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PROCESSES UNDERLYING ONE FORM OF SYNAPTIC PLASTICITY: FACILITATION

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SUMMARY

Facilitation is one of the most prevalent forms of synaptic plasticity, and is often invoked as a quality which is important in the nervous system's ability to generate adaptive behavior. The squid giant synapse provides an excellent opportunity to explore the biophysical mechanism of synaptic facilitation. Previous studies showed that facilitation is not due to changes in presynaptic action potentials or after-potentials. Evidence summarized here indicates that facilitation is also not a consequence of presynaptic calcium channel properties, nor is it a reflection of growing increments in presynaptic calcium concentration with repeated activity. Moreover, arsenazo III absorbance microspectrophotometry has revealed a residual calcium following presynaptic activity, and injection of calcium presynaptically facilitates spike-evoked transmitter release. A nonlinear relation between calcium and transmitter release is demonstrated, and this plus a mathematical model of diffusive calcium movements within the presynaptic terminal account for both the time course of transmitter release and the magnitude and decay of facilitation following an action potential.

INTRODUCTION

Significance of Facilitation

Synapses, and especially chemically transmitting synapses, do not transmit information in a static fashion. Instead, the efficacy with which a presynaptic action potential influences the firing of the postsynaptic neuron is often a strong function of the level of

preceding presynaptic activity. One of the most prominent forms of short-term synaptic plasticity at many synapses is synaptic facilitation, or homosynaptic facilitation, which refers to the enhancement of the strength of synaptic transmission following activity in the presynaptic neuron. Ever since this phenomenon was first described by Feng in 1940, it has attracted the attention of neurobiologists and psychologists. In the last four decades, hundreds of the former have concentrated on its mechanism and integrative function, while since the nineteenth century dozens of the latter have speculated on its role in adaptive behaviors such as conditioned reflexes and higher forms of learning (Ramon y Cajal, 1894; Freud, 1954; Hebb, 1958; Young, 1964; Deutsch, 1971; Rosenzweig et al., 1972; Thompson et al., 1972; Eccles, 1973; Mark, 1974). Recently, a special form of heterosynaptic facilitation, involving the presynaptic facilitatory effect of one pathway upon the efficacy of transmission in another pathway, has been implicated in behavioral sensitization, a simple form of learning sharing some of the characteristics of conditioning (Klein et al., 1981).

Hypothetical Mechanisms of Synaptic Facilitation

In every synapse in which a quantal analysis has been performed, facilitation has been found to be due to an increase in the amount of transmitter released by a nerve impulse (reviewed in Zucker, 1973). Two hypotheses about the mechanism for this presynaptic process enjoyed considerable popularity until recently: 1) the pre-spike hypothesis, which states that more transmitter is released by larger or broader presynaptic action potentials, and 2) the after-potential hypothesis, which proposes that facilitation is a consequence of long-lasting after-potentials in nerve terminals. However, in every synapse at which these hypotheses have been critically tested they have been shown to be false (Zucker, 1974a,c; Zucker and Lara-Estrella, 1979). The most elegant demonstration that facilitation can occur in the absence of changes in presynaptic action potential and resting potential is the study by Charlton and Bittner (1978a,b) on the squid giant synapse, where they observed directly with presynaptic intracellular recording from the nerve terminal the electrical events occurring during synaptic facilitation.

Two other hypotheses of the mechanism of facilitation have received more attention recently: 1) the calcium channel hypothesis, which assumes that facilitation is a property of calcium channels, such that they admit more calcium ions to successive depolarizations or repeated identical action potentials, and 2) the residual calcium hypothesis, which supposes that facilitation arises from the action of residual presynaptic intracellular calcium remaining after presynaptic electrical activity.

