Presynaptic Calcium in Transmitter Release and Posttetanic Potentiation[a]

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Until recently, study of the role of calcium in transmitter release has been limited to electrophysiological recording of postsynaptic responses while altering the calcium influx into presynaptic terminals, either by changing external calcium concentration or by manipulating