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Decline in Calcium Cooperativity as the Basis of Facilitation at the Squid Giant Synapse

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The role of Ca in transmitter release and facilitation has been examined at the squid giant synapse by evaluating the effect of changes in the external concentration of Ca on the excitatory postsynaptic potential (EPSP). Changes in Ca were achieved by means of an arterial perfusion technique, circumventing the diffusion barrier that exists between the synapse and the bathing medium.

Transmitter release was found to follow a high power (n) of the external Ca at low nonsaturating Ca concentrations and low stimulus frequencies: 4.0 ± 0.1 (mean ± SE) in 22 experiments. The value of n was not fixed, however, but declined at stimulus frequencies above 0.3 Hz to 2.9 at 1 Hz, 2.3 at 10 Hz, 1.7 at 50 Hz, and 1.1 at 80 Hz. This decline in n was due to a greater proportional facilitation of the EPSP at lower Ca levels. In a separate experiment, facilitation was determined at a fixed low Ca level over a wide range of stimulus frequencies. A plot of the maximum percentage increase in the EPSP against the stimulus frequency described three "steps," with plateaus occuring at 0.3-10 Hz, 10-50 Hz, and over 80 Hz. The timing of these steps corresponds to the stimulus frequencies at which the cooperativity declines.

The results of this study indicate that the cooperative action of four Ca ions are required to trigger the transmitter release mechanism, and that the phenomenon of facilitation involves a reduction in Ca cooperativity. It is proposed that Ca ions can bind up to four receptors on the release site and that binding results in the opening of "gates." The opening of all four gates triggers transmitter release. Opening one to three gates results in the partial activation of the release site, so that fewer additional Ca ions are required to trigger transmitter release. According to this model, each of the observed "steps" reflects the time required for the closure of one, two, or three gates.

The invasion of the nerve terminal by an action potential initiates a sequence of events culminating in the release of the transmitter substance. These events include depolarization, opening of voltage-sensitive Ca channels, influx of Ca ions, and finally, by an as-yet-unknown mechanism, Ca activation of the transmitter quantum release sites. The number of transmitter quanta released per impulse is not constant, however, but may be enhanced by prior impulses in the nerve terminal, a process termed "facilitation." The mechanisms underlying facilitation are of particular interest owing to their potential importance as a basic means of modulating transmitter release from the nerve terminal.

Facilitation occurs in a wide range of synapse types (e.g., Atwood, 1976; Balnave and Gage, 1974; Bittner and Sewell, 1976; Katz and Miledi, 1968; Kuno, 1964; Magelby and Zengle, 1982; Muir and Porter, 1973). Previous studies have established that facilitation is a presynaptic phenomenon involving the enhancement of one or more of the steps between action potential invasion of the nerve terminal and discharge of the transmitter substance. Possible mechanisms include an increase in the amplitude or duration of the action potential, an increase in voltage dependence of Ca influx, maintained elevated cytoplasmic Ca levels (the "residual Ca hypothesis"), and increased sensitivity of the release sites to activation by Ca.

Action potential amplitude and duration changes during facilitation have been examined in the squid giant synapse nerve terminal but could not account for enhanced transmitter release (Charlton and Bittner, 1978b; Miledi and Slater, 1966; Takeuchi and Takeuchi, 1962). A progressive increase in Ca entry per impulse was recently regarded as a strong possibility, but facilitation has been shown to occur without a significant change in the Ca current (Charlton et al., 1982). From these studies it can be concluded that facilitation must involve the enhancement of a step in the transmitter release process that lies between Ca influx and discharge of the transmitter quantum.

In this study the squid stellate ganglion giant synapse preparation has been used to examine the relation of external Ca concentration to transmitter release, and to further examine the presynaptic events that underlie facilitation. This preparation is well suited to the exploration of basic synaptic processes due to the long-term stability of recording (Bullock, 1948) and the well-established and distinguishing aspect of this preparation, the large presynaptic terminal that allows direct microelectrode penetration (Bullock and Hagiwara, 1957; Hagiwara and Tasaki, 1958). In addition, facilitation has been demonstrated at this synapse (Bloedel et al., 1966; Charlton and Bittner, 1978a, b; Charlton et al., 1982; Katz and Miledi, 1967; Kusano and Landau, 1975; Miledi and Slater, 1966; Takeuchi and Takeuchi, 1962) and is particularly marked at low external Ca levels (Charlton and Bittner, 1978a; Kusano and Landau, 1975; see also below).

The squid giant synapse has one technical drawback: Examination of the effects of changes in external substances on synaptic transmission is impeded by a marked diffusion barrier between the synapse and the external medium (Bryant, 1958), which particularly hampers studies of Ca. Thus, equilibration times for changes in Ca levels range from 30 min to nearly 2 hr (Katz and Miledi, 1970; Lester, 1970; Miledi and Slater, 1966). The method of perfusing the ganglion via the aortic blood supply (Stanley, 1985a; Stanley and Adelman, 1984) used in the present study essentially eliminates this problem, and allows the rapid introduction of ions and other substances to the synapse.

