Correlation of Transmitter Release with Membrane Properties of the Presynaptic Fiber of the Squid Giant Synapse

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ABSTRACT Depolarization of the presynaptic terminal by current produced a postsynaptic potential (PSP) which increased with increasing presynaptic polarization and then reached a plateau. Iontophoretic injection of tetraethylammonium ions (TEA) into the presynaptic axon near the terminal produced a prolonged presynaptic spike. The resulting PSP is increased in size and its time course closely followed that of the presynaptic spike. The presynaptic fiber no longer exhibited rectification and strong depolarizations revealed that the PSP reached a maximum with about 110 mv depolarization. Further depolarization produced a decrease in PSP amplitude and finally transmission was blocked. However, a PSP then always appeared on withdrawal of the depolarizing current. Under the conditions of these experiments, the PSP could be considered a direct measure of transmitter release. Bathing the TEA-injected synapse with concentrations of tetrodotoxin (TTX) sufficient to block spike activity in both pre- and postsynaptic axons did not greatly modify postsynaptic electrogenesis. However, doubling TTX concentration reversibly blocked PSP. Thus the permeability changes to Na and K accompanying the spike do not appear necessary for transmitter release. Some other processes related to the level of presynaptic polarization must be involved to explain the data. The inhibition of transmitter release by strong depolarizations appears to be related to Ca action. A membrane Ca current may also be necessary for normal transmitter release.

INTRODUCTION

The excitation-secretion coupling process by which transmitter is released following spike activity of the presynaptic axon is a problem of considerable importance. Katz (1962) and Katz and Miledi (1965 *a*) have postulated that Ca is directly involved in the transmitter release mechanism at the frog neuro-

muscular junction, but the detailed mechanism of Ca action has not been elucidated. Following the work of Hagiwara and Tasaki (1958) and of Takeuchi and Takeuchi (1962), it has been generally held that the release of transmitter is proportional to depolarization of the presynaptic terminal produced by the spike. These workers reported that augmentation of the spike amplitude in the presynaptic axon of the squid caused augmentation of the excitatory postsynaptic potential (PSP). On the other hand, it has been observed that spontaneous miniature PSP's recorded from vertebrates (Furukawa, Sasaoka, and Hosoya, 1959; Ogura, Watanabe, and Mori, 1964; Elmqvist and Feldman, 1956; Kao and Nishiyama, 1965) or invertebrates (Reuben and Grundfest, 1960; Ozeki, Freeman, and Grundfest, 1966; Miledi, 1966) are unaffected when spike electrogenesis of the axon is blocked by tetrodotoxin (TTX) or saxitoxin. Under such conditions very brief depolarizing stimuli applied to the nerve terminal of frog (Katz and Miledi, 1965 *b*, 1967 *a*) or crustacean (Ozeki, Freeman, and Grundfest, 1966) neuromuscular junctions or the squid giant synapse (Kusano and Nakajima, 1962, unpublished; Bloedel, Gage, Llinás, and Quastel, 1966; Katz and Miledi, 1966) still evoke PSP's. These findings indicate that excitation-secretion coupling does not require an increased Na conductance of the presynaptic axon membrane, since the process of Na activation is eliminated specifically by the toxins (Narahashi, Moore, and Scott, 1964; Nakamura, Nakajima, and Grundfest, 1965; Takata, Moore, Kao, and Fuhrman, 1966; Hille, 1966). The conductance increase due to K activation is still present, however, after treatment with these toxins.

It is well-known that K activation is diminished or blocked by the tetraethylammonium ion (TEA) (Hagiwara and Saito, 1959) but the processes of Na activation and inactivation are generally unaffected by TEA (Hille, 1967; Koppenhöfer, 1967) and as a result the action potential is prolonged in many electrically excitable tissues. The TEA action is effective in squid axons only when it is applied intracellularly (Tasaki and Hagiwara, 1957; Armstrong and Binstock, 1965). Taking advantage of this response of the squid axon membrane to TEA, experiments were carried out to investigate the mechanism of transmitter release at the squid giant synapse. The presynaptic spike configuration, altered by means of electrophoretically injected TEA, was correlated with the size and duration of the PSP. The difficulty of the experimental procedure is compensated for by the fact that possible actions of TEA on the postsynaptic membrane (Koketsu, 1958; Stovner, 1958) are avoided by the presynaptic injection. Experiments were also carried out to study the effects of TTX on the transmitter release from the TEA-injected presynapse. Preliminary accounts of the present findings have been published (Kusano, Livengood, and Werman, 1967; Werman, Livengood, and Kusano, 1967).

MATERIAL AND METHODS

The common squid (*Loligo pealei*), available at the Marine Biological Laboratory in Woods Hole, Massachusetts, was used for these experiments during the summers of 1962, 1963, and 1966. The technique of dissection of the stellate ganglia was essentially similar to that of Bullock (1948). The pre- and postganglionic nerve ends were tied with fine silk threads and the preparation was then mounted in the experimental chamber. The chamber, which was made either of Lucite or of Parawax, consisted essentially of three compartments connected by a narrow groove running the length of the chamber. The stellate ganglion was placed in the center compartment with the pre- and postganglionic nerves extending along the groove into the outer compartments. The outer compartments were then used for either external stimulation or external recording, employing silver wire electrode pairs embedded in each of the outer compartments. The preparation was stabilized mechanically in the recording chamber by applying a slight tension to the threads tied to the nerve ends. The final exposure of the synaptic area was carried out in the recording chamber while continuously monitoring synaptic transmission. The accessory presynaptic fiber was pinched with fine forceps at the level of the stellate ganglion to eliminate activity of the proximal synapse of the giant axon (Bryant, 1959) and only the distal synapse was studied.

Microelectrodes used in these experiments were 3 M KCl or 1 M K citrate electrodes for potential recordings or for current application; 1 M tetraethylammonium chloride (pH 7.0–7.4) electrodes for iontophoresing TEA into the presynaptic site. In some cases three electrodes were inserted into presynaptic sites, the first for recording, the second for current application, and the third for iontophoresis. In general, however, the iontophoretic TEA electrode was also used for polarizing the presynaptic membrane. Postsynaptically, two electrodes were inserted for recording and polarization, respectively. The sites of presynaptically inserted electrodes were always less than 1.0 mm from the closed end of the presynaptic fiber and the recording and polarizing or iontophoresing electrodes were within 50 μ of each other. Two postsynaptic electrodes were also placed less than 500 μ from the synaptic region. The preparation was continuously perfused with cooled oxygenated seawater (18–22°C). In certain experiments, different concentrations of tetraethylammonium chloride (5, 10, and 20 mM) were made up in seawater and these were then used as the bathing solutions. In other experiments, TTX (up to 10^{-6} g/ml) was added to the bathing solution after TEA was iontophoresed into the presynaptic axon. Recording and stimulating techniques were otherwise conventional.

