Further Study of the Relationship between Pre- and Postsynaptic Potentials in the Squid Giant Synapse

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ABSTRACT The minimal presynaptic depolarization (MPD) for producing a detectable postsynaptic potential (PSP) was lower than 25 mv in normal or tetrodotoxin (TTX)-containing seawater. The MPD was about 10 mv when a small amount of tetraethylammonium ions (TEA) was injected into the presynaptic terminal. Application of linearly increasing depolarizing current to the normal presynaptic terminal at times produced a PSP before a presynaptic spike was evoked; the rate of rise of the resulting PSP was much slower than that of a PSP triggered by the normal presynaptic spike. A brief depolarizing pulse that preceded the presynaptic spike in normal seawater or the initial transient presynaptic depolarization in TTX decreased the PSP. It increased the PSP when it was applied during the spike or initial transient depolarization. Hyperpolarizing pulses had the reverse effect. The Off-PSP was also modified by inserting pulses at an initial part of the recovery phase of the strong presynaptic depolarization. These results indicate further that increases in Na^+ and K^+ conductance during presynaptic spike activity are not a requirement for transmitter release; the rate of release of transmitter can be controlled by electrical manipulation of the presynaptic terminal; there is a superficial correspondence between the time courses of presynaptic depolarization and the resulting PSP. Thus presynaptic depolarization appears to be only the first step in the series of events constituting excitation–transmitter release coupling. It may not be a necessary step for the release mechanism.

INTRODUCTION

Further studies on the process of excitation–transmitter release coupling at the squid giant synapse have been carried out by correlating electrical changes in the presynaptic terminal and the resulting postsynaptic electrogenesis. Since the biochemical nature of the transmitter in this synapse is not yet

known and direct measurement of the transmitter output from the terminal as a result of presynaptic activity is not possible, the postsynaptic potential (PSP) has been considered to be a direct index of transmitter release in the present experiments. This paper will present evidence that presynaptic depolarization may be essential to the initial process of excitation–transmitter release coupling, but that it may only be an indirect trigger process for the transmitter release mechanism. It has long been suggested that an increase in an inward movement of Ca^{++} during depolarization at the presynaptic terminal is directly responsible for the transmitter release (cf. Hodgkin and Keynes, 1957; Katz, 1962). Preliminary accounts of the present findings have been reported (Kusano, 1967 *a* and *b*).

MATERIALS AND METHODS

The stellate ganglion of common squid (*Loligo pealei*) was used throughout the experiments. Details of the dissecting technique (Bullock, 1948), experimental arrangement, and both pre- and postsynaptic intracellular recording and stimulating techniques have been described in a previous paper (Kusano et al., 1967 *b*). Various rates of linearly increasing or decreasing current pulses as well as square current pulses, or combinations of these were applied to the presynaptic terminal intracellularly to produce various shapes of presynaptic depolarization. Only the distal giant synapse was studied in most experiments, but in a few cases the proximal synapse was employed. The results obtained from the two synapses were essentially identical in these experiments. Most of the experiments were carried out by perfusing the preparation continuously with cooled, oxygenated seawater (16–20°C). Artificial seawater (423 mM NaCl, 9 mM KCl, 9.27 mM $CaCl_2$, 22.94 mM $MgCl_2$, 25.5 mM $MgSO_4$, 2.15 mM $NaHCO_3$) with a pH range of 7.5–8.0 was employed for some experiments. Electrical characteristics of the giant synapse obtained in natural and artificial seawater were almost identical. Various salines modified with respect to ionic composition were also employed and will be described in the text. The distance across the synapse between the sites of presynaptically inserted electrodes and the electrodes in the postsynaptic fiber was kept within 1 mm. When a lower temperature was desired, the preparation together with the perfusing saline was cooled by a Peltier-effect cooling device. Iontophoretic injection of TEA into the presynaptic terminal, external application of TTX (10^{-6} g/ml), or both treatments were employed in a number of experiments. The purpose of these treatments was to diminish development of delayed rectification by K activation when presynaptic depolarization was applied and to diminish spike activities in both pre- and postsynaptic axons, respectively (Kusano et al., 1967 *b*). In some preparations Ca^{++} and Cs^{+} were also electrophoretically introduced into the presynaptic terminal.

RESULTS

Electrical Characteristics of Giant Synapse

Some of the electrical characteristics obtained from more than 26 preparations at an average temperature of 18.6°C were as follows: pre- and post-

synaptic resting potentials were −58.7 mv and −59.6 mv, respectively; amplitudes of action potentials were 80.3 mv and 91.7 mv; firing levels were 22.3 mv and 19.4 mv; effective resistances were 62.0 KΩ and 12.0 KΩ; and membrane time constants were 2.0 msec and 1.6 msec. The synaptic potential was >20 mv with a maximum rate of rise of >25.0 v/sec, a rise time to peak of 1.0 msec, a half-decay time of 1.12 msec, and a synaptic delay time of 0.72 msec (measured from the maximum rate of rise of the presynaptic spike to the point of rise of PSP). The spike firing levels in both pre- and postsynaptic axons were somewhat higher than in the giant axon preparation of *Loligo pealei* which is known to be about 15 mv (Hagiwara and Oomura, 1958). The difference may have been caused by the slight damage due to an extensive cleaning of the pre- and postsynaptic region. This factor may also be reflected in action potential amplitudes in both the pre- and postsynaptic axons. Repetitive spike firing activity in the normal presynaptic axon, examined by intracellular square current application, was seen in 2 out of 26 preparations. On the other hand, the postsynaptic giant axon showed repetitive spike firing activity in all preparations examined.

Relationship between Pre- and Postsynaptic Potentials in Normal, TTX-Perfused, and Presynaptically TEA-Injected Synapses

NORMAL SYNAPSE The relation between directly applied presynaptic depolarization and PSP amplitude in the normal synapse has already been described (Fig. 2 A in Kusano et al., 1967 *b*). Fig. 1 shows the relationship between amplitude of PSP and postsynaptic membrane potential in a preparation in seawater when different amounts of presynaptic depolarization were applied. It was expected that the effective conductance increase in the postsynaptic fiber was further augmented by the larger PSP's which were produced by the larger peak presynaptic depolarizations. This increase could be due to the difference in size of activated postsynaptic area which reflects the amount of transmitter released. Moreover, the postsynaptic membrane is not an isopotential system, therefore the different lines do not converge at one point which may be called the equilibrium potential of the PSP (Hagiwara and Tasaki, 1958).