Both these hypotheses have received some positive experimental support. Measurements of calcium influx into neurons, using the non-

linear calcium-sensitive photoprotein aequorin, suggested that the calcium channels do themselves facilitate (Stinnakre and Tauc, 1973; Eckert et al., 1977; Lux and Heyer, 1977; Stinnakre, 1977). However, measurements of calcium influx using voltage-clamp recording of calcium current or the linear calcium-sensitive dye arsenazo III indicate that calcium channels do not facilitate (Akaike et al., 1978; Tillotson & Horn, 1978; Connor, 1979; Ahmed and Connor, 1979; Smith and Zucker, 1980; Gorman and Thomas, 1980). Unfortunately, none of these studies was performed on presynaptic membrane calcium channels, but rather on molluscan cell body membranes.

The idea that residual presynaptic calcium is responsible for synaptic facilitation was propounded by Katz and Miledi (1965). Several lines of evidence support this hypothesis: 1) Action potentials occurring in a calcium-free medium not only fail to release transmitter, but they also fail to facilitate subsequent transmitter release to later action potentials occurring after restoration of external calcium (Katz and Miledi, 1968). 2) Treatments believed to increase presynaptic calcium concentration, such as exposure to metabolic inhibitors (Alnaes and Rahamimoff, 1975) or fusion with calcium-containing liposomes (Rahamimoff et al., 1978) sometimes increase spike-evoked transmitter release. 3) Exposure to low calcium medium, which may reduce presynaptic calcium during spike activity, reduces the post-tetanic increase in miniature EPSP freauency, which also is thought to arise from residual calcium (Erulkar and Rahamimoff, 1978). Two sub-hypotheses have been been proposed to explain how residual calcium may lead to facilitation: 1) If there is a nonlinear relation between internal calcium and transmitter release, as suggested by a nonlinear relation between external calcium and release (Dodge and Rahamimoff, 1967), then residual calcium will shift the release vs. calcium system to a steeper part of the operating curve. Now a given increment of presynaptic calcium due to the constant influx in an action potential will be more effective in enhancing release, while the same nonlinearity will minimize the effectiveness of residual calcium on spontaneous release rate (Katz and Miledi, 1968; Miledi and Thies, 1971). I call this the nonlinear release version of the residual calcium hypothesis. 2) Alternatively, the calcium entering during an action potential may act at one locus to phasically release transmitter, while residual calcium may act at a different locus to condition subsequent phasic releases and cause facilitation (Balnave and Gage, 1974). I call this the two-site version of the residual calcium hypothesis.

The remainder of this report will focus on two issues: First, I shall summarize experimental results collected at the Marine Biological Laboratory with the collaboration of Drs. Milton Charlton and Stephen Smith, which support a role for presynaptic residual calcium in generating synaptic facilitation at the giant synapse in the stellate ganglion of the squid. The full details of this work have been presented elsewhere (Charlton et al., 1981). Second, I shall

present a theoretical model, developed in collaboration with Dr. Norman Stockbridge, of how the internal diffusion of residual calcium away from the plasma membrane can account for the magnitude and kinetics of facilitation at this synapse. Details of this model may be found in Dr. Stockbridge's Ph.D. dissertation (Stockbridge, 1981).

EXPERIMENTAL SUPPORT FOR THE RESIDUAL CALCIUM HYPOTHESIS

Presynaptic Calcium Currents Do Not Facilitate to Repeated Depolarizations

Before proceeding to a test of the residual calcium hypothesis, we explored the possibility that the calcium channels at the presynaptic terminal of the squid giant synapse exhibit intrinsic facilitatory properties. To do this, we used the three-electrode voltage clamp configuration of Adrian et al. (1970) to record the local membrane currents through the end of the presynaptic terminal. Sodium and potassium currents were blocked with external tetrodotoxin and 3,4-diaminopyridine and with presynaptic iontophoresis of tetraethylammonium. Paired brief depolarizing pulses were delivered to the presynaptic terminal and adjusted to evoke subthreshold facilitating EPSPs in the postsynaptic giant axon. Fig. 1A shows the currents recorded during depolarizing pulses. These currents include brief capacitance transients due to the charge and discharge of the presynaptic membrane during the onset and offset of the depolarizations, a large inward calcium current turning on during each pulse, and a superimposed smaller leak current due to the resting potassium and chloride permeability of the membrane. The current trace in Fig. 1B shows the leak and capacitance currents with inverted polarity during two hyperpolarizing pulses of equal magnitude. In Fig. 1C, leak and capacitance currents are also shown alone during depolarizing pulses in low-calcium sea water containing the calcium channel blocker cadmium. Comparison of Figs. 1A-C shows that the inward calcium current that turns on during each depolarization is identical, although the second pulse releases nearly twice as much transmitter as the first. The perfectly linear parts of the capacitance and leak currents can be removed by averaging equal numbers of responses to depolarizing and hyperpolarizing pulses (Fig. 1D), and again the calcium currents appear constant. There was no suggestion of calcium channel facilitation in nine similar experiments, except that in one instance when a very brief (less than 1 msec) interval separated pulses, we observed that the calcium current activated slightly faster during the second pulse. This interval is unphysiological in that it is less than the absolute refractory period, and when longer intervals of over 1 msec were used, no such effect was observed, while facilitation of transmitter release was substantial. Apparently, this effect represents a small priming of activation of calcium channels, probably due to the calcium channels not having returned to their resting state, although they were fully closed. It

