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Role of Residual Calcium in Synaptic Depression and Posttetanic Potentiation: Fast and Slow Calcium Signaling in Nerve Terminals

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Summary

Trains of action potentials evoked rises in presynaptic Ca^{2+} concentration ($[Ca^{2+}]_i$) at the squid giant synapse. These increases in $[Ca^{2+}]_i$ were spatially nonuniform during the trains, but rapidly equilibrated after the trains and slowly declined over hundreds of seconds. The trains also elicited synaptic depression and augmentation, both of which developed during stimulation and declined within a few seconds afterward. Microinjection of the Ca^{2+} buffer EGTA into presynaptic terminals had no effect on transmitter release or synaptic depression. However, EGTA injection effectively blocked both the persistent Ca^{2+} signals and augmentation. These results suggest that transmitter release is triggered by a large, brief, and sharply localized rise in $[Ca^{2+}]_i$, while augmentation is produced by a smaller, slower, and more diffuse rise in $[Ca^{2+}]_i$.

Introduction

Many lines of evidence indicate that a rise in the Ca^{2+} concentration ($[Ca^{2+}]_i$) within presynaptic terminals couples electrical excitation to secretion of neurotransmitters. This evidence includes demonstrations that elevation of $[Ca^{2+}]_i$ triggers transmitter secretion (Miledi, 1973; Alnaes and Rahamimoff, 1975); introduction of Ca^{2+} buffers into presynaptic terminals blocks transmitter secretion (Kretz et al., 1982; Adler et al., 1991); and action potentials elevate presynaptic $[Ca^{2+}]_i$ (Llinas and Nicholson, 1975; Miledi and Parker, 1981; Charlton et al., 1982). These and other findings leave little doubt that Ca^{2+} acts as an essential presynaptic second messenger for the triggering of transmitter release (Katz, 1969; Augustine et al., 1987).

However, one anomalous conclusion that emerges from measurements of presynaptic $[Ca^{2+}]_i$ is that action potential–evoked Ca^{2+} signals and transmitter release have very different time courses: elevated Ca^{2+} levels, in the range of submicromolar concentrations, persist for many seconds following an action potential, while transmitter release typically terminates within a few milliseconds (Barrett and Stevens, 1972). This kinetic discrepancy can be understood by assuming that steep gradients of $[Ca^{2+}]_i$ occur in presynaptic terminals during electrical excitation (Zucker and Stockbridge, 1983; Simon and Llinas, 1985; Smith et al., 1988) and that very high local $[Ca^{2+}]_i$, briefly reaching levels that may be as much as hundreds of micromolar, is needed to trigger transmitter release (Simon and Llinas, 1985; Roberts et al., 1990; Verhage et al., 1991; Adler et al., 1991). In this case, the small residual Ca^{2+} signals occurring after an action potential would be too small to trigger release.

Although the residual presynaptic Ca^{2+} signal evoked by action potentials may be too small to trigger release, it is possible that this small and slow signal could have other physiological roles. In this paper we test the possibility that the residual Ca^{2+} signals are involved in mediating slow, plastic changes in synaptic efficacy. For this purpose, we have employed the "giant" synapse of squid, whose unusually large presynaptic terminal permits direct measurement and manipulation of presynaptic $[Ca^{2+}]_i$. We have studied the depression (Kusano and Landau, 1975) and potentiation (Magleby and Zengel, 1975) of transmitter release that occur during and after a train of presynaptic action potentials. We find that while depression does not appear to be due to a persistence of elevated presynaptic $[Ca^{2+}]_i$, potentiation is caused by this slow, residual presynaptic Ca^{2+} signal. Thus, the Ca^{2+} influx caused by an action potential has multiple functions: the large, local elevation of $[Ca^{2+}]_i$ triggers transmitter release, while the smaller, more global residual Ca^{2+} signal activates reactions that augment synaptic transmitter release.

Results

Video Imaging of Presynaptic Ca^{2+} Signals

Real-time ratio imaging was used to examine the spatial and temporal distribution of the increases in $[Ca^{2+}]_i$ produced by brief trains of presynaptic action potentials. These increases depended upon the presence of external Ca^{2+} and, thus, were due to Ca^{2+} influx into the terminal. As reported previously, pronounced gradients of $[Ca^{2+}]_i$ occurred during the trains due to the restriction of the sources of Ca^{2+}—the voltage-gated Ca^{2+} channels—to the small region of the presynaptic terminal closest to the postsynaptic cell (Smith et al., 1988; Augustine et al., 1989; Smith et al., unpublished data). These gradients are illustrated in Figure 1A with images taken from the area indicated in the inset of Figure 1B. During the train of action potentials, $[Ca^{2+}]_i$ was highest in the presynaptic regions closest to the postsynaptic axon (Figure 1A, top row). As the train progressed, $[Ca^{2+}]_i$ gradually rose throughout the terminal, but the lateral gradients of $[Ca^{2+}]_i$ were main-