In the previous study and in the present work, it was shown by using square current pulse application, that the minimal presynaptic depolarization (MPD) for initiating the first detectable PSP was not obtained because the PSP never appeared before the presynaptic axon reached threshold. Thus the voltage region between threshold and the smallest presynaptic spike which accompanied the first detectable PSP could not be examined.

By applying linearly increasing current pulses, it was hoped to accommodate normal spike electrogenesis at the presynaptic terminal and to see the relation between the slowly increasing presynaptic depolarization and the

PSP (Auerbach and Bennett, 1967). In most of the normal synapses examined, accommodation of spike generation of the presynaptic terminal was not seen and firing of a presynaptic spike preceded the PSP. Typical results are shown in Fig. 2, *A1–B2*. The critical spike firing level was slightly elevated, but the presynaptic spike always originated from a local response (Hagiwara and Oomura, 1958). In *B1* a small PSP with a slow rate of rise occurring near the end of the applied current appeared without a presynaptic spike. In some preparations (*C1–2*) the amplitude of the PSP component increased markedly when the intensity of presynaptically applied square current was increased (compare with postsynaptic responses in *C1* and *C2*). When the

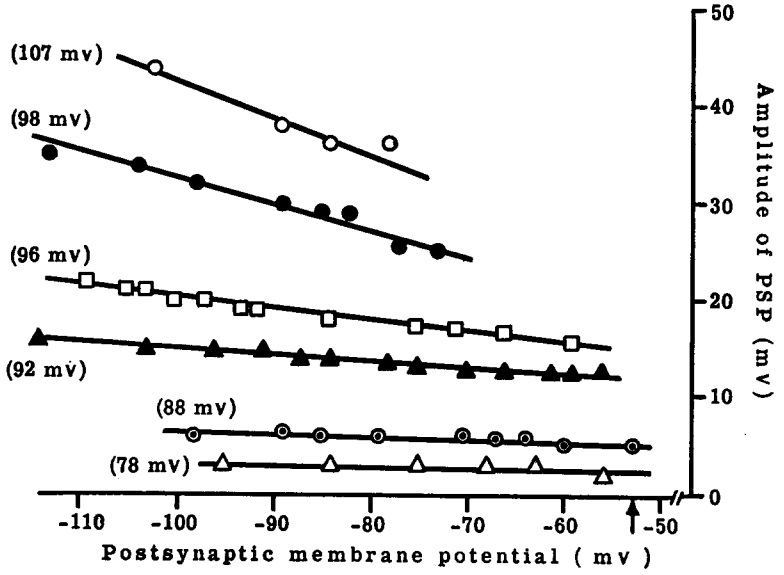

FIGURE 1. Relationship of PSP amplitude and postsynaptic membrane potential at various presynaptic peak depolarizations (78–107 mv). Single preparation. Arrow shows the resting postsynaptic membrane potential.

duration of the linearly increasing current was short, the results were similar to those of short square current pulse application and the presynaptic spike was followed by a PSP (*D1*). In *D1*, a 20 mv PSP initiated by a 65 mv presynaptic spike at about threshold is shown. The time course of this PSP is that generally seen in a typical PSP triggered by a normal presynaptic spike. When a linearly increasing current of about 12 msec duration was applied, the critical firing level for the normal presynaptic spike increased from 25 to about 40 mv. By increasing presynaptic depolarization gradually, the PSP was initiated in the absence of a presynaptic spike firing (*D2–4*). The PSP time course was characterized by a much slower than normal rate of rise. When the presynaptic membrane potential reached 40 mv, a PSP of 30 mv

was observed (*D3*). Thus, a much larger PSP was initiated by the smaller amount of presynaptic depolarization in comparison to the PSP produced by the threshold spike (compare *D1* with *D3*). A large PSP induced a post-

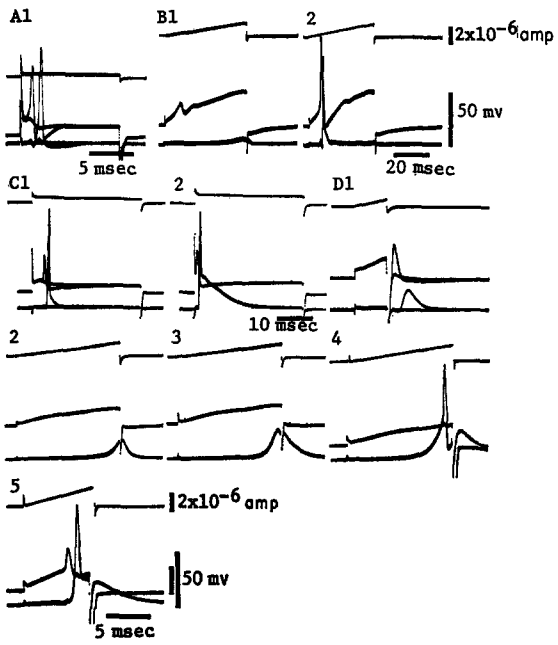

FIGURE 2. Pre- and postsynaptic response to the application of linearly increasing current pulse in the presynaptic terminal. Data from two different preparations are shown: *A1–B2* were obtained from a single preparation which showed the most common case and *C1–D5* were obtained from another preparation. *A1*, pre- (middle trace) and postsynaptic (lower trace) responses to subthreshold and threshold presynaptic depolarization by square current pulse (upper trace). *B1–2*, subthreshold and threshold presynaptic depolarization induced by linearly increasing current of about 40 msec duration and the subsequent postsynaptic response. *C1*, responses to subthreshold and threshold depolarization by presynaptically applied square current pulse. *C2*, responses to suprathreshold presynaptic depolarization. *D1*, responses to threshold presynaptic depolarization induced by a linearly increasing current pulse of 5 msec duration (two superimposed traces). *D2–4*, increasing intensities of linearly increasing current of 12 msec duration and the resulting pre- and postsynaptic responses. *D5*, current duration shortened, same final intensity as *D4*. In *D5* the 50 mv calibration sign to the left is for presynaptic recording and that at the right is for postsynaptic recording (*C1–D5*). Voltage calibration signs in subsequent figures follow this convention. The position of the base line of the presynaptic potential recordings was intentionally moved for purposes of clarity.

synaptic spike before the presynaptic depolarization reached threshold (*D4*). Because of the very slow rate of rise of PSP, sometimes slower than the time course of the falling phase of PSP, it was impossible to measure MPD, synaptic latency, and PSP rise time. When presynaptic depolarization reached 20

mv, a postsynaptic potential of 1 mv deflection could be measured. In *D5* the presynaptic spike was generated by raising the rate of linearly increasing current to the same final intensity as that in *D4*. When the presynaptic spike occurred (*D5*) the repolarization following the postsynaptic spike was less than in *D4*, indicating that the PSP was larger.

