PRESYNAPTIC REGULATION OF NEUROTRANSMITTER RELEASE: A HANDBOOK

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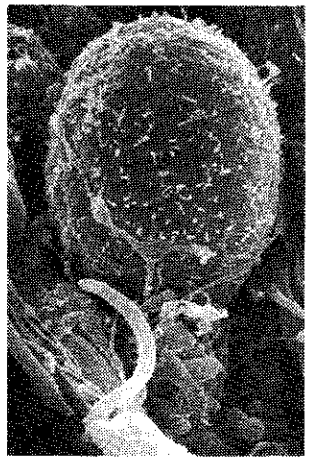
Volume I



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THE ROLE OF CALCIUM IN REGULATING NEUROTRANSMITTER RELEASE IN THE SQUID GIANT SYNAPSE

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SUMMARY

The squid giant synapse has revealed more about the regulation of transmitter release than any other central nervous synapse. This review summarizes the following topics:

1. Structure and function of the squid giant synapse.
2. Identity and postsynaptic effect of the transmitter.
3. Fatigue or depression of transmitter release.
4. Synaptic facilitation.
5. Input-output characteristic.
6. Role of calcium in transmitter release.
7. Voltage clamp of the presynaptic current.
8. Dependence of transmitter release upon calcium influx.
9. Stoichiometry of calcium action.
10. Mechanism of calcium action.
11. Voltage-dependence of transmitter release.
12. Mathematical models of calcium and transmitter release.
13. Presynaptic calcium domains and transmitter release.

1. INTRODUCTION

Fifty years ago, J.Z. Young (1936,1939) described a remarkable synaptic contact between very large neurons in the squid stellate ganglion. Its large size permits penetration with multiple microelectrodes in both the presynaptic terminal and postsynaptic cell. This enables the simultaneous recording of electrical activity on both sides of the synapse, allows voltage clamp recording of both pre- and postsynaptic membrane currents, and provides access to the presynaptic apparatus for pharmacological agents as well as substances that can be used to chemically detect presynaptic events such as calcium concentration changes. No other synapse permits such diverse experimental manipulations, especially in the presynaptic terminal. Indeed, no other synapse has provided as much information about the basic physiology of synaptic transmission in general and about transmitter release in particular.

A presynaptic giant fiber enters the stellate ganglion through its pallial nerve and terminates on each of several postsynaptic giant motor neurons which fan out in the stellate nerves to excite contraction of the body wall (Fig. 1A). At the most posterior synapse, the presynaptic terminal can be as large as 1 mm long, and 50 μm in diameter along its major elliptical axis. The postsynaptic neuron is formed from the fusion of hundreds of cells to form

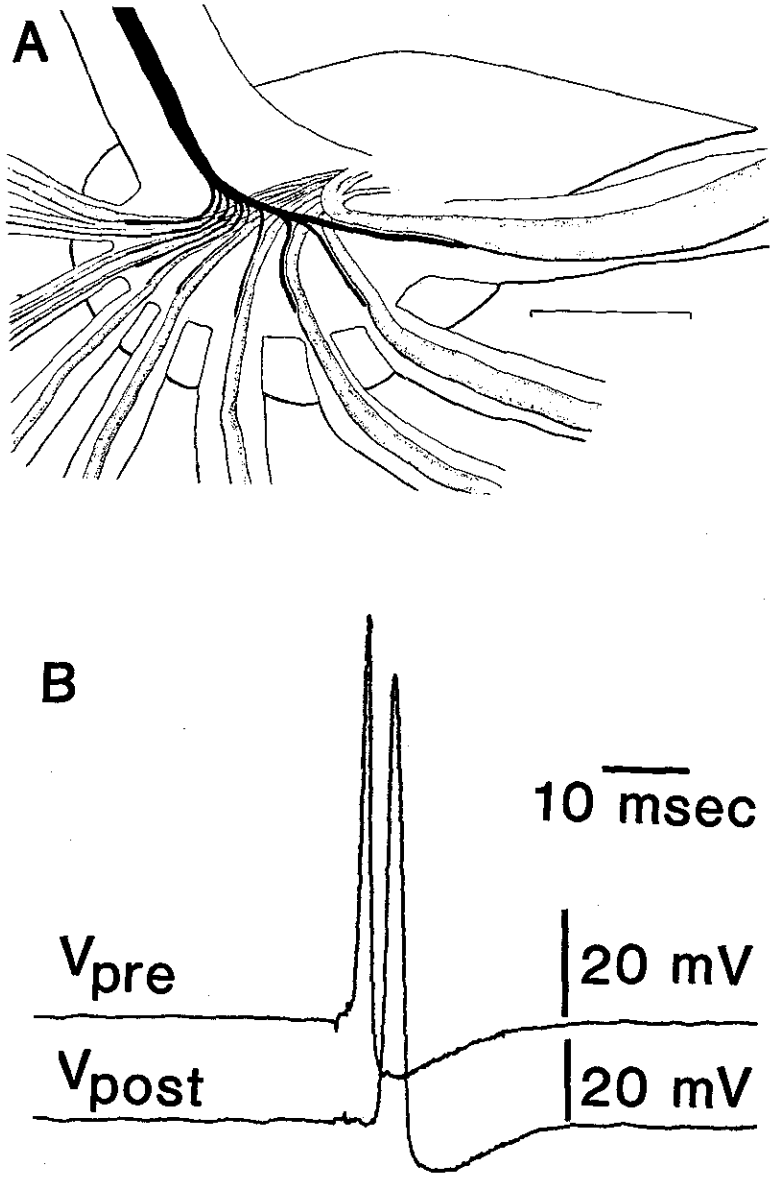


Fig. 1: A, drawing of the squid stellate ganglion. The presynaptic giant axon, shown in black, enters in the pallial nerve and makes contact with a postsynaptic giant axon, shown as stippled, exiting in each stellate nerve. Scale mark 1 mm. Adapted from Young, 1938. B, simultaneous intracellular recording from the presynaptic terminal and largest postsynaptic axon, during orthodromic transmission across the synapse.

an axon that is often 200 μm wide at the distal end of the synapse, and this axon widens to nearly 1 mm on its way to the periphery. This is the same axon made famous by voltage clamp studies of membrane currents underlying action potentials (Hodgkin et al., 1952). The giant synapse is the last central synapse in the neural circuit responsible for eliciting escape responses to a variety of sensory stimuli (Young, 1938).

Anatomical studies of this synapse show it to consist of contacts between the presynaptic terminal and about 10,000 small protrusions of the postsynaptic axon (Hama, 1962; Castejon and Villegas, 1964; Young, 1973; Martin and Miledi, 1975; Martin, 1977; Pumplin and Reese, 1978; Martin and Miledi, 1986). Each of these synaptic contact regions, or "active zones" contains a cluster of presynaptic vesicles and intramembranous particles thought to correspond to quantal units of transmission and calcium channels, respectively (Pumplin, Reese and Llinás, 1981). The postsynaptic membrane is electron dense at the active zone and contains a high density of intramembranous particles, probably corresponding to postsynaptic receptors to the neurotransmitter.

2. GENERAL PROPERTIES OF THE GIANT SYNAPSE

Bullock and Hagiwara (1957) were the first to record intracellularly from the squid giant synapse (Fig. 1B). They found transmission to be unidirectional with a synaptic delay of about 1 ms at 14°C, indicative of chemical transmission. Synaptic transmission was sensitive to anoxia and was blocked by drugs affecting transmission at other chemical synapses (Bryant, 1958), and was highly temperature-sensitive (Weight and Erulkar, 1976; Llinás et al. 1987). Hagiwara and Tasaki (1958) measured the electrical coupling across the giant synapse and found it to be negligible. They used an A.C. bridge circuit to measure postsynaptic conductance increase during the excitatory postsynaptic potential (EPSP). They also used the voltage clamp technique to measure the synaptic current underlying the EPSP. This method uses a high-gain feedback amplifier to sense deviations of the membrane potential from some desired "command potential." The feedback amplifier is used to pass current across the membrane so as to reduce these deviations to a negligible level. This current is the postsynaptic current required to hold the postsynaptic potential at the command level. Changes in synaptic conductance are reflected as changes in the synaptic current measured when the voltage clamp circuit is connected. Hagiwara and Tasaki (1958) found that this current was of an appropriate magnitude and time course to generate the

depolarizing phase of the normal EPSP. A small subsequent hyperpolarizing phase or "undershoot" (Bullock and Hagiwara, 1957) is expected from activation of the delayed rectifier by the depolarizing phase of the EPSP (Joyner and Westerfield, 1982), and is blocked by intracellular injection of tetraethylammonium ions into the postsynaptic axon (Miledi, 1969; Westerfield and Joyner, 1982).

The postsynaptic current exhibits a reversal potential (Fig. 2A) characteristic of a conductance increase to ions with an equilibrium potential of approximately zero millivolts (Hagiwara and Tasaki, 1958; Katz and Miledi, 1967). Gage and Moore (1969) measured this reversal potential at +45 mv and found it to depend in a nearly Nernstian manner on the sodium concentration. In a later study, Llinás, et al., (1974) restricted postsynaptic current to the immediate subsynaptic region with intra-axonal oil droplets, and found a reversal potential of +20 mV, similar to the estimate by Miledi (1969). Llinás et al. (1974) also found the postsynaptic conductance change to be voltage-independent. Since the EPSP reversal potential is less than the sodium equilibrium potential, potassium current is thought to contribute to the EPSP. Kusano et al., (1975) used postsynaptic injection of the calcium-sensitive photoprotein aequorin to demonstrate that calcium current also is involved in generating the EPSP.

As at other chemical synapses, transmitter release appears to be quantized in multimolecular units. Miledi (1967) and Mann and Joyner (1978) observed miniature excitatory post-synaptic potentials (MEPSPs) that were about 25 μV in amplitude in particularly small synapses. Thus a normal EPSP is composed of roughly 5000 quanta. The frequency of these MEPSPs increased with temperature, presynaptic depolarization, and with the addition of La^{3+}, as at the neuromuscular junction. When spike-evoked synaptic transmission was depressed by adding La^{3+}, or when release was elicited by small presynaptic depolarizations, the EPSP was seen to fluctuate roughly according to Poisson statistics (Fig. 2B). The quanta composing the EPSP are usually regarded as the reflection of the fusion of synaptic vesicles with the presynaptic membrane and the release of their neurotransmitter contents by exocytosis, a phenomenon described by the vesicle fusion hypothesis (see Whittaker, this volume); this hypothesis is supported by the finding that the presynaptic capacitance increases with stimulation when transmitter release is permitted by leaving calcium in the medium (Gillespie, 1979), indicating an increase in membrane area.