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It is reported here that, at low stimulus frequencies, the quantity of transmitter released from the nerve terminal is related to the fourth power of the external Ca concentration. This observation is consistent with the hypothesis that the cooperative action of four Ca ions is necessary to induce activation of the transmitter release site (Dodge and Rahamimoff, 1967). In addition, it is suggested that the underlying mechanism of facilitation involves a decline in Ca cooperativity, so that fewer ions are needed to activate the release process. Some of these results have been presented previously in abstract form (Stanley, 1985b, c).

Materials and Methods

Experiments were carried out on squid (*Loligo pealii*) of less than 14 cm mantle length. The dissection and perfusion techniques have been described in detail (Stanley and Adelman, 1984). In brief, the squid was decapitated and the internal organs exposed by section up the midventral mantle. The aorta, which is located between the digestive gland and its enclosing muscular tube, was cannulated just rostral to the exit of the common stellate artery. The right stellate artery was ligated and the left stellate ganglion was removed together with the cannulated aorta and a piece of the intervening digestive gland muscle. The preparation was mounted in a chamber and continually perfused with artificial sea water (composition in mM: NaCl, 430; KCl, 10; $CaCl_2$, 10; $MgSO_4$, 50; HEPES, 10; pH 7.3) under O_2 pressure (18 psi). The O_2 served both to maintain the preparation and to force the perfusate through the blood system. Room temperature was regulated so that the preparation remained at 15–19°C. Under these conditions the synapse could be maintained for several hours of study.

Conventional KCl-filled microelectrodes (5–12 MΩ) were used for intracellular recording or current passing from the pre- or postsynaptic giant axons. Output from the microelectrode was led to a unity-gain amplifier (WPI S-7000), a storage oscilloscope, and a six-channel FM tape recorder. The prenerve, containing the presynaptic second-order giant axon, was stimulated by means of a pair of silver wire electrodes. Evoked potentials were photographed directly from the oscilloscope screen on Polaroid film. Since EPSPs were of small amplitude (mostly <10 mV), and the transmitter reversal potential is well into the positive range (Llinas et al., 1974; Miledi, 1969), it was not necessary to correct EPSP amplitudes for nonlinearity of summation.

Perfusion solutions were changed by manually turning the tap on a micro-stopcock (Hamilton) to allow one of four or five alternative solutions to flow into the ganglion. All solutions were maintained at the same O_2 pressure to avoid mechanical disturbance of the microelectrode on switching from one solution to another. The Ca concentrations in the test solutions ranged from 1.0 to 10 mM while adjusting the NaCl concentration to maintain osmolarity. Raising or lowering the Ca concentration in the perfusate resulted in a change in the EPSP that began within seconds and generally reached equilibrium within 1 min (Fig. 1). A minimum of 2 min was allowed between Ca changes to ensure full equilibration.

Results

Effect of external Ca concentration on EPSP amplitude

At low stimulus frequencies (<0.3 Hz), the amplitude of the EPSP was found to be highly dependent on the Ca concentration in the perfusate. At an external Ca concentration of less than 0.5 mM, the EPSP was below the level of detection of the recording system. Between 1 and 4 mM Ca, the EPSP increased markedly with an increase in external Ca (Fig. 2).

Above 4–5 mM Ca, the EPSP exceeded the postsynaptic giant axon threshold and its amplitude was masked by the action potential. In experiments necessitating high external Ca levels, the EPSP was brought below action potential threshold by a hyperpolarizing current pulse delivered from a second intracellular microelectrode. At Ca levels above approximately 5 mM, EPSPs were of large amplitude and very prone to decline after multiple stimuli, presumably due to a depletion of transmitter reserves in the nerve terminal. In order to control for depletion at higher Ca levels, we adopted an experimental protocol that included a return to a control external Ca level between each test Ca level (Fig. 3). The EPSP at each Ca test level was expressed as a percentage of the mean of the 8 mM Ca level immediately prior to and after the test Ca level.

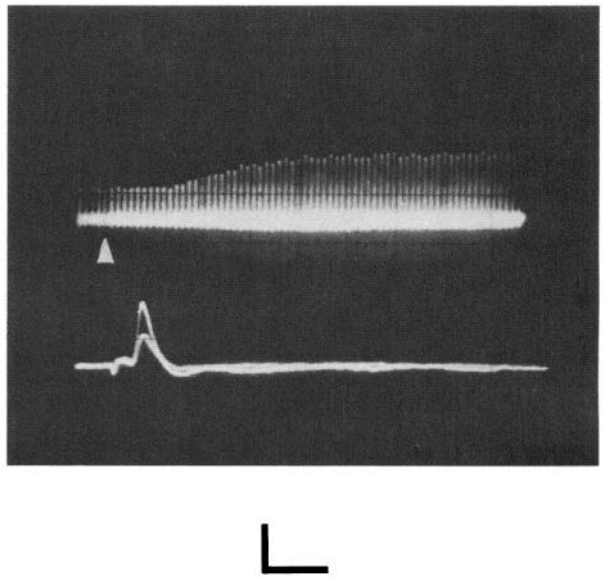

Figure 1. Time course of EPSP amplitude change following an increase in the Ca ion concentration in the perfusion medium. *a,* EPSPs evoked at a stimulus frequency of 1 Hz and recorded intracellularly from the postsynaptic giant axon. At the *arrowhead* the perfusion solution was changed from 1.0 to 1.5 mM Ca. Note the rapid change of the EPSP to a larger value. The initial and final EPSPs are superimposed at a fast sweep speed in *b*. Calibration: *Upper trace,* 10 sec and 1 mV; *lower trace,* 10 msec and 1 mV.