TTX-PERFUSED SYNAPSE By using square current pulses, Bloedel et al. (1966; 1967) reported an MPD of between 75 and 100 mv, Katz and Miledi (1966) obtained 30 to 50 mv, and Kusano et al. (1967 *b*) reported less than 50 mv as MPD. These differences are primarily due to differences in the proximity of the pre- and postsynaptic electrodes to the synaptic area. In the present experiments, special care was taken to decrease these distances, particularly by placing the presynaptic electrodes within the last branch of the presynaptic fiber, which is the "*synaptic area.*" A typical result, which is quite similar to those reported previously by Katz and Miledi (1966; 1967 *c*), is shown in Fig. 3. The MPD was below 25 mv. Although the maximum rate of increase of the PSP, produced by a 10 mv increment of presynaptic depolarization in the experiment partially shown in *B1–3*, is about 5 mv (filled circles) in the graph, the largest value of the maximum increase obtained in this series of experiments was about 18 mv (open circles). The maximum PSP amplitude obtained by the application of square current pulse was 37 mv at the pre- and postsynaptic resting potentials of 60 mv and the minimum presynaptic depolarization which produced a maximum PSP amplitude was 70 mv. These values for the rate of increase and amplitude of the PSP were further augmented by giving a conditioning presynaptic hyperpolarization or by increasing external Ca^{++}, and consequently the latter value was lowered (Bloedel et al., 1967; Katz and Miledi, 1967 *c*; Kusano et al., 1967 *b*). In *A1–3* and *B1–3* a comparison is shown of the presynaptically applied current intensity, the resulting presynaptic peak depolarization, and the PSP before and after TTX application. In the normal synapse (*A1–3*), when about 1×10^{-6} amp of current was injected into the presynaptic terminal the threshold spike of about 70 mv was initiated and the resulting PSP was about 16 mv. After TTX (10^{-6} g/ml) perfusion (*B1–3*), 5.4×10^{-6} amp of current was required to produce a 70 mv presynaptic peak depolarization and the resulting PSP amplitude was approximately the same (compare *A2* with *B2*). The time courses of PSP's initiated both by the normal spike and by the peak transient depolarization in the TTX-perfused synapse are quite similar (compare *A1–2* with *B1–2*). This is probably related to the similarity of the time courses of both types of depolarization (Katz and Miledi, 1967 *c*).

TEA-INJECTED SYNAPSE The details of the relation between presynaptic depolarization and the PSP in the synapse presynaptically injected with large amounts of TEA have been described in a previous paper (Kusano et al., 1967 *b*). As in the case of the normal synapse, it was generally not possible

to measure MPD by applying square current pulses because of spike generation. In a few cases, however, a lower MPD than the level of presynaptic spike firing was obtained shortly after TEA had been injected. Fig. 4 shows a

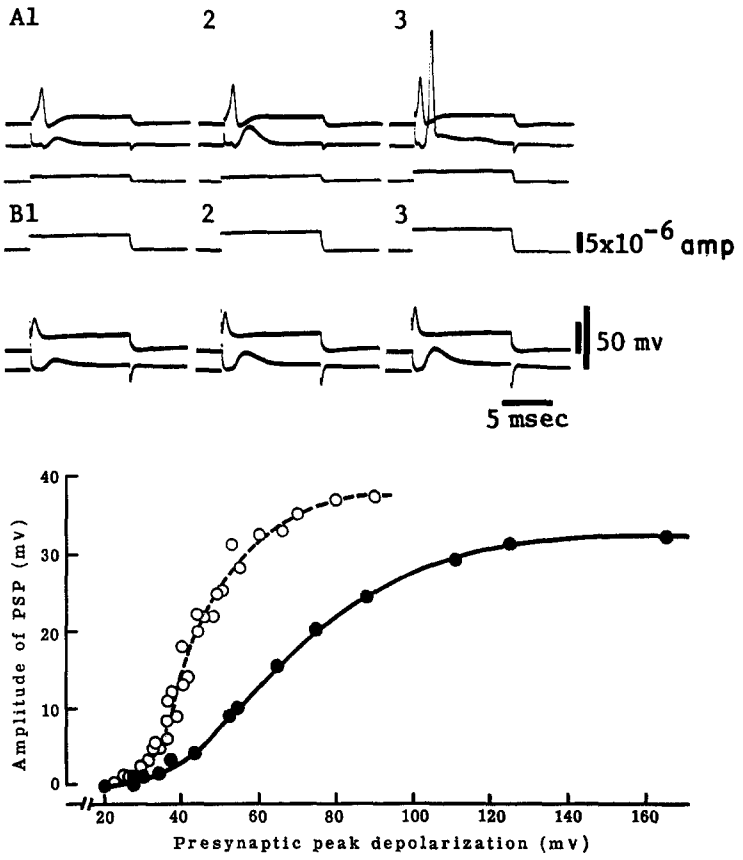

FIGURE 3. Example of the relationship between presynaptic depolarization and PSP amplitude in the normal and TTX-perfused synapse. *A1–3*, pre- (upper trace) and postsynaptic (middle trace) potential changes in response to directly applied presynaptic current (bottom trace) in the normal synapse. *B1–3*, same preparation. The same relationship obtained after perfusion with 10^{-6} g/ml TTX. Presynaptically applied currents are shown on the top trace. Two different examples are shown in the graph. The relation illustrated by filled circles was obtained by experiments as partially shown in *B1–3*; open circles were obtained from another preparation perfused with TTX. Both pre- and postsynaptic resting potentials in these two cases were 60 mv.

case of an exceptionally low MPD. In this figure, *A1–2* illustrate control conditions before TEA injection and *B1–6* were obtained after TEA injection. The presynaptic resting potential, firing level (20 mv), and effective resistance were almost identical to the control condition. The duration of the presynaptic spike was, however, doubled. Presynaptic recordings (*B1–6*)

suggest that considerable diminution of delayed rectification in the presynaptic terminal occurred (Kusano et al., 1967 *b*). The graph shows that the MPD in this case is clearly lower than 10 mv at the threshold for discrimination of a 1 mv PSP and suggests an absolute threshold of only a few milli-