Glutamate (Miledi, 1967), but not acetylcholine (Stanley, 1984), depolarizes the postsynaptic membrane, and is therefore a candidate for the natural neurotransmitter. Like the EPSP, the postsynaptic response to

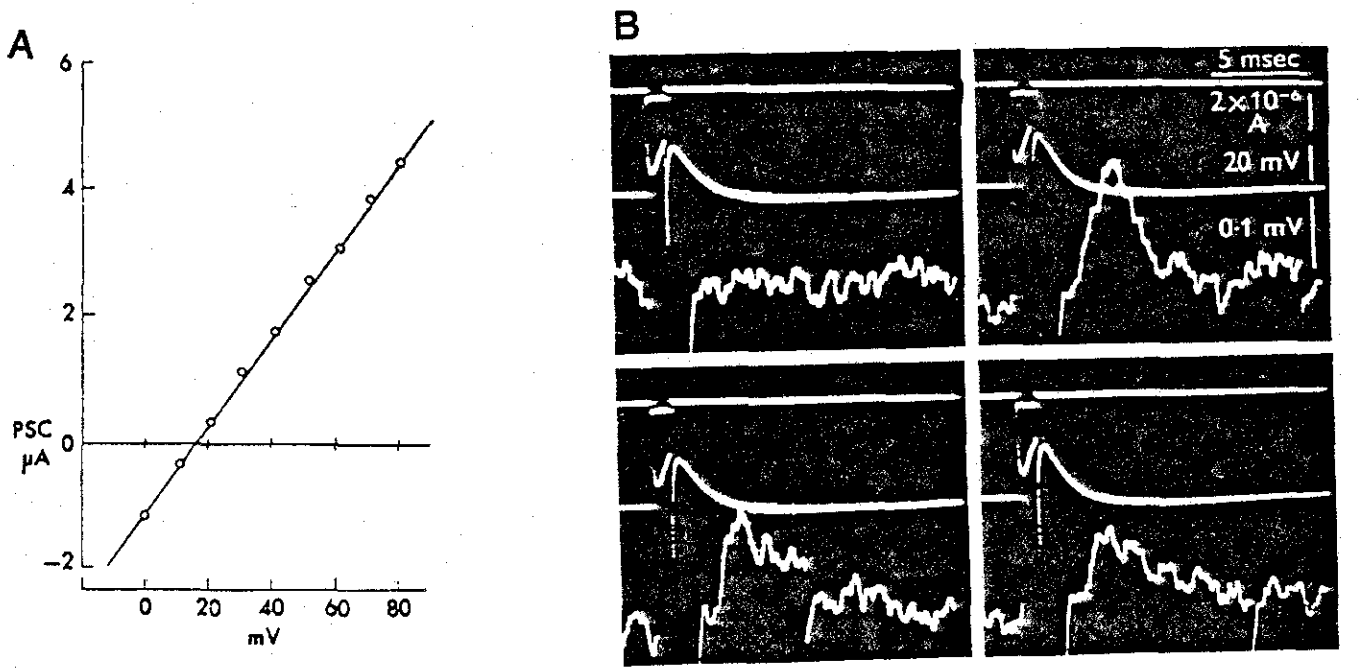


Fig. 2: A, relationship between postsynaptic current in voltage-clamped axon and postsynaptic membrane potential, showing reversal potential of +15 mV. Adapted from Linás et al., 1974. B, fluctuating postsynaptic potentials generated by small presynaptic depolarizations and recorded in a selected small postsynaptic axon. Traces from top show current amplitude, presynaptic potential, and postsynaptic potential. Failures and single quantal responses are shown. Adapted from Miledi, 1967.

glutamate is mediated by sodium, potassium, and calcium ions (Eusebi et al., 1985; Adams and Gillespie, 1988). Miledi (1969) and Adams and Gillespie (1988) reported that the reversal potential of the glutamate response was somewhat less than that for the EPSP, suggesting that glutamate may not be the transmitter, or that postsynaptic and extrasynaptic receptors are not identical. The Hill coefficient for glutamate action is 2.0 suggsting that two glutamate molecules must bind to the receptor to activate it (Adams and Gillespie, 1988). Bath-applied glutamate depresses the EPSP, perhaps due to desensitization of the receptors (Kelly and Gage, 1969). The glutamate receptor agonist kainic acid also depolarizes the postsynaptic axon, and desensitizes the synaptic response to the normal transmitter (de Santis et al., 1978). Finally, the glutamate receptor antagonist Joro spider toxin blocks the EPSP without affecting the presynaptic spike (Kawai et al., 1983; Saito et al., 1985). Thus, the bulk of the evidence still favors glutamate or a similar substance as the transmitter at this synapse.

3. SYNAPTIC DEPRESSION

In the first extracellular recordings of synaptic responses to presynaptic action potentials, Bullock (1948) reported a profound fatigue or depression of transmission to repeated activation. During a train of presynaptic action potentials, the postsynaptic EPSP got smaller and smaller until it dropped below threshold and no longer excited a postsynaptic spike. This decline in the size of the EPSP during repeated activation is called synaptic depression.

3.1. Depletion Model

Horn and Wright (1970) showed that during synaptic depression, the EPSP decrement led directly to a loss of action potential transmission across the synapse without any change in the threshold for initiating a postsynaptic spike. Erulkar and Weight (1977) showed that depression is not caused by potassium accumulation in the extracellular space following action potentials. Kusano and Landau (1975) analyzed synaptic depression in response to repeated presynaptic spikes. They found a nearly linear relationship between the percentage reduction of successive EPSPs and the total amount of transmitter released by prior EPSPs in a brief tetanus. As more transmitter was released, either by prolonging the train, raising the calcium in the medium, or stimulating the presynaptic terminal with a larger depolarization, the following EPSP was more depressed. Depression behaved as if each

impulse liberated a fraction of a releasable store, about 5%, which recovered slowly to its initial level, with a time constant of about 5 sec. In trains of more than 5 to 10 spikes, depression accumulated less than expected, as if a process of enhanced refilling or mobilization of the releasable store was triggered. Thus depression of transmission at this synapse is usually described in terms of a model of depletion and slow replenishment of a releasable store of transmitter.

3.2. Diminished Calcium Influx Model

An alternative explanation for depression is that it is due to a decline in the calcium influx to successive action potentials. The presynaptic spike itself does not change during depression (Fig. 3A) (Takeuchi and Takeuchi, 1962). Blocking spikes with tetrodotoxin and tetraethylammonium, Katz and Miledi (1967) showed that repeated constant presynaptic depolarizing current pulses still elicited EPSPs showing depression. Moreover, when the presynaptic terminal is voltage clamped, calcium currents may be recorded to brief depolarizations repeated at a frequency that leads to depression of EPSPs (Charlton et al., 1982). These calcium currents remain constant while the EPSPs decline (Fig. 3B). The increments in intracellular calcium concentration (Ca_{in}) caused by calcium influx during successive depolarizations may also be monitored by injecting the calcium-sensitive dye arsenazo III into presynaptic terminals and measuring the absorbance of the terminal at a wavelength sensitive to the formation of arsenazo-calcium complexes (Miledi and Parker, 1981; Charlton et al., 1982). These increments in Ca_{in} are also constant in response to successive action potentials leading to synaptic depression. Therefore, depression must arise at some later stage in the release process, and depletion of releasable transmitter remains the most popular hypothesis for this process.

4. SYNAPTIC FACILITATION

Superimposed on depression is the much briefer process of synaptic facilitation. Facilitation decays with fast and slow components, with time constants of about 10 and 50 ms (Charlton and Bittner, 1978a) Facilitation accumulates during a tetanus, as if successive spikes each contribute an increment to some facilitating substance or process.

Takeuchi and Takeuchi (1962) were the first to study the mechanism of facilitation at the squid giant synapse. They found that it was accompanied

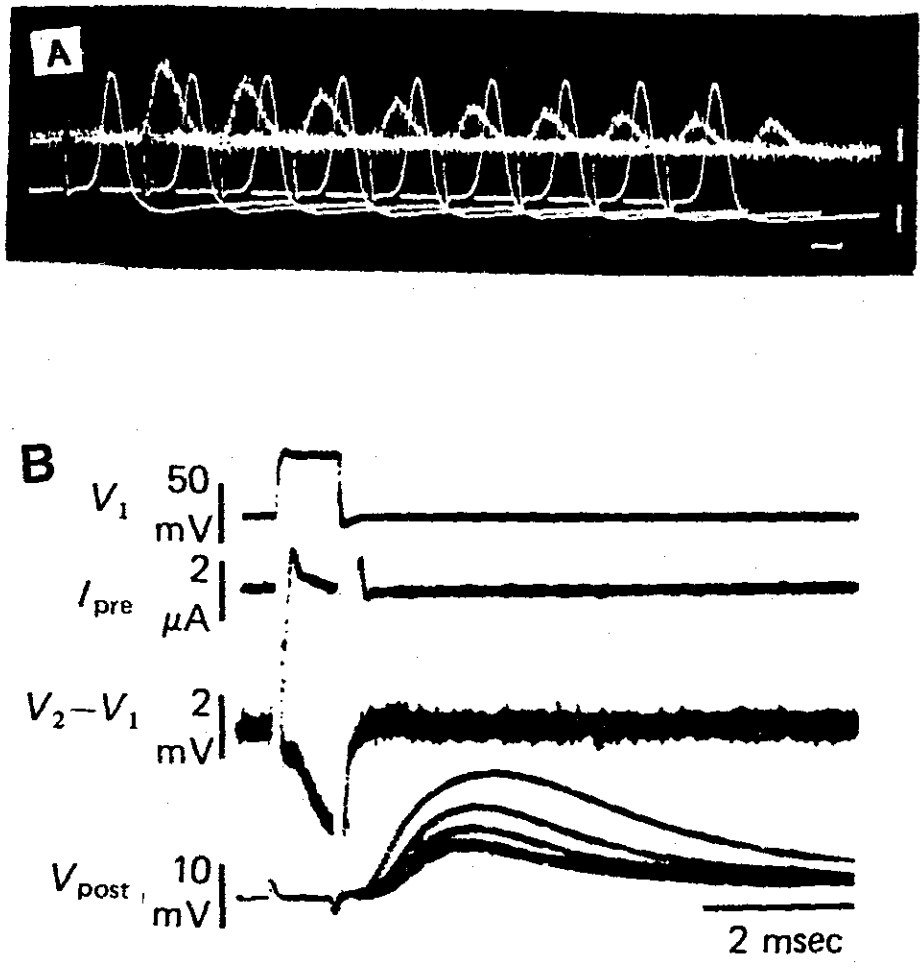


Fig. 3: A, synaptic depression elicited by repetitive stimulation at 2 Hz (superimposed records). Top trace shows postsynaptic current in voltage-clamped giant axon. Lower trace is presynaptic potential. Calibrations: 1 μA, 20 mV, 1 ms. Adapted from Takeuchi and Takeuchi, 1962. B, presynaptic depolarizing pulses repeated at 4 Hz generate EPSPs showing depression. The presynaptic calcium current remains constant. Traces from top show presynaptic potential, total presynaptic voltage-clamp current (dominated by leakage current), local presynaptic current (proportional to voltage gradient in terminal using three-electrode voltage clamp and dominated by calcium current), and postsynaptic potential. Adapted from Charlton et al., 1982.

by an increase in presynaptic spike amplitude, which they felt was the immediate cause of the phenomenon. However, Miledi and Slater (1966) found that facilitation often occurs in response to constant or even smaller presynaptic action potentials (at short intervals). Similarly, Bloedel et al., (1966) could elicit facilitating EPSPs with constant depolarizing current pulses separated by a brief interval when sodium and potassium currents were blocked pharmacologically.

Charlton and Bittner (1978b) performed additional experiments on the origin of facilitation. They found that facilitation could be observed in the absence of growth in action potential amplitude, which normally contributed at most a small component of facilitation at the beginning of a train of spikes. Since hyperpolarization increases transmitter release evoked by a spike, they considered whether facilitation could be caused by the hyperpolarizing afterpotentials following action potentials. They found that removal of the hyperpolarizing afterpotentials by injection of steady hyperpolarizing current reduced facilitation by only a small amount (Fig. 4A). Furthermore, the increase in transmitter release caused by hyperpolarization similar to the hyperpolarizing afterpotential was much less than that occurring during facilitation. Therefore, they concluded that only a small part of facilitation could be attributed to the effect of hyperpolarizing afterpotentials.

