transmitter release.

INTRODUCTION

The release of transmitter substances from nerve terminals is usually accompanied by structural changes: a reduction in the number of synaptic vesicles and an increase in the area of the presynaptic membrane (Heuser & Reese 1973; Model et al. 1975; Ceccarelli et al. 1972, 1973; Pysh & Wiley 1972, 1974). The present experiments were done to determine the passive electrical properties of the presynaptic terminal of the squid giant synapse, and to look for changes during stimulation that might be associated with transmitter release. Two microelectrodes were inserted into the presynaptic terminal and constant current pulses used to stimulate the synapse and to determine the terminal resistance, time constant and capacitance. A brief account of this work has already been given (Gillespie 1978).

METHODS

All of the experiments were done on the distal or ‘giant’ synapse in the stellate ganglion of adult squid of the species Alloteuthis subulata (Lamarck, 1798) (mantle lengths 8–15 cm).

The dissection procedure was essentially that described by Miledi (1967) for the removal of the stellate ganglion along with a small piece of mantle. In suitable preparations, presynaptic terminals 500 μm long and 40 μm in diameter could be visualized clearly by using a combination of oblique and transmitted light. To
avoid contraction of the remaining mantle when the synapse was activated, the
giant motor axons were crushed individually 1–2 mm away from the ganglion.
The dissection was done in oxygenated sea water at a temperature between
20 and 22 °C.

The preparation was mounted in a perspex bath (volume 1 ml) and the bathing
solution changed continuously at a rate of 1–5 ml/min. In most experiments the
bathing solution was filtered sea water. Solutions containing cobalt chloride and
lanthanum chloride were made by adding the appropriate amount of crystalline
salt to filtered sea water. Zero calcium artificial sea water had the composition
470 mM NaCl, 66 mM MgCl₂ and 5 mM Tris, made isotonic to sea water with sucrose
(pH 7.7). All solutions were bubbled with 100 % oxygen since it has been reported
that this synapse fails rapidly if deprived of O₂ (Bryant 1958). The experiments
were done at room temperature (20–22 °C).

Recording microelectrodes were filled with 3 M KCl and had resistances between
15 and 25 MΩ and tip potentials less than 5 mV. Current passing electrodes were
filled with 1.8 M potassium citrate and had resistances between 3 and 8 MΩ.
Membrane potentials were measured by using a Grass preamplifier (Model P 18)
with negative capacity compensation. The total current injected was measured
as the voltage drop across a 4.7 kΩ resistor between the bath and ground.

The recording and current passing electrodes were put into the presynaptic
terminal in the region where it makes contact with the last stellar nerve (see inset
of figure 1). The current electrode was also used to stimulate. Invariably the current
electrode was placed distal to the last branch of the presynaptic axon and the
recording electrode placed distal to the current electrode.

Results

The effect of stimulation on the terminal capacity and resistance

The potential change recorded in a non-stimulated presynaptic terminal during
a hyperpolarizing current pulse is shown in figure 1a. Because of uncertainties
about the distribution of current within the terminal, the exact structure of the
terminal and its dimensions, it is not possible to calculate specific electrical con­
stants with the present two microelectrode technique. If it is assumed that the
preparation behaves like a simple resistance and capacitance in parallel then it is
possible to determine a terminal resistance (Rₜ), a terminal time constant (τₜ)
and a terminal capacity (Cₜ). The terminal resistance is defined as the resulting
steady-state potential change divided by the total applied current. In this terminal,
Rₜ is 86.5 kΩ. The rising phase of the potential change usually followed an approxi­
mately exponential time course. Figure 1d (○) shows a semi-logarithmic plot of
the potential change in (a). From such a plot the terminal time constant was taken
to be the time required for the potential to reach 63 % of its final value. In this
case τₜ is 0.48 ms. Thus the terminal capacitance, given by the ratio τₜ/Rₜ, is
5.5 nF.
Stimulation of presynaptic terminals