A simple plot of EPSP amplitude against external Ca resulted in a sigmoid relation (Fig. 3*d*). Replotting the data on logarithmic coordinates gave a straight line at low external Ca levels (typically less than 5 mM), while at higher levels the plot became progressively less steep (Fig. 3*e*). This was interpreted as evidence for a power relation between EPSP amplitude and external Ca concentrations with saturation at high Ca levels. Thus, subsequent experiments were carried out primarily in the nonsaturating, 1–4 mM external Ca range.

In 22 experiments using 17 preparations the slope (n) of the log/log relation between external Ca (1–5 mM) and the EPSP amplitude was 4.0 ± 0.1, with a range of 3.5–4.9. It was concluded that the EPSP amplitude is related to the fourth power of the external Ca concentration at low, nonsaturating Ca levels and low stimulus frequencies.

Effect of external Ca on the presynaptic action potential

In order to relate the EPSP amplitude to the quantity of Ca that enters the terminal with each impulse, it is necessary that the action potential amplitude not change significantly with external Ca. No significant change could be detected in the presynaptic giant axon action potential amplitude or duration over the range of 2–8 mM external Ca (Fig. 4), consistent with previous observations (Katz and Miledi, 1970). Since the EPSP amplitude and external Ca can be demonstrated to show a steep dependence with an n of 4 over a far smaller Ca range (e.g., 2–3 mM), it is unlikely that a change in the presynaptic action potential amplitude is a major factor in this relationship.

Effect of an increased stimulus frequency on the relation of external Ca to the amplitude of the EPSP

In some of the experiments exploring the relationship between external Ca and EPSP amplitude, the stimulus frequency was 1

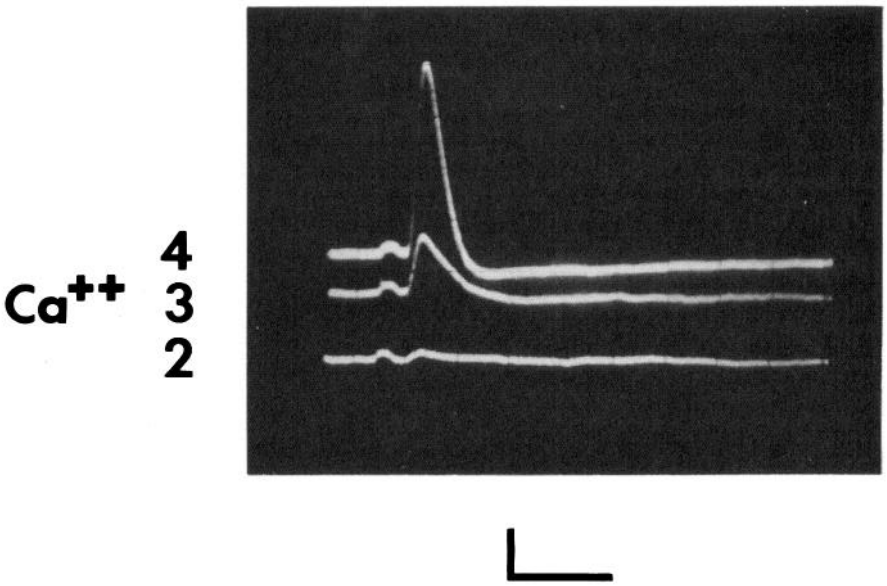

Figure 2. Effect of changing external Ca concentration on the EPSP. Each trace shows a single EPSP evoked at Ca concentrations ranging from 2 to 4 mM. Calibration, 10 msec and 2 mV.

Hz or higher. It was noted that in these cases the n value was consistently lower. The effect of stimulus frequency on n was therefore tested over the frequency range of 0.1–80 Hz. One to two stimulus frequencies were tested in each experiment. The external Ca concentration was equilibrated and a stimulus train was given until the EPSP reached a maximum facilitated value. The external Ca concentration was then changed and the stimulus train repeated. The initial EPSP in the train served as an unstimulated control for each stimulus train.

Increasing the stimulus frequency changed the relationship between the EPSP amplitude and the external Ca: The log–log plot became progressively less steep (Fig. 5). This change was due to the unequal facilitation of the EPSP at different Ca levels. For example, at a stimulus frequency of 10 Hz there was a proportionally greater facilitation of the EPSP at 2 mM Ca than at 3 mM (Fig. 6).