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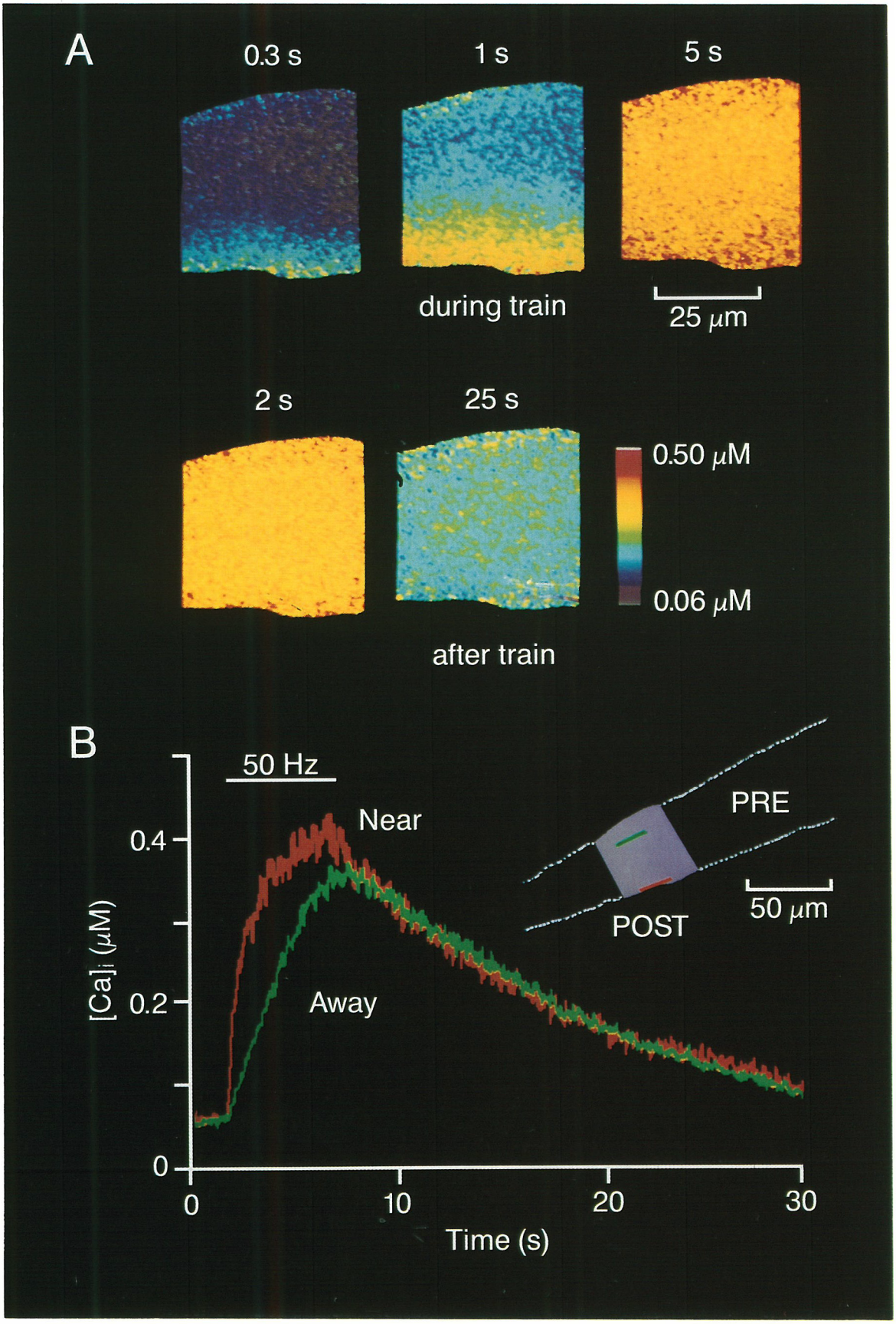
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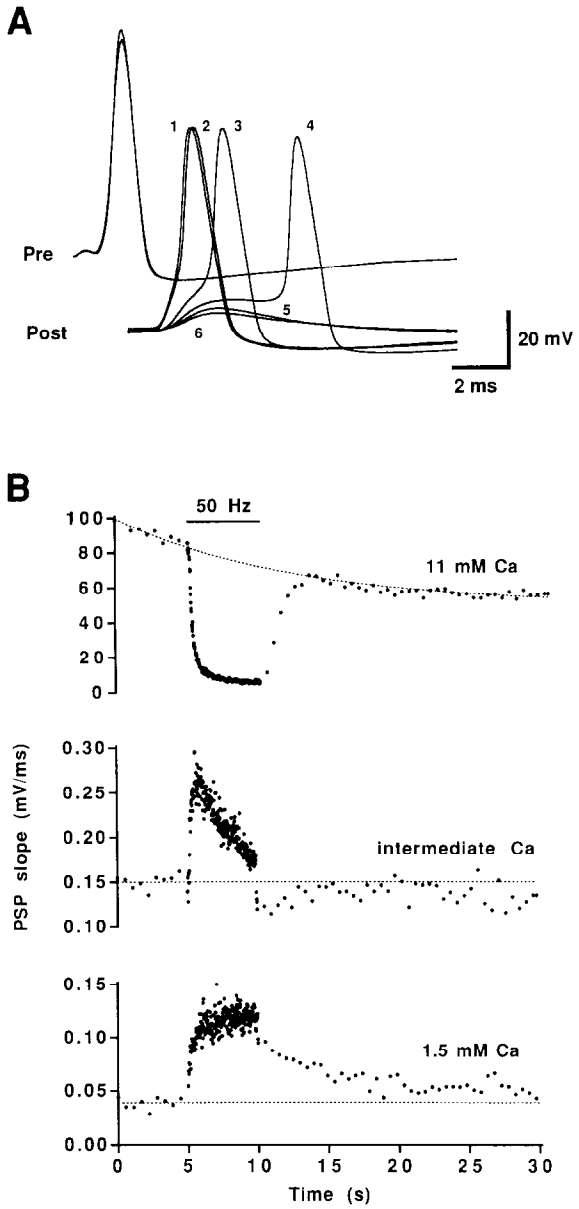


Figure 2. Electrical Activity Induces Synaptic Plasticity at the Squid Giant Synapse

(A) Presynaptic action potentials evoking PSPs. At the beginning of a train of presynaptic action potentials (50 Hz, 5 s), PSPs were suprathreshold and generated action potentials. Under normal ionic conditions (11 mM Ca^{2+}), PSPs quickly became subthreshold as presynaptic activity persisted and synaptic activity was reversibly depressed. Numbers on PSP traces indicate responses measured before (#1) and 40, 100, 160, 220, and 1000 ms into (#2–6) the train.

(B) The upper graph illustrates the time course of synaptic depression produced by a train. The rate of rise of the PSPs was taken as a measure of the magnitude of synaptic transmission and was plotted as a function of time. Note that some depression occurred before the train during the 2 Hz stimulation that preceded the 50 Hz train. Lowering $[Ca^{2+}]_o$ caused the synapse to exhibit both augmentation and depression (middle graph). With an external Ca^{2+} concentration of about 1.5 mM, augmentation during and after the train could be clearly observed (lower graph).

tained. Following the end of the presynaptic electrical activity, $[Ca^{2+}]_i$ stopped rising and the spatial gradients dissipated (Figure 1A, bottom row). $[Ca^{2+}]_i$ then slowly declined, presumably because it was being removed from the terminal by active removal mechanisms (Zipser et al., 1991, Biophys. J., abstract).

The time course of the $[Ca^{2+}]_i$ change produced by a train of action potentials is shown graphically in Figure 1B. This graph compares the Ca^{2+} signals produced in the two regions of the presynaptic terminal indicated by the colored bars of the inset in Figure 1B. $[Ca^{2+}]_i$ rose more slowly and to lower levels in the region distant from the postsynaptic cell (Figure 1B, inset, green bar), presumably because Ca^{2+} was diffusing from the active zones into the interior of the terminal. Spatial equilibration across the width of the terminal required 1–2 s, as indicated by the convergence of the two curves shown in Figure 1B. There are also modest longitudinal gradients of $[Ca^{2+}]_i$ that are obvious in measurements using a lower magnification lens. These will be described elsewhere (Smith et al., unpublished data). Throughout the presynaptic terminal, $[Ca^{2+}]_i$ required several hundred seconds to return fully to its prestimulus levels (Miledi and Parker, 1981; Charlton et al., 1982; Zipser et al., 1991, Biophys. J., abstract).

In summary, presynaptic $[Ca^{2+}]_i$ can be seen to rise to concentrations of a fraction of a micromolar for many seconds following a train of action potentials. There are pronounced lateral gradients of $[Ca^{2+}]_i$ during the train, but these rapidly dissipate following the termination of electrical activity. We next examined the properties of synaptic transmission during such trains of action potentials.