Figure 1. The effect of stimulation on the terminal resistance, time constant and capacity. (a)–(c) Tracings of the total current injected into a presynaptic terminal (upper traces) and the resulting potential change (lower traces): (a) before stimulation; (b) immediately after stimulation for 30 s at 10 Hz; (c) 28 s after stimulation had stopped. Horizontal calibration, 1 ms; vertical calibration 10 mV and 0.213 µA. (d) Semi-logarithmic plots of the rising part of the potential change in the resting terminal (○), after stimulation (●) and after 28 s with no stimulation (○). The points were obtained by measuring the difference (ΔV) between the potential at time t and the steady-state value. The solid lines were fitted by regression analysis to (a) and (b). Section (e) shows the change in terminal capacity before, during and after stimulation: values are shown for the resting terminal, before and after stimulation (○), and during stimulation (●). Stimulation was begun at time zero. The inset shows a diagram of the preparation; the presynaptic terminal (pre.), postsynaptic terminal (post.), current electrode (I) and recording electrode (V). Terminal P3, table 1; temperature 20 °C.

The presynaptic terminal was then stimulated at a frequency of 10 Hz. Each stimulus resulted in a postsynaptic action potential. Figure 1b shows the potential change during a hyperpolarizing current pulse immediately after the 300th stimulus. \( R_t \) is now 86.7 kΩ; in this case little different from the rested terminal. However, \( \tau_t \) (figure 1d (●)) is increased to 0.66 ms corresponding to an increase in \( C_t \) of 2.1 to 7.6 nF. Figure 1c shows a third hyperpolarizing pulse given 28 s after the end of stimulation. \( R_t \) and \( \tau_t \) are now 85.8 kΩ and 0.51 ms respectively, corresponding to a value of \( C_t \) of 5.9 nF. Apparently the terminal capacity increases during stimulation and afterwards during inactivity returns to its resting value.
Figure 2. The effect of stimulus frequency on the change in terminal capacity. (a), (b), (d), (e) Tracings of the total current injected into the presynaptic terminal (upper traces) and the resulting potential change. (a), (d) In the rested terminal; (b), (e), immediately after stimulation for 36 s at 10 and 20 Hz respectively. (c) Semi-logarithmic plots of the rising part of the potential change in the resting terminal (○) in (a) and after stimulation (●) in (b); similar plots for (d) and (e) are shown in (f). The points were obtained as for figure 1 d and the lines fitted by regression analysis. Horizontal calibration, 1 ms; vertical calibration 10 mV and 0.213 μA. (g) The time course of the change in terminal capacity for each frequency of stimulation. Resting values before and after stimulation are shown for 10 Hz (○) and 20 Hz (▲). Stimulation was begun at time zero and shown for 10 Hz (●) and for 20 Hz (▲). The solid lines were drawn arbitrarily, the uppermost having twice the slope of the lower. Terminal P8 and P8', table 1; temperature 20 °C.
The effect of repetitive stimulation on $R_t$, $\tau_t$ and $C_t$ in eight other experiments is shown in table 1. In every case $\tau_t$ is increased after stimulation. The effect on $R_t$ was more variable, being increased in some terminals more than others. $C_t$ was increased in all cases. By using paired 't' tests it was found that the increase in $C_t$ was highly significant ($p < 0.001$) and that the increase in $R_t$ was also significant ($p < 0.02$).

The time course of the change in capacity

The time course was determined by interposing hyperpolarizing current pulses during the stimulus train and determining $R_t$ as $\tau_t$ as before. Figure 1e shows the result from the same terminal as in figure 1a–d. Stimulation was begun at time zero (measurements shown as filled circles) and $C_t$ was found to increase in an approximately linear fashion throughout the period of stimulation.

Stimulation was then stopped, whereupon $C_t$ returned to its resting value with an approximately exponential time course (time constant 19 s). Table 1 also shows the time constant of recovery ($\tau_r$) in five other terminals.

For short periods of stimulation (less than 30 s), the time course of the increase in $C_t$ was found to depend on the frequency of stimulation. Figure 2 illustrates such a result. Figure 2a and b respectively show records from the rested terminal and immediately after 36 s stimulation at 10 Hz. As in the previous experiment the rising phase of the potential change is slower after stimulation than before (figure 2c) (terminal P8, table 1). Figure 2d–f show corresponding traces and plots for the same terminal at rest and immediately after 36 s stimulation at 20 Hz (terminal P8', table 1). The time course of the change in terminal capacity at both frequencies is shown in figure 2g. As can be seen, doubling the rate of stimulation approximately doubles the rate of increase of $C_t$. This corresponds to a constant increment in $C_t$ of approximately 5 pF per presynaptic action potential.