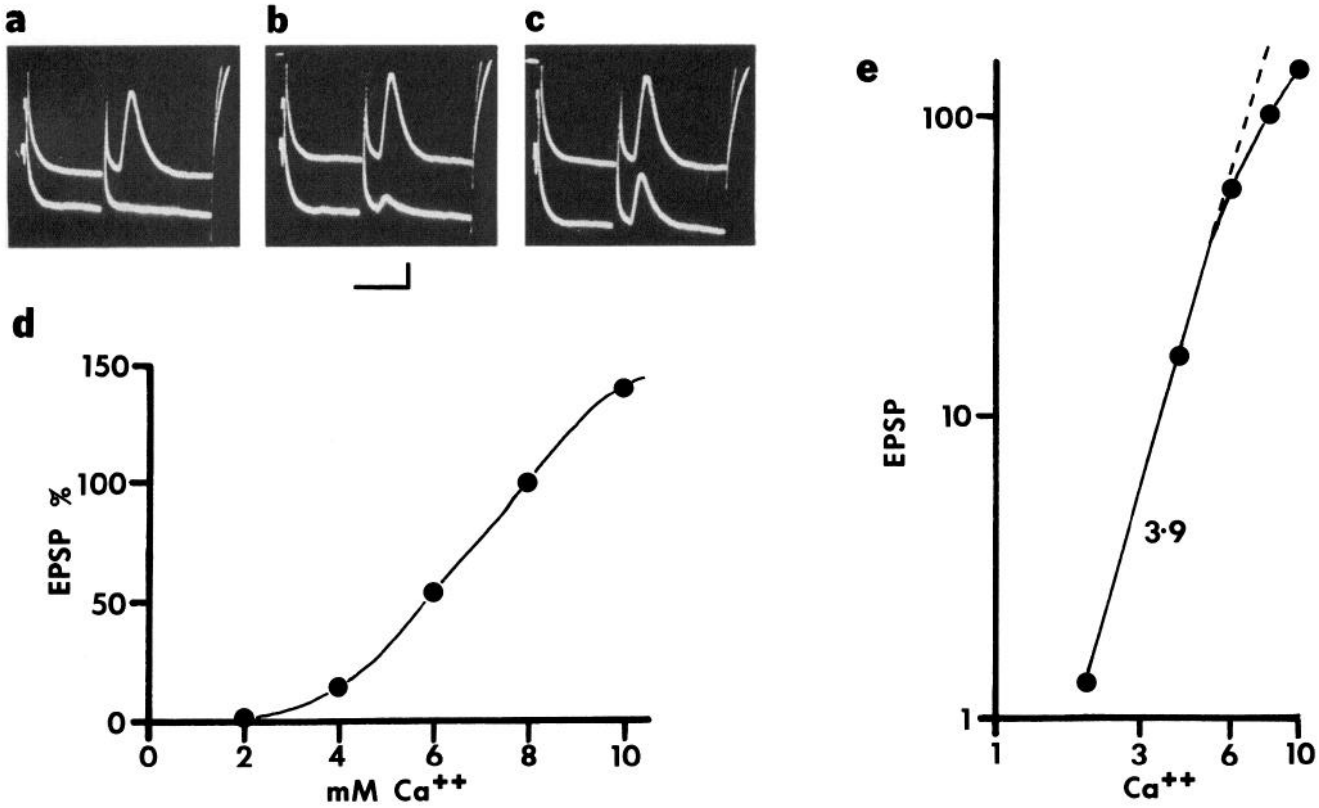

Figure 3. Effect of changing external Ca on EPSP amplitude. EPSPs were recorded intracellularly from the postsynaptic giant axon while stimulating the prenerve. *a–c,* Each shows two traces of EPSPs recorded at a control 8 mM external Ca (*upper*) and one test Ca concentration: 2 mM (*a*), 4 mM (*b*), 6 mM (*c*). A hyperpolarizing pulse of constant amplitude, delivered by means of a second intracellular microelectrode, maintained the EPSPs below action potential threshold. Calibration, 10 msec and 1 mV. Single EPSP amplitudes were expressed as a percentage of the amplitude at the control external Ca of 8 mM and were plotted against the external Ca concentration on linear (*d*) and double-logarithmic (*e*) coordinates.

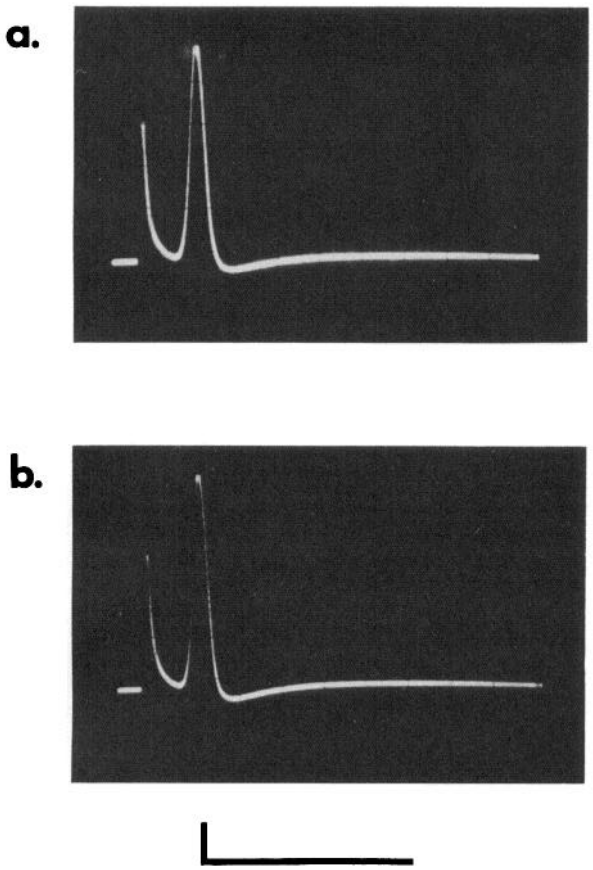

Figure 4. Action potentials recorded intracellularly from the presynaptic giant axon during perfusion with 2 mM (*a*) and 8 mM (*b*) external Ca. Calibration, 10 msec and 20 mV.