RESULTS

Effects of Presynaptic Polarization on Synaptic Transmission before TEA Injection

It appeared worthwhile to repeat and extend some of the earlier observations on the relationship of presynaptic spike size to transmitter release (Hagiwara

and Tasaki, 1958; Takeuchi and Takeuchi, 1962; Miledi and Slater, 1966). The results summarized in Figs. 1 and 2 were obtained from a single preparation except for Figs. 1 D1–7 and 2 C1–2 and 2 D. In the experiments shown in Fig. 1 the presynaptic axon was stimulated with external electrodes and the conducted presynaptic spike amplitude (upper traces) was modified at the terminal region by brief intracellularly applied current (B, C) or by a steady current (D). In B and C (lower traces) a brief constant hyperpolarization was applied postsynaptically in order to prevent the PSP from reaching the firing level for the spike and thereby initiating depolarizing conductance changes of the postsynaptic axon. When the presynaptic axon was depolarized (B1–4, D4–7), using current delivered through 3 M KCl or 1 M K citrate-filled electrodes, the presynaptic spike amplitude decreased markedly and the PSP amplitude decreased. When the presynaptic axon was hyperpolarized, however, the overshoot of the spike peak did not change (C1–2, D1–2). The increase in PSP with hyperpolarization is manifested by attainment of the postsynaptic critical firing level (C2) or by a progressive shortening of the latency of the postsynaptic spike (D4–1) with increasing presynaptic hyperpolarization. However, the latency always remained greater than 0.5 msec. The amplitude of the PSP was almost linearly related to the total amplitude of the presynaptic spike (A). The measurements were limited by the amount of hyperpolarization that could be applied to the presynaptic terminal before invasion of the propagated spike was blocked. At this stage, even though the overshoot of the spike was clearly diminished (Takeuchi and Takeuchi, 1962), the PSP was maximal.

The relation between the amplitude of presynaptic spikes from a directly applied depolarizing current and the PSP is shown in Fig. 2. The peak voltage of the directly elicited presynaptic spike was taken as the measure of the presynaptic depolarization. The peak voltage of the presynaptic spike was increased by increasing the applied current (B). The PSP amplitude at first increased very steeply with increases in the peak spike voltage but further presynaptic depolarization resulted in decreasing increments in PSP amplitude. Finally, the PSP amplitude appeared to saturate when the peak of the presynaptic spike reached approximately 110 mv (Fig. 2 A). Further polarization was difficult to apply because of the large delayed rectification due to K activation (Hodgkin and Huxley, 1952). The saturation of the PSP is not due to approach of the PSP to its equilibrium potential because the postsynaptic axon was strongly hyperpolarized. Under these conditions, the amplitude of the PSP can be considered a measure of transmitter release. Thus, the process of transmitter release appears to have been saturated. No significant changes in PSP time course and synaptic delay were found during these experiments.

Fig. 2 C2 shows an example in which strong depolarization caused repetitive firing of the presynaptic axon spike and the corresponding postsynaptic re-

sponses. The second postsynaptic response has a slower rate of rise than the initial response, indicating the diminished transmitter release triggered by the smaller presynaptic spike. A series of superimposed traces from another experi-

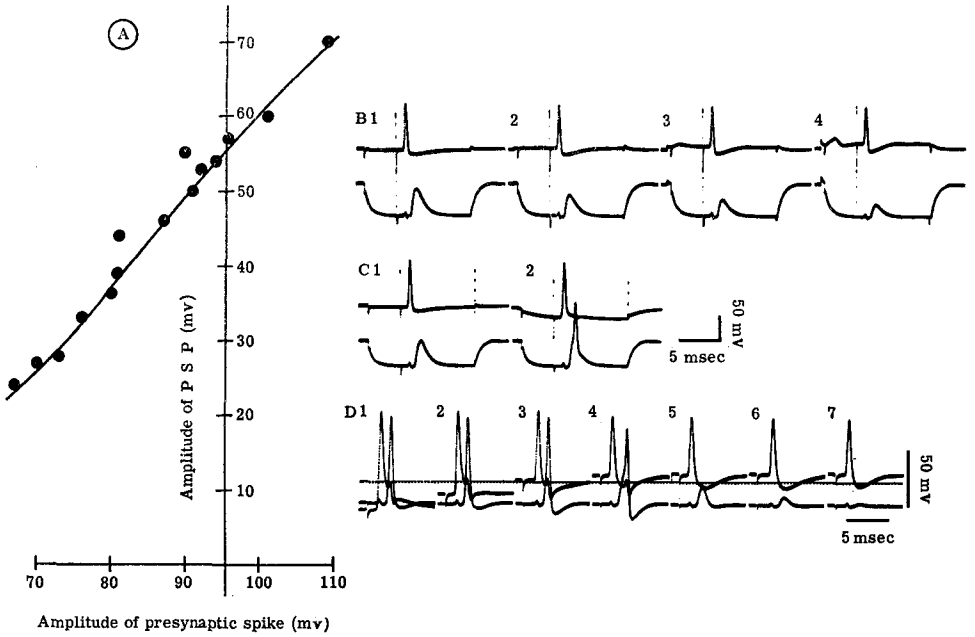

FIGURE 1. Effects on PSP of presynaptic polarization during a conducted spike. A, relationship between the amplitude of presynaptic spike (abscissa) and the amplitude of the PSP (ordinate). Sample records are presented in B1-4 and C1-2. Resting potential, −54 mv for presynaptic fiber and −45 mv for postsynaptic fiber. Prefiber spike amplitude was 96 mv at the resting level. Amplitudes of PSP's were obtained with 64 mv hyperpolarization postsynaptically to prevent spike electrogenesis. B1-4, the upper trace shows a progressive decline in the amplitude of the presynaptic action potential with increasing depolarizing pulse amplitude. The lower trace shows the corresponding decrease in PSP amplitude. C1-2 effect of presynaptic hyperpolarization. C1, control response obtained without presynaptic hyperpolarization. C2, a small amount of presynaptic hyperpolarization produced an increase in PSP amplitude sufficient to produce a postsynaptic spike. D1-7, effects of sustained presynaptic polarization on synaptic transmission in another preparation. D3 is approximately the control level (dotted line), D1-2 are hyperpolarizations, and D4-7, depolarizations. The upper trace, except in frame D1, shows the responses of the presynaptic spike, and the lower trace shows the corresponding effects produced on the postsynaptic spike and PSP. The trace positions are reversed in frame D1.

ment is shown in Fig. 2 D. Increasing presynaptic depolarization results in increased spikes occurring with progressively briefer latency. The PSP is similarly increased and its latency is shortened.