In some fibres in which stimulation was prolonged (more than 30 s), the increase in capacitance was not maintained throughout the period of stimulation. Figure 3 shows one such experiment. The resting capacitance of this terminal (terminal P1, table 1) was about 3.8 nF ($\circ$). Stimulation at 10 Hz was begun at time zero and $C_t$ determined after every 100 stimuli. As before, the terminal capacity increased during stimulation ($\bullet$). The increase was approximately linear for the first 300 impulses; however, continued stimulation did not result in a further increase, rather $C_t$ decreased slowly during the remainder of the period of stimulation. At the end of stimulation ($\circ$), $C_t$ quickly returned to its resting level.

It is possible that these changes are associated either with the electrical activity in the presynaptic terminal or with transmitter release. Experiments were then done to look for the change in capacity under conditions where nerve impulses do not release transmitter and when transmitter is released in the absence of nerve impulses.
Removal of external calcium

When calcium ions are absent from the bathing solution, transmitter release from this synapse is abolished but the presynaptic action potential remains unaffected (Miledi & Slater 1966). The effect of repetitive stimulation on the capacity of terminals bathed in artificial sea water containing no calcium (0 Ca a.s.w.) was therefore determined. Figure 4 shows the result of such an experiment. The procedure was as follows. The terminal was first bathed in normal sea water and the resting terminal capacity determined (○). As a control the terminal was then stimulated at 10 Hz and \( C_t \) determined after every 100 stimuli (●). In this terminal (terminal A, table 2), as in that of figure 3, stimulation resulted in a rapid increase in \( C_t \) which was not maintained. After stimulation the terminal was allowed to recover for several minutes in normal sea water and then bathed in 0 Ca a.s.w. for 20 min. The resting capacitance was then redetermined (▲); although this was unchanged, the increase in \( C_t \) invariably seen in normal sea water in response to stimulation was now absent. Since in calcium-containing sea water the increase in \( C_t \) could be repeatedly observed it seems unlikely that the absence of an increase in \( C_t \) during stimulation in 0 Ca a.s.w. is due to deterioration of the synapse. Experiments were tried in which the preparation was returned to normal sea water. All of these experiments were unsuccessful, as it proved difficult to keep the micro-electrodes in the terminal for such periods of time. Table 2 shows the results obtained from this and three other terminals. In each, reducing the extracellular calcium abolished the increase in \( C_t \) usually observed during stimulation.
(In the rested state (control) and after repetitive stimulation (stimulated).)

<table>
<thead>
<tr>
<th>fibre</th>
<th>$a$ (μm)</th>
<th>$l$ (μm)</th>
<th>$l'$ (μm)</th>
<th>r.p. (mV)</th>
<th>$R_{t}$ (kΩ)</th>
<th>$\tau_{t}$ (ms)</th>
<th>$C_{t}$ (nF)</th>
<th>no. stim.</th>
<th>r.p. (mV)</th>
<th>$R_{t}$ (kΩ)</th>
<th>$\tau_{t}$ (ms)</th>
<th>$C_{t}$ (nF)</th>
<th>$C_{t, \text{stim.}} / C_{t, \text{cont.}}$</th>
<th>$\tau_{r}$ (s)</th>
</tr>
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<tbody>
<tr>
<td>P1</td>
<td>19</td>
<td>278</td>
<td>342$^\dagger$</td>
<td>-57</td>
<td>141</td>
<td>0.53</td>
<td>3.8</td>
<td>300$^\dagger$</td>
<td>-60</td>
<td>144</td>
<td>1.06</td>
<td>7.4</td>
<td>1.99</td>
<td>—</td>
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<tr>
<td>P2</td>
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<td>352</td>
<td>185</td>
<td>-68</td>
<td>98</td>
<td>0.52</td>
<td>6.3</td>
<td>300$^\dagger$</td>
<td>-64</td>
<td>97</td>
<td>0.94</td>
<td>9.7</td>
<td>1.52</td>
<td>19</td>
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<tr>
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<td>185</td>
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<td>86.5</td>
<td>0.48</td>
<td>5.5</td>
<td>250$^\dagger$</td>
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<td>7.6</td>
<td>1.13</td>
<td>16</td>
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<td>430$^\dagger$</td>
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<td>330$^\dagger$</td>
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<td>0.56</td>
<td>4.3</td>
<td>300$^\dagger$</td>
<td>-58</td>
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<td>270$^\dagger$</td>
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<td>79</td>
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<td>275$^\dagger$</td>
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<td>270$^\dagger$</td>
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<td>130</td>
<td>0.59</td>
<td>4.4</td>
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<td>100</td>
<td>-60</td>
<td>106</td>
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<td>-60</td>
<td>106</td>
<td>3.15</td>
<td>29.7</td>
<td>1.25</td>
<td>60</td>
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</table>