The most popular hypothesis for synaptic facilitation is the residual calcium theory of Rahamimoff (1968), Katz and Miledi (1968) and Miledi and Thies (1971). This theory proposes that a residual calcium from prior activity lingers at release sites for many milliseconds after a spike. Evidence summarized below suggests a highly nonlinear relation between Ca_{in} and transmitter release. Then residual calcium should elicit only a minimal increase in transmitter release, expressed as an increase in the frequency of the release of quanta causing miniature EPSPs (Barrett and Stevens, 1972; Zengel and Magleby, 1981; Zucker and Lara-Estrella, 1983). However, the same nonlinearity predicts that summation of residual calcium with calcium influx in subsequent spikes will generate a substantial facilitation.

A somewhat surprising finding of Charlton and Bittner (1978b) was that changing the amount of transmitter release by evoking spikes with small or large depolarizing pulses had large effects on the size of the EPSP evoked by the spike and pulse, but little effect on the size of a subsequent EPSP evoked by an orthodromic presynaptic action potential. One might expect that large changes in EPSP amplitude must reflect large changes in calcium influx to the first spike. This would cause large changes in residual calcium and large effects on facilitation. However, if transmitter release depends on calcium influx raised to a higher power, slightly different spikes triggered by different

pulse amplitudes will be accompanied by small changes in calcium influx that will cause large changes in transmitter release. The small changes in calcium influx will result in slightly different levels of residual calcium, and this will have only minimal effects on an EPSP evoked by a subsequent spike. A formal treatment of this problem appears in Zucker (1987).

Evidence that residual calcium is somehow related to facilitation is provided by the experiments of Charlton et al. (1982). They found that presynaptic injection of calcium ions facilitated transmitter release in response to action potentials that were unaffected by the procedure (Fig. 4D). This effect was only seen when transmitter release was restricted to the region into which calcium was injected by bathing the terminal in calcium-free medium and flowing external calcium over the terminal near the injection electrode. Calcium injection affects only a small portion of the terminal, and its effect is lost when the whole synapse is transmitting (Miledi and Slater, 1966; Kusano, 1970). These results implicate calcium in facilitation, but they do not indicate whether residual calcium acts only by nonlinear summation with calcium entering in an action potential, or by some other effect on the secretory machinery.

A reduction in the exponent of the power-law dependence of transmitter release on external calcium at high frequencies of stimulation has also been explained in terms of the residual calcium hypothesis (Stanley, 1986), where some calcium binding sites become saturated with elevated residual calcium as frequency increases. However, the results could also be explained as effects of synaptic depression and of saturation of the release mechanism.

Charlton et al. (1982) considered the possibility that facilitation is caused by an increase in the change in Ca_{in} at transmitter release sites. They found that presynaptic calcium currents obtained by voltage-clamping the terminal remained constant during repeated brief depolarizations that elicited facilitating EPSPs (Fig. 4B). When calcium concentration changes in the terminal were measured by arsenazo spectrophotometry, these also remained constant while EPSPs facilitated (Fig. 4C). Since the rising phase of the arsenazo signal occurs during the millisecond long depolarization, this must arise from Ca_{in} before it has had time to diffuse away from sites where transmitter is released. The arsenazo signal may not reflect the true time course in the calcium concentration change at release sites, because of the kinetics of the calcium binding to arsenazo (Fogelson and Zucker, 1985; but see Baylor et al., 1982). Nevertheless, changes in the calcium increment would still be detected as changes in the arsenazo absorbance signal. Thus the increments of calcium concentration at release sites to successive spike-like depolarizations

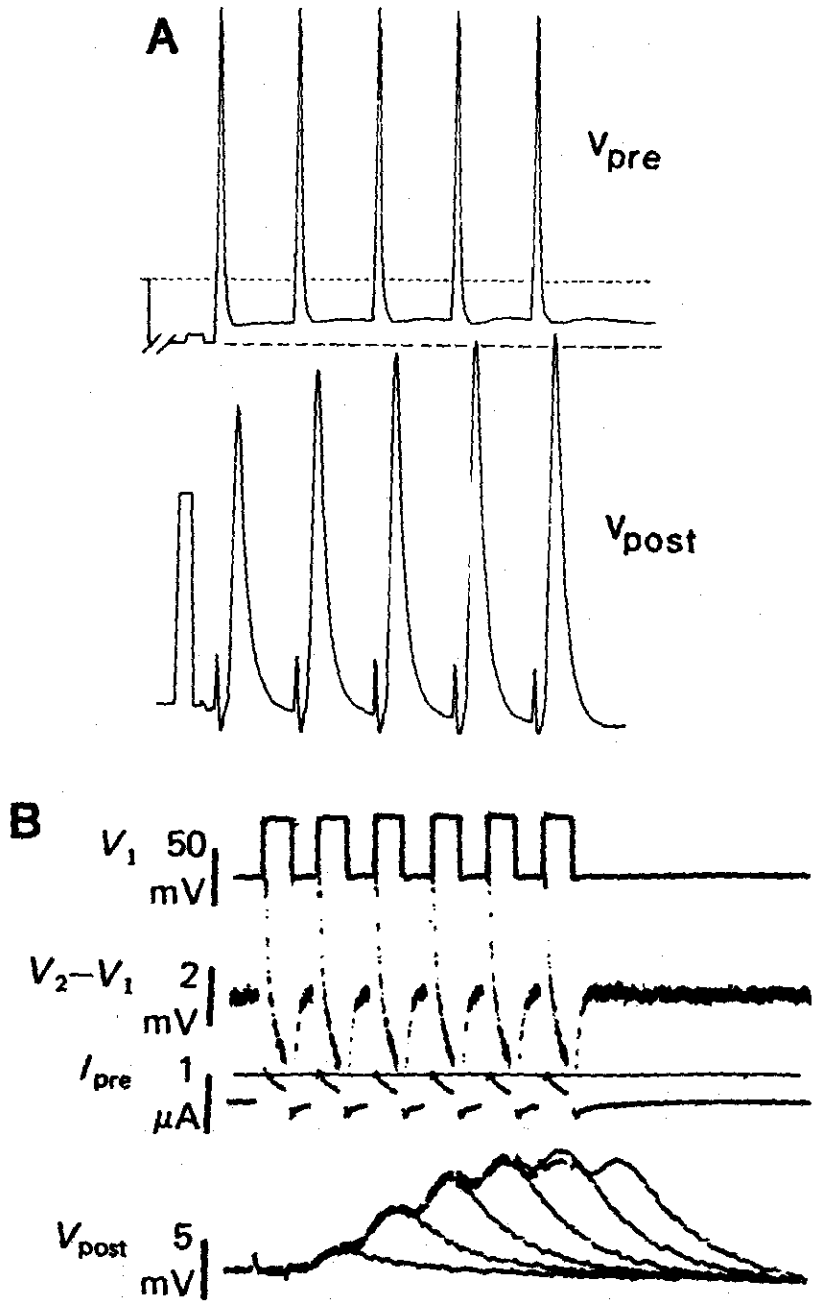
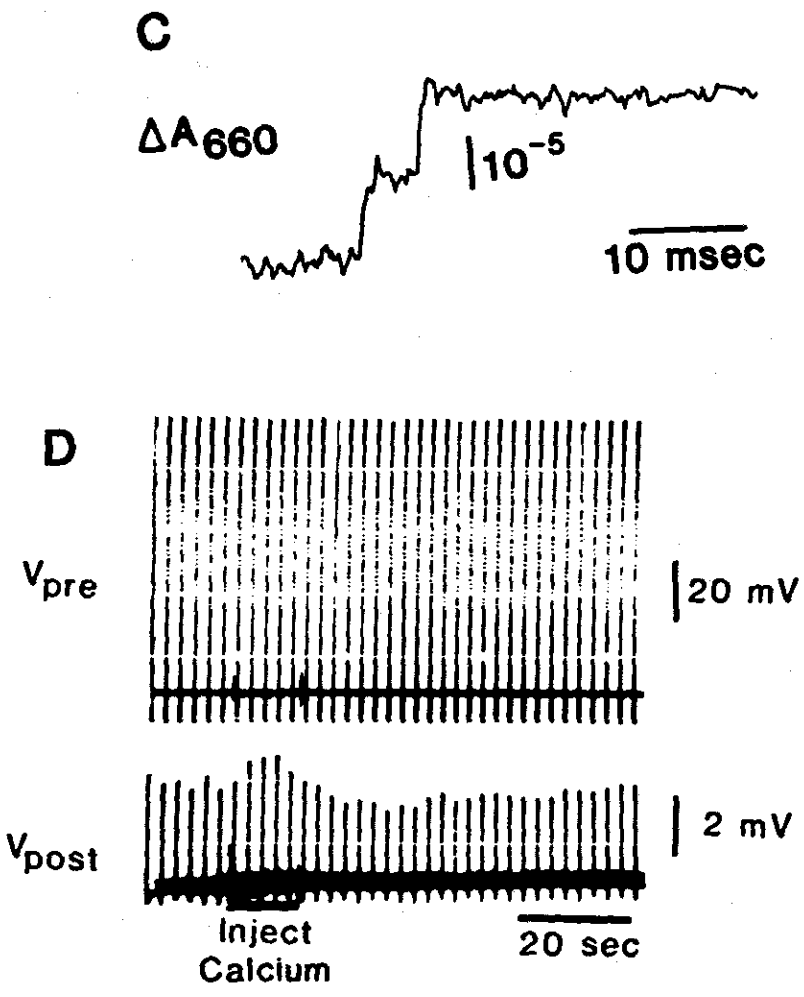


Fig. 4: A, synaptic facilitation produced by presynaptic action potentials repeated at 100 Hz, using a low-calcium medium to reduce depression. The presynaptic



spikes became slightly smaller with repetition, and the hyperpolarizing afterpotentials were abolished by hyperpolarizing the terminal from its resting level (dotted line) to the level of the dashed line. The square calibration pulse on both records is 2 mV and 2 ms. Adapted from Charlton and Bittner, 1978b. B, calcium currents in the presynaptic terminal, seen best in the local current trace, are constant while the EPSPs grow during synaptic facilitation. A thin line is drawn through the peak calcium current in each pulse. Trace identification is the same as in Fig. 3B. C, the increments in presynaptic calcium concentration to two presynaptic action potentials separated by 7 ms are identical, as measured by arsenazo spectrophotometry. The absorbance calibration corresponds roughly to 50 nM calcium. D, injection of calcium into the presynaptic terminal facilitates spike-evoked transmitter release, if release is restricted to the region of the terminal where calcium is injected. B to D adapted from Charlton et al., 1982.

appear to be constant, and facilitation cannot arise from increases in these increments.

Another process affecting transmitter release at many synapses is potentiation or post-tetanic potentiation. This gradual increase in release evoked by a large number of action potentials, which decays slowly after a tetanus, is not observed at the squid giant synapse, probably because it is impossible to discern in the presence of strong depression. At other synapses potentiation has been attributed partly to sodium accumulation in the presynaptic terminals (Atwood et al., 1975; Rahamimoff et al., 1980). An interesting finding in this regard is that the presynaptic injection of sodium potentiates release elicited by spikes at the squid giant synapse, even though the spikes are smaller because of the reduced trans-membrane sodium gradient (Charlton and Atwood, 1977a). Elevating internal sodium shifted the input-output curve to lower presynaptic potentials, as if the sensitivity of transmitter release to calcium influx or the calcium influx itself increased. It is also possible that sodium injection raises resting Ca_{in} making potentiation operate much like facilitation. Recent evidence from neuromuscular junctions indicates that post-tetanic potentiation is accompanied by a rise in presynaptic Ca_{in} (Delaney et al., 1989).