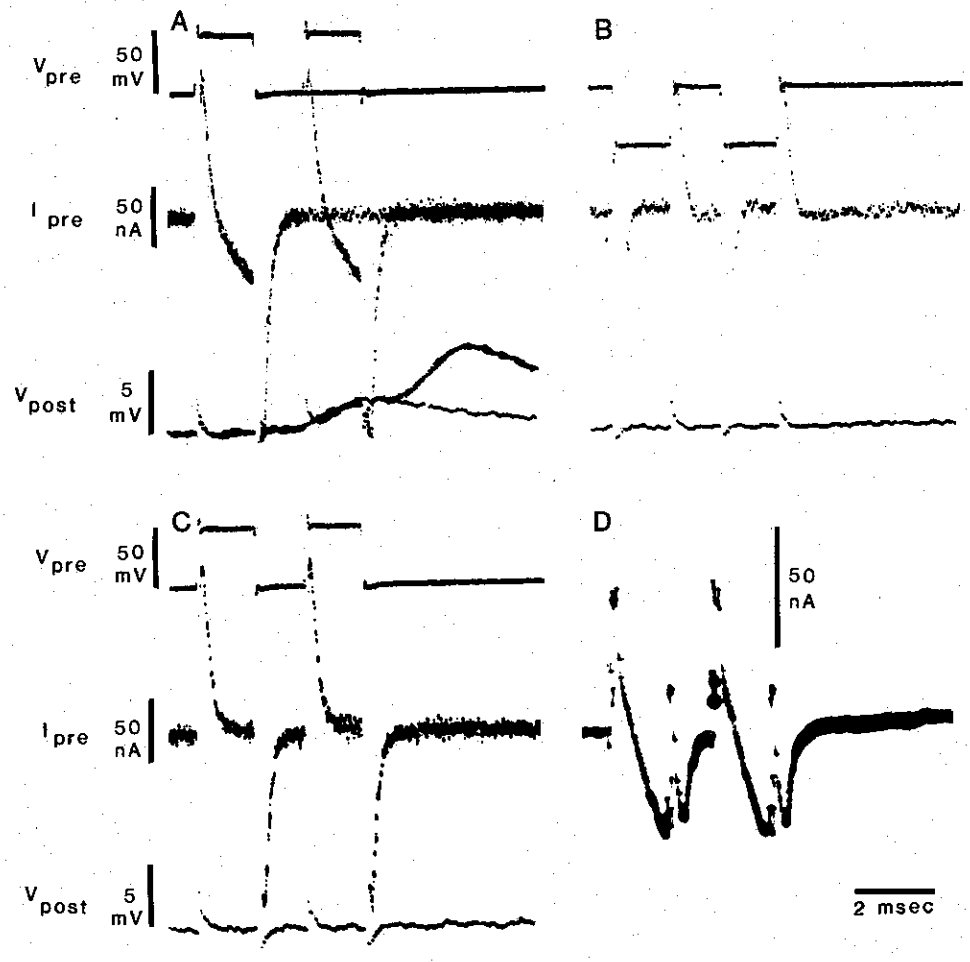

Fig. 1. Non-facilitating calcium currents in the presynaptic terminal. A: Superimposed records of presynaptic calcium, leak, and capacitance currents and postsyaptic potentials to both single and paired paired presynaptic depolarizations. B: Presynaptic leak and capacitance currents to paired hyperpolarizations. C: Presynaptic leak and capacitance currents after blocking calcium current with 1 mM cadmium in sea water with only 1 mM calcium. D: Presynaptic calcium current, obtained by averaging currents to 8 paired equal and opposite pulses as in A and B. Abbreviations: V_{pre}, presynaptic voltage-clamped potential; I_{pre}, presynaptic membrane current; V_{post}, postsynaptic potential.

is likely that under normal circumstances, facilitation of calcium channels is not involved in synaptic facilitation.

There is a Nonlinear Relation Between Calcium Influx and Synaptic Transmission

It is well known that elevating depolarization amplitude to about 0 mV absolute membrane potential increases both the amplitude of the presynaptic calcium current and the postsynaptic response (Llinas et al., 1981a,b). Facilitation, in contrast, apparently increases synaptic transmission without any concurrent increase in calcium influx. According to the nonlinear release version of the residual calcium hypothesis, this could arise from a nonlinear relation between internal calcium and transmitter release. We explored this possibility by measuring the relationship between presynaptic calcium current and transmitter release to brief depolarizing pulses of different amplitude. In three preparations, we plotted peak amplitude of presynaptic calcium current vs. peak amplitude of EPSP (after Martin correction), or peak amplitude of calcium current