mean ± s.e.

-63 ± 2 109 ± 7 0.73 ± 0.15 6.87 ± 1.49

TABLE 2. THE EFFECT OF STIMULATION ON THE ELECTRICAL PROPERTIES OF PRESYNAPTIC TERMINALS BATHED IN ARTIFICIAL SEA WATER CONTAINING NO CALCIUM

<table>
<thead>
<tr>
<th>fibre</th>
<th>$a$ (μm)</th>
<th>$l$ (μm)</th>
<th>$l'$ (μm)</th>
<th>r.p. (mV)</th>
<th>$R_{t}$ (kΩ)</th>
<th>$\tau_{t}$ (ms)</th>
<th>$C_{t}$ (nF)</th>
<th>no. stim.</th>
<th>r.p. (mV)</th>
<th>$R_{t}$ (kΩ)</th>
<th>$\tau_{t}$ (ms)</th>
<th>$C_{t}$ (nF)</th>
<th>$C_{t, \text{stim.}} / C_{t, \text{cont.}}$</th>
<th>$\tau_{r}$ (s)</th>
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<td>A</td>
<td>28</td>
<td>351</td>
<td>185</td>
<td>-66</td>
<td>73.2</td>
<td>0.48</td>
<td>6.6</td>
<td>250</td>
<td>-58</td>
<td>77.6</td>
<td>0.51</td>
<td>6.6</td>
<td>1.00</td>
<td>—</td>
</tr>
<tr>
<td>B</td>
<td>41</td>
<td>315</td>
<td>200$^\dagger$</td>
<td>-58</td>
<td>80.1</td>
<td>0.60</td>
<td>7.6</td>
<td>200</td>
<td>-50</td>
<td>84.2</td>
<td>0.63</td>
<td>7.5</td>
<td>0.99</td>
<td>—</td>
</tr>
<tr>
<td>C</td>
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<td>148</td>
<td>300$^\dagger$</td>
<td>-67</td>
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<td>0.95</td>
<td>12.5</td>
<td>1000</td>
<td>-66</td>
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<td>0.88</td>
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<td>0.61</td>
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mean ± s.e.

-60 ± 2 96.2 ± 19.8 0.66 ± 0.1 ± 1.8

Symbols and abbreviations to tables 1 and 2: $a$, terminal radius; $l$, distance between recording and stimulating electrode; $l'$, distance between recording electrode and the end of the terminal; r.p., resting potential; no. stim., number of stimuli; $R_{t}$, terminal resistance; $\tau_{t}$, terminal time constant; $C_{t}$, terminal capacitance; $C_{t, \text{stim.}} / C_{t, \text{cont.}}$ ratio of capacitance after stimulation to that before stimulation; $\tau_{r}$, time constant for the return of the terminal capacity from the value after stimulation to the control value. $P8'$ and $P8''$ are two experimental runs in the same terminal.