5. TRANSMISSION WITH AND WITHOUT SPIKES: INPUT-OUTPUT CHARACTERISTIC

A small reduction in the amplitude of the presynaptic action potential, caused by mild depolarization of the presynaptic terminal, is accompanied by a large reduction in the size of the EPSP; mild presynaptic hyperpolarization has the opposite effect. This was noted even in the earliest studies on the squid giant synapse using intracellular electrodes (Bullock and Hagiwara, 1957; Hagiwara and Tasaki, 1958; Takeuchi and Takeuchi, 1962; Miledi and Slater, 1966; Kusano et al., 1967). EPSP amplitude depended nearly exponentially on spike amplitude, with a 30 mV increase in spike amplitude causing a ten-fold increase in EPSP.

Takeuchi and Takeuchi (1962) and Kusano et al. (1967) tried to distinguish whether the size of the EPSP was determined by the peak level of depolarization reached by the action potential or by the difference between the peak and resting levels (amplitude) of the presynaptic spike. Tonic depolarization decreases both peak and amplitude of orthodromic spike, as well as EPSP size, so this procedure does not distinguish between the possibilities. Spikes triggered directly by brief depolarizations reach a higher

peak with larger pulses, and the EPSP also increases, indicating that the peak presynaptic spike depolarization is important. However, tonic hyperpolarization leads to spikes of greater amplitude whose peak level may be the same or even slightly reduced, yet the EPSPs still grow, indicating that the amplitude of the spike is also important.

An important advance in the study of synaptic transmission was brought about by the dissociation of transmitter release from the movements of sodium and potassium ions underlying the presynaptic action potential. Kusano (1968) described postsynaptic responses to slowly increasing presynaptic current that avoided an action potential by relying on threshold accommodation to ramp depolarizations. However, only the discovery of agents that block sodium and potassium channels allowed synaptic transmission to be totally dissociated from spike electrogenesis. Using tetrodotoxin (TTX) in the bath to block voltage-dependent sodium current, and intracellular injection of tetraethylammonium (TEA) to block presynaptic delayed rectification due to potassium channels, a number of workers were able to study the dependence of EPSP amplitude on presynaptic potential (Bloedel et al., 1966; Katz and Miledi, 1966, 1967; Gage, 1967; Kusano et al., 1967; Kusano, 1968). Transmitter release was again found to be very sensitive to the level of peak depolarization, with a dependence similar to that seen earlier with action potentials of different amplitude. When care was taken to place presynaptic electrodes directly adjacent to the synaptic contact zone and particularly healthy preparations were used, the sensitivity was found to reach a ten-fold increase in PSP amplitude per 10 mV depolarization (Fig. 5) (Katz and Miledi, 1966, 1967; Kusano, 1968). With well placed electrodes and the presynaptic space constant prolonged by TEA, transmitter release could be stimulated with a depolarization of only 10 mV (Kusano, 1968). More recently, Charlton and Atwood (1977b) were able to elicit transmitter release with depolarizations of only a few mV even without the use of TEA.

6. THE ROLE OF CALCIUM IN TRANSMITTER RELEASE

6.1. External Calcium Required for Transmitter Release

Much recent effort has focussed on the role calcium plays in transmitter release, as reviewed by Augustine et al. (1987). Early workers (Bryant, 1958; Takeuchi and Takeuchi, 1962) observed that raising Ca_o increased synaptic transmission, and magnesium had the opposite effet. Miledi and Slater (1966) found that external calcium was essential for transmission, and that calcium

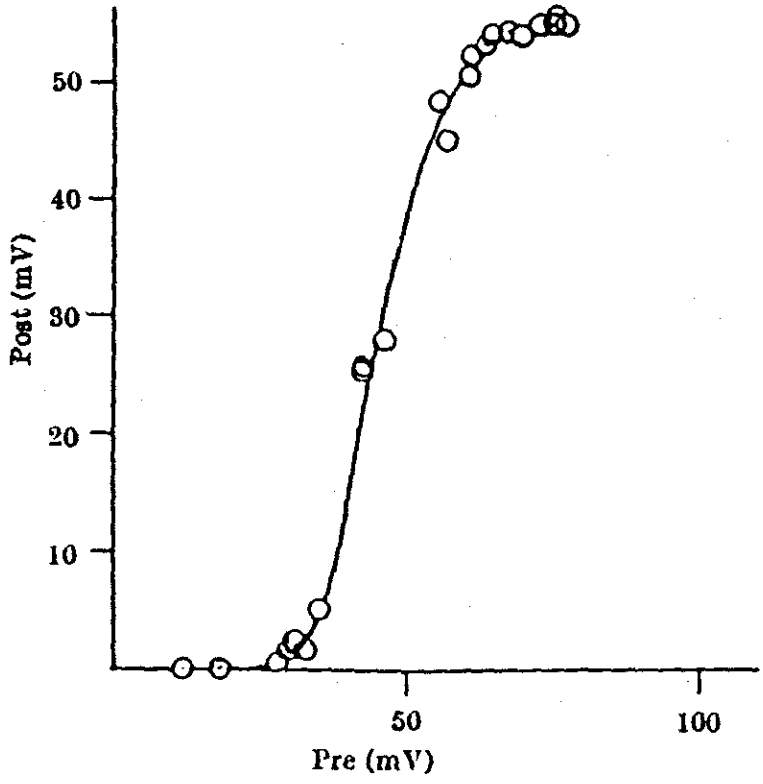


Fig. 5: Input-output curve relating postsynaptic responses to presynaptic potential, when the terminal is depolarized with 1 ms pulses and spikes are blocked with tetrodotoxin. Adapted from Katz and Miledi, 1966.

had to be present outside the nerve terminal at the moment of the presynaptic action potential. Lester (1970) and Katz and Miledi (1970) reported a highly nonlinear relationship between transmitter release and Ca_o, with release following up to the fourth power of Ca_o. In studies of transmission without action potentials, Katz and Miledi (1967) and Kusano (1970) found that increasing Ca_o shifted the input-output curve such that a given depolarization was more effective in releasing transmitter. Barium and strontium substituted only weakly for calcium in supporting transmission (Katz and Miledi, 1969a), where magnesium, manganese, and lanthanum antagonized release (Miledi, 1971).

6.2. Site of Action of Calcium

An important question was: Where was calcium acting to support transmitter release, externally or inside the presynaptic terminal? Early attempts at injecting calcium presynaptically did not restore transmission in a calcium-free medium (Miledi and Slater, 1966), and sometimes even depressed transmission when external calcium was present (Kusano, 1970). This was initially regarded as contradicting the idea that calcium might act at an intracellular site, although it was recognized that calcium might be rapidly sequestered and might reach only a small portion of the presynaptic apparatus.

One indication that calcium was acting internally to release transmitter was the existance of an off-EPSP (Katz and Miledi, 1967; Kusano et al., 1967; Kusano, 1968). When spikes were blocked and the terminal depolarized to a level approaching the calcium equilibrium potential, transmitter release was depressed during the pulse, and occurred only at the cessation of the depolarization (Fig. 6A). This peculiar result was explained as reflecting a dual action of depolarization: the increased opening of calcium channels as the membrane was depolarized up to about 0 mV, followed by the retardation of calcium entry as the calcium equilibrium potential was approached with further depolarization. Then calcium influx would occur only after the pulse, when the driving force for calcium influx was again increased and before the calcium channels could close. It might be objected that the reduction of pulse-evoked transmitter release following prior depolarization indicates that calcium channels inactivate, in which case they would not open at the end of a long large depolarization. However, Katz and Miledi (1971) showed that such an effect of prior depolarization on subsequently evoked release was due to potassium activation and was blocked by TEA injection.

The discovery of action potentials that were supported by calcium influx into the presynaptic terminals when sodium and potassium currents were blocked (Katz and Miledi, 1969b) provided further evidence for presynaptic calcium channels and an intracellular site of calcium action. The demonstration that calcium injection could directly evoke the release of quanta (Miledi, 1973) also supported an intracellular locus of action. Finally, a rise in Ca_{in} in the presynaptic terminal during action potentials was demonstrated directly, using at first the photoprotein aequorin (Llinás, Blinks and Nicholson, 1972), then the metallochromic indicator arsenazo III (Miledi and Parker, 1981; Charlton et al., 1982), and finally calcium-selective microelectrodes (Deitmer et al., 1983). Like transmitter release, calcium influx could be suppressed

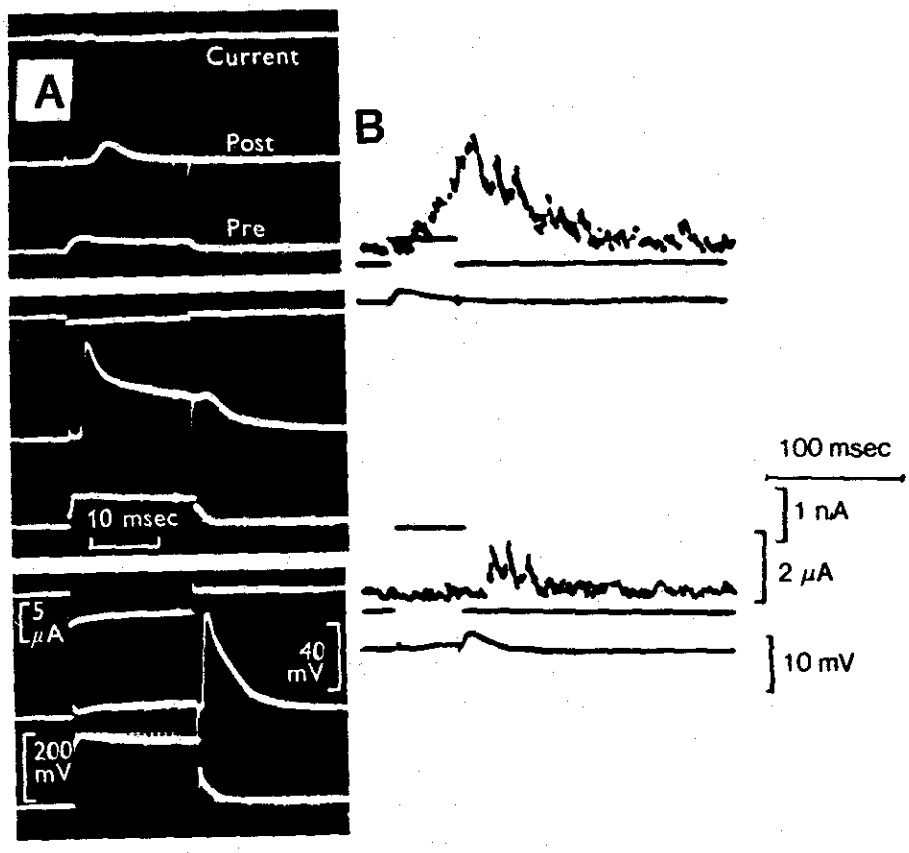


Fig. 6: A, postsynaptic responses to presynaptic depolarizing currents of increasing amplitude when sodium and potassium conductance are blocked with TTX and TEA. Large pulses suppress transmitter release until the end of the pulse (off-EPSP). Adapted from Katz and Miledi, 1967, B, postsynaptic responses and presynaptic calcium concentration changes detected with the photoprotein aequorin to small and large depolarizing pulses. Traces from top show aequorin luminescence monitored as current in a photomultiplier tube, presynaptic current used to depolarize the terminal, and postsynaptic potential. Adapted from Llinás and Nicholson, 1975.

until the end of a large depolarization (Fig. 6B) (Llinás and Nicholson, 1975). These intracellular calcium transients were confined to the presynaptic terminal itself (Llinás, 1984), with no detectable influx occurring in the main body of the presynaptic axon. Recently, Smith and Augustine (1988), and Smith et al. (1988) have confirmed this finding using the fluorescent calcium indicator fura-2. They have shown additionally that the calcium influx is confined to the synaptic surface in contact with the postsynaptic axon, further restricting the location of the calcium channels to near sites of transmitter release.