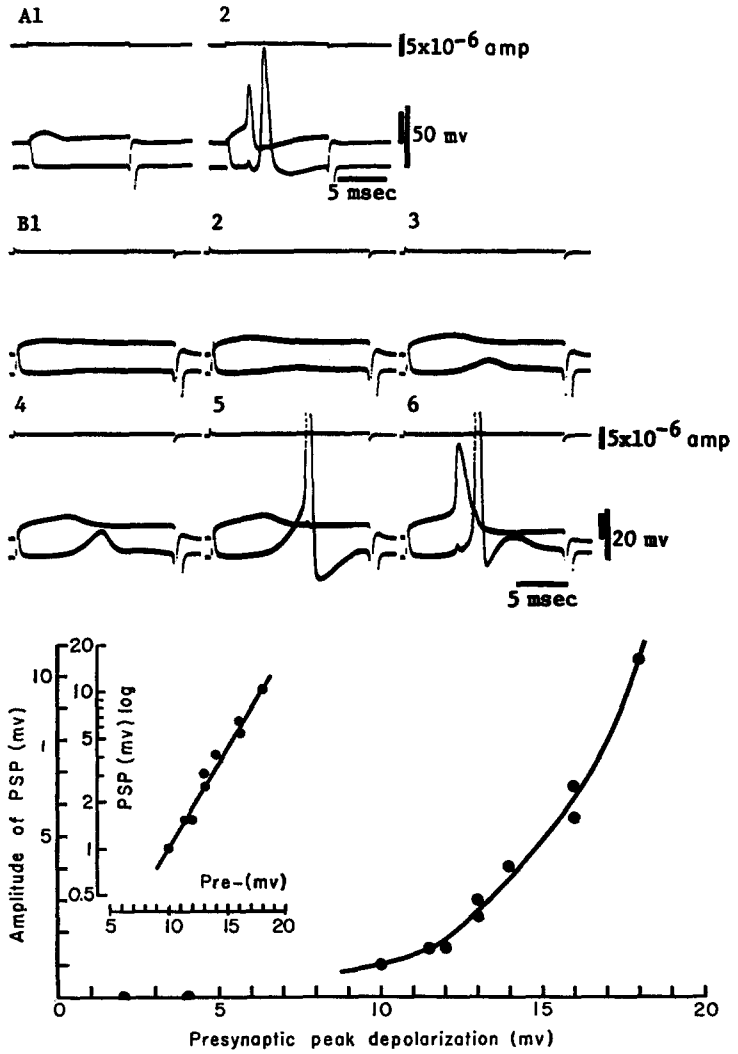

FIGURE 4. Effects on PSP of iontophoretically injecting small amounts of TEA into the presynaptic terminal. The TEA current was less than 1×10^{-6} amp for 10 min. The distance between pre- and postsynaptic electrodes was less than 600 μ; this was the closest electrode arrangement in this series of experiments. *A1–2*, control responses at subthreshold (*A1*) and at threshold (*A2*) presynaptic depolarization. *B1–6*, after TEA injection. Upper trace monitors the presynaptic depolarizing current, middle trace presynaptic potential change, and bottom trace the resulting postsynaptic responses. Graph: relation between presynaptic peak depolarization and amplitude of PSP. Inset graph is in semilogarithmic scale.

volts. The inset graph is a semilogarithmic plot of PSP amplitude versus presynaptic peak depolarization, which indicates the relation is exponential at this range. Before TEA injection, a PSP was not identifiable, even at 16 mv of presynaptic peak depolarization (*A1*). The results indicate, therefore, that TEA greatly enhances secretory activity at the presynaptic terminal (Kusano et al., 1967 *b*).

In preparations in which TEA was injected in larger amounts, some additional data, not previously reported, were found (Fig. 5). When the presynaptic terminal was depolarized more than 100 mv for more than 30 msec a prolonged On-PSP and an Off-PSP of shorter duration appeared (Kusano et al., 1967 *b*). The Off-PSP as well as On-PSP reached the threshold spike firing level of the postsynaptic axon (*A1*). Sometimes a prolonged On-PSP generated repetitive postsynaptic spike firing at a rate of about 100/sec during the plateau of the presynaptic depolarization (*A2*). Further increase of

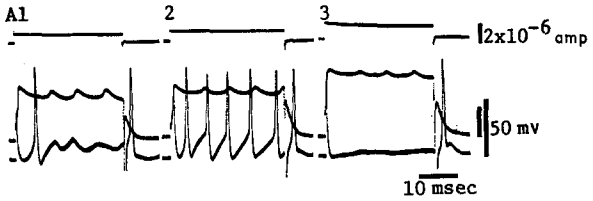

FIGURE 5. Postsynaptic responses to strong depolarization in the TEA-injected presynaptic terminal. *A1–3*, increasing presynaptic depolarization. Upper trace monitors applied current, middle trace is presynaptic potential, and bottom trace is postsynaptic potential recordings.

presynaptic depolarization showed only an Off-postsynaptic spike and the On-PSP was diminished (*A3*). The presence of spikes, in most cases does not allow measurement of the level of MPD or the PSP time course and its amplitude changes. The spike was therefore eliminated with TTX applied in concentrations of up to 10^{-6} g/ml. In a previous paper (Kusano et al., 1967 *b*), we reported the case of a completely reversible abolition of PSP when a large amount of TEA was injected presynaptically and followed by TTX perfusion. Similar reversible abolition of the PSP was seen in two preparations in the present study, but in most cases the PSP persisted for a long time. The cause of these differences in effect was not clarified. The combined treatment with TEA and TTX (Fig. 6) abolished the delayed rectification which is present in the presynaptic fiber when TTX alone is applied (Fig. 3). The MPD was less than 30 mv and the PSP was prolonged (Fig. 6). The duration of the On-PSP is primarily determined by the amplitude and duration of presynaptic depolarization within a limited range. As seen in Fig. 7, the PSP was not sustained but declined slowly when the presynaptic depolariza-

tion was sustained for more than 30 msec at a level higher than 50 mv. Further increase of the duration of presynaptic depolarization did not alter the peak of the PSP. However, a small potential (less than 3 mv) persisted until the end of the polarizing current. The peak of the large PSP became some-