Electrical Activity Causes Plastic Changes in Synaptic Transmission

Single presynaptic action potentials normally evoked so much transmitter release that the resultant postsynaptic potential (PSP) was suprathreshold and elicited an action potential in the postsynaptic axon (Figure 2A). However, a train of presynaptic action potentials caused complex changes in transmission. In normal ionic conditions (11 mM external Ca^{2+}), re-

Figure 1. Presynaptic $[Ca^{2+}]_i$ Changes Produced by Trains of Action Potentials (5 s, 50 Hz) as Recorded with Video Imaging

The inset in (B) shows a schematic representation of the part of the giant synapse on which the microscope was focused. Pre- and postsynaptic structures run parallel to each other and do not overlap. The presynaptic area that was chosen for optical recording is indicated as the gray shaded region. (A) Time-dependent changes in presynaptic $[Ca^{2+}]_i$ during and after a train of action potentials in this area at the times indicated. The changes in $[Ca^{2+}]_i$ are encoded in the pseudocolor scale shown below. (B) Changes in presynaptic $[Ca^{2+}]_i$ plotted against time. Ca^{2+} signals from two different regions of the presynaptic terminal (indicated by colored bars in the inset of [B]) are compared.

petitive presynaptic activity led to a reversible depression of synaptic transmission (Figure 2A). These changes in synaptic transmission were quantified by measuring the rate of rise of the PSP (see, e.g., Miledi and Slater, 1966; Augustine and Charlton, 1986). An example of the kinetics of development of depression during a train of action potentials and of recovery from depression after the train is shown in Figure 2B (top). Low frequency stimulation (2 Hz) produced a modest decrease in PSP amplitude and rate of rise evident as a slowly declining baseline. Increasing the stimulus frequency to 50 Hz for 5 s reduced synaptic transmission by about 95%; on the average, such a stimulus reduced transmission by 92% ± 5% (mean ± SD, n = 19). The development of depression during the high frequency train could be described as the sum of two exponentials, with time constants of 106 ± 52 ms and 900 ± 305 ms (mean ± SD, n = 19). On the average, the fast component was 2.9 ± 1.5 (mean ± SD, n = 19) times larger than the slow component. Following the train, depression recovered in an exponential fashion over several seconds. Although the time constant for this exponential recovery was quite variable from preparation to preparation, the mean time constant was 3.9 ± 2.8 s (mean ± SD, n = 26). This value for the rate of recovery from depression is in reasonable agreement with that reported by Kusano and Landau (1975).

As observed at other synapses (del Castillo and Katz, 1954; Betz, 1970; Zucker, 1972), depression at the squid synapse depends upon the magnitude of synaptic transmission (Kusano and Landau, 1975). By lowering the external Ca^{2+} concentration ($[Ca^{2+}]_o$), transmission at the squid synapse could be greatly reduced (e.g., Katz and Miledi, 1970; Lester, 1970; Stanley, 1986; Augustine and Charlton, 1986). Under these conditions, the amount of synaptic depression is also appreciably reduced (Figure 2B, middle). As depression was reduced, an underlying potentiation of synaptic transmission could be observed during and following a train of action potentials. With an $[Ca^{2+}]_o$ of approximately 1.5 mM, this tetanic and posttetanic potentiation of synaptic transmission could be observed in relative isolation (Figure 2B, bottom). During a 5 s, 50 Hz train, potentiation increased synaptic transmission by an average of 134% ± 9% (mean ± SD, n = 26). The magnitude of the potentiation was rather seasonal, being most evident during months when transmission at the synapses was most robust (i.e., June and September, during the June–September squid season). The development of potentiation during such trains could be described by a single exponential with a time constant of 720 ± 338 ms (mean ± SD, n = 26). Following the end of these trains, synaptic transmission also recovered with an exponential time course. The mean time constant of 2.5 ± 1.1 s (mean ± SD, n = 26) for this exponential recovery defines this potentiation as the augmentation phase of tetanic and posttetanic potentiation (Magleby and Zengel, 1976).

Thus, repetitive presynaptic electrical activity evokes at least two relatively slow forms of plasticity at the squid synapse: depression and augmentation. Depression predominates under normal physiological conditions, while augmentation is most evident under conditions where synaptic transmission is reduced to approximately 1/1000 of its normal strength. We examined these two forms of plasticity in relative isolation by studying synapses bathed in saline containing either normal or lowered $[Ca^{2+}]_o$. With these experimental approaches we could address the possible role of the residual presynaptic Ca^{2+} signal in mediating these two forms of plasticity.

Role of Residual Ca^{2+} Signals in Depression and Augmentation

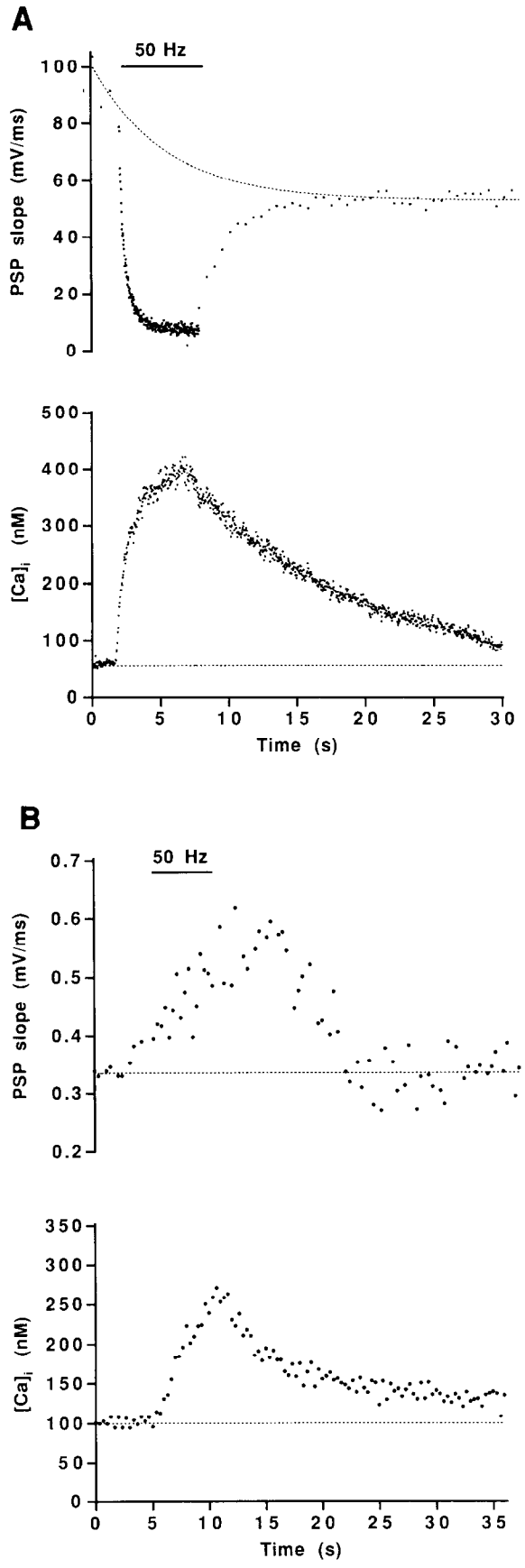
To evaluate the possible role of the long-lasting, residual $[Ca^{2+}]_i$ changes in mediating depression and augmentation, we have asked two questions: Is there a temporal correlation between the $[Ca^{2+}]_i$ changes and the time course of these two forms of plasticity? and does selective removal of the residual Ca^{2+} signal eliminate these two forms of plasticity?