The effect of stimulus frequency on EPSP amplitude was evident at rates as slow as 1 Hz, where the log–log plot gave a slope of 2.9 ± 0.2, $n = 3$ (Table 1; Fig. 5). Thus, at this stimulus frequency the EPSP amplitude was related to approximately the third power of the external Ca concentration. At higher stimulus frequencies the power function declined further to 2.3 (10 Hz) and 1.8 (30 Hz) (Table 1). Above 30 Hz, the plot of log EPSP amplitude to log external Ca did not always fit a straight line at the higher levels of the 1–5 mM Ca range. At lower external Ca levels (1–3 mM), a reasonable linear relation was observed even at higher stimulus frequencies, and slopes of the log–log plot were 1.65 (50 Hz) and 1.1 (80 Hz) (Table 1; Fig. 5).

Effect of stimulus frequency on facilitation of the EPSP

The relationship between facilitation and stimulus frequency was examined at a fixed, low external Ca level. The prenerve was stimulated with trains of impulses at a wide range of frequencies and the maximum facilitation of the EPSP was determined at each frequency. This experiment was carried out at a low Ca level since the above results indicate that under these conditions facilitation is most evident and transmitter depletion may be largely avoided. Preliminary experiments established that at 2 or 3 mM external Ca, the initial EPSP was sufficiently large to measure with reasonable accuracy, but cumulative release was low enough to avoid significant depletion.

In this experiment, the prenerve was stimulated with trains of stimuli at stimulus frequencies ranging from 0.06 to 80 Hz. Stimulus trains were given at 2 min intervals to allow recovery from fatigue and facilitation. The stimulus train was continued until maximum facilitation of the EPSP was reached, ranging from 20 to 30 sec at low frequencies to a few seconds at high frequencies. At a stimulus frequency of 0.1 Hz, the EPSP amplitude was maintained for many minutes without any apparent facilitation, but at frequencies above 0.3 Hz, facilitation was evident (Fig. 7). At frequencies of up to 10–20 Hz, the EPSP increased progressively, reaching a plateau that could be maintained for many seconds of continuous stimulation (Fig. 6). At higher frequencies, facilitation was usually followed by depression of transmission, which was attributed to depletion of releasable transmitter quanta.

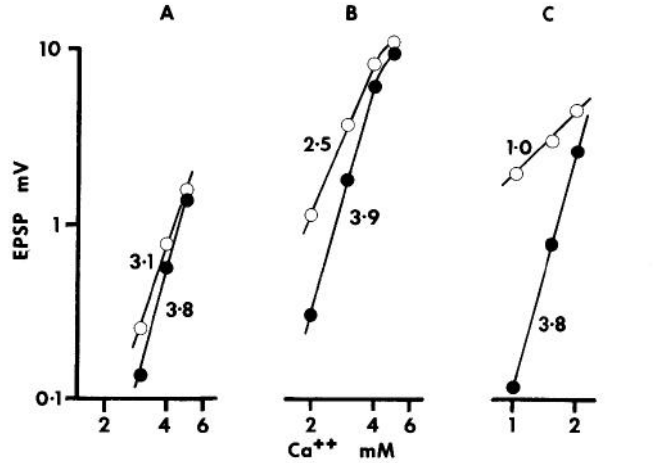

Figure 5. Plot of EPSP amplitude against external Ca at different stimulus frequencies in three different experiments: *A*, 1.0 Hz; *B*, 10 Hz; *C*, 80 Hz. The prenerve was stimulated with a train of impulses at each external Ca concentration and stimulus frequency. Trains were given at intervals of at least 2 min. The first EPSP in each stimulus train is denoted by the *filled circles* and plotted against Ca. A straight line through these values gives a slope close to 4. The *open circles* show the amplitude of the maximum facilitated EPSP at a point in the stimulus train where the EPSPs had reached a steady state or, at high stimulus frequencies, immediately prior to fatigue.

The mean amplitude of three of the maximum facilitated EPSPs was expressed as a percentage of the initial EPSP and plotted against the stimulus frequency. Attempts to fit the data by a smooth sigmoid line were only very approximate; a far better visual fit was obtained in each case by a line that depicted three "steps" (Fig. 7). This interpretation was supported by the repeatability of the steps in different experiments. The significance of these steps in relation to the above findings is discussed below.