Figs. 1 A and 2 A both indicate that depolarization of the presynaptic axon

is a requirement for excitation-secretion coupling. However, the saturation seen in Fig. 2 A indicates a nonlinearity in the relationship which may represent initiation of an antagonistic "second process" by strong depolarization. Effects due to the modification of the total spike amplitude cannot be ruled

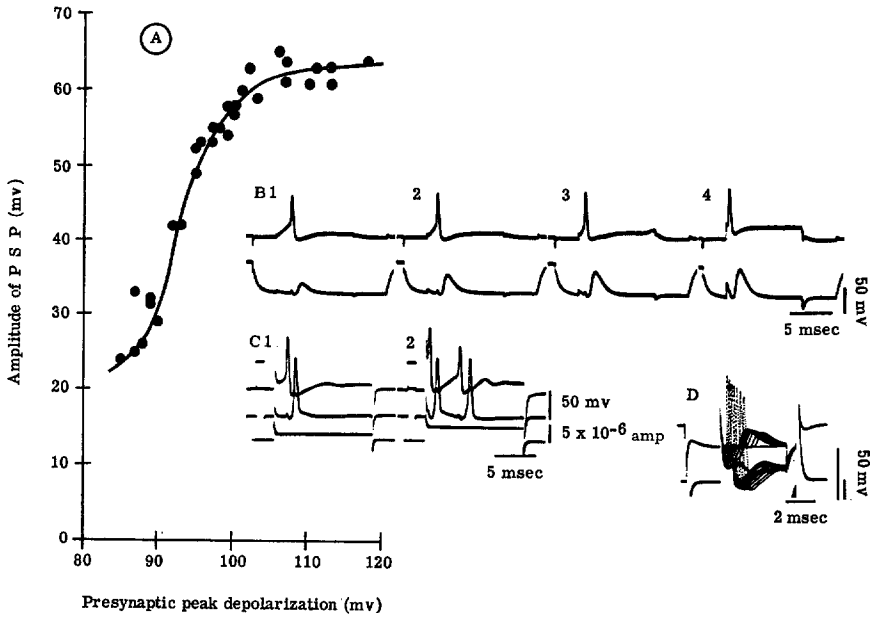

FIGURE 2. Relationship of directly evoked presynaptic spike peak amplitude to PSP amplitude. A, the relationship between peak amplitude of a directly evoked presynaptic spike and the PSP amplitude. B1-4, upper trace shows increasing levels of presynaptic peak depolarization with increasing levels of directly applied current. Lower trace shows the corresponding effect produced on the PSP amplitude in the 64 mv hyperpolarized postsynaptic axon. C1-2, in another preparation, repetitive firing of the presynaptic spike produced by increasing intensity of depolarizing current. Upper trace shows presynaptic responses, middle trace postsynaptic electrogenesis, and bottom trace monitors the applied current. The small transient preceding the second postsynaptic spike in C2 represents capacitative pickup of the presynaptic spike. D, another preparation. Directly evoked presynaptic spike on lower trace, PSP on upper. 50 mv calibration sign to left is for presynaptic recording and that at right is for the postsynaptic recording. All voltage calibration signs in subsequent figures follow this convention.

out. For example, superimposing a brief (less than 200 μsec) depolarizing pulse on the presynaptic spike increases the amplitude of the PSP, while a strong brief hyperpolarization during the falling phase of the spike diminishes or suppresses the PSP (unpublished data). An analogous phenomenon has also been reported in the frog neuromuscular junction (Katz and Miledi, 1967 a).

Effects of External Application of TEA on Synaptic Transmission

In seawater containing 5, 10, or 20 mM TEA chloride, both pre- and postfiber resting potentials, membrane resistances, and spike firing levels did not change significantly (Tasaki and Hagiwara, 1957). Synaptic transmission was maintained in 20 mM TEA media for at least 20 min during which time the following results were encountered: a decrease in amplitude and a slight prolongation of the duration of both pre- and postsynaptic action potentials; a slight lengthening of synaptic delay (from 0.7 to 0.9 msec); and a gradual decrease in amplitude of PSP without significant change in time course (Bryant, 1958). No recovery from these minimal changes was seen after the TEA-treated preparation was bathed in normal seawater for 1 hr.

Iontophoretic Injection of TEA into the Presynaptic Terminal

PRESYNAPTIC SPIKE AND MEMBRANE PROPERTIES It was necessary to inject a large amount of TEA current into the presynaptic terminal to produce a typical prolonged TEA spike. The absolute amount of injected TEA could not be determined because the transport number of TEA was not known. Preparations which produced typical prolonged TEA spikes were obtained when a relatively weak iontophoretic current (up to about 1×10^{-6} amp) was sustained for a long period (3-5 hr). A stronger current employed for a shorter period was less successful in this respect. It is likely that following a prolonged slow injection the TEA was dispersed over a considerable volume of the presynaptic region. Typical results are shown in Fig. 3, in which A1 to A4 were taken during 7 hr of TEA injection. The records of the responses in both pre- and postsynaptic axons to external stimulation of the presynaptic axon were made during temporary removal of the background TEA current. The most prominent effect of the TEA was slowing of the falling phase of the action potential. Although the rise time (particularly the 90–100% level) of the action potential was delayed, the maximum rate of rise of the action potential usually changed less than 30%. Sometimes marked secondary elevations were superimposed on the prolonged falling phase of the TEA spike as shown later (see Fig. 9 B2). Because of drift of the recording system and possible changes in microelectrode characteristics during such long experiments the resting potential changes could not be measured accurately. There appeared to be, however, only a small decrease, at most, in the presynaptic resting potential. In general, a marked prolongation of the spike duration was often associated with a slight reduction in the spike height as reported by Tasaki and Hagiwara (1957). The normal spike duration of about 0.9 msec (time between the beginning of the action potential to the point of intersection of the spike falling

phase with the base line) was increased to a maximum of 73 msec. The prolonged TEA spike showed a decreased undershoot. In the case of Fig. 3 the hyperpolarizing after-potential peak before TEA injection was 16 mv below the level of the resting potential. When the action potential was prolonged by TEA injection as seen in A4, the undershoot was decreased to 7 mv. Fig. 3 B1–4, shows directly evoked spikes initiated close to the TEA-iontophoresing electrode, at almost the same time as those recorded in A1 to A4 respectively. The additional change in the response to direct stimulation during TEA injection was a gradual prolongation of the utilization time for spike initiation tested by square pulse current, suggesting gradual elimination of depolarizing membrane rectification by TEA.