Temporal Relationship between $[Ca^{2+}]_i$ and Changes in Synaptic Efficacy

The temporal relationship between the changes in $[Ca^{2+}]_i$ and synaptic transmission produced by trains of presynaptic action potentials was examined with simultaneous measurements of presynaptic $[Ca^{2+}]_i$ and PSPs. Synapses bathed in normal $[Ca^{2+}]_o$ responded to trains of action potentials with rises in $[Ca^{2+}]_i$. These signals were approximately 10 times higher than the resting $[Ca^{2+}]_i$ and declined over many seconds. Under these conditions, depression caused PSPs to decline with a time course similar to that of the rising phase of the Ca^{2+} signal (Figure 3A). However, recovery from depression was much more rapid than recovery of the Ca^{2+} signal. For the example shown in Figure 3A, the time required for half-recovery of the PSPs was 3 s, compared with 8.5 s for the Ca^{2+} signals. Synapses bathed in low $[Ca^{2+}]_o$ (1.5 mM) produced more modest rises in $[Ca^{2+}]_i$, never reaching 1 μM during the standard 5 s, 50 Hz train, and augmentation was visible during and after the train (Figure 3B). In preparations where depression was not evident, augmentation continued to increase throughout the train, while $[Ca^{2+}]_i$ tended to increase less rapidly near the end of the train. Augmentation also decayed more rapidly than $[Ca^{2+}]_i$ after the train. For the experiment of Figure 3B, the time required for half-recovery of the PSP was about 6 s, compared with about 9 s for the Ca^{2+} signal. Thus, the time course of the presynaptic Ca^{2+} signal was not identical to that of either synaptic depression or augmentation.

Effect of Blocking the Residual Ca^{2+} Signal on Synaptic Plasticity

As an alternative means of evaluating the physiological function of the residual Ca^{2+} signal, the Ca^{2+} buffer EGTA was microinjected into the presynaptic terminal. Because of the relatively slow kinetics of this buffer, it does not block action potential–evoked



transmitter release when injected into the presynaptic terminal of the squid synapse (Adams et al., 1985; for details see Adler et al., 1991).

Microinjection of EGTA into the presynaptic terminal completely abolished the rise in $[Ca^{2+}]_i$ normally detected with the video imaging system. There was no measurable elevation of $[Ca^{2+}]_i$ during trains of action potentials, even in the portion of the nerve terminal closest to the postsynaptic axon, where the rise normally is largest (Figure 4A). The EGTA injection abolished both the $[Ca^{2+}]_i$ rise and the prolonged residuum of elevated $[Ca^{2+}]_i$ that normally follows an action potential train (Figure 4B). The fact that EGTA blocked the long-lasting $[Ca^{2+}]_i$ rise without affecting evoked transmitter release (or the local rise in $[Ca^{2+}]_i$ that triggers release) indicates that this Ca^{2+} buffer can be used as a high-pass temporal and spatial filter for Ca^{2+} signals. Such injections of EGTA can be used to determine what happens to synaptic depression and augmentation when the residual Ca^{2+} signal is eliminated. We found that injection of EGTA did not reduce synaptic depression (Figure 5A). This result, which was found in all 5 replicates of this experiment, indicates that the residual Ca^{2+} signal produced by tetani does not cause synaptic depression. However, close inspection of the kinetics of depression indicated that EGTA injection slightly sped up the onset of depression (Figure 5B).

In contrast, microinjection of EGTA had a dramatic effect on augmentation. Injections of EGTA that did not alter action potential-evoked PSPs or synaptic depression completely abolished augmentation (Figure 6). Both the increase in PSP rate of rise normally observed during the train (tetanic augmentation) and the slowly decaying augmentation that outlasted the train (posttetanic augmentation) were completely abolished. This effect was seen in 4 different experiments in which tetani were given both before and following EGTA injection (as in Figure 6) and in an additional 3 experiments in which trains were given only after EGTA injection, and no augmentation was evident. The abolition of augmentation by EGTA indicates that the residual Ca^{2+} signal is responsible for producing augmentation, despite the apparent temporal discrepancy between the time courses of the Ca^{2+} signal and augmentation. Furthermore, it suggests that the slight hastening of depression following EGTA injection is due to the removal of superimposed augmentation, rather than being a direct effect upon the kinetics of depression.

Figure 3. Presynaptic Ca^{2+} Signals and Synaptic Plasticity

Changes in $[Ca^{2+}]_i$ were recorded during and after synaptic depression (A) and augmentation (B), in regions close to the postsynaptic axon (as shown by the red bar in the inset of Figure 1B). The experiment of (A) was performed on a synapse bathed in saline containing 11 mM Ca^{2+}, while that of (B) was performed on another synapse bathed in low (1.5 mM) Ca^{2+}.