vs. initial slope of EPSP (which is a better measure of synaptic conductance change), or the area of the calcium current during a depolarization, including the tail current following the pulse, vs. EPSP area. In all cases, the data fit a power-law relationship such that when plotted on log-log co-ordinates, most points fall nearly on a straight line with slope of about 2. Two examples of this sort of plot of presynaptic calcium current area vs. EPSP area are shown in Fig. 2. This is a milder nonlinearity than that reported for some synapses (e.g., Dodge and Rahamimoff, 1965), but a steeper one that that reported for others (e.g., Zucker, 1974b). However, it is close to that expected from the relation between external calcium and transmitter release at the squid giant synapse (Katz anf Miledi, 1970; Lester, 1970).

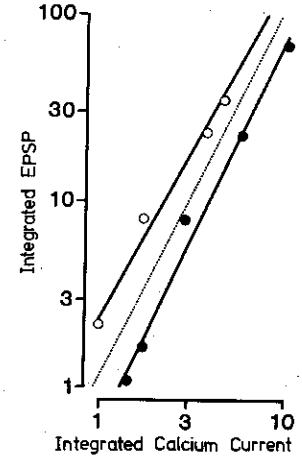

Fig. 2. Relation between transmitter release, measured as EPSP area, and integral of presynaptic calcium current, plotted on logarithmic co-ordinates. Data are from two synapses; units are arbitrary. Dotted line represents a power of 2.

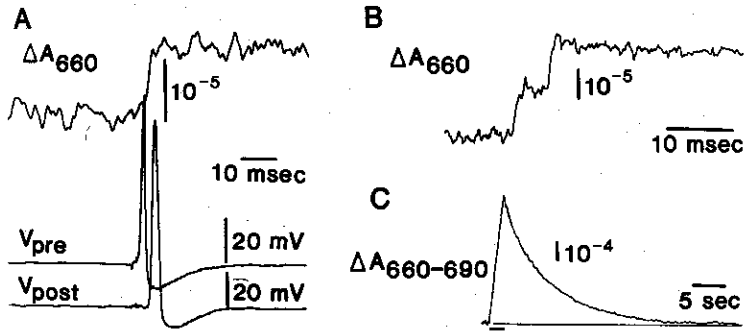

Fig. 3. Absorbance changes at 660 nm (ΔA_{660}) in 3 presynaptic terminals filled with about 0.25 mM arsenazo III. A: calcium concentration change and postsynaptic response accompanying one presynaptic action potential. B: Presynaptic calcium concentration transients accompanying paired action potentials. C: Presynaptic calcium concentration change elicited by a 33 Hz train of impulses for the duration of the open bar. Here differential absorbance change at 660 and 690 nm is displayed. Absorbance records are averages of 64 responses in A and 16 in B and C. ΔA of 10^{-5} corresponds roughly to a calcium concentration change of 50 nM.