In some cases, following prolonged application of sustained TEA current,

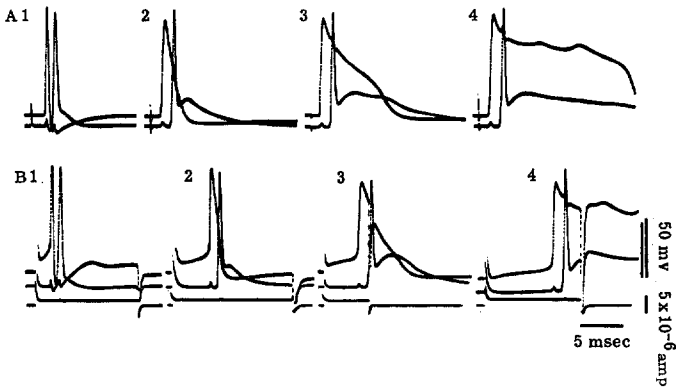

FIGURE 3. Effects of presynaptically injected TEA on the time course of presynaptic spike and postsynaptic response. A1-4, presynaptic spikes indirectly evoked during progressive TEA administration. The top trace shows the presynaptic response and the second trace, the postsynaptic response. B1-4, presynaptic spikes directly evoked by threshold stimulation; records made at almost the same times as those of A1-4. The lowest trace monitors the stimulating current.

the presynaptic fiber membrane suddenly depolarized, and repetitive firing was initiated. This firing persisted even after removal of the TEA current. In Fig. 4 A such repetitive firing of the presynaptic axon (35 mv, 8 msec duration at a frequency of about 25 cps) is shown. These small presynaptic spikes triggered small PSP's of about 15 mv which were below the firing level of the postsynaptic axon. This condition continued for several minutes during which PSP's of constant amplitude always followed each presynaptic spike. The resting potential of the presynaptic fiber did not quite return to the level that existed before the onset of repetitive firing, but the subsequent large and prolonged presynaptic spikes were still capable of triggering postsynaptic spike electrogenesis (Fig. 4 B1 and B2). After such repetitive firing of the presynaptic axon, the presynaptic axon membrane began to generate action

potentials in response to both inward and outward current application and the effective membrane resistance of the presynaptic fiber was seen to have decreased significantly.

The relationship of presynaptic polarization to PSP size has already been discussed in the control condition (Figs. 1 and 2). In general it was not possible to apply a large steady-state depolarization of over 30 mv because of the marked delayed rectification exhibited. However, in the TEA-injected preparation, it was possible to polarize the presynaptic membrane to a level many times as high. Fig. 5 demonstrates the change produced in the steady-state current-voltage relations of the presynaptic fiber by TEA injection. The depolarizing membrane rectification due to delayed K activation was diminished by TEA injection in the presynaptic fiber while the behavior of the postsynaptic fiber was unchanged. There was no significant change in the spike firing level, but both below and above the firing level the membrane behaved

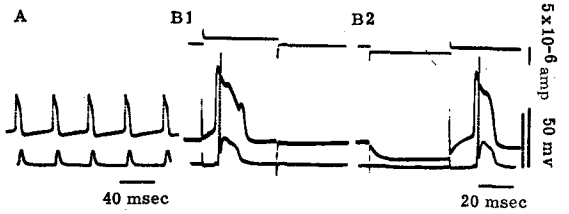

FIGURE 4. Repetitive firing of the presynaptic spike during TEA iontophoresis. A, the upper trace shows repetitive firing of the presynaptic spike. The lower trace shows the corresponding PSP's. B1-2, responses to directly applied depolarization (B1) and hyperpolarization (B2) after the recovery from condition in A. See text for further discussion.

in an ohmic fashion at steady state. Such membrane behavior was seen more frequently after large TEA currents had been injected for a long time.

It was not always possible to produce typically prolonged TEA spikes. When a strong iontophoretic current was applied within a short period, conduction of the presynaptic spike into the region of the TEA electrode was blocked and the membrane potential was depolarized to some extent. The directly elicited spike became smaller than that of the control and spike duration was not prolonged significantly (Fig. 6). Nevertheless the small, brief spike and subsequent depolarization during applied current elicited a large, prolonged PSP. The increase and prolongation of the PSP depended on the magnitude and duration of the presynaptic depolarization.

POSTSYNAPTIC ELECTROGENESIS The electrical properties of the postsynaptic giant fiber membrane, such as rectification and resting resistance (Fig. 5), membrane time constant, threshold firing level, and amplitude of the spike, and amplitude of the hyperpolarizing after-potential of the antidromic postsynaptic axon spikes (Fig. 7 B) were not modified by injecting TEA into the

presynaptic fiber. As in the control condition there was no significant amplitude difference between the orthodromic and antidromic spikes of the postsynaptic fiber (Fig. 7 A, B). However, the over-all postsynaptic electrogenesis was

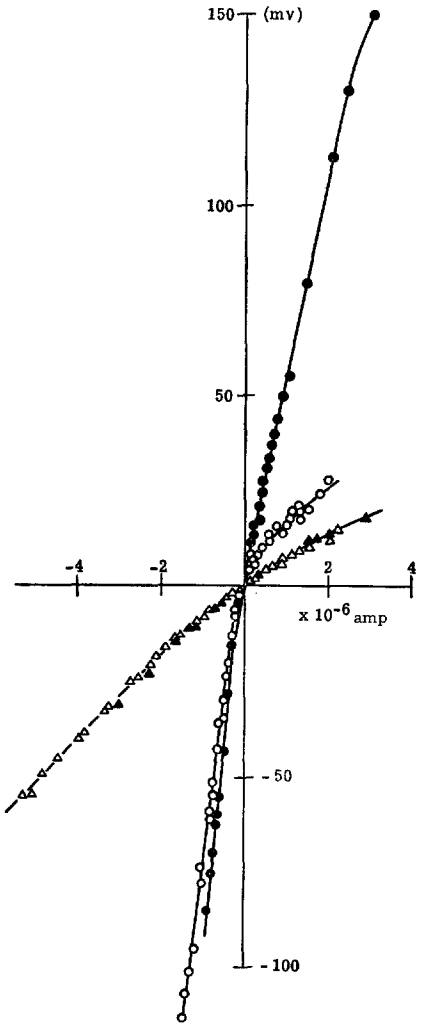

FIGURE 5. Current-voltage relations of presynaptic (circles) and postsynaptic fibers (triangles) were obtained before (open) and after (filled) TEA was injected into the presynaptic site. The depolarizations represent the steady-state conditions.

modified considerably, reflecting the effect of TEA on the excitation-secretion process of the presynaptic terminals. First of all, the postsynaptic response lost the hyperpolarizing undershoot which normally terminates the spike electrogenesis of the squid axon. This change occurred even before the presynaptic spike had been markedly prolonged or had lost its hyperpolarizing undershoot (Fig. 3 A1), indicating that the TEA injection had a marked early effect in prolonging the secretory process and hence the duration of the PSP. The PSP

became still more prolonged and larger as the presynaptic spike became more prolonged and exhibited an apparent maximum after the termination of the postsynaptic spike (Fig. 3 A2–4). The indentation in this after-depolarization caused by the PSP is probably due, however, to a manifestation of the conductance increase which results from the K activation of the spike electrogenesis. The amplitude of the peak in the PSP sometimes exceeded 30 mv, indicating that TEA injection increases the amount as well as the duration of the secretory activity in the presynaptic terminal. The prolonged PSP was associated with an increased conductance of the postsynaptic membrane, manifested both by reduction in the IR drop produced by pulses of current (Fig. 7 A) and by incomplete invasion of an antidromic spike (Fig. 7 C1, C2).