Discussion

Calcium cooperativity at the giant synapse

Several previous reports have focused on the relationship between Ca and transmitter release at the squid giant synapse, but these studies fail to arrive at a consensus for n at this synapse, with values including 1 (Llinas et al., 1981), 2 (Augustine and Eckert, 1984; Charlton et al., 1982), 2.5 (Katz and Miledi, 1970),

Table 1. Pooled results for the slope of the plot of ln(EPSP) against ln(Ca) at different stimulus frequencies[a]

Hz	0.1	0.5	1.0	10	20	30	50	80
n	4.0	3.4	2.9	2.3	2.0	1.8	1.7	1.1
SE	0.4	0.5	0.2	0.7	—	—	0.1	0.1
N	22	5	3	6	1	1	6	6

[a] Hz, stimulus frequency; *n*, mean slope of ln(EPSP amplitude)/ln(Ca) plot; SE, standard error; *N*, number of determinations.

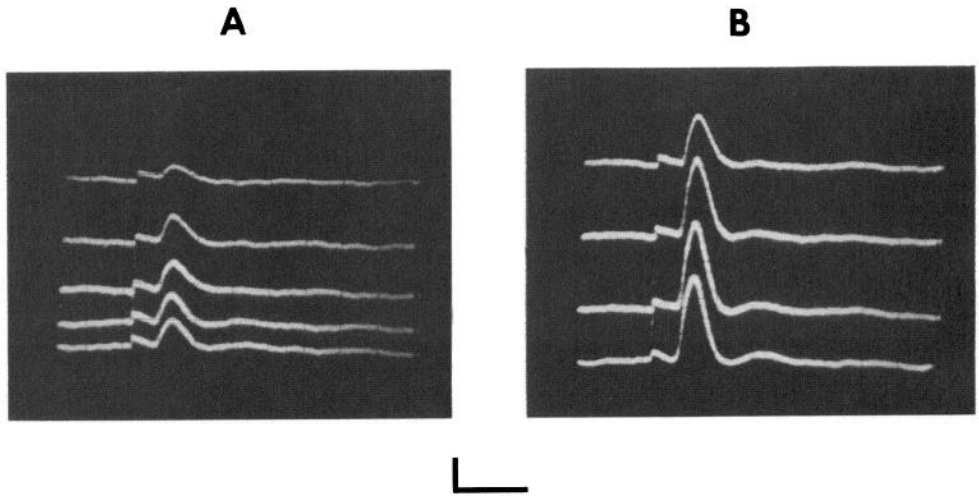

Figure 6. Facilitation of the EPSP during stimulation at a frequency of 10 Hz and perfusion with 2 mM (*A*) and 3mM (*B*) Ca. Each panel shows the first response in the stimulus train (*top*) and EPSPs recorded at 5 sec intervals. Note the greater proportional facilitation at the lower Ca level. Note also that the amplitude of the facilitated EPSP is maintained for many seconds of 10 Hz stimulation without evidence of fatigue or depression. Calibration, 10 msec and 1 mV.

3 (Smith et al., 1985), and 3.5 (Lester, 1970). In general, these studies can be divided between two basically different experimental strategies, both of which attempt to determine the Ca dependence of the transmitter release mechanism. These strategies are (1) to maintain a constant external Ca level while varying the driving force for Ca influx (a voltage step) or (2) to change the external Ca level and maintain the driving force for Ca influx into the terminal constant (usually the action potential), as in the present study.

The first of these strategies, controlling Ca influx by a voltage pulse, has resulted in n values ranging from 1 (Llinas et al., 1981) to 3 (Smith et al., 1985). The interpretation of these experiments is complicated by the involvement of two factors in determining the amplitude of the gross observed Ca current: the number of Ca channels opened, and the quantity of current passing through each channel. Both factors change over the voltage step ranges tested, but they may not have the same effect on transmitter release. In particular, if individual Ca channels are closely associated with the transmitter release sites, consistent with recent findings (Llinas et al., 1981; Pumplin et al., 1981), the opening of one Ca channel could allow sufficient Ca influx to activate one release site—at least over part of the membrane potential range tested. If so, the relationship between gross Ca current and transmitter release would be linear, since it reflects the recruitment of Ca channels rather than the relationship between internal Ca and the transmitter release mechanism.

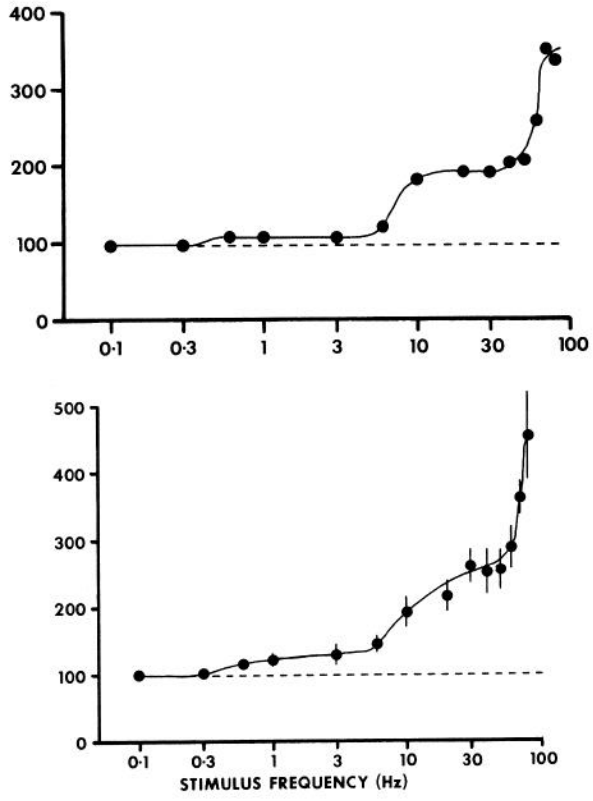

Figure 7. Plot of maximum facilitation against stimulus frequency at 2 mM Ca. At each stimulus frequency the maximum facilitated EPSP was expressed as a percentage of the amplitude of the first EPSP in the train. *Upper plot,* Results obtained in a single experiment; *Lower plot,* four pooled experiments. *Bars* denote SE.