Intracellular Calcium Changes to Repeated Action Potentials Are Constant and Long-Lasting

The above results show that calcium currents do not facilitate during spike-like depolarizations evoking facilitating EPSPs, but do not exclude the possibility that the presynaptic intracellular calcium concentration increments accompanying successive action potentials may facilitate, due for example to a saturation of internal calcium buffers. We measured intracellular calcium concentration changes during electrical activity by filling the presynaptic terminal with the calcium-sensitive dye arsenazo III and recording absorbance changes at several wavelengths of light passing through the terminal with a microspectrophotometer. Low levels of arsenazo III were used, so that the dye interfered very little with normal presynaptic calcium regulation, as indicated by the normal level of suprathreshold synaptic transmission characteristic of this synapse when it is activated in normal calcium medium and by full-size action potentials. Fig. 3A shows a typical presynaptic calcium signal from a giant nerve terminal during a presynaptic action potential, which elicits a suprathreshold postsynaptic spike. The intracellular calcium rises rapidly during the falling phase of the presynaptic spike, when most of the calcium enters the terminal (Llinas et al., 1981b), and the calcium level remains high for several seconds following the

action potential. When two presynaptic action potentials are elicited in close succession, the EPSPs display a net depression, due to depletion of transmitter stores at high release levels in normal calcium medium with full-size action potentials. Nevertheless, facilitation still occurs normally, although it is masked by depression (Kusano and Landau, 1975; Charlton and Bittner, 1978a). Fig. 3B shows that the calcium increment caused by the second action potential evoking synaptic facilitation is identical to the calcium signal accompanying the first action potential. Finally, trains of presynaptic action potentials elicit a linearly rising absorbance change in the arsenazo-filled terminal, again indicating that facilitation of transmitter release occurs in the absence of any facilitation of transient changes in presynaptic calcium concentration (Fig. 3C). The records of Fig. 3 also show that there is indeed a residual calcium in the nerve terminal which persists long after presynaptic activity has ended (see also Miledi and Parker, 1981).

Injecting Calcium Presynaptically Facilitates Transmission

Until now no evidence has been adduced at any synapse that an increase in presynaptic calcium facilitates spike-evoked transmitter release without affecting the action potential. To test this possibility, we injected calcium by inter-barrel micro-iontophoresis into the presynaptic terminal and observed very little effect on spike-evoked release. It occurred to us that injection of calcium from one point is likely to influence only a small part of the giant synapse, which is about 1 mm long. We modified the experiment by working in a low-calcium medium to block transmission from most of the terminal and by restoring transmission locally with a focal extracellular calcium pipette near the same part of the terminal into which we were injecting calcium. Now only the part of the synapse we could

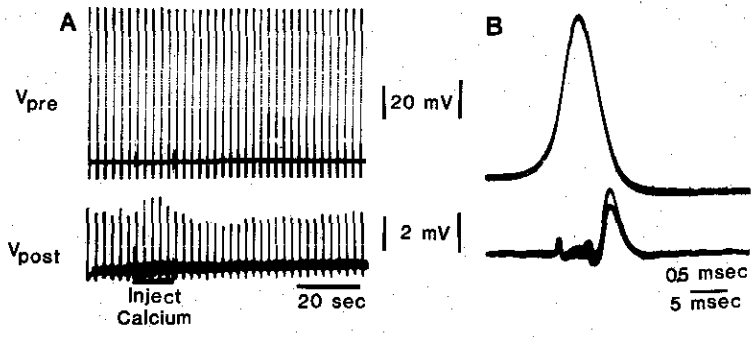

Fig. 4. Effect of presynaptic calcium injection on spike-evoked transmitter release. A: The presynaptic axon was stimulated every 3 seconds, and calcium was injected during the horizontal bar. B: Superimposed presynaptic action potentials and EPSPs in response to one stimulus before and one during presynaptic calcium injection.

influence intracellularly was transmitting in response to action potentials, and we regularly found that elevating intracellular calcium enough to increase the miniature EPSP frequency and elicit a noisy small postsynaptic depolarization (Miledi, 1973) invariably also facilitated spike-evoked release without having any effect whatever on the presynaptic action potential (Fig. 4). Apparently residual calcium exists and can cause facilitation.