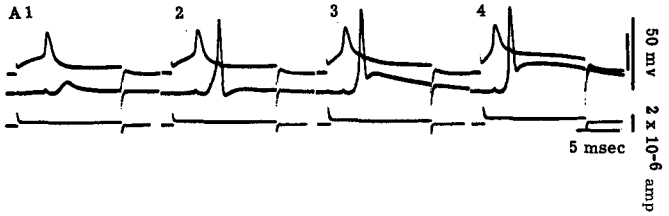

FIGURE 6. Effect of administration of high intensity TEA on presynaptic spike and PSP's. The directly evoked presynaptic spike is only slightly prolonged (upper trace), but it is now possible to produce prolonged presynaptic depolarization using direct stimulation. PSP in middle trace is increased and prolonged (A2-4) by increasing the intensity of presynaptic depolarization. Bottom trace monitors the applied current.

While the apparent maximum of the PSP occurring after the falling phase of the postsynaptic spike sometimes exceeded that of the PSP of the control response (Fig. 3) and the firing level from a second spike directly evoked by applied depolarizing current, no postsynaptic repetitive firing was seen during a prolonged large PSP. It may be surmised that the large size and prolonged PSP induced Na inactivation and K activation, both actions serving to depress spike initiation. The brief PSP's evoked by repetitive activity of the untreated presynaptic fiber did initiate repetitive response in the postsynaptic fiber (Fig. 2 C2).

When a long depolarizing pulse was applied presynaptically the duration of the PSP was markedly prolonged (see Figs. 6 A3, 4; 10 A4–6; 12 A1–3, C1). The PSP followed the time course of the depolarizing pulse, ending a few milliseconds after the pulse. On the other hand application of a depolarizing pulse of various strengths and shorter duration (about 1 msec) did not change the PSP duration significantly.

An external stimulus applied to the presynaptic fiber, however, produced a presynaptic spike of fairly constant duration and duration of the postsynaptic potential closely followed the duration of the presynaptic spike under these

conditions. The maximum duration of the presynaptic spike was more than 70 msec and the duration of the PSP was also prolonged to about the same duration (Table I). Although the correspondence of the time course of the PSP to that of the prolonged presynaptic TEA spike suggested the possibility of electrical coupling across the synapse, this did not occur. In the normal synapse electrical coupling had already been ruled out (Hagiwara and Tasaki, 1958; Takeuchi and Takeuchi, 1962; Miledi and Slater, 1966). Under control conditions transmission sufficient to elicit postsynaptic spikes could be elicited by 1/sec presynaptic stimulation. But after TEA injection, it was necessary to

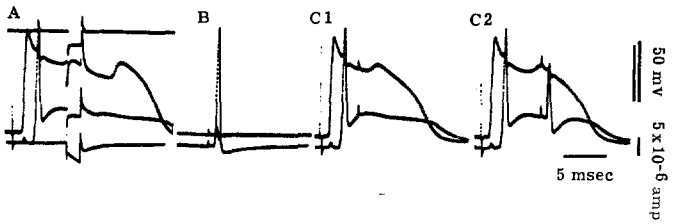

FIGURE 7. Conductance increase during prolonged postsynaptic potential. A, conductance increase of postsynaptic fiber was tested by a current pulse. Top trace monitors current; middle trace shows presynaptic response to external stimulation; bottom trace shows the membrane response of the postsynaptic fiber to applied current in the absence of stimulation and the response to the same current during the prolonged PSP in two superimposed traces. B, antidromic postsynaptic spike in synaptic region. Note absence of depolarizing after-potential. C1, failure of antidromic postsynaptic spike to invade synaptic region during a prolonged PSP. A slightly stronger stimulus was applied to the postsynaptic fiber than in B. C2, an antidromically invading postsynaptic spike during a prolonged PSP is greatly attenuated by the conductance increase of the postsynaptic membrane.

reduce the frequency of presynaptic stimulation to 1/30 sec to maintain the PSP amplitude. This may be the result of transmitter depletion due to increased transmitter release per impulse. Repetitive stimulation at frequencies greater than $\frac{1}{10}$ sec produced progressive decrease in the PSP amplitude without great change in either the presynaptic spike or the PSP duration.

Repetitive firings of shorter duration spike components superimposed on the plateau phase of the TEA spike showed slight or imperceptible effects on postsynaptic electrogenesis (Figs. 3 A4; 4 B; 7 A, C1; 8; 9; 10 A6). The small size of such effects probably results from the depression of transmitter release by background depolarization.

SYNAPTIC DELAY There was, in general, a progressive increase in synaptic delay during the course of TEA injection. In A1 to A4 of Fig. 3, synaptic delay increased from 0.7 msec to 1.0 msec. Maximum synaptic delay obtained by TEA presynaptic injection was 2.0 msec. However, a control experiment without TEA for such long periods was not carried out, and recovery

from TEA injection was not seen. In most preparations, synaptic delay progressively increases with time, finally leading to block of transmission. The relationship of TEA to the increase in synaptic delay thus remains uncertain.

The maximum rate of rise of the presynaptic spike changed only slightly during TEA injection and could not account for the progressive synaptic delay. Nor was there any correlation between synaptic delay and the duration of the presynaptic spike. The duration of the TEA spike can be reversibly lengthened by superimposing a sustained outward current, especially during the middle of the time course of TEA injection. Synaptic delay time was therefore compared using prolonged presynaptic TEA spikes and shorter presynaptic TEA spikes (Fig. 9 B1, 2) and no change in delay was observed. When the presynaptic spike became almost rectangular in shape after the injection of TEA, the PSP was evoked during the early plateau phase when the presynaptic spike was decaying with a maximum rate of 3 v/sec (Fig. 8). There is a delay

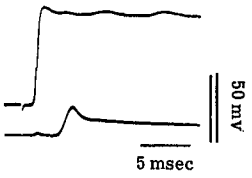

FIGURE 8. Relation between time course of an almost rectangular TEA presynaptic spike and the resulting PSP.

time of 1.8 msec from the point of maximum rate of rise and 1.3 msec from the peak of the presynaptic spike in this example. Thus, all these measurements show no obvious relation between synaptic delay and the time course of the falling phase of the presynaptic spike.