Katz and Miledi (1969b) have suggested that, in addition to triggering transmitter release, prolonged presynaptic calcium accumulation may secondarily block or inactivate release. Recently, Adams et al., (1985) have found that mitochondrial inhibitors and agents blocking calcium extrusion reduce spike-evoked transmission. Since the effects were partially reversed by EGTA injection to buffer calcium, the results were attributed to an inhibitory action of presynaptic calcium on transmitter release. To what extent this is related to a depletion of transmitter stores due to continuous transmitter release, or to a reduction in calcium influx during spikes, remains to be determined.

7. VOLTAGE CLAMP OF PRESYNAPTIC CALCIUM CURRENT

The report by Llinás et al., (1976a) that the presynaptic calcium current could be measured by voltage clamping the terminal began a methodological revolution in the study of the function of this synapse. Now it was really possible to relate transmitter release directly to the influx of calcium into the presynaptic terminal. The first step was to characterize the dependence of calcium current on membrane potential, when sodium current was blocked with TTX and potassium current was blocked with 4-aminopyridine (Llinás et al., 1976) and sometimes also with intracellular injection of TEA. Calcium currents were initially recorded as total membrane currents using the two-electrode voltage clamp. These currents included a large leak current into the presynaptic axon which had to be subtracted. In later experiments (Llinás, 1977; Llinás et al., 1981a), the calcium current flowing through the well-controlled terminal region was measured using the three-electrode voltage clamp technique developed for short fibers (Adrian, Chandler and Hodgkin, 1970). Calcium influx was seen to increase sigmoidally in time during a depolarizing pulse (Fig. 7A). At the end of the pulse, the sudden increase in driving force caused a precipitous increase in calcium influx, which decayed rapidly as the calcium channels closed. Large depolarizations

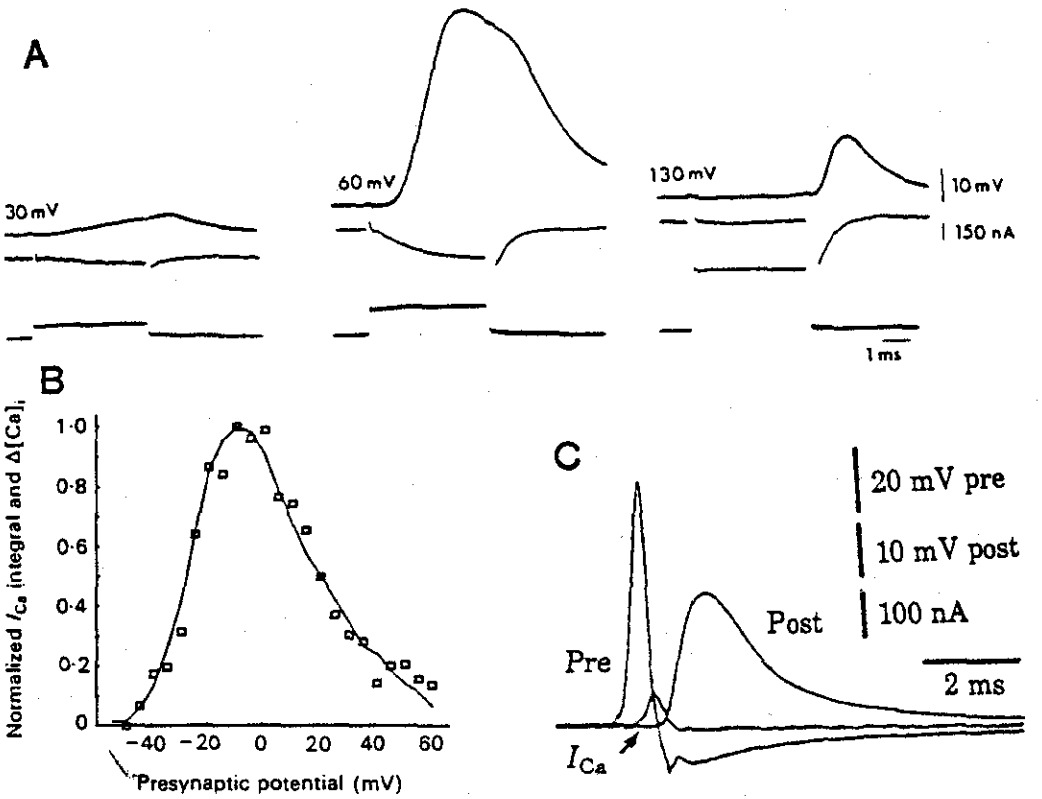


Fig. 7: A, postsynaptic responses (top trace) and presynaptic calcium currents (middle trace) to presynaptic depolarizations (bottom trace) under voltage clamp. Numerals indicate amplitude of depolarization. Adapted from Llinás et al., 1981b. B, integrated calcium current (curve) and calcium concentration changes measured with arsenazo (symbols) to 6-ms depolarizations of varying amplitude. Adapted from Augustine et al., 1985b. C, voltage clamp of the presynaptic terminal with an action potential waveform, with the accompanying presynaptic calcium current and postsynaptic response. Adapted from Llinás et al., 1982.

approaching the calcium equilibrium potential suppressed calcium influx during the pulse, but were still followed by a large tail current. This behavior is similar to that of the activation of sodium current, and could be described by a mathematical formulation (Llinás et al., 1976a, 1981a; Llinás, 1977) similar to that used by Hodgkin and Huxley (1952) to describe gating of the sodium channels in the postsynaptic giant axon.

The properties of activated calcium current through open channels were also studied. Calcium influx was seen to level off as the membrane potential was hyperpolarized below the resting potential. Calcium influx was approximately linear with respect to Ca_o, and tended to saturate at high calcium levels. This latter result is important because it shows that the nonlinear relation between transmitter release and Ca_o does not arise from the dependence of calcium influx upon concentration. To account for these results, a single energy barrier model of the calcium conductance was employed. Unlike sodium current, calcium current did not inactivate appreciably during brief depolarizations, although a slow calcium-dependent inactivation, that would be negligible in a normal action potential, has been observed in presynaptic calcium currents (Augustine et al., 1981; Augustine and Eckert, 1984a).

Augustine et al., (1985a) have described a different method for measuring calcium influx into the presynaptic terminal under voltage clamp. In addition to measuring calcium current, they recorded the absorbance changes due to the formation of calcium-arsenazo III complexes when the dye was injected into the terminal. The absorbance signal, which reports the total amount of calcium entering the cell and reacting with arsenazo, was similar to the time-integral of the calcium current in its voltage-dependence (Fig. 7B).

8. DEPENDENCE OF TRANSMITTER RELEASE UPON CALCIUM INFLUX

8.1. Presynaptic Depolarization, Calcium Influx and Transmitter Release

One of the most exciting outcomes of these voltage clamp experiments is the ability to relate postsynaptic responses to presynaptic calcium currents (Llinás et al., 1981b). Accordingly a close correspondance was found between the time- and voltage-dependence of both presynaptic calcium influx and postsynaptic response (Fig. 7A). The steep rise in EPSP with small depolarizations, the suppression of transmitter release at large potentials, and the development of an off-EPSP, were all seen to reflect the behavior of the

presynaptic calcium current and tail current. These results provide the strongest evidence that transmitter release is triggered directly by a rise in Ca_{in}. Brief depolarizations elicit a larger calcium influx at the end of the pulse than during the pulse. Thus transmitter release is activated primarily by the calcium influx in the tail current. Peak depolarization increases calcium influx by opening more calcium channels, while increasing the resting potential increases the calcium influx in the tail current by increasing the driving force. Therefore, the dependence of transmitter release on both action potential peak and amplitude is expected. By clamping the presynaptic terminal with a recorded waveform of an action potential (Llinás, Sugimori and Simon, 1982), the exact dependence of transmission on spike duration and amplitude could be observed. The EPSP was the same as for a real action potential (Fig. 7C), further dissociating transmitter release from the sodium and potassium currents underlying action potential generation. Transmission was confirmed to depend mainly on the calcium influx occurring during the falling phase of the spike.

During moderately long (40 ms) depolarizations, transmitter release declined, while calcium current was maintained. Thus the drop in transmission seen previously to long pulses (Fig. 6A) (Katz and Miledi, 1967; Kusano et al., 1967; Kusano, 1968) must be attributed to some fatigue in the release process. This is presumably related to synaptic depression to repeated spikes, and perhaps due to depletion of an immediately releasable store of presynaptic vesicles.

An interesting result of these experiments was the discovery (Llinás et al., 1976a, 1981b; Llinás, 1977) that transmitter release followed the activation of calcium current during a pulse by about 200-400 μs at 18°C (Fig. 7A). The off-EPSP occurring at the end of the pulse when calcium channels are already activated was also about 200 μs. Thus the normal synaptic delay consists of an initial component due to the activation of calcium current, and a second component reflecting the action of calcium on the release process. Both components are highly temperature-sensitive (Charlton and Atwood, 1979).

8.2. Transmitter Release and Other Divalent Cations

Voltage clamp of the presynaptic terminal was also used to study synaptic transmission when other divalent cations replaced calcium (Augustine and Eckert, 1984b). As with spike-evoked release (Katz and Miledi, 1969a), strontium and barium were marginally effective in supporting release. Transmission was reduced to about 1% when strontium replaced calcium, and to about 0.1% in barium medium. The presynaptic currents carried by these

ions were very similar, with calcium and strontium currents indistinguishable in amplitude and time course, and barium currents about 20% larger. Since miniature EPSPs were unaffected by ion substitution, the results reflect differences in the ability of different ions to initiate neurosecretion once they enter the terminal.

9. STOICHIOMETRY OF CALCIUM ACTION

One puzzling result of the initial voltage-clamp experiments was a nearly linear relationship between postsynaptic response and presynaptic calcium current when using different amplitudes of depolarization (Llinás et al., 1976a, 1981b, 1982; Llinás, 1977). Moreover, transmitter release for a given depolarizing pulse was reported to depend weakly on Ca_o (Llinas et al., 1981b). These results were quite surprising, in view of the finding that spike-evoked release depends on Ca_o raised to a power of about 3 or 4 (Fig. 8A) (Lester, 1970; Katz and Miledi, 1970).

Recent improvements in experimental methodology have helped to reconcile this discrepancy. Thus, Augustine et al. (1985a) discovered that calcium influx into the presynaptic terminal is not spatially uniform. Using localized arsenazo spectrophotometry, they found that the proximal part of the synapse is less depolarized than the distal terminal, resulting in a nonuniform rate of neurosecretion, which is particularly serious for large depolarizations. With increasing depolarization, the calcium equilibrium potential is approached distally and distal transmitter release declines, while attenuated depolarization proximally is still opening more calcium channels and releasing more transmitter proximally. This complication severely distorts the relationship between transmitter release and calcium current. This problem was solved by using an external calcium pipette in a calcium-free medium to restrict calcium influx and transmitter release to the distal portion of terminal, whose voltage was well controlled by the clamp configuration. Saturation of the postsynaptic response, as the postsynaptic equilibrium potential was approached by large EPSPs, was avoided by voltage-clamping the postsynaptic axon at the synapse and recording postsynaptic current. Synaptic depression during a depolarization was minimized by measuring presynaptic calcium current and postsynaptic current before its peak and subsequent decline due to depression. This also eliminated the tail current and off-EPSP from consideration. These refinements revealed calcium currents similar to those recorded previously (Llinás et al., 1987), but restricted transmitter release to the well clamped terminal region. This also

enabled the observation of a power-law relationship with exponent about 3 (Fig. 8B) between postsynaptic response and calcium current for different size depolarizations (Smith et al., 1985; Augustine et al., 1985b; Augustine et al., 1988b).