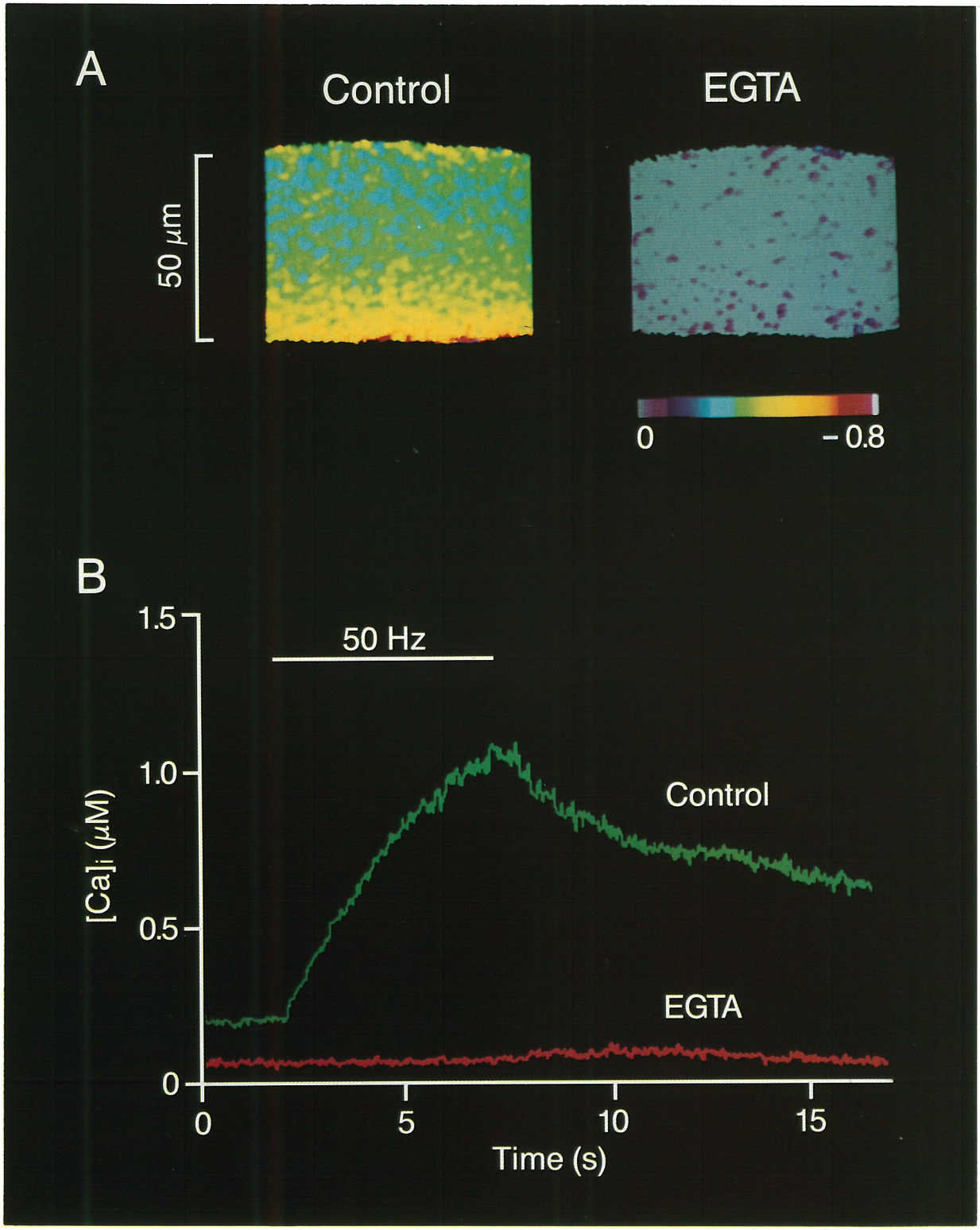


Figure 4. Effect of EGTA on Presynaptic Ca^{2+} Signals

(A) The control image (left) shows the $[Ca^{2+}]_i$ gradients present at the end of a 5 s long train (50 Hz) of presynaptic action potentials. Following iontophoretic injection of EGTA, no stimulus-induced changes in $[Ca^{2+}]_i$ could be detected (right) even though synaptic transmission was unaffected by EGTA. The calibration bar indicates the range of $\Delta F/F_o$ (see Experimental Procedures) displayed in the two images; this range corresponds to $[Ca^{2+}]_i$ values of 0.19–5 μM in the control image and 0.06–2.7 μM in the EGTA image.

(B) Comparison of the time course of Ca^{2+} signals measured before and after EGTA injection. These Ca^{2+} signals were recorded from a presynaptic region similar to that indicated by the red bar in the inset of Figure 1B.

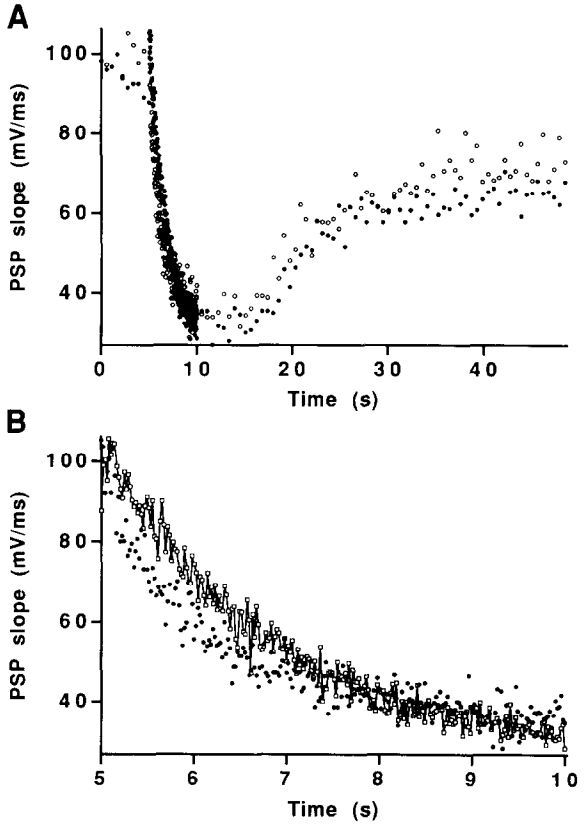


Figure 5. Effect of EGTA on Synaptic Depression

PSP rate of rise was plotted against time with and without EGTA injection into the presynaptic terminal. (A) EGTA affected neither the amount of depression nor the recovery from depression. (B) Depression sped up slightly when EGTA was injected into the presynaptic terminal, as shown on an expanded time scale. Presynaptic stimulation was for 5 s at 50 Hz. Solid circles, EGTA; open symbols, control.

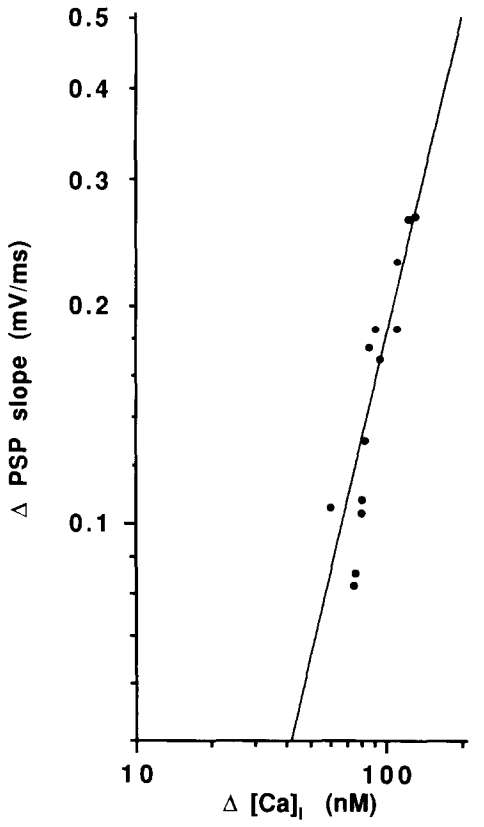


Figure 7. Dependence of Augmentation on $[Ca^{2+}]_i$

The relationship between PSPs and presynaptic $[Ca^{2+}]_i$ for 6 s following presynaptic stimulation (5 s, 50 Hz) is illustrated using a double logarithmic scale. PSPs were recorded every 500 ms. Values for PSP slope and $[Ca^{2+}]_i$ before tetanic stimulation have been subtracted. A power function with an exponent of 1.55 fits the data (solid line).