Some of the effects of TEA injection into presynaptic fibers on synaptic transmission activity are summarized in Table I.

Changes in PSP Time Course Produced by Hyperpolarizing Pulses Superimposed on the TEA Presynaptic Spike

The membrane resistance at the peak of the TEA spike was greatly reduced and then gradually recovered to nearly the resting value during later stages of the action potential plateau (Tasaki and Hagiwara, 1957; Koketsu, Cerf, and Nishi, 1959). Such a prolonged TEA spike could be shortened by superimposing a short inward current pulse during the plateau. Records A2–4 in Fig. 9 show resistance changes during the TEA spike plateau measured by the IR drop produced by inward current pulses of 1.2×10^{-6} amp for 2 msec. In A3 and A4 these pulses shortened the presynaptic spike. The relation between PSP time course and the duration of the presynaptic spike was examined using this method. When the control TEA spike (A1) was shortened (A2–4), the PSP time course was correspondingly shortened in each case. In general, the shortened PSP time course was still much longer than the time course of

TABLE I

CHANGES IN PRE- AND POSTSYNAPTIC SPIKE, RESTING POTENTIAL, AND SYNAPTIC POTENTIAL PRODUCED BY PRESYNAPTICALLY INJECTED TEA*

Preparation [No.]	Presynaptic fiber			Synaptic potential‡		Postsynaptic fiber		
		Action potential					Action potential§	
	R.P.	Amplitude	Duration	Delay	Duration‖	R.P.	Amplitude	Duration
	mv	*mv*	*msec*	*msec*	*msec*	*mv*	*mv*	*msec*
1	−63 to −61	75 to 77	1.0 to 73	0.7 to 2.0	70	−52 to −50	80 to 80	1.0 to 1.0
2	−60 to −56	103 to 87	0.8 to 20	0.7 to 1.0	26.5	−50 to −66	106 to 105	0.8 to 0.8
3	−54 to −54	87 to 84	0.6 to 2.0	0.6 to 1.1	¶	−45 to −36	84 to 70	0.6 to 1.0
4	−66 to −55	105 to 95	0.6 to 3.6	0.6 to 1.2	5.8	−64 to −55	104 to 82	0.6 to 1.1
5	−63 to −55	98 to 76	0.7 to 43	0.8 to 1.3	47	−61 to −54	105 to 100	0.9 to 0.9
Mean	−61.2 to −57.2	93.6 to 83.8	0.74 to 28.5	0.68 to 1.3	37.4	−52 to −52.2	95.8 to 87.4	0.72 to 0.96

The strength of TEA-iontophoresing current and injection time varied in each case (1–7 hr). The presynaptic action potentials were all elicited by brief external stimulation since the PSP duration can be increased by prolonged depolarization of the TEA-injected presynaptic terminal.

* Values given show change from control to maximum response after TEA.

‡ Indirect responses in all cases.

§ Antidromic spike.

‖ Only measured after TEA injection without hyperpolarization.

¶ Only responded to direct stimulation.

the PSP before TEA, and followed the time course of the presynaptic spike. Sustained depolarizations were sometimes recorded postsynaptically after abolition of a presynaptic TEA spike by anodal shocks (B3, 4).

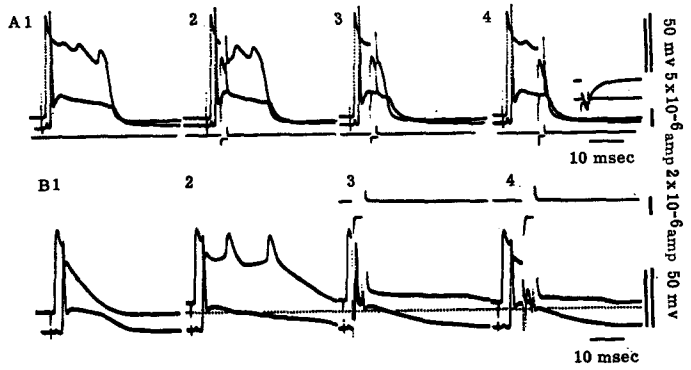

FIGURE 9. The effect on the PSP time course of shortening the presynaptic TEA spike by hyperpolarizing pulses. A1, control for this sequence. Spikes are evoked by external stimuli. Upper trace is presynaptic, middle is postsynaptic, and lower trace monitors current pulse applied presynaptically. A2-4, constant intensity (shown in A4, inset) hyperpolarizing pulse was applied at different times during the plateau of the TEA spike. B1, control response in a different preparation. B2-4, the presynaptic membrane was depolarized about 8 mv (dotted line represents resting potential) by sustained TEA current application, and a prolonged TEA spike was initiated (B2). B3-4, strong inward currents were injected during the plateau of the prolonged TEA spike with the resulting partial abolition of further response. In this case, the PSP time course could not be shortened as much as expected and the PSP time course was much slower than expected from the membrane time constant of the postsynaptic fiber. Note the sustained, small presynaptic depolarizations after abolition. Current is monitored in upper trace.

Relation between Amplitudes of Presynaptic Depolarization in TEA-Injected Prefiber and PSP

In the presence of spike electrogenesis, it was difficult to estimate the threshold membrane potential of the presynaptic terminal for measurable PSP production. No PSP was seen with depolarizations of the TEA-injected presynaptic fiber below the spike threshold. Thus, the smallest effective depolarization was that produced by the barely threshold spike. In the case of Fig. 10, this amplitude was 62 mv from the resting potential level (−55 mv). The data obtained from several TEA-injected preparations show that the minimal amplitude of the presynaptic spike for measurable PSP production was the amplitude of the resting potential. Often, however, the threshold presynaptic spike response to directly applied depolarization produced a PSP with a smaller amplitude than that following a conducted spike. A failure of complete invasion of the presynaptic terminal by the smallest direct spike may be

responsible for this difference (Hagiwara and Tasaki, 1958). Increasing presynaptic depolarization resulted in an increase in PSP amplitude and generation of a postsynaptic spike (Fig. 6).