Similar methodological refinements were used to study the relationship between transmitter release to a depolarizing pulse and Ca_o (Augustine and Charlton, 1986). The external calcium pipette cannot be used to alter the Ca_o between several levels in one preparation. Instead, this was done by combining arterial perfusion (Stanley and Adelman, 1984) and bath perfusion to rapidly and accurately change the calcium level. Presynaptic calcium influx was found to depend linearly upon Ca_o, saturating at high levels (Fig. 8C). On the other hand, a power-law relationship with exponent up to 4 was observed between postsynaptic current and Ca_o. A similar relationship was found between postsynaptic current and presynaptic calcium current for different size pulses at low levels of external calcium using the same techniques. Thus all indications pointed to a high-power-law relationship between transmitter release and calcium influx. Since Ca_{in} changes measured spectrophotometrically are directly related to calcium current (Augustine et al., 1985a), the nonlinearity must reside in the activation of transmitter release by Ca_{in}.

Another possible indication of a high stoichiometry of calcium action is a stepwise increase in facilitated transmitter release as spike frequency increases (Stanley, 1986). This might indicate the progressive saturation of multiple calcium binding sites involved in transmitter release. However, the fact that facilitation accumulates with several apparent kinetic components may also reflect the existence of several calcium removal processes in the presynaptic terminal, and the fact that diffusion of calcium away from release sites is not a first order process (Fogelson and Zucker, 1985).

10. MECHANISM OF CALCIUM ACTION

Exactly how multiple calcium ions elicit transmitter release remains a mystery. It is usually assumed that calcium binds with a protein on the plasmalemma or the vesicle membrane to initiate exocytosis. Calcium binding sites have been observed ultrastructurally at both sites in the squid giant synapse (Hillman and Llinás, 1974). Since calmodulin binds four calcium ions, it is often regarded as a likely calcium receptor in neurotransmission (Reichardt and Kelly, 1983), but the evidence for its involvement is equivocal at best (Publicover, 1985).

Recent evidence from the squid giant synapse indicates that calcium is

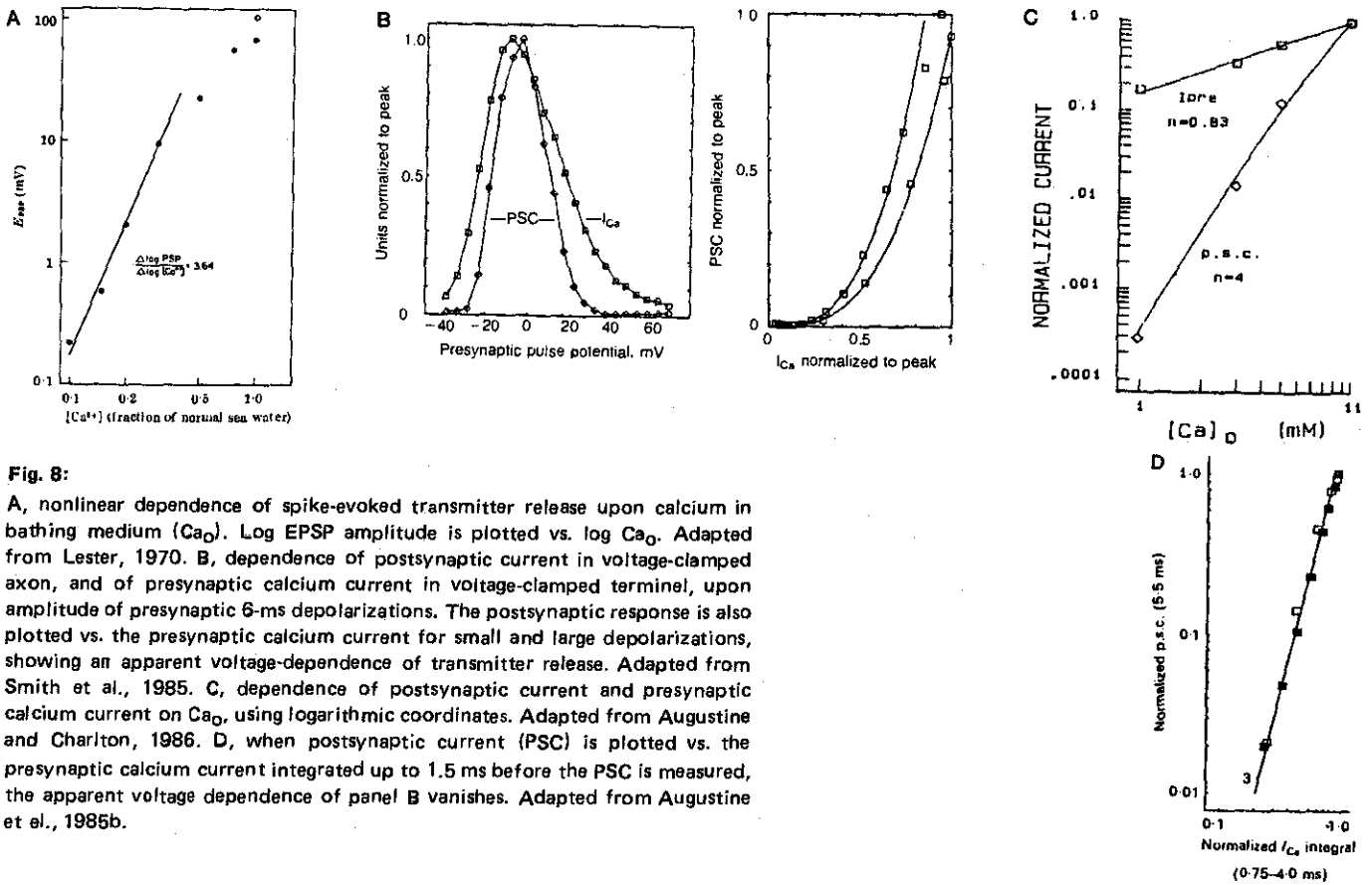


Fig. 8:

A, nonlinear dependence of spike-evoked transmitter release upon calcium in bathing medium (Ca_o). Log EPSP amplitude is plotted vs. log Ca_o. Adapted from Lester, 1970. B, dependence of postsynaptic current in voltage-clamped axon, and of presynaptic calcium current in voltage-clamped terminal, upon amplitude of presynaptic 6-ms depolarizations. The postsynaptic response is also plotted vs. the presynaptic calcium current for small and large depolarizations, showing an apparent voltage-dependence of transmitter release. Adapted from Smith et al., 1985. C, dependence of postsynaptic current and presynaptic calcium current on Ca_o, using logarithmic coordinates. Adapted from Augustine and Charlton, 1986. D, when postsynaptic current (PSC) is plotted vs. the presynaptic calcium current integrated up to 1.5 ms before the PSC is measured, the apparent voltage dependence of panel B vanishes. Adapted from Augustine et al., 1985b.

likely to influence some aspect of transmitter release via a calmodulin action (Llinás et al., 1985). Presynaptic injection of calcium-calmodulin-dependent protein kinase II enhanced transmitter release elicited by voltage-clamp depolarizations. Presynaptic calcium currents were unaffected. Synapsin I is a presynaptic protein phosphorylated by this kinase, which is also present presynaptically. When dephosphorylated synapsin I was injected, it diminished transmitter release, while the phosphorylated form was without effect. Effects of kinase and synapsin I developed slowly (over a course of minutes), suggesting a modulation rather than a mediation of the transmitter release process by the calcium-calmodulin dependent phosphorylation of synapsin I. The selective association of synapsin I with synaptic vesicles (Navone et al., 1984) suggests that it may regulate the availability of transmitter for release in a calcium-dependent manner, and play a role in processes such as facilitation, potentiation, and depression.

An interesting hypothesis for calcium action in triggering exocytosis has been proposed by Stanley and Ehrenstein (1985). They suggest that multiple calcium ions activate potassium channels in vesicles, which with anion channels lead to osmotic swelling and fusion of vesicles. Unfortunately however, efforts to verify this hypothesis by injecting blockers of calcium-activated potassium channels into the presynaptic terminal failed to reveal the presence of such channels (Augustine et al., 1988a).

11. VOLTAGE-DEPENDENCE OF TRANSMITTER RELEASE

11.1 Effect of Presynaptic Potential on Transmitter Release

Another curious result of the early voltage clamp studies was the finding that transmitter release seemed to be influenced directly by the presynaptic potential. A given level of calcium influx can be achieved by small depolarizations, opening few calcium channels, and by large depolarizations, opening many channels with reduced calcium current due to approach to its equilibrium potential. Thus one may relate transmitter release to presynaptic calcium current for rising and falling limbs of the bell shaped curves of calcium current and postsynaptic response vs. presynaptic potential (Fig. 8B). Llinas et al. (1981b) found that large depolarizations evoked substantially larger postsynaptic responses than small depolarizations causing the same calcium influx. This was taken as evidence for a direct influence of presynaptic potential on release mechanisms.

Using the methodological improvements described above, Smith et al.

(1985) and Augustine et al. (1985b) restricted calcium influx and transmitter release to the well-clamped distal portion of the terminal, clamped the postsynaptic neuron, and plotted postsynaptic current vs. presynaptic calcium current (or its integral). As a consequence, very little difference appeared between the rising and falling limbs of the dependence of transmitter release upon calcium current. When synaptic delay was taken into account, and transmitter release rate was measured shortly after the end-point of integrating calcium current, the apparent voltage dependence was markedly diminished (Fig. 8D). Thus any direct effect of voltage during a pulse on release is small, if it exists at all. Recently, evidence that voltage directly influences transmitter release at the neuromuscular junction (Parnas and Parnas, 1986) has also been questioned on methodological grounds (Zucker et al., 1986). Finally, the separate roles of calcium and voltage in eliciting transmitter release have been explored at synapses between cultured neurons (Zucker and Haydon, 1988). Voltage clamp and calcium buffering by photodynamic chelators were used to independently control presynaptic potential and calcium concentration. Transmitter release was evoked by elevating presynaptic calcium activity, but was completely unaffected by presynaptic potential.

11.2. Effect of Prior Polarization on Release

Prior polarization may also modulate transmitter release. Such an effect is quite distinct from the enhancement of transmitter release by steady hyperpolarization, due to increase in the calcium tail current. Initial reports that prior hyperpolarization enhances transmitter release to a subsequent depolarization (Miledi and Slater, 1966; Katz and Miledi, 1967; Gage, 1967), were shown to reflect effects of potential on length constant and the spread of subsequent depolarization (Katz and Miledi, 1971). Later reports (Katz and Miledi, 1971), in which delayed rectification was blocked pharmacologically, indicate that prior depolarization enhances release to a subsequent depolarization. A preliminary report (Simon et al., 1983) suggests that this effect was partly due to an increase in calcium current, which was primed by the prior depolarization. Another component appears to be independent of effects on calcium current, and may represent a direct effect of voltage on the release process. Another possibility is that small calcium currents during the prior depolarization (Charlton and Atwood, 1977b) raise the resting calcium level, which facilitates transmitter release by summing with calcium entry during the following pulse.