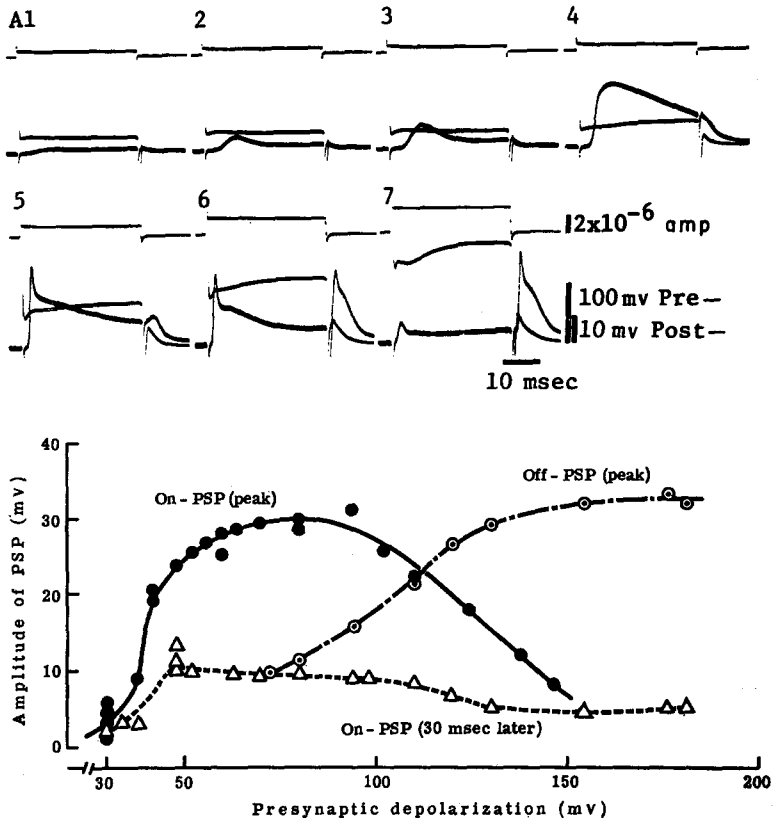

FIGURE 6. Relation between presynaptic depolarization and PSP amplitude after presynaptically injecting TEA and then perfusing externally with 10^{-6} g/ml TTX in seawater. TEA injection (2×10^{-7} amp) was carried out for about 3 hr. *A1–7*, upper trace, presynaptically applied square current pulses. Base lines of pre- and postsynaptic potential recording are superimposed. The relationship between amplitude of PSP and amount of presynaptic depolarization is shown in the graph. Resting potential, 59 mv for prefiber and 58 mv for postfiber.

what spiky, probably due to development of delayed rectification in the postsynaptic membrane. The relation between presynaptic depolarization and amplitude change of PSP is shown in the graph of Fig. 6. The peak amplitudes of On-PSP and Off-PSP are about the same (Katz and Miledi, 1967 *c*) if an adequate duration of presynaptic depolarization is applied. The minimal presynaptic depolarization for initiating the Off-PSP was about 70 mv,

or the same amount of depolarization which initiated a maximum On-PSP. The peak of the Off-PSP appeared at about 150 mv presynaptic depolarization, a value at which the On-PSP was almost abolished.

In a few preparations, external Ca^{++} concentration was varied from 58 mM (Mg-free modified seawater) to Ca^{++}-free to test the hypothetical mecha-

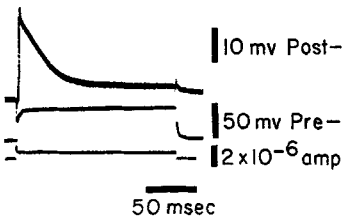

FIGURE 7. Relation between steady presynaptic depolarization of about 50 mv for 150 msec (middle trace) and the time course of the resulting PSP (upper trace). The bottom trace monitors the presynaptically applied current. Same preparation as Fig. 5.

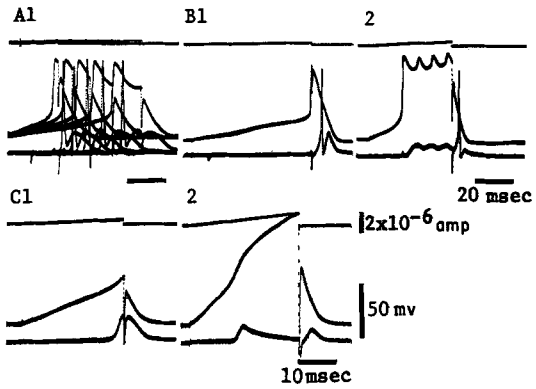

FIGURE 8. Effects of applying linearly increasing current to the TEA-injected presynaptic terminal. *A1*, superimposed recordings of pre- and postsynaptic responses to various durations of five different rates of linearly increasing current pulses; final intensity constant in all sweeps. The final intensity of the shortest pulse (10 msec) was a little higher than threshold. This intensity was kept constant but the pulse duration was successively increased to 14, 17, 24, and 32 msec. *B1–2*, the rate of rise and final intensity of current was increased from threshold (*B1*) to suprathreshold (*B2*). *C1–2*, after 10^{-6} g/ml TTX perfusion, two different rates of linearly increasing current were applied; pulse duration constant. Note On- and Off-PSP production in *C2*. Time scale in *A1* is same as *C1–2*.

nism in which Ca^{++} permeability increase is necessary for the generation of both On- and Off-PSP's (Katz and Miledi, 1967 *c*; Kusano et al., 1967 *b*). Generation of both On- and Off-PSP's could not be separated, but both depended upon external Ca^{++} concentration. In a low-Ca^{++} medium, however, the Off-PSP could not often be generated while a small On-PSP still persisted. Electrophoretic injection of Ca^{++} into the presynaptic terminal (Miledi and Slater, 1966) was also carried out. The amount of Ca^{++} injected when the evoked presynaptic spike became a graded response could not be measured accurately. A diminution of the PSP which was evoked by presynaptic depolarization was then observed.

Linearly increasing current pulses were also applied to the TEA-injected presynaptic terminal. One example of these results is shown in Fig. 8. Since the firing level of the spike did not change significantly from the control, a smaller current intensity than the control (Fig. 2 *B1–2*) could cause spike firing (Fig. 8 *A1*) in the presynaptic terminal, indicating the diminution of delayed rectification. In *B1* the PSP caused by the threshold presynaptic spike was enough to trigger a postsynaptic spike although it may be a mixture of On- and Off-PSP's. In *B2* the plateau level of the presynaptic spike by suprathreshold depolarization was sustained at about 70 mv during linearly increasing current application, but the On-PSP did not evoke a spike. A postsynaptic spike occurred when the current was terminated and probably was due to summation of both On- and Off-PSP's. In *C1–2*, the preparation was perfused with TTX. The PSP shown in *C1* may be a summated form of On- and Off-PSP's. However, series recording showed the On-PSP tends to decrease during the increasingly large presynaptic depolarization as shown in *C2*.