Further increase in presynaptic depolarization resulted in further increase in PSP amplitude from the level of threshold depolarization to a depolar-

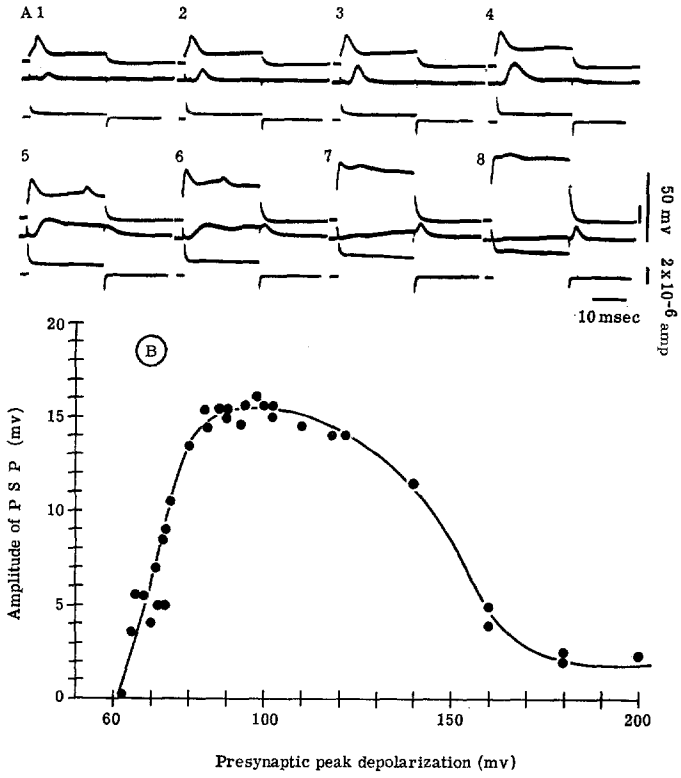

FIGURE 10. A1-8, relation between directly applied presynaptic depolarization and PSP amplitude. Top trace is the presynaptic response, middle trace is the postsynaptic response, and bottom trace monitors the current applied to the presynaptic axon. See text for discussion. B, relation between peak presynaptic depolarization (abscissa) and PSP amplitude (ordinate).

ization level of about 120 mv. In Fig. 10 B, the PSP reached its maximum amplitude when the presynaptic terminal was depolarized to about 100 mv. This level varied from preparation to preparation (80–120 mv), apparently related to the amplitude of the presynaptic spike and resting potential level. The rising portion of the curve, from 60 to 100 mv depolarization, is seen to correspond to the relationship found in the control case, using directly evoked spikes (Fig. 2 A). Beyond this maximum level, the PSP amplitude decreased and finally was completely blocked during strong depolarization (Fig. 10 A8).

The "turning-off" of transmitter release with increasing presynaptic depolarization was completely graded and must represent the mobilization of a process which qualitatively competes with the normal mechanism of transmitter release. The PSP component, however, then appeared after the cessation of depolarization (A7–8). Moreover, during the phase of graded decreasing PSP production, the break PSP added to the reduced make PSP (A6–7). After the termination of a large depolarization the break PSP was always evoked with a fairly constant latency (about 1.0 msec). The time course of this break PSP did not depend on the duration of the depolarization applied to the presynaptic terminal and it was very similar in time course to the PSP (maximum rate of rise of 19 v/sec, rise time of 1 msec, maximum rate of fall of 8 v/sec, and half decay time of 1 msec) shown in A2 and A3, in which presynaptic spikes were generated by suprathreshold stimuli.

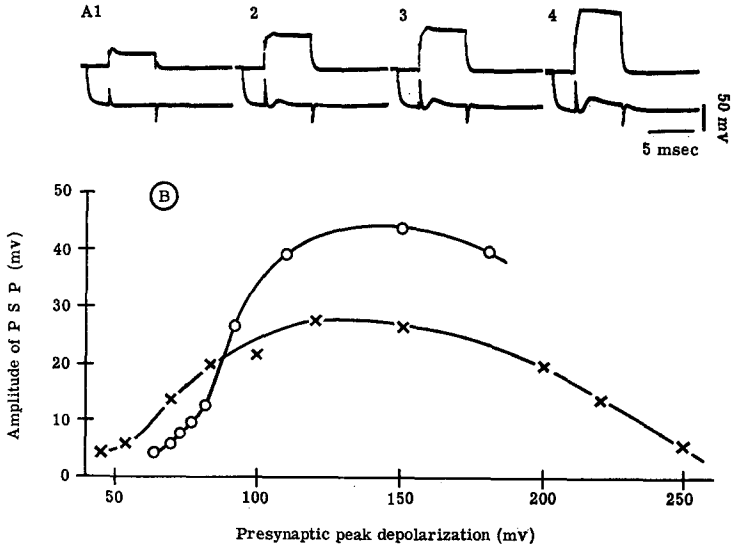

FIGURE 11. Effect of TTX (5×10^{-7} g/ml) on presynaptically injected TEA preparation. A1-4, relation between presynaptically applied depolarization (upper trace) and PSP amplitude (lower trace). PSP was measured in the 73 mv hyperpolarized postsynaptic axon. B, relation between presynaptic depolarization (abscissa) and changes in amplitude of the PSP (ordinate) before (circle) and after TTX (x) was administered. Resting potential of the presynaptic fiber after TTX administration was −54 mv.

Effect of Tetrodotoxin on Transmitter Release in the Presynaptically Injected TEA preparation

When a concentration of TTX (5×10^{-7}–10^{-6} g/ml) adequate to completely eliminate spike electrogenesis in both pre- and postsynaptic fibers was applied

externally to the presynaptically injected TEA preparation, either of two results was obtained. The first is shown in Fig. 11. In this case transmitter release could be demonstrated and the PSP time course as well as the form of the relation between applied presynaptic depolarization and PSP amplitude, was almost the same as that of the control (TEA-treated preparation). The most striking effects noted were a reduction in the threshold membrane potential of the presynaptic terminal for PSP production, accompanied by a small increase in PSP for small depolarizations (Fig. 11 B). These data clearly showed that the level of threshold membrane depolarization of the presynaptic

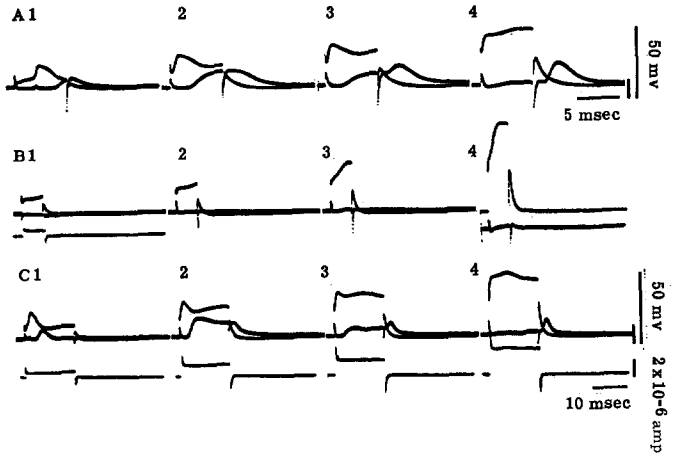

FIGURE 12. Effect of TTX (10^{-6} g/ml) on presynaptically injected TEA preparation. A1-4, control. B1-4, with TTX in the bathing medium. Both pre- and postsynaptic responses abolished. See text for discussion. C1-4, recovery from TTX after 30 min perfusion by seawater.

terminal for measurable PSP (for transmitter release) was below the zero reference level. The depression of the PSP maximum noted after TTX may not be completely the result of TTX perfusion since the decrease in amplitude of PSP in relation to repetitive strong presynaptic depolarizations was seen even in the absence of TTX.