$^\dagger$ Stimulation at 10 Hz; $^\ddagger$ stimulation at 20 Hz; $^\ddagger$ estimated values.
Cobalt ions in the bathing solution can block evoked transmitter release (Stinnakre 1977; Stinnakre & Tauc 1973). An effect of cobalt on the increase in terminal capacity during stimulation was therefore looked for. Figure 5a and b respectively show records of the potential change record during a hyperpolarizing current pulse in a rested terminal and after stimulation for 30 s at 20 Hz. $R_t$ before stimulation was found to be 106 kΩ and after stimulation was 115 kΩ. The rising phase of each potential change is plotted semilogarithmically in figure 5c, and as can be seen the terminal time constant is little affected by stimulation. Consequently the terminal capacity does not increase during stimulation. Figure 5d shows values for $C_t$ before, during and after stimulation. The resting capacity is about 7.1 nF and no change could be detected throughout the period of stimulation (same terminal as figure 2). A similar result was obtained in two other terminals. The resting
stimulation of presynaptic terminals

Figure 6. The effect of lanthanum ions on the resting terminal capacity. (a), (b) Traces of the total current injected (upper traces) and the resulting potential change (lower traces) for a terminal bathed in sea water (a) and after 50 s exposure to sea water plus 5 mM lanthanum ions. Horizontal calibration 1 ms; vertical calibration 10 mV and 0.213 pA. (c) Semi-logarithmic plots of the rising part of the potential change in sea water (○) and sea water plus lanthanum (●); the lines were fitted by regression analysis. Sea water containing lanthanum was run into the bath at time zero. (d) The time course of the effect of lanthanum on the resting terminal capacity. Values in sea water are shown by ○ and in sea water plus 5 mM lanthanum ●. Terminal 47; temperature 22 °C.

The effect of lanthanum ions

In motor nerve terminals (Heuser & Miledi 1971) and in the squid giant synapse (Mann & Joyner 1978) exposed to lanthanum, there is an increase in the rate of spontaneous transmitter release. Changes in the resting terminal capacitance were then looked for in terminals exposed to 5 mM lanthanum. The result obtained from one such experiment is shown in figure 6. Figure 6a and b respectively show the potential change recorded in a terminal bathed in sea water and sea water plus 5 mM lanthanum. Figure 6c shows semilogarithmic plots of the potential capacitance in figure 2 can be compared with that in figure 5d. It can be seen that the resting capacitance increased from about 5 to 7 nF after exposure to cobalt.
change in figure 6a and b. It was found that $\tau_t$ increases after exposure to lanthanum. $C_t$ is also increased, and figure 6d shows the time course of the change in capacity upon exposure to lanthanum. In sea water the resting capacitance is approximately 7.7 nF. Lanthanum was added at the time zero and $C_t$ determined at the times shown. After 25 s, $C_t$ was observed to have increased and to reach a maximum of 10 nF after about 60 s. Thereafter $C_t$ fell slowly despite the continued presence of lanthanum. Such a transient increase was observed in two other terminals. The effect of lanthanum on the terminal resistance was more variable, either increasing slowly (two fibres) or remaining unchanged (one fibre) throughout the exposure to lanthanum.

Stimulation of the postsynaptic cell

The effect of repetitive stimulation on the resistance and capacitance of the postsynaptic cell was also determined. Both electrodes were inserted into the postsynaptic cell adjacent to the synapse and hyperpolarizing current pulses used to measure the membrane resistance and time constant. The postsynaptic cell was stimulated by using depolarizing current pulses, each pulse resulting in an action potential. In seven postsynaptic cells so examined, repetitive stimulation had little effect on the resting cable properties. The mean ratio of capacitance after stimulation to that before was $1.03 \pm 0.01$ (± s.e.) and not significantly different from 1.00.

The effect of repetitive stimulation on the postsynaptic potential

It has been reported that this synapse fatigues rapidly (Bullock 1948; Hagiwara & Tasaki 1958; Katz & Miledi 1967). It is therefore of some interest to determine the output of transmitter when the terminal is stimulated at the frequencies used in the present experiments. Figure 7a–c show records of postsynaptic potentials recorded at two different gains: (a) in a rested terminal, (b) after 500 stimuli at 10 Hz and (c) about 30 s after the period repetitive stimulation was stopped. In each record an action potential arises from a postsynaptic potential. Following the action potential in the rested terminal, there is a large after-depolarization which is usually attributed to the postsynaptic potential. Comparing figure 7a and 7b, it is evident that after stimulation the rate of rise of the postsynaptic potential is reduced and the after depolarization is replaced by an after-hyperpolarization.