The second strategy for relating Ca to transmitter release, that is, changing the external Ca level, has also resulted in a range of n values, from 2.5 (Katz and Miledi, 1970) to 4 (this study). With this approach, the number of Ca channels activated with each impulse presumably remains reasonably constant, since the action potential does not change (see also Katz and Miledi, 1970). The amplitude of the Ca current through each channel may be presumed to be linearly related to the external Ca concentration (although this assumption requires confirmation). Thus, changes in transmitter release may be expected to reflect changes in Ca influx, even if the Ca channel is closely associated with the release site.

The range of n values obtained in different reports using this experimental strategy can probably be attributed to the problem of Ca access (see the introduction). A detailed study by Lester (1970), however, obtained an estimate of n of 3.5 despite these technical difficulties. Thus, the conclusion of the present study is that the Ca dependence of transmitter release at the squid giant synapse is highly nonlinear, with a power relation of about 4. This finding is consistent with previous studies at the frog neuromuscular junction (Andreu and Barrett, 1980; Dodge and Rahamimoff, 1967) and several other preparations.

Significance of multiple Ca ion involvement in release

To what step in the sequence of events involved in synaptic transmission can the cooperative action of four Ca ions be attributed? Ca cooperativity could be due to (1) Ca entry into the terminal (Cooke et al., 1973; Parnas et al., 1982); (2) buffering in the cytoplasm (Nachsen and Drapeau, 1982); or (3) multiple ion activation of the release site (Dodge and Rahamimoff, 1967). Although these causes are theoretically possible, cooperativity in Ca entry into the terminal has not been seen (Llinas et al., 1981; Nachshen and Blaustein, 1980), and the cytoplasmic buff-

ering hypothesis lacks experimental support. In particular, the buffering model predicts a decline in n at very low external Ca levels (Nachsen and Drapeau, 1982), but no such decline has been reported (Andreu and Barrett, 1980). Thus, the suggestion of Dodge and Rahamimoff (1967), that Ca cooperativity occurs at the level of the release site, remains the most likely hypothesis. Their suggestion was that the n of 4 was due to the requirement for the activation of four (or actually a minimum of four) Ca receptors on the transmitter release site.

Ca cooperativity and facilitation

The power relation of external Ca to EPSP amplitude was found to depend on the stimulus frequency. This finding is consistent with previous results on the frog neuromuscular junction, where it is reported that the dependence of Ca transmitter release at very low quantal contents declines from an n of 4 to lower values at high stimulus frequencies (Andreu and Barrett, 1980). In the giant synapse this decline was evident at stimulus frequencies as low as 0.5 Hz, when a value for n of 3.4 was obtained. Further decline in n occurred with increasing stimulus frequency, reaching 1.1 at a frequency of 80 Hz.

The simplest explanation for the decline in Ca cooperativity with increasing stimulus frequency is that free Ca ions accumulate in the nerve terminal (Alnaes and Rahamimoff, 1975; Charlton et al., 1982; Katz and Miledi, 1968; Rahamimoff, 1968; Zucker, 1974; Zucker and Stockbridge, 1983), which results in the equilibrium saturation of a fraction of the Ca receptors on the release site. This hypothesis would explain facilitation that involved a moderate reduction in cooperativity, since, during subsequent impulses, fewer additional Ca ions would need to bind to achieve the four required for release site activation.

A large decline to unity cooperativity is difficult to account for with the free Ca hypothesis. Such a decline would require the equilibrium saturation of a mean of three of the four Ca receptors on the release site during the interval between impulses. Since the residual Ca is free, actual binding would vary between zero and four Ca ions, with a relatively high probability of four ions bound. Each time this occurred, however, the release sites would be activated, and hence the terminal might be expected to quickly become nonfunctional because of the depletion of releasable transmitter. That this does not occur—the apparent cooperativity declines to close to one at a stimulus frequency of 80 Hz without inactivation of the terminal—favors a facilitation mechanism that cannot in itself induce release. Thus, although it remains possible that the equilibrium binding of accumulated free Ca plays a role in facilitation, this hypothesis appears to be insufficient to totally explain the phenomenon. It should be noted that there would be no such limitation if transmitter release could not be triggered by Ca alone, but also required membrane depolarization (Dudel et al., 1983).

A simple model of transmitter release that accounts for facilitation, without suffering from the above limitation, can be devised. The components of this model are a transient increase of Ca and a release site with four Ca receptors. It is supposed that binding of Ca to each of the receptors on the release site triggers a subsequent event, which I will term the opening of a "gate," to distinguish it from the binding and dissociation of Ca itself (see below). In this model, all four gates must be opened to activate transmitter release. In addition, it is suggested that the gates (S1–S4) close at different rates, so that S1 closes very slowly while S4 closes much more rapidly, with S2 and S3 intermediate.