The second result is shown in Fig. 12. With twice the dose of TTX used in the experiment of Fig. 11, the PSP was completely blocked (B1–4). This phenomenon could be reversed by washing away the TTX with seawater (C1–4). Unfortunately, the concentration of intracellular TEA could not be determined and compared in the two cases.

DISCUSSION

The foregoing experiments demonstrate three facts about the relationship between the electrical activity and the process of transmitter secretion at the pre-

synaptic terminal; (*a*) presynaptic injection of TEA prolongs the presynaptic action potential and increases the PSP amplitude and its duration; (*b*) the release of transmitter is clearly related to the event which occurs at a certain level of presynaptic depolarization, reaching a maximum and then declining with progressively greater depolarization of the terminal; (*c*) the dependence of the transmitter secretion mechanism on presynaptic polarization cannot be entirely explained by either Na or K conductance increases.

The most obvious effect of TEA injection is the prolongation of the spike. Changes in action potential configuration and other electrical characteristics obtained with TEA injected electrophoretically into a presynaptic fiber were quite similar in every respect to those found in earlier work in which TEA salt solution was directly introduced into the axoplasm of the squid giant axon (Tasaki and Hagiwara, 1957). Their study and the work of a number of other investigators studying various excitable membranes show that the main effect of TEA on the electrically excitable membrane is elimination of the K activation process. The production of prolonged spikes is in part a consequence of this elimination of K activation. Since the PSP represents a direct measure of both the transmitter released by the presynaptic terminal and the postsynaptic membrane sensitivity, one can conclude that presynaptically injected TEA increases the amount of transmitter release, at least partially, by modifying electrically excitable presynaptic axon membrane characteristics. Similar augmentation of the frog end plate potential (Koketsu, 1958; Katz and Miledi, 1967 *c*) and the excitatory postsynaptic potential in the toad motoneuron (Washizu, 1959) by extracellular TEA application has been seen. However, the present results do not discriminate between the possibilities that a prolonged PSP is a consequence of prolonged transmitter release or of some other possible effects; for example inhibition of transmitter inactivation as a direct result of presynaptic depolarization. It is also uncertain as to whether or not TEA affects the synthesis of the transmitter.

Since the presynaptic terminal is a closed axonal ending, it is possible, even in the physiological state, that there is no net inward current across the terminal during orthodromic invasion by a spike. There certainly is no likelihood of net inward current by the presynaptic depolarization after TTX application. Yet, transmitter release has been demonstrated in many preparations. Thus, inward Na current is not necessary for the release of transmitter (Ozeki, Freeman, and Grundfest, 1966).

On the other hand, the activation of K current through the terminal by depolarization might be responsible for transmitter release (Ozeki, Freeman, and Grundfest, 1966). It would then be expected that transmitter release should be diminished and delayed when evoked by the TEA spike, corresponding to the changes in K activation produced by TEA injection. This clearly is not the case. The site of application of TEA was limited by diffusion from the

orifice of the TEA microelectrode. Under these conditions it is uncertain whether or not the effects of TEA are the same at the site of transmitter release as they are a short distance away where they are actually measured. It is clear, however, that any partial effects would be in the same direction: the reduction of K activation. Since PSP is actually augmented by TEA, it would appear that K current is not responsible for transmitter release.

The completely reversible block of PSP occurring when high concentrations of TTX are applied to TEA-injected preparations raises the possibility that the activation process of the synaptic membrane may also be altered. It has been reported that after TTX depresses spike electrogenesis, with time the receptor potential of the cat Pacinian corpuscle is also diminished (Ozeki and Sato, 1965). However, no known case of TTX action on a synaptic membrane has been demonstrated. The reduction of Na and K currents has been confirmed in voltage clamp experiments in the squid giant axon with 20 mM TEA intracellularly and 400 mM TTX extracellularly.[1] Recently, it has been shown that TTX blocks not only Na current but also inward current carried by other ions including Ca and K under specific conditions (Tasaki, Singer, and Watanabe, 1966; Watanabe, Tasaki, Singer, and Lerman, 1967). It is possible that the higher concentrations of TTX exert a secondary effect on the excitation-secretion coupling process.

In view of the dependence of transmitter release on extracellular Ca levels (Feng, 1936 *a*, *b*; del Castillo and Stark, 1952; del Castillo and Engbaek, 1954; Katz, 1962; Katz and Miledi, 1965 *a*, 1966, 1967 *a*; Miledi and Slater, 1966), it might well be that transmitter release is triggered by a depolarization of a specialized terminal membrane to a level sufficient to change the membrane properties, producing an increased Ca ion permeability. The inward flow of Ca would somehow result in transmitter release. Hodgkin and Keynes (1957) have speculated that Ca ions enter the terminal membrane during the impulse and cause disruption of bound transmitter.

The nature of the mechanism inhibiting transmitter release with strong depolarizations (Figs. 10–12) would then be explained by the potential barrier to the inward movement of Ca ions down its concentration gradient that such depolarizations produce. Thus, the inward Ca ion movement just below threshold for transmitter release (42 mv in Fig. 11 B) should equal that when transmission is completely blocked by strong depolarization. If the effects of the potential barrier disappeared more rapidly following withdrawal of depolarization than did the permeability increase to Ca ions, the break transmitter release would be explained. A similar explanation has been proposed by Katz and Miledi (1966, 1967 *b*).

[1] Narahashi, T. 1966. Personal communication.

These experiments are a continuation of work done by K. Kusano jointly with S. Nakajima in 1962 and with H. Gainer in 1963 at the Laboratory of Neurophysiology, Department of Neurology, Columbia University.

We wish to express our gratitude to Dr. H. Grundfest for his advice during the experiments and his criticism of the manuscript, and to Dr. R. A. Davidoff for correcting the English in the early form of the draft.

Tetrodotoxin was kindly provided by Sankyo Company, Tokyo, Japan.

This work was supported in part by grants from the National Institutes of Health, United States Public Health Service (NB-06968-01) to K. K., Career Award (NB14, 815-02) to R. W., and (F. R. 05371) to the Institute of Psychiatric Research.

Received for publication 12 May 1967.

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