THEORETICAL MODEL OF SYNAPTIC FACILITATION DUE TO PRESYNAPTIC DIFFUSION OF RESIDUAL CALCIUM

The above results suggest strongly that synaptic facilitation is due to some action of residual calcium on the release process, and that changes in the presynaptic action potential, resting potential, calcium channels, and size of calcium concentration increments are not involved. However, two important questions remain: 1) How can residual calcium persist for so long (many seconds) in the terminal, while transmitter release decays in a millisecond or two following a presynaptic spike and facilitation decays within a few tens of milliseconds? 2) Can the nonlinear release version of the residual calcium hypothesis explain the kinetic properties of release and facilitation, or must a two-site version be invoked?

With regard to the first question, it must be realized that our measure of residual calcium, using arsenazo III, detects free calcium anywhere in the cytoplasm in the last 3/4 mm of the presynaptic terminal. However, only the calcium ions in the immediate vicinity of transmitter release sites, immediately beneath the presynaptic plasma membrane, can influence transmitter release to an action potential. Facilitation is a short-lived process that is maximal immediately following an action potential. In contrast to post-tetanic potentiation, facilitation does not develop slowly, and cannot be due to changes in transmitter metabolism or to mobilization of stores. Thus, the residual calcium we detect with arsenazo anywhere in the terminal is not the same calcium that can influence transmission and facilitation. What we need is a measurement of the calcium level just beneath the membrane at transmitter release sites. This is not available.

Indeed, from earlier studies of the movement of calcium within cells (Smith and Zucker, 1980 and references therein), we expect that calcium entering a cell through surface membrane channels will rapidly diffuse into the interior of the cell after the channels close, quickly diluting the submembrane calcium concentration, but still being detected by arsenazo. Thus, calcium at release sites ought to decline much more rapidly than the arsenazo signal, which only reports the removal of free calcium from cytoplasm as a whole. Diffusion is just the sort of higher order process postulated in 1968 by Katz and Miledi to remove calcium rapidly from the presynaptic transmitter release sites.

To reconcile the arsenazo data with the characteristics of transmitter release and facilitation, I have used a computational model of calcium diffusion within a cell, originally developed for spherical symmetry by Thompson (1976) and Smith (1978). More recently, I have collaborated with Dr. Norman Stockbridge, who developed a similar model with cylindrical geometry (Stockbridge, 1981). The latter model presumes that calcium ions enter a cylindrical process at the surface, and then diffuse inward. The model includes binding to fixed cytoplasmic sites to account for the slow diffusion of calcium in cytoplasm. Calcium is removed from the system by a surface membrane calcium transport system, and a resting inward calcium current is included to yield a non-zero calcium level in steady state. The parameters of the system are adjusted to accord with physiological measurements of the relevant processes. Thus it is assumed that 1/40 of cytoplasmic calcium remains free while the remaining 97.5% is rapidly bound. This is similar to measurements of the rapid binding of calcium by cytoplasm (Brinley et al., 1977, 1978; Baker and Schlaepfer, 1978). A surface pump rate of 2×10^{-4} cm/sec was selected. This is within the range reported by Blaustein (1976) and DiPolo (1976). A surface leak of 2 f$\underline{M}/cm^2$.sec was selected, to yield a resting intracellular calcium concentration of 10 n$\underline{M}$ (DiPolo et al., 1976). The calcium influx during an action potential was represented as 25 p$\underline{M}/cm^2$.sec for 1 msec, as measured under voltage clamp for spike-like depolarizations by us and Llinas et al. (1981a). The squid giant presynaptic terminal was represented as an infinitely long circular cylinder having a 0.025 mm radius. These parameters were used to drive the diffusion equation in cylindrical co-ordinates, and the equation was solved numerically for the spatial distribution of intracellular calcium as a function of time during and following one or more presynaptic action potentials.