Ionic Requirements for Producing PSP

Besides the necessity of external Ca^{++} for transmitter release, the presence of Na^+ was essential for producing a postsynaptic conductance increase, even in the TTX-perfused synapse. Total replacement of Na^+ with $Tris^+$ or $choline^+$ eliminated the PSP within a few minutes. The PSP fully recovered when the preparation was replaced with control saline containing TTX. In Li^+ saline, the PSP was maintained for more than 30 min, but then gradually diminished in amplitude. However, the nature of the effects on transmitter release of these ions could not be investigated at present.

Repetitive Presynaptic Firing and Corresponding PSP's

Internal application of Cs^+, like TEA, into the squid giant axon is known to prolong spike duration (Sjodin, 1966), and to greatly increase PSP amplitude by modifying transmitter secretory activity in the terminal of lobster neuromuscular junction (Gainer et al., 1967). Experiments on Cs^+ injection into the presynaptic terminal were preliminary in nature. The duration of the presynaptic spike was not prolonged, perhaps due to an insufficient inection of Cs^+ (less than 1×10^{-6} amp for up to 2 hr). However, in some preparations repetitive firing was produced in the presynaptic terminal during depolarization (Fig. 9). Although it was not clear whether this phenomenon was caused by the direct action of Cs^+, the correlation of pre- and postsynaptic potentials during repetitive firing was studied. Repetitive presynaptic firing was also seen during TEA injection (Kusano et al., 1967 *b*). A one-to-one relation of both pre- and postsynaptic axon spikes was seen up to about 130 cps (*A1*), when presynaptic depolarizations of about 150 msec duration were applied at 1 min intervals. Upon further increase of presynaptic depolarization, the number of postsynaptic spikes decreased with diminishing ampli-

tude (*A2*). The PSP's were summated and formed a plateau. In *A2* the plateau level of the summated PSP's reached about 40 mv. When the interval between applied depolarization was shortened the PSP amplitude gradually decreased during repetitive presynaptic activity, possibly from transmitter exhaustion (*A3*). When the postsynaptic fiber was directly stimulated by a square current pulse of 150 msec duration, high frequency repetitive firing of the postsynaptic spike appeared (*B1*). This postsynaptic repetitive spike firing had no effect on the presynaptic fiber (*B1*). In *C1–2*, correlation between the amplitude of presynaptic spikes during repetitive firing and corresponding

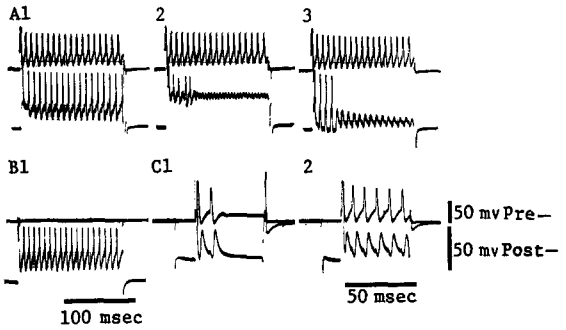

FIGURE 9. Repetitive firing of the presynaptic axon with following PSP's. Data was obtained after Cs^+ was injected presynaptically. Current electrode used for direct presynaptic depolarization contained 1 M CsCl. *A1–3*, presynaptic repetitive spike firing produced by 150 msec duration of suprathreshold depolarizing current (upper trace) and the corresponding postsynaptic response (lower trace). *A2* obtained when presynaptic depolarizing current had been increased above that in *A1*. A capacitative pick-up of the repetitive firing of the presynaptic spikes is seen in the postsynaptic trace. *A3* was obtained after a short interval between stimulations. *B1*, repetitive spike firing in the postsynaptic fiber produced by direct stimulation. *C1–2*, postsynaptic membrane was hyperpolarized about 45 mv during repetitive firing of the presynaptic spike. Pre- and postsynaptic resting membrane potential levels were superimposed. See text for explanation.

PSP's is shown under postsynaptic hyperpolarization to block postsynaptic spike firing. The second presynaptic spike was clearly smaller than the first, but the corresponding PSP was about the same height (*C1*). Successive presynaptic spikes gradually decreased in amplitude, but each corresponding PSP peak was almost the same and did not decrease markedly (*C2*) in height. The phenomenon was quite different from the one we saw when presynaptic depolarization was applied during a conducted presynaptic spike (cf. Fig. 1 in Kusano et al., 1967 *b*). Furthermore, the phenomenon shown in *C1–2* is also different from the observation of Takeuchi and Takeuchi (1962), in which facilitation of the PSP's in this synapse was found when two conducted presynaptic spikes were brought to the terminal at short intervals. This

has been considered in relation to the increase of the second presynaptic spike height.

Effects of Presynaptically Applied Brief Pulses on the Transmitter Release

INTERACTION BETWEEN BRIEF PULSES AND THE PRESYNAPTIC SPIKE Katz and Miledi (1967 *a*) modified transmitter release from the nerve terminal of the frog neuromuscular junction by superimposing electric pulses on the presynaptic action potential. Similarly the effects on the PSP amplitude of application of brief de- or hyperpolarizing pulses preceding, during, and subse-

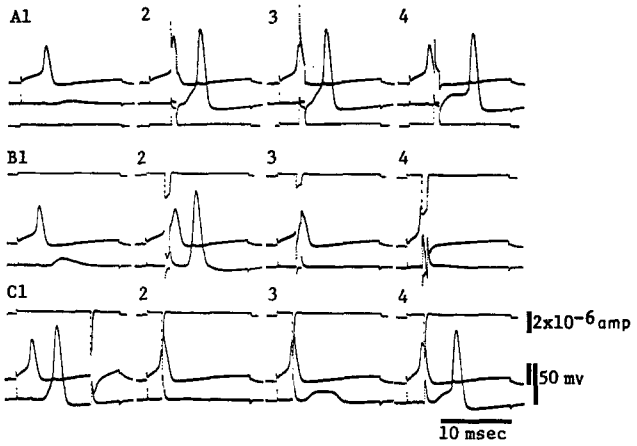

FIGURE 10. Effects on PSP amplitude of brief depolarizing or hyperpolarizing pulses delivered during the various phases of directly evoked presynaptic spike (16°C). The control values of presynaptic peak depolarization in each column were 93 mv (*A1*), 95 mv (*B1*), and 100 mv (*C1*), respectively. *A2–4*, a depolarizing pulse (600 μsec) was applied at three different times. *B2–4*, various intensities of hyperpolarizing pulses (600 μsec) were applied. *C1–4*, a constant intensity hyperpolarizing pulse (100 μsec) was applied.