More recent results (Llinás et al., 1983; Llinás et al., 1987) suggest that presynaptic depolarization can enhance release to presynaptic calcium injection when calcium influx is blocked pharmacologically. Since presynaptic injection of calcium can activate potassium channels (Augustine and Eckert, 1982a,b), subsequent depolarization might lead to additional postsynaptic depolarization due to extracellular potassium accumulation (Erulkar and Weight, 1977). Clearly, the issue of modulation of transmitter release by presynaptic potential requires further study.

12. EARLY MODELS OF CALCIUM AND TRANSMITTER RELEASE

The ability to measure fairly accurately such presynaptic events as calcium influx, changes in Ca_{in}, and time courses of transmitter release, synaptic depression, and synaptic facilitation, has sparked several efforts to relate these events to each other quantitatively using mathematical models of hypothetical synaptic mechanisms.

12.1. The Model of Llinás and Co-Workers

An early model (Llinás et al., 1976a) and later refinement (Llinás et al., 1981b) attempted to relate transmitter release to calcium influx. It was assumed that calcium concentration at active sites equilibrated rapidly after calcium channels open, because such sites are in the immediate vicinity of calcium channel mouths. Thus intracellular calcium at release sites was taken as proportional to macroscopic calcium current. Calcium was supposed to bind rapidly to a vesicle fusion factor, which then transformed to an active form that triggers exocytosis after a fixed delay (Llinás et al., 1982). Calcium could dissociate slowly and irreversibly, representing calcium uptake and buffering, from either active or inactive forms of the vesicle fusion factor. To account for what appeared to be a strong voltage dependence of release, rate constants for fusion factor activation, calcium unbinding, and exocytosis were all assumed to be voltage-dependent. Exocytosis depleted the store of available vesicles, leading to a decline in release to maintained depolarization. Transmitter release was allowed to open postsynaptic channels with a limited lifetime. The resultant conductance change drove synaptic current through an electrical equivalent of the postsynaptic cell to generate the EPSP, which was shunted by a time- and voltage-dependent postsynaptic potassium conductance.

This model provided a remarkably accurate fit between predicted and observed calcium current and postsynaptic response for various presynaptic

depolarizations. However, a large number of free parameters were available to adjust the fit, and many structural details of the model were quite speculative. Subsequently, many of the quantitative aspects of the data predicted by this model, such as a nearly linear dependence of transmitter release on calcium influx and an apparently high voltage dependence of release, have been revised using improved methodology. Nevertheless, like the Hodgkin and Huxley (1952) model, it provided an excellent quantitative description of experimental results within a theoretically plausible framework.

12.2. The Model of Zucker and Stockbridge

Another early model (Zucker and Stockbridge, 1983), considered the effects of calcium diffusion away from the presynaptic membrane, as well as cytoplasmic calcium binding and active calcium extrusion, in determining the time course of submembrane calcium following an action potential (Fig. 9). In this model, too, submembrane or active calcium concentration changes were simply linearly dependent upon calcium influx. Using the best data available at the time (Llinás et al., 1981b; Charlton et al., 1982), this model assumed only a square-law relationship between calcium and transmitter release. Nevertheless, calcium diffused radially away from the membrane rapidly enough to terminate transmitter release within 1-2 msec after an action potential. A small residual submembrane calcium was sufficient to lead to synaptic facilitation of appropriate amplitude and time course tested by a second action potential. Finally, calcium was removed by extrusion at a rate similar to that indicated by arsenazo spectrophotometry (Charlton et al., 1982). This model was less comprehensive in mechanistic detail than the model of Llinás et al., (1981b). Instead of attempting to account for the mechanism of transmitter release and the form of the postsynaptic response, it accounted only for the time courses of phasic calcium changes triggering transmitter release, and the magnitude and time courses of facilitation and total presynaptic calcium. However, this model simulated real physical processes, all of which had been independently measured in squid axons. There were no free parameters.

13. CALCIUM DOMAINS AND TRANSMITTER RELEASE

13.1. Problem: The Cooperativity of Calcium in Inducing Release

An important new insight into presynaptic function was the recognition by Chad and Eckert (1984) and Simon et al., (1984) that calcium is not

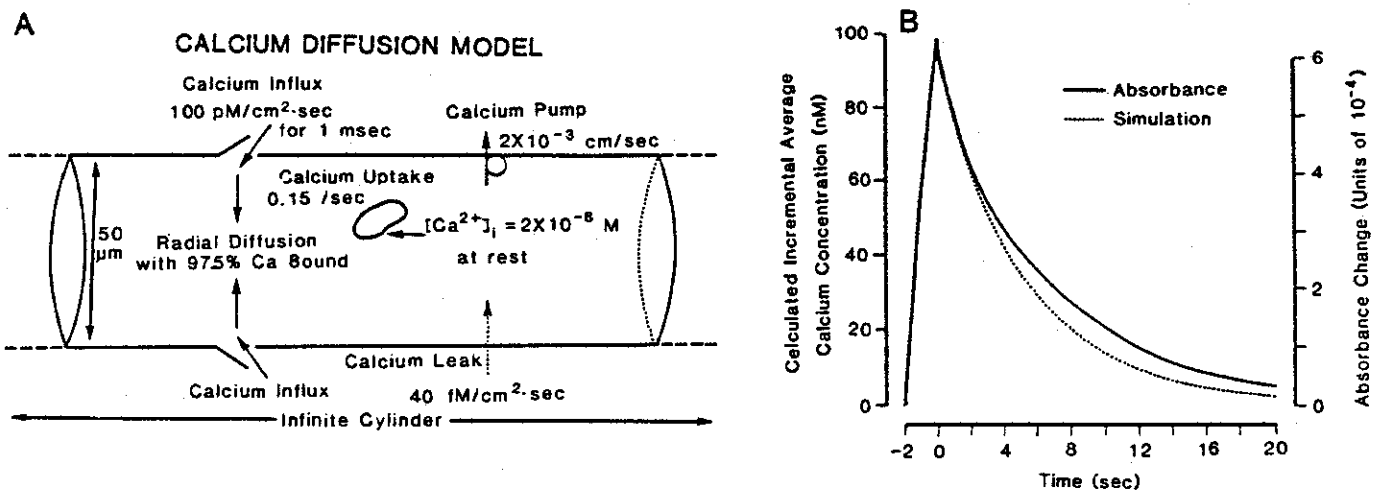


Fig. 9: Simulations of presynaptic calcium movements and transmitter release using a simple model (panel A) of uniform calcium influx, radial diffusion with binding, extrusion and uptake. B, predicted and observed changes in average presynaptic unbound calcium concentration during a 2-s 33-Hz tetanus.

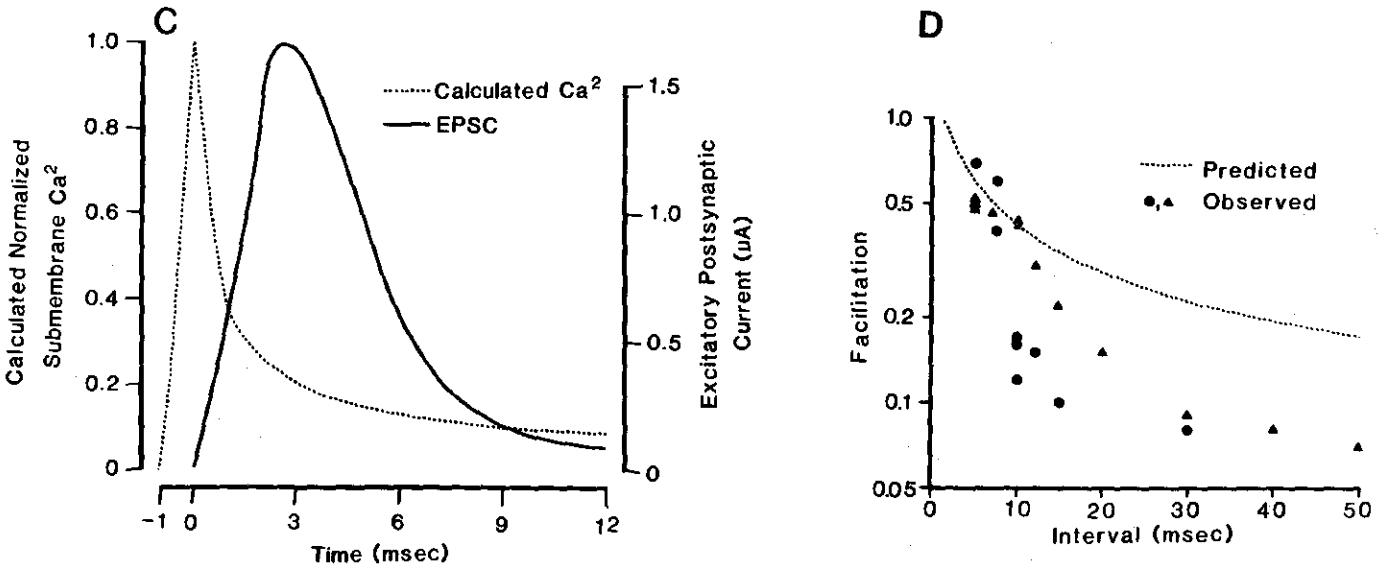


Fig. 9 (cont.): C, Submembrane calcium raised to the second power is compared to the time course of the postsynaptic current, which lags the release of transmitter. D, predicted and observed decay of synaptic facilitation following a single action potential. Adapted from Zucker and Stockbridge, 1983.

distributed uniformly underneath the membrane during an action potential, but is rather concentrated into domains of ions surrounding single calcium channels. The synaptic delay is too short for these calcium domains to collapse before transmitter release occurs. Thus calcium must act locally to release transmitter in the immediate vicinity of open channel mouths.

A consequence of this situation is that increasing depolarization recruits transmitter release from more calcium domains, and there should be a linear relationship between transmitter release and presynaptic calcium current (Simon and Llinás, 1985). If calcium acts cooperatively to trigger release, then the drop in calcium influx through each channel with increased depolarization as the calcium equilibrium potential is approached will cause the curve of transmitter release vs. calcium current to assume a concave downward shape. For the same reason, transmitter release to large depolarizations will be less than release to small depolarizations, for a given calcium influx. How, then, can the observed concave upward power-law shape of the experimental data be explained? How does the apparent enhancement of release by depolarization arise?

13.2. Solutions:

13.2.1. The Nearest Neighbor Model of Simon and Llinás

Simon and Llinás (1985) proposed that several calcium domains must open simultaneously directly under a vesicle to trigger exocytosis, with calcium binding at each of several sites on a vesicle. The probability of calcium binding is a function of the peak calcium concentration attained at the calcium channel mouth. This requirement that several neighboring calcium channels open simultaneously at the correct points under a vesicle is one way to generate the observed relationship between calcium current and release. Simon and Llinás (1985) proposed that a single calcium ion binds at each binding site on a vesicle. This proposal was based on the belief that if multiple calcium ions react at binding sites, the expressed stoichiometry of the dependence of release upon calcium influx should drop as resting calcium is raised and some calcium binding sites become occupied. Simon (1984) did not observe such a change in stoichiometry between release and calcium current when resting calcium was increased by a depolarized hold potential. However, Stanley (1986) did observe a reduced dependence of release upon Ca_o when spike frequency was increased, suggesting that a high calcium cooperativity at binding sites does exist whose expression depends on resting calcium level. Moreover, Augustine and Charlton (1986) observed a reduction

in the exponent relating transmitter release to calcium current when Ca_o was raised, again suggesting a saturation of multiple calcium binding sites within calcium domains.