The effect of the terminal was stimulated infrequently, the rate of rise of the postsynaptic potential was about 70 V/s ($\odot$). At time zero the terminal was stimulated at 10 Hz and a record of every 25th postsynaptic response taken. The rate of rise of the postsynaptic potential fell continuously during the period of repetitive stimulation ($\bullet$), suggesting that the transmitter output was falling. The observed rise in capacity at this time, however, was approximately linear. It is therefore possible that the increase in capacity and transmitter release are not directly related. It is also possible that the slow fall in capacitance observed after
the initial rise during prolonged stimulation (figures 3 and 4) may be in some way related to synaptic fatigue. At the end of stimulation, the rate of rise of the postsynaptic potential returned to its resting level with an approximately exponential time course. The time constant for recovery in this experiment was 33 s. In three other experiments the time constants of recovery were 33, 32 and 91 s. It is interesting to note that the return of $C_t$ to its resting value after stimulation is also exponential with a time constant of about 32 s.

**Figure 7.** The effect of repetitive stimulation on the postsynaptic potential. (a)–(c) The postsynaptic response recorded at two different gains; (a), when the terminal is rested; (b), after 500 stimuli at 10 Hz; (c), 30 s after the end of repetitive stimulation. The current electrode was in the presynaptic terminal and the recording electrode in the postsynaptic cell. The rate of rise of the postsynaptic potential was measured from the high gain record (uppermost trace) and taken to be the steepest part of the record immediately after the stimulus artefact. (d) The change, in another fibre, in the rate of rise of the postsynaptic potential during stimulation (●) at 10 Hz. The resting value and the time course of recovery are shown (○). Terminals 57 and 58; temperature 22 °C.

**Discussion**

The changes observed in the passive electrical properties of the presynaptic terminal as a result of repetitive stimulation have not hitherto been reported. The absence of these changes in terminals bathed in artificial sea water containing no calcium and in sea water containing cobalt suggests that they are associated with transmitter release rather than the electrical activity in the nerve terminal. The absence of any change in the cable properties of the postsynaptic cell during
stimulation supports this idea as does the finding that lanthanum, which releases transmitter, also causes an increase in terminal capacity.

Several explanations for the increase in capacity have been considered. First, there may be changes in the membrane thickness or the membrane dielectric. From the present experiments it is not possible to discount this explanation, but it seems to be unlikely. Secondly, the volume of an axon has been observed to increase after prolonged repetitive stimulation (Hill 1950). It is possible that an increase in terminal area due to this phenomenon may lead to an increase in capacity which may account for a small part of the observed change.

A more likely explanation for the increase in terminal capacity is an increase in membrane area of the terminal. Support for this suggestion comes from ultrastructural studies at other synapses, which show increases in terminal area following repetitive stimulation (Heuser & Reese 1973; Ceccarelli et al. 1972, 1973; Pysh & Wiley 1972, 1974; Model et al. 1975). This increase in area may be due to the incorporation of synaptic vesicles into the presynaptic membrane.

It is generally accepted that these synaptic vesicles are associated with transmitter release, stimulation causing the vesicles to fuse with the presynaptic membrane emptying their contents into the synaptic cleft (for a review see Heuser 1977). There is evidence, however (see Birks 1974; Marchbanks 1976, 1977; Osborne 1977; Tauc 1977), to suggest that the transmitter is not released from vesicles but that it may leave the cell via gated pores in the presynaptic membrane. The present results alone cannot distinguish between these hypotheses for the mechanism of transmitter release, but they do suggest that at some stage in synaptic transmission vesicles may be incorporated into the presynaptic membrane.

The increase in the terminal resistance that is observed during stimulation is not consistent with an increase in the membrane area; the resistance should decrease as the membrane area increases. There is no obvious explanation for the increase in resistance at the present time. It is possible that the intracellular resistance could change as a result of the morphological changes that are associated with transmitter release. If this were the case then it would alter the values of the membrane resistance and capacitance determined in the present analysis.

It seems likely that the observed changes in the electrical properties are associated with synaptic transmission. However, the precise nature and magnitude of the changes in electrical properties of the presynaptic terminal during stimulation cannot be determined from the present experiments. It is likely that several factors are involved, namely changes in terminal area, changes in membrane resistance, thickness and dielectric and changes in intracellular resistivity.

I should like to thank Dr H. Meves and Professor R. Miledi, F.R.S., for reading and commenting on the manuscript. This work was supported by a grant from the Welcome Trust.
References