quent to the presynaptic spike were investigated. Examples are shown in Figs. 10 and 11. The amplitude of the control PSP's in Fig. 10 was obtained by controlling the peak presynaptic depolarization (*A1*, *B1*, and *C1*). The smaller PSP induced by smaller presynaptic depolarization was more easily modified by imposed pulses. Applied pulse durations ranged between about 100 to less than 600 μsec. The shorter pulses required a larger current intensity to modify PSP amplitude. Superimposed depolarizing pulses during the directly evoked presynaptic spike increased the PSP (*A2–4*) and superimposed brief hyperpolarizing pulses diminished the PSP (*B3–4* and *C2–4*), whether or not the amplitude of the presynaptic spike peak was altered. In contrast, a hyperpolarizing pulse applied just before the beginning of the presynaptic spike produced a marked increase in PSP amplitude, although the amplitude of the presynaptic spike and the rate of rise at times were smaller

(*B2*). The application of subthreshold depolarizing pulses preceding the spike decreased the PSP amplitude. In Fig. 11, the effects on the PSP amplitude of a hyperpolarizing pulse, applied at various times during the falling phase of the conducted presynaptic spike and at various times preceding the conducted presynaptic spike, are shown by superimposed recordings. In *A*, the largest PSP is the control, which was obtained when the pulse was not ap-

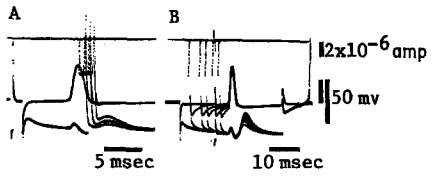

FIGURE 11. Effects on PSP amplitude of a constant-intensity, 300 μsec hyperpolarizing pulse applied at various times after the peak of the conducted presynaptic spike (*A*) and of a constant-intensity 100 μsec hyperpolarizing pulse applied at various times preceding the conducted presynaptic spike (*B*). Postsynaptic axon was hyperpolarized more than 30 mv to prevent postsynaptic spike firing. Temperature 4°C, distal synapse. See text for explanation.

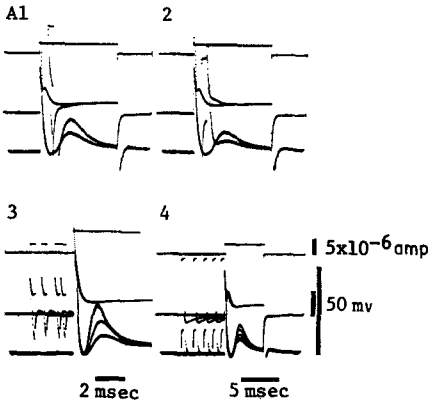

FIGURE 12. Interaction between presynaptically-applied, brief depolarizing and hyperpolarizing pulses on the PSP amplitude in 10^{-6} g/ml TTX-perfused synapse at 14°C. *A1*, effect of depolarizing pulse superimposed on the falling phase of transient presynaptic depolarization. The smaller PSP is the control response. *A2*, effect of hyperpolarizing pulse superimposed on the falling phase of transient presynaptic depolarization. The larger PSP is the control response obtained without interaction. *A3*, effects on the PSP of a small presynaptic depolarizing pulse, which preceded a larger and longer presynaptic depolarizing pulse. The interval between pulses was varied and responses were superimposed; the shortest interval between pulses gave the largest diminution in PSP amplitude. The largest PSP was obtained without a preceding short depolarizing pulse. *A4*, effects of a small presynaptic hyperpolarizing pulse, which preceded a testing presynaptic depolarizing pulse; responses were superimposed. When the interval between pulses was the shortest the PSP amplitude was maximum. The smallest PSP is the control response without a conditioning hyperpolarizing pulse.

plied. The most attenuated PSP was obtained when the pulse just followed the spike peak. Pulses applied later decreased the PSP amplitude less. In *B*, the smallest PSP is the control which was obtained when the pulse was not applied. The pulse closest to the spike increased the PSP amplitude to a maximum value. The amplitude of PSP was increased less when the interval between pulse and spike was increased.

INTERACTION BETWEEN DE- AND HYPERPOLARIZING PULSES IN THE TTX-PERFUSED SYNAPSE In the absence of both pre- and postsynaptic spikes under

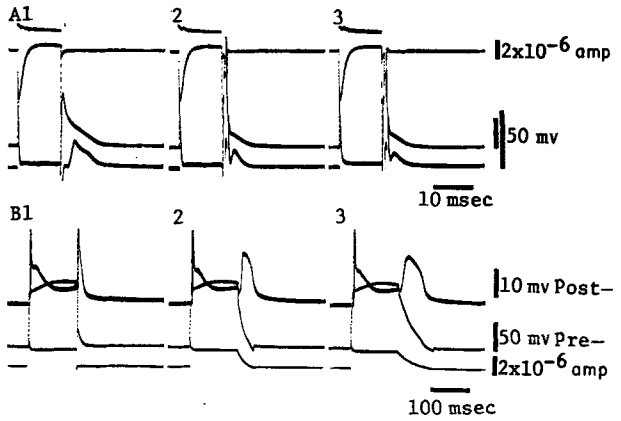

FIGURE 13. Changes in Off-PSP produced by altering the time course of the falling phase of strong presynaptic depolarization by a superimposed hyperpolarizing or depolarizing pulse. *A1–3*, the recovery time course of a strong presynaptic depolarization was shortened by adding brief, strong hyperpolarizing pulses. *A1* is the control response without a hyperpolarizing pulse. Presynaptic depolarization was kept constant. Upper trace is applied current, middle trace presynaptic potential, and bottom trace postsynaptic response. *B1–3*, the recovery time course of a strong presynaptic depolarization was prolonged by slowly decreasing the current. *B1* is the control response; presynaptic depolarization was kept constant. The upper trace is the postsynaptic response, the middle trace the presynaptic potential, and the bottom trace presynaptically applied current.

the influence of TTX, interaction between pulses in either of two directions for PSP production was studied (Fig. 12). A strong hyperpolarizing pulse applied during the falling phase of transient depolarization diminished the PSP (*A1*). On the other hand, application of depolarizing pulses during the same period increased the PSP (*A2*). When small depolarizing test pulses, which were smaller than the pulses required for transmitter release, preceded the conditioning depolarizing pulse, the PSP was decreased. A hyperpolarizing conditioning pulse preceding the depolarizing testing pulse induced an increment of PSP amplitude. This was seen even when the peak of the testing presynaptic depolarization became slightly lower. The effect of preceding pulses was maximum when the interval between the test and conditioning pulses was the shortest.