Relationship between $[Ca^{2+}]_i$ and Augmentation

Given that the residual Ca^{2+} signal appears to cause augmentation, our combined measurements of augmentation and $[Ca^{2+}]_i$ allow us to define the $[Ca^{2+}]_i$ requirements for augmentation. For this purpose, we quantified the relationship between $[Ca^{2+}]_i$ and the changes in PSP rate of rise associated with augmentation. This relationship was not examined in detail for responses recorded during trains of presynaptic action potentials, because it is impossible to define the contribution of augmentation if any concurrent facilitation or depression is present. For signals measured after trains, augmentation occurred at presynaptic $[Ca^{2+}]_i$ levels on the order of 1 μM or less. At these times there was a nonlinear relationship between the posttetanic rise in $[Ca^{2+}]_i$ and the amount of augmentation, measured as the change in PSP rate of rise. When plotted on logarithmic coordinates, this relationship could be described as a power function with an exponent greater than 1. For the experiment shown in Figure 7, this exponent was approximately 1.5. This suggests that augmentation is produced by effector molecules that have a high affinity for Ca^{2+}. It also suggests that multiple Ca^{2+} binding events may be required in order to produce augmentation.

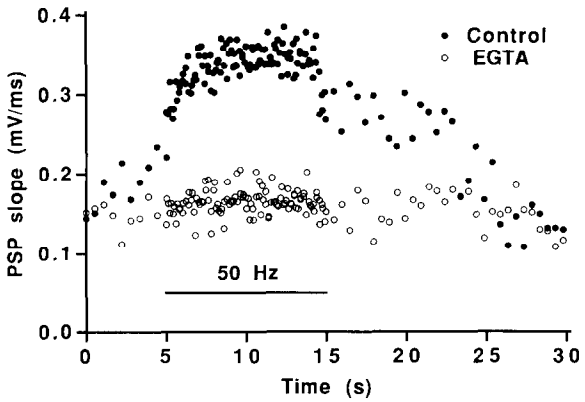


Figure 6. Effect of EGTA on Synaptic Augmentation

Tetanic and posttanic augmentation was measured with 1.5 mM external Ca^{2+}. Following EGTA injection, augmentation was completely blocked. Presynaptic stimulation was for 5 s at 50 Hz.

Discussion

In this paper we have described the changes in pre-

synaptic $[Ca^{2+}]_i$ and synaptic transmission produced at the squid giant synapse by brief bouts of presynaptic electrical activity. We have shown that presynaptic $[Ca^{2+}]_i$ rises in a spatially nonuniform manner during action potentials, rapidly equilibrates after the action potentials stop, and then slowly declines over many seconds. We have also shown that such bouts of electrical activity elicit at least two forms of synaptic plasticity: depression and augmentation, a form of tetanic and posttetanic potentiation. Although the time courses of neither depression nor augmentation match those of the long-lasting presynaptic Ca^{2+} signal, it appears that augmentation is caused by this Ca^{2+} signal because microinjection of the Ca^{2+} buffer EGTA into the presynaptic terminal blocks both the Ca^{2+} signal and the augmentation. $[Ca^{2+}]_i$ levels less than 1 μM apparently can produce augmentation, and there is a nonlinear relationship between the rise in $[Ca^{2+}]_i$ and the amount of augmentation. This nonlinear relationship accounts for the kinetic differences between presynaptic $[Ca^{2+}]_i$ changes and augmentation.

Presynaptic Mechanisms of Depression and Augmentation

There have been previous suggestions that synaptic depression may be related to long-lasting changes in presynaptic $[Ca^{2+}]_i$. First, elevation of presynaptic $[Ca^{2+}]_i$ depresses synaptic transmission (Miledi and Slater, 1966; Charlton et al., 1982; Adams et al., 1985). Second, it has been suggested that "mobilization" of releasable transmitter quanta is enhanced during tetani of action potentials (Kusano and Landau, 1975). Given that such tetani produce a rise in $[Ca^{2+}]_i$ (Figure 1) and that depression is thought to be due to a temporary depletion of releasable quanta (Betz, 1970; Zucker, 1972; Kusano and Landau, 1975), it is plausible that the tetanic rise in $[Ca^{2+}]_i$ is responsible for recovery from synaptic depression. However, our finding that elimination of the residual Ca^{2+} signal, by injection of EGTA, did not affect depression indicates that the measured residual $[Ca^{2+}]_i$ is not the signal for recovery from depression.

Our results provide evidence that tetanic and posttetanic potentiation are present at the squid giant synapse. With the relatively brief trains that we employed, we could evoke only the relatively large and rapid augmentation phase of these forms of plasticity (Magleby and Zengel, 1976). It is likely that stimulation with larger numbers of presynaptic action potentials would have allowed us to see the slower, smaller "potentiation" phase as well. The augmentation that we have examined is clearly distinct from the more rapid process of synaptic facilitation, which has decay time constants in the millisecond range at this synapse (Charlton and Bittner, 1978).

Many previous studies have suggested that both augmentation and potentiation are due to residual presynaptic Ca^{2+} signals (e.g., Rosenthal, 1969; Weinreich, 1971; Magleby and Zengel, 1975; Erulkar and Rahamimoff, 1978; Lev-Tov and Rahamimoff, 1980; Zengel and Magleby, 1981; Zucker and Lara-Estrella, 1983; Kretz et al., 1982; Connor et al., 1986; Delaney et al., 1989). Our experiments extend these earlier studies by both directly measuring and manipulating presynaptic $[Ca^{2+}]_i$ during augmentation. The strongest piece of evidence in favor of the hypothesis is that presynaptic injection of EGTA blocks both the measured Ca^{2+} signal and augmentation. This complements (and helps explain) the observation of Kretz et al. (1982) that injection of EGTA into the cell body of an Aplysia neuron reduces the slower potentiation phase of posttetanic potentiation.