Fig. 5 shows the behavior of this model in reponse to one or two spikes. In part A, the calcium concentration was integrated throughout the volume of the terminal, to yield an average cytoplasmic concentration as a function of time during and after a single action potential. This corresponds to the arsenazo measurement of intracellular calcium. It may be seen that the model predicts rather well the rate of removal of calcium from cytoplasm detected by arsenazo. The predicted peak in spatially averaged calcium concentration reached during an impulse is slightly lower than we estimate from our arsenazo calibrations (Fig. 3), but we must emphasize that the arsenazo calibration is extremely rough, due to uncertainties in path length, stray light, intracellular arsenazo concentration, and chemical factors affecting the calcium-arsenazo reaction. The rate of calcium removal is set largely by the rate of the calcium extrusion pump, and is nearly unaffected by other parameters.

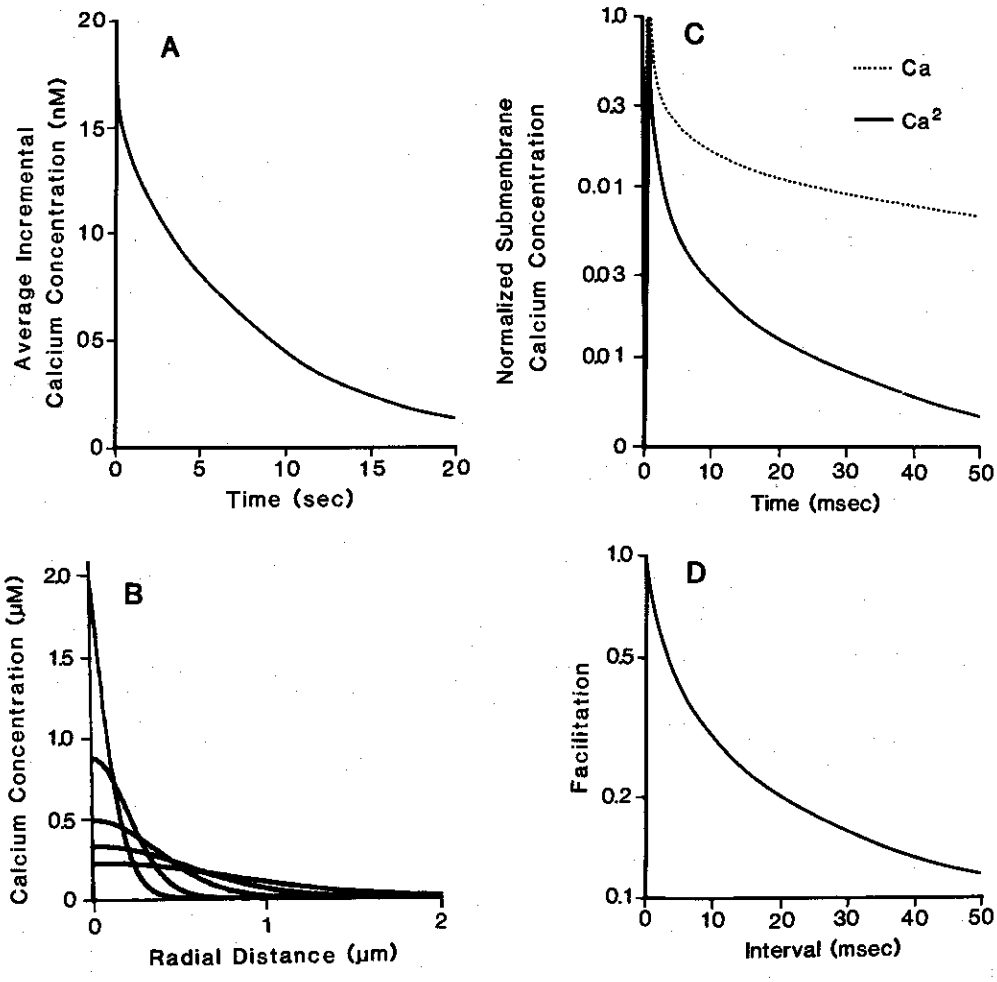

Fig. 5. Predictions of a diffusion model of calcium movements in the presynaptic terminal. A: Average increase in calcium concentration during and after an action potential at time 0. B: Presynaptic calcium concentration vs. distance from the membrane at (from the top) 0, 1, 4, 9, and 19 msec after the end of the action potential. C: Logarithm of submembrane calcium concentration, and its square, during and after a single spike, expressed as a fraction of the peak value at the end of the spike. 1.0 on the ordinate corresponds to 2.21 μM calcium and its square. D: Predicted logarithm of facilitation for two spikes separated by the interval shown on the abscissa. Facilitation is the fractional increase of the second EPSP compared to the first.

However, it is not this average calcium that triggers the release of neurotransmitter, but rather the calcium concentration just underneath the membrane. The calcium entering at the membrane surface diffuses rapidly into the interior of the terminal, so that the initially sharp gradient of calcium at the surface is almost entirely dissipated after about 10 msec (Fig. 5B). Fig. 5C shows the calcium concentration in the 10 nm thick shell just below the plasma membrane as a function of time during and after a nerve impulse (1 synaptic vesicle has a radius of about 25 nm). The submembrane calcium falls to about 50% in 1.5 msec. Since transmitter release depends on the square of the submembrane calcium concentration (see above), it seems more relevant to compare the time course of the square of the predicted submembrane calcium concentration to the time course of the release of transmitter. The latter can be estimated to be about as fast as the rate of decline of postsynaptic current under voltage clamp following a presynaptic action potential. This synaptic current declines with a half time of about 1 msec. The turn-off of