ALTERATION OF ON- AND OFF-PSP'S BY BRIEF PRESYNAPTIC MEMBRANE POTENTIAL CHANGE IN TEA-INJECTED SYNAPSE Pulses applied in TEA-injected presynapses gave results similar to those seen in Fig. 10. An adequate depolarizing pulse applied during the falling phase of the prolonged TEA spike increased the amplitude and especially the duration of the PSP. However, when a depolarizing pulse was applied during the plateau of the TEA spike, no effect was seen even when the membrane was depolarized to more than 50 mv. When the presynaptic terminal was strongly depolarized so as to inhibit transmitter release, the effect of additional pulses was reversed. A certain intensity and duration of hyperpolarizing pulses now induced the PSP while depolarizing pulses had no effect.

The amplitude and the time course of Off-PSP evoked by strong presynaptic depolarization were also altered when pulses were applied during the falling phase of the depolarization (Fig. 13). The records of *A2–3* show that strong brief hyperpolarizing pulses diminish the Off-PSP compared to that of the control response (*A1*). When the time course of the falling phase of a strongly depolarized presynaptic membrane was prolonged by adding a slowly declining, depolarizing current, the peak amplitude of the Off-PSP was decreased, but its duration was more prolonged (*B2–3*) than that of the control responses (*B1*).

DISCUSSION

The minimal presynaptic depolarization, by application of current, required for initiating the first detectable PSP in the normal or TTX-perfused squid giant synapse was lower than 25 mv. The proximity of pre- and postsynaptic electrodes across the synapse was a most important factor for obtaining the smallest values. This level of MPD obtained by square current pulse application is a little higher than or about the same as the critical spike firing level at the normal presynaptic terminal. Therefore, in most cases no PSP could be detected in the postsynaptic fiber before presynaptic spike generation. However, a number of considerations appear to suggest that a much smaller value of presynaptic depolarization than the measured MPD is sufficient to trigger transmitter release. The measured MPD represents the effective potential change across the presynaptic membrane and may not give an accurate picture of the IR drop across that portion of the terminal membrane where transmitter is released. There are also factors involved in the postsynaptic structure. Spontaneous miniature PSP's (Takeuchi and Takeuchi, 1962; Miledi, 1966 and 1967) were not recorded from the present giant synapse preparation, probably due to very low effective resistance of the postsynaptic axon. Thus about 1 mv of the discrimination threshold for PSP from the noise level of the present recording system may have been too high.

The necessity of Na^+ or K^+ permeability increase as a possibly linked process during excitation-transmitter release coupling in the squid giant synapse

has been ruled out by studying the PSP in the TTX-perfused synapse and the effects of presynaptic injection of TEA (Bloedel et al., 1966; Katz and Miledi, 1966 and 1967 *c*; Kusano et al, 1967 *a* and *b*). It is also doubtful that the low value of the MPD is sufficient to produce sudden increases of Na^+ or K^+ permeability across the presynaptic terminal membrane. Figs. 2–4 provide additional evidence to support this idea. Furthermore, acetylcholine release from the frog nerve terminal has been demonstrated in the complete absence of external Na^+ (del Castillo and Katz, 1954, 1955; Koketsu and Nishi, 1959). Douglas and Rubin (1963) have shown that Na^+, K^+, and Cl^- are not required for evoking catecholamine secretion from the chromaffin cells of the mammalian adrenal medulla. In the squid giant synapse preparation, the transmitter itself has not been identified and its release has not yet been demonstrated except by postsynaptic membrane potential changes produced by presynaptic depolarization. There is some indication, however, that the presynaptic membrane potential change may be a secondary factor for release of transmitter, as shown in Fig. 4, in which presynaptic TEA injection lowered the MPD to about 10 mv, and thus there is no consistent threshold (see also Miledi, 1967). The possible cause of this TEA action is obscure at present. In the frog neuromuscular junction as well as in other chemical synapses, the transmitter is spontaneously released from the nerve terminal (Katz, 1962).

In view of the close correlation of both time courses of the PSP and presynaptic depolarization, it can be said that the rate of rise of PSP is controlled to some extent by the rate and amount of presynaptic depolarization which controls the rate and amount of transmitter secretion. However, postsynaptic conductance changes and receptor sensitivity to the transmitter are also involved. Since electrical constants of the postsynaptic membrane do not seem to be altered in most of the present experiments, various changes in the time course of PSP, which indicate changes in transmitter activity, must be due to the rate and amount of transmitter secretion. It should be noted here that the apparent correlation between the pre- and postsynaptic potentials may be a superficial one since a greater rate of rise of the presynaptic depolarization depresses the PSP.

It has been shown that facilitation of the PSP's can be observed in the synapse perfused with TTX (Bloedel et al., 1966) when two identical amplitudes of presynaptic depolarizations of 1 msec duration are applied at short intervals, as well as when the untreated synapse is examined by conducted presynaptic spikes (Takeuchi and Takeuchi, 1962). Bloedel et al. (1966) proposed that the phenomenon seen in their case is due to the facilitation of some process of the transmitter release, rather than to changes in the amplitude of the presynaptic depolarization or in membrane current. Data shown in Fig. 9 would appear to support their idea.

Figs. 10–13 show that the release of transmitter can be controlled elec-

trically by subsequent or preceding brief changes in the membrane potential of the presynaptic terminal, even after the presynaptic spike has reached a maximum. This would seem to indicate that the process of transmitter release involves an electrochemical reaction which is not simply or directly dependent upon an absolute membrane potential level or on membrane current.

Data supporting the hypothesis that Ca^{++} permeability increase or accumulation of Ca^{++}, induced by arrival of the presynaptic spike at the terminal or by direct depolarization of the terminal itself, may be linked to the transmitter release process, are accumulating (Frankenhaeuser and Hodgkin, 1957; Hodgkin and Keynes, 1957; Katz and Miledi, 1967 *b*; Miledi and Slater, 1966; Gage, 1967). Generation of both On- and Off-PSP's requires the presence of extracellular Ca^{++}, not intracellular Ca. Tasaki et al. (1967) recently demonstrated the indispensability of extracellular Ca^{++} for maintaining excitability of the squid giant axon membrane and the significant increase in Ca^{++} influx with excitation. However, little information on any fundamental chemical reaction of Ca^{++} within the excitable membrane during depolarization or hyperpolarization is available, especially at the membrane of the presynaptic terminal. The present paper suggests that a certain magnitude of depolarization of the presynaptic terminal may be the first step in excitation–transmitter release coupling, but it is questionable whether depolarization is a necessary step for the release mechanism. It has been reported that catecholamine secretion is related to the uptake of Ca^{++}, rather than any change in chromaffin cell membrane potential (Douglas et al., 1967).

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