Assuming that calcium acts linearly at each binding site, Simon and Llinás (1985) were unable to account for facilitation by the usual residual calcium model of summation with calcium influx, nor for the highly nonlinear dependence of release upon Ca_o. They proposed that facilitation arises from a slowly developing secondary action of calcium on the availability of releasable quanta, but this contrasts with the fact that facilitation is maximal immediately after an action potential (Charlton and Bittner, 1978a). They also proposed that external calcium increases transmitter release nonlinearly by increasing the resting level of Ca_{in} and altering the facilitatory process, but this fails to account for the very rapid time course of external calcium action (Miledi and Slater, 1966).

The mathematical formulation of the idea of Simon and Llinás (1985) that multiple calcium domains are required for release of a vesicle of transmitter is given in Simon (1984). On p. 59, the following equation expresses the dependence of the rate of vesicular release ($REL_{vesicle}$) on the probability of a calcium channel opening near a vesicle (P_{op}), the number of such channels needed to activate a vesicle (N), the intracellular calcium concentration change at a vesicular calcium binding site resulting from a calcium channel opening (Ca_{in}), the number of calcium ions needed to activate each binding site on a vesicle (n), and a proportionality factor (P_{rel}):

$$REL_{vesicle} = P_{rel} * (Ca_{in})^n * (P_{op})^N.$$

This equation predicts a linear relation between external calcium (which determines Ca_{in}) and transmitter release if $n = 1$, contrary to many observations. It also leads to a facilitation due to residual calcium which persists only as long as does transmitter release after a spike, if $n = 1$. However, this equation is correct only if the rise in intracellular calcium occurs independently of the opening of calcium channels, and is a joint probability of two unrelated events – calcium channel opening and a rise in Ca_{in}. But the rise in calcium is not independent of channel opening; on the contrary, it is caused by calcium channels opening.

13.2.2. An Alternative Formulation

A more appropriate formulation of the nearest neighbor model might be as follows: A vesicular binding site is activated only if bound by n calcium ions.

The probability that n calcium ions react with a vesicular binding site is proportional to the nth power of the calcium concentration reached if a channel opens, $(Ca_{in})^n$), times the probability that a channel opens (P_{op}). A vesicle is released only if it is activated by n calcium ions at each of N sites, so the preceding product must be raised to the Nth power. This gives

$$REL_{vesicle} = P_{rel} * (P_{op} * (Ca_{in})^n)^N.$$

Now release depends nonlinearly (with power N) on external calcium even if only one calcium ion binds each vesicular binding site (n=1), and residual calcium will add to the peak calcium at binding sites near calcium channels (Ca_{in}) to nonlinearly facilitate subsequent responses after phasic release has subsided. This reformulation of the Simon and Llinás (1985) model of transmitter release governed by N neighboring calcium channels appears to alleviate its difficulty in predicting the calcium dependence of transmitter release and kinetics of facilitation.

Another accomplishment of the nearest neighbor requirement is that it explains qualitatively the apparent voltage dependence of release described by Llinás et al. (1981b). In this model, the voltage dependence arises from the fact that at low depolarizations, few calcium channels open and few vesicles will be influenced by sufficient neighboring open calcium channels. At large depolarizations, many channels open, and many more vesicles are activated by calcim entering through channels near each of the required binding sites. Even though calcium influx is reduced through each channel, so that the probability of each calcium cloud being strong enough to achieve calcium binding is lower, the increased number of calcium domains overcomes this effect, and transmitter release shows a positive voltage dependence. The important point is that this arises entirely from the different distributions of calcium domains at different voltages, rather than from any actual voltage dependence of any step in transmitter release.

Simon and Llinás (1985) suggest that vesicle depletion will also be more severe at low depolarizations, with denser calcium domains more likely to release vesicles in their vicinity, than at high depolarizations with weaker calcium influx per channel. This can also contribute to an apparent voltage dependence of transmitter release.

13.2.3. The Model of Domain Overlap of Fogelson and Zucker

A similar model was developed independently by Fogelson and Zucker

(1985). Again, attention was focused on the nonuniform distribution of calcium under the membrane into domains surrounding calcium channel mouths (Fig. 10A). Two important differences distinguish this model from that of Simon and Llinás (1985):

1) Calcium was assumed to act cooperatively at one locus on a vesicle or the plasma membrane, rather than at several independent sites contributing to vesicle fusion.

2) Release was assumed to occur about 50 nm away from calcium channel mouths, consistent with experimental observations at neuromuscular junctions (Heuser et al., 1979). In this model, calcium binding sites are never exposed to the intense calcium concentration peaks immediately under calcium channel mouths. Instead, they are affected only by the shoulders of the calcium domains. As depolarization increases and more domains open closer to each other, these shoulders overlap so that the calcium concentration at calcium binding sites rises. Since calcium acts cooperatively, this leads to the nonlinear dependence of release on macroscopic calcium current. A small apparent voltage dependence of release (Smith et al., 1985; Augustine et al., 1985b) is also predicted, because the overlap of calcium domains at high depolarizations more than compensates the reduced calcium entry due to reduced driving force (Fig. 10B) (Zucker and Fogelson, 1986). As in the nearest neighbor model, this apparent voltage dependence arises from the different arrangements of calcium domains at different potentials, not from any truly voltage-dependent step in the release of transmitter.

This model also predicts a nonlinear dependence of transmitter release on Ca_o (Katz and Miledi, 1970; Lester, 1970; Augustine and Charlton, 1986). Moreover, it accounts for the magnitude and time course of facilitation, not only following one spike, but even in a tetanus of 100 action potentials (Fogelson and Zucker, 1985), using the standard residual calcium hypothesis, and predicting residual calcium resulting from diffusion, extrusion, and binding after influx through calcium channel arrays (Fig. 10C). The residual calcium predicted by this model is similar to that predicted by the simpler model of calcium entering uniformly across the membrane (Zucker and Stockbridge, 1983). However, the peak calcium concentration at release sites during an action potential is much higher, necessitating a higher cooperativity (4 or 5 rather than only 2) to account for facilitation from residual calcium. This stoichiometry is consistent with the most recent estimates of the dependence of transmitter release upon calcium current or Ca_o concentration (Augustine and Charlton, 1986; Augustine et al., 1988b).

An interesting finding of this model is that the rapid diffusion of calcium away from calcium channel clusters is needed to pull active calcium down

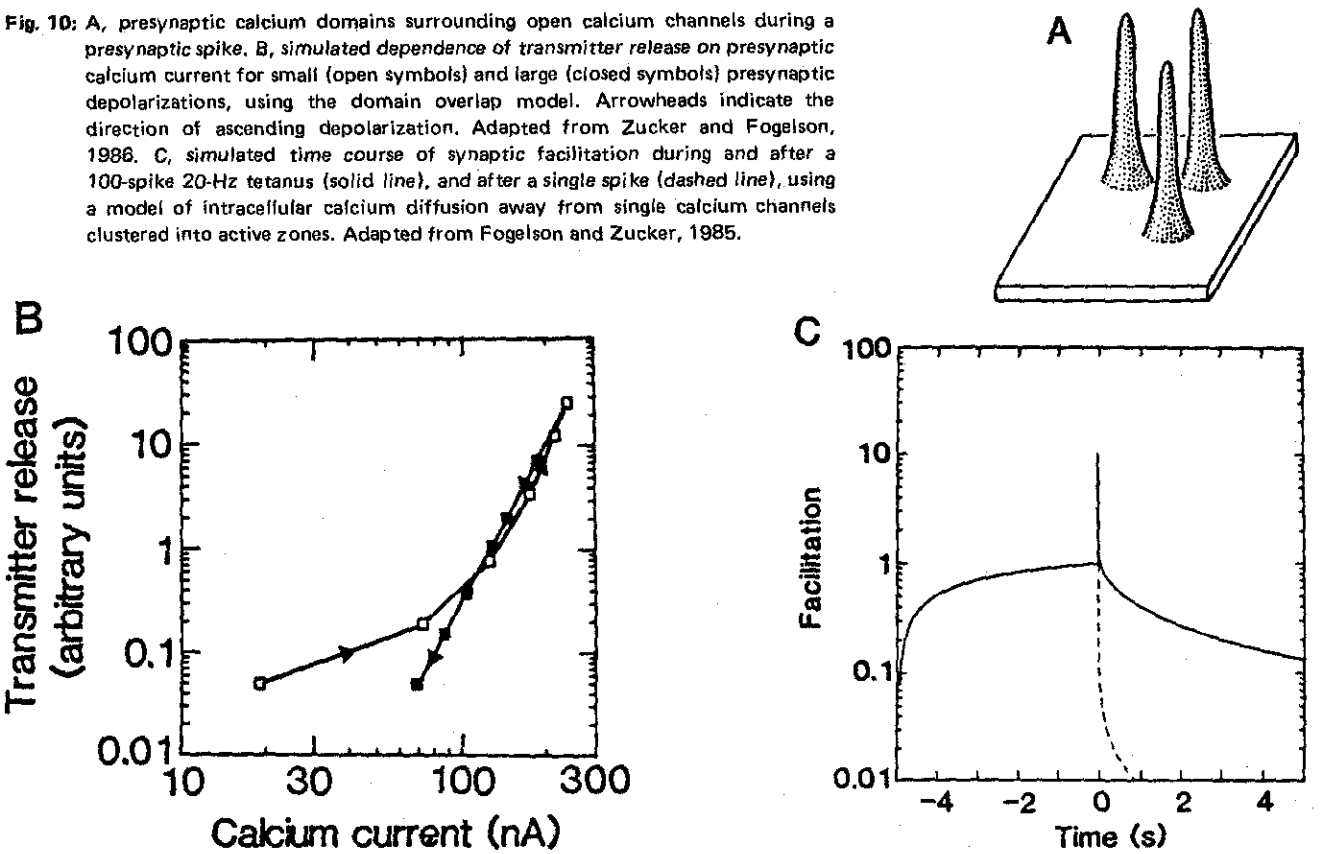


Fig. 10: A, presynaptic calcium domains surrounding open calcium channels during a presynaptic spike. B, simulated dependence of transmitter release on presynaptic calcium current for small (open symbols) and large (closed symbols) presynaptic depolarizations, using the domain overlap model. Arrowheads indicate the direction of ascending depolarization. Adapted from Zucker and Fogelson, 1986. C, simulated time course of synaptic facilitation during and after a 100-spike 20-Hz tetanus (solid line), and after a single spike (dashed line), using a model of intracellular calcium diffusion away from single calcium channels clustered into active zones. Adapted from Fogelson and Zucker, 1985.

rapidly following spikes in a tetanus, so that transmitter release is still rapidly terminated (Fogelson and Zucker, 1985). This may be why calcium channels and vesicles are clustered into release sites or active zones. Dispersing channels uniformly over the plasma membrane, or allowing calcium to cross the membrane uniformly, does not drive calcium away from release sites quickly enough to preserve phasic release to tetanic spikes. Likewise, the nearest neighbor model of Simon and Llinás (1985) and the domain overlap model of Zucker and Fogelson (1986) both require calcium channels to be clustered in release sites to account for the relationship between transmitter release and calcium current.

14. CONCLUSIONS

These mathematical models are still quite crude, and none of them even approaches a definitive description of presynaptic events. Nevertheless, they have provided valuable new insights into the presynaptic roles of calcium in releasing transmitter, and provided useful frameworks for trying to explain the role of calcium in neurotransmitter release. These models, and the techniques of measuring calcium influx, measuring and controlling Cain (Tsien and Zucker, 1986), and monitoring transmitter release are undergoing constant refinement. Biochemical probes of the release apparatus are just beginning to find application in understanding physiological function at the squid giant synapse. Well formulated and testable hypotheses of the biophysical mechanism of exocytosis are just beginning to be devised. These represent the pathways of present and future research, which shows no sign of abating.

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