The influx of Ca into the nerve terminal will bind to zero to four receptors on each release site, resulting in a distribution of release sites with zero to four gates opened. Release sites with four gates opened will discharge their transmitter quantum, whereas those with one to three will not. Whether the transmitter release due to a subsequent impulse will be facilitated depends on the stimulus interval. If the impulse occurs at a delay that is longer than the time required for closing of the slowest gate, S1, release will not change. If, however, the interval is shorter than this time, release sites that bound at least one Ca ion will only require three additional ions to activate transmitter release. Thus, release is facilitated, and the cooperativity will decline to 3. Similarly, further facilitation and decline in cooperativity will occur if the stimulus interval is shorter than the closing time of gates S2 and S3. Note that in this model, the experimentally observed decline in cooperativity with facilitation is only apparent: Under all conditions, four gates must be open to trigger transmitter release.

Presumably, the minimum Ca cooperativity should be 1, when three out of the four gates are continually open, since without one available gate it would not be possible to link transmitter release to Ca influx—assuming independent release site activation.

In contrast to the residual Ca hypothesis, with this model a decline in the cooperativity to 1 would not result in an indiscriminate resting transmitter release. An additional advantage is that the duration of the EPSP would not be expected to show marked changes, even during maximum facilitation, since transmitter release would still be terminated abruptly by the removal of excess Ca.

The model can be tested by determining the degree of facilitation at different stimulation rates, at a constant external Ca level. Facilitation should not occur at stimulus intervals longer than the time taken for recovery of S1. Above this frequency, facilitation should increase with stimulus frequency, and maximum facilitation should occur when the stimulus interval exceeds the recovery rate of S3. Thus, a plot of facilitation against stimulus frequency should show a sigmoid relation. A sigmoid curve could indeed be fitted to the experimental data, but the plot showed further detail: A far better visual fit was obtained by a line depicting three steps. It is suggested that each of the observed steps reflects the stimulus frequency at which the stimulus interval exceeds the time required to close one of the Ca-triggered gates, S1–S3. If so, the number of gates that are effectively in a continuously open state (s) at any stimulus frequency can simply be determined as the number of steps climbed.

According to the model discussed above, the power relation between external Ca and the EPSP amplitude, n, is an estimate of the number of closed gates on the release sites. Thus, for the model to be consistent, the number of closed gates, n, and the number of already opened gates, s, should sum to 4 at any stimulus frequency.

A comparison of n and s at different stimulus frequencies shows that this is indeed the case. At long stimulus intervals, $n = 4$, which corresponds to four available Ca receptors, and s is taken to be 0. At 1 Hz, $n = 2.9$, $s = 1$, and the sum is 3.9. Similarly, at 10, 50, and 80 Hz, the sums of n plus s are 3.8, 4.2, and 4.1, respectively.

Perhaps the simplest molecular interpretation of the above model is that the slow recovery of the S1–S3 gates reflects the slow dissociation of Ca from its receptors on the release site. An attempt to model the experimental data on this basis was only partially successful, however, depicting the decline in cooperativity observed with increasing stimulus frequency, but not the steps. Thus, if the above model proves correct, the delayed recovery of the Ca-activated gates would seem to have a more complex molecular basis.

Facilitation and neural processes

Differences in the capability of showing facilitation between synaptic preparations are also of interest. Some synapses show little or no release in response to a single stimulus, but a large degree of facilitation. In contrast, other synapses, such as the

squid giant synapse at normal external Ca (usually taken to be 10 mM), show little facilitation, very large responses to a single stimulus, and rapid depletion (Kusano and Landau, 1975). At low Ca levels, however, the giant synapse is more typical of the former type (see above). Thus, differences in the capability of showing facilitation at different synapses may simply reflect different ratios of Ca influx to release site density, rather than different fundamental facilitatory mechanisms. It should also be noted that in nerve terminals containing two (or more) releasable substances, some selection over which is released could be achieved by different capabilities for facilitation by their respective release sites.

The significance of facilitation in neural processes may extend beyond the enhancing effect of one impulse on another. Facilitation may also be important as a final step in other processes of transmitter release enhancement. According to the above model, any influence on the nerve terminal that reduces the number of Ca ions required for release site activation will enhance transmitter release. Conversely, an increase in Ca receptors (it remains at least theoretically possible that a fifth or more of the receptors could be revealed) would inhibit release. Thus, regulation of transmitter release Ca cooperativity is an additional potential mechanism whereby the strength of synaptic contacts, and hence the direction of impulse conduction in complex neural systems, can be modulated.

Note added in proof. Since the original submission of this paper, we have presented a simple model of exocytosis that can account for some of the observations discussed in this study [Stanley, E. F., and G. Ehrenstein (1985) A model for exocytosis based on the opening of calcium-activated potassium channels in vesicles. Life Sci. *37:* 1985–1995]. According to this model, the "memory" associated with facilitation is not due to Ca accumulation in the cytoplasm, but to the buildup of osmolarity in the synaptic vesicles themselves.

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