transmitter release is probably the rate limiting factor in determining this decay rate, because when transmitter release is artificially shut off almost instantaneously by suddenly stepping the presynaptic membrane potential to the calcium equilibrium potential after a 1 msec moderate depolarization, the postsynaptic current is shortened to less than that following a regular impulse or depolarization (R. Llinas, S. Simon, and M. Sugimori, personal communication). Thus, the decline of the postsynaptic current probably is similar in time course to the decline in transmitter release. From Fig. 5C, it is also similar to the decline of the square in submembrane calcium concentration predicted by the diffusion model.

Finally, Stockbridge and I have used this model to predict the magnitude and time course of synaptic facilitation following a single impulse. We have driven the model with two action potentials separated by various intervals, and estimated the peak submembrane calcium concentration reached by the second peak. If we suppose the amount of transmitter released by a nerve impulse is related to the square of this calcium concentration, then we may predict the facilitation of the second EPSP (expressed as the fractional increase in EPSP size with respect to the first EPSP) by plotting the fractional increase of the peak squared submembrane calcium concentration reached during the second spike with respect to that reached during the first. This prediction is shown in Fig. 5D. Noting the semi-logarithmic co-ordinates, the curve may be described as showing a peak facilitation of about 100%, with a prominent early decline lasting about 10 msec and a slower and smaller component lasting a few tens of milliseconds. This is almost exactly how facilitation actually behaves at the squid giant synapse (Charlton and Bittner, 1978a).

CONCLUSIONS

From this and earlier work, we may conclude that synaptic facilitation is not caused primarily by changes in the presynaptic action potential, membrane potential, calcium channels, or calcium buffering. Rather, synaptic facilitation may be accounted for quantitatively as a consequence of a residual presynaptic calcium following nerve activity, operating on a nonlinear dependence of transmitter release on calcium to amplify the effect of a constant calcium influx accompanying nerve impulses. A final proof of this hypothesis must await the development of techniques to measure directly and selectively the submembrane calcium concentration following nerve activity, and to manipulate submembrane calcium accurately so as to measure precisely the dependence of transmitter release on submembrane calcium. Until then, the nonlinear release version of the residual calcium hypothesis will remain only our best hypothesis for the mechanism of this fascinating and ubiquitous form of synaptic plasticity.

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