Our results contrast with those of Tanabe and Kijima (1989), who reported that loading frog nerve terminals with the Ca^{2+} chelator BAPTA-AM did not block augmentation. One reason for this discrepancy might be that in their experiments BAPTA-AM was present at a low concentration and was saturated with Ca^{2+} early in the tetanus. An alternative explanation is that EGTA, but not BAPTA, releases protons upon binding Ca^{2+} (e.g., Ahmed and Connor, 1980) and that the resultant change in pH is responsible for the block of augmentation. A simple calculation suggests that such a rise in pH should be quite small, due to cytoplasmic pH buffering. EGTA buffering of the ~1 μM rise in $[Ca^{2+}]_i$ produced by a train of action potentials (as in Figure 4B) would be associated with a total binding of perhaps 100 μM Ca^{2+} to EGTA (assuming that only 1/100 of the total influx of Ca^{2+} appears as free Ca^{2+}; e.g., Ahmed and Connor, 1988). Since EGTA releases 2 protons for each Ca^{2+} bound, this would release 200 μM H^+. With a buffering power of 10 milliequivalent H^+ per pH unit per liter for squid axoplasm (Roos and Boron, 1981), this would yield a change in pH of only 0.02 units. This is consistent with the small changes in intracellular pH found in EGTA-loaded snail neurons during Ca^{2+} entry (Ahmed and Connor, 1980). It is unlikely that such a small change in pH would affect augmentation, particularly since transmission at the squid synapse is insensitive to presynaptic pH changes in the range of 6.3–7.8 (Adams and Thomas, 1989, J. Physiol., abstract; Zipser, Deitmer, and Augustine, unpublished data).

Another piece of evidence in support of the hypothesis that residual Ca^{2+} signals cause augmentation is that both $[Ca^{2+}]_i$ and augmentation rise during the tetanus and decline afterward. This is in line with previous work on potentiation in Aplysia by Connor et al. (1986) and in crayfish by Delaney et al. (1989). However, at the squid synapse there is a temporal discrepancy between these two signals: augmentation declines more rapidly than the measured Ca^{2+} signal. There are two possible explanations for this discrepancy. First, it is possible that the molecule(s) responsible for mediating augmentation is located very close to the release site, so that it is in the vicinity of local $[Ca^{2+}]_i$ gradients too steep to be detected with our imaging technique (see below). This seems unlikely because augmentation lasts for several seconds after a train

(Figure 3B), while lateral $[Ca^{2+}]_i$ gradients dissipate within 1–2 s (Figure 1B). The second possible explanation is that the disparities in time courses are due to a nonlinear relationship between the change in $[Ca^{2+}]_i$ and the amount of augmentation. Although our measurements of the $[Ca^{2+}]_i$ dependence of augmentation suggest that this is the case for the squid synapse, at the crayfish neuromuscular synapse (Delaney et al., 1989) and hippocampal synapses (Delaney et al., 1991, Biophys. J., abstract) there appears to be a linear relationship between presynaptic $[Ca^{2+}]_i$ and the magnitude of the potentiation phase of posttetanic potentiation. This difference could be due to distortion of the $[Ca^{2+}]_i$ measurements at the squid synapse, caused by very local $[Ca^{2+}]_i$ gradients (see above) or to differences in mechanisms between the different forms of plasticity at these different synapses. For example, it is possible that the Ca^{2+} binding step is rate-limiting for potentiation at the crayfish and hippocampal synapses, but a subsequent step determines the time course of augmentation of squid.

Regardless of the precise stoichiometric relationship between $[Ca^{2+}]_i$ and augmentation, our results provide strong support for the idea that residual presynaptic Ca^{2+} signals are responsible for augmentation. Other forms of synaptic plasticity, such as facilitation (Katz and Miledi, 1968; Charlton et al., 1982) and the potentiation phase of posttetanic potentiation (Erulkar and Rahamimoff, 1978; Lev-Tov and Rahamimoff, 1980; Kretz et al., 1982; Delaney et al., 1989), also appear to require a presynaptic Ca^{2+} signal. However, rises in postsynaptic $[Ca^{2+}]_i$ apparently serve as signals for certain other forms of synaptic plasticity, such as long-term potentiation of transmission at hippocampal synapses (Smith, 1987; Collingridge and Singer, 1990) and long-term synaptic depression in the cerebellum (Sakurai, 1990; Konnerth et al., submitted).

As originally formulated for synaptic facilitation by Katz and Miledi (1968), the residual Ca^{2+} theory was based on prolonged and partial occupancy of a receptor that binds multiple Ca^{2+} ions and is capable of triggering transmitter release only when all of its binding sites are occupied. In the specific case of synaptic augmentation, it is also possible to propose an alternative mechanism in which the residual Ca^{2+} signal activates other reactions, separate from the reaction that triggers transmitter release, and these secondary reactions then lead to augmentation. Support for this latter possibility comes from the several differences between transmitter release and augmentation. Aside from the obvious differences in kinetics (millisecond versus second), transmitter release apparently requires $[Ca^{2+}]_i$ on the order of 100 μM (Roberts et al., 1990; Adler et al., 1991), while augmentation seems to occur at $[Ca^{2+}]_i$ below 1 μM (Figure 7). Furthermore, transmitter release and augmentation appear to have different selectivities for divalent cations (Zengel and Magleby, 1981; Augustine and Eckert, 1984). Still other differences in Ca^{2+} stoichiometry (Dudel, 1981; Delaney et al., 1989) and developmental appearance (Ohmori et al., 1981) distinguish the potentiation phase of posttetanic potentiation from transmitter release. Thus, we favor a mechanism in which augmentation is produced by one or more reactions different from those responsible for mediating transmitter release.

Multiple Ca^{2+} Signaling Pathways in Presynaptic Terminals

Implicit in the above proposal of separate Ca^{2+}-dependent mechanisms for triggering transmitter release and its augmentation is the concept that multiple Ca^{2+} signaling pathways coexist in presynaptic terminals. The transmitter release event apparently is triggered by a very localized rise in $[Ca^{2+}]_i$ that can reach very high levels (Roberts et al., 1990; Adler et al., 1991). Two of our observations lend further support to this idea. First, we find that following a train of action potentials, $[Ca^{2+}]_i$ persists at levels on the order of 0.5–1 μM after evoked transmitter release has ceased. This indicates that $[Ca^{2+}]_i$ higher than 0.5–1 μM is required to cause release at the rates produced by action potentials. Second, we have found that EGTA-injected presynaptic terminals have no measurable Ca^{2+} signal (Figure 4) yet release transmitter (Figure 6; Adams et al., 1985; Adler et al., 1991). This demonstrates that the local Ca^{2+} signal for transmitter release is too spatially restricted to be visible with the video imaging methods we employed. The Ca^{2+} signal for augmentation appears to be smaller, slower, and more spatially distributed. Diffusion can yield both signals from a single source: the high local Ca^{2+} signal can occur only in the immediate vicinity of an open channel, while the slower, small, more distant Ca^{2+} signal can result from diffusional dilution of the former signal (Chad and Eckert, 1984; Simon and Llinas, 1985; Zucker and Fogelson, 1986; Smith and Augustine, 1988; Gutnick et al., 1989). Diffusion will tend to terminate the transmitter release process soon after the Ca^{2+} channel closes (if the receptor for release has a relatively low affinity for Ca^{2+}), while the slower residual Ca^{2+} signal will linger and could coordinate a spectrum of slower reactions that prepares the presynaptic terminal for the next bout of transmitter release (Llinas et al., 1991). Synaptic augmentation may be one expression of such reactions.

Experimental Procedures

Preparation and Electrophysiological Methods

Stellate ganglia of squid (Loligo pealei) were isolated as described by Augustine and Eckert (1984). They were cooled to 15°C via a peltier cooling device and incubated in squid saline (455 mM NaCl, 54 mM $MgCl_2$, 11 mM $CaCl_2$, 10 mM KCl, 3 mM $NaHCO_3$, 10 mM NaHEPES [pH 7.2]). Low-Ca^{2+} saline was prepared by replacing $CaCl_2$ with an equivalent amount of $MgCl_2$. Conventional microelectrode techniques were used to examine synaptic transmission at the giant synapse. Pre- and postsynaptic neurons were visually identified, and each was impaled with one or more electrodes. One microelectrode was inserted into the giant presynaptic neuron, ~1 mm proximal to the synaptic terminal; injection of current through this electrode was used to evoke presynaptic action potentials directly. This electrode usually was filled with 3 M KCl, but in some experiments it instead contained

0.5 M EGTA and was used both to stimulate and to inject this Ca^{2+} buffer iontophoretically into the presynaptic neuron. In these experiments, 100 nA of current was passed for approximately 10 min, which should have injected more than 1 mM EGTA into the entire presynaptic neuron (Adler et al., 1991). A second microelectrode was filled with 3 M KCl and was inserted within the synaptic region of the postsynaptic neuron. This electrode was used to monitor changes in PSPs evoked by the presynaptic action potentials. These signals were acquired with PCLAMP (Axon Instruments) running on an IBM-AT computer and analyzed with AUTESP, a waveform analysis program written by H. Zucker (Max-Planck-Institute for Psychiatry, Munich, Germany). A numerical Marquardt algorithm in the Igor program (Wave Metrics, Inc., OR) was used to fit exponential functions to extracted data.

Video Imaging of Ca^{2+}

In parallel with electrophysiological measurements of synaptic transmission, video imaging methods were used to measure presynaptic $[Ca^{2+}]_i$. For these measurements, the fluorescent indicator fura-2 (Grynkiewicz et al., 1985) was iontophoretically injected into the presynaptic terminal. Injection was accomplished by filling a third microelectrode with a solution containing 1 mM fura-2 and 100 mM KCl and inserting this microelectrode proximal to the presynaptic terminal to avoid dye concentration gradients within the terminal (Miledi and Parker, 1981). Fura-2 was injected iontophoretically with 100 nA of hyperpolarizing current, passed for 30–120 min to yield a final concentration of approximately 100 μM within the presynaptic terminal (Smith et al., 1988).

Digital imaging microscopy was used to obtain two-dimensional maps of presynaptic $[Ca^{2+}]_i$. The methods used have been described in detail by Smith et al. (1988). In brief, an upright epifluorescence microscope (Zeiss Axioskop) equipped with a 40×, water immersion objective (Zeiss) and a 75 W xenon illumination lamp was used to examine the fluorescence signal of the presynaptic terminal. This signal was detected with a SIT video camera (Cohu), and the video images were stored in real time (30 Hz) on an analog optical memory disk recorder (Panasonic TQ 2026F). The images were then digitized and analyzed off-line, using a Matrox MVP-AT frame grabber installed in an AST Premium 286 computer. Software written by Dr. Stephen Smith (Stanford University) was used to process the images.

To measure the changes in fura-2 fluorescence associated with synaptic transmission, the single wavelength ratio technique was employed (Smith et al., 1988; Kasai and Augustine, 1990). With this technique, the fura-2 is excited with a single excitation wavelength (380 nm, bandpass 10 nm) and the time-dependent changes in fluorescence intensity are measured for every pixel within the image by subtracting fluorescence signals measured during and following a stimulus (F_t) from those measured before the stimulus (F_o). The changes in fluorescence (ΔF) are then divided by a background-corrected version of F_o to yield a ratio image. As long as the path length and dye concentration are constant during the measurement period, this ratio yields information comparable to that obtained with the more conventional two-wavelength ratio method (Tsien and Poenie, 1986), but has the advantage of higher time resolution (~10 Hz with our camera; Neher and Augustine, 1991).

Converting the fluorescence ratio signals into $[Ca^{2+}]_i$ values requires knowledge of the resting $[Ca^{2+}]_i$ of the terminal (Neher and Augustine, 1991). This was determined before a presynaptic stimulus episode by alternately exciting the fura-2 with 380 nm and 360 nm light. The ratio of the emitted light was then converted to $[Ca^{2+}]_i$ by the following equation:

$$[Ca^{2+}]_{rest} = K'_D(R - R_{min})/(R_{max} - R), \quad (1)$$

where R is the measured fluorescence ratio (360 emission/380 emission), K'_D is the effective Ca^{2+} dissociation constant of fura-2 within the cell, and R_{min} and R_{max} represent the values of R under conditions of minimal and maximal association with Ca^{2+} (Grynkiewicz et al., 1985; Neher, 1989). The constants, K'_D, R_{min}, and R_{max} were determined by extracting cytoplasm from squid axons and mixing the axoplasm with equal volumes of solutions containing 5 μM fura-2, 400 mM KCl, and 40 mM Na-HEPES (pH 7.2). These solutions were then set to either low Ca^{2+} ($<10^{-8}$ M) with 20 mM EGTA, high Ca^{2+} with 20 mM $CaCl_2$, or intermediate Ca^{2+} ($\sim 6.7 \times 10^{-7}$ M, assuming that EGTA binds Ca^{2+} with a K_D of 3.4×10^{-7} M under these conditions [Adler et al., 1991]) with 20 mM EGTA mixed with 13.3 mM $CaCl_2$. The three unknown constants were then calculated from the values of R for the three conditions (Neher, 1989). For our particular conditions, K'_D was 1.75×10^{-6} M, R_{min} was 0.227, and R_{max} was 1.369.

The time-dependent changes in the single-wavelength emission ratio, $(\Delta F)/F_o$, were then converted to time-dependent presynaptic Ca^{2+} signals (Ca^{2+}_t) with a rearrangement of Equation 3 of Neher and Augustine (1991):

$$Ca^{2+}_t = (AK_D - K'_D)/(1 - A) \quad (2)$$

where $A = [(\Delta F)/F_o][(K'_D + [Ca^{2+}]_{rest})/(K_D + [Ca^{2+}]_{rest})]$, and K_D is the absolute Ca^{2+} dissociation constant for fura-2 within the cell. Since $K_D = K'_D(R_{min}/R_{max})$ for the case when 360 nm is one of the two wavelengths used to excite fura-2 (Neher and Augustine, 1991), under our conditions K_D was 2.91×10^{-7} M. This K_D value is more than 2-fold lower than a previous determination under ionic conditions appropriate for marine organisms (Poenie et al., 1985). However, at least part of this difference is due to the fact that our calibration solutions, but not those used previously, contain cytoplasm, which substantially lowers the K_D for fura-2 (e.g., Augustine and Neher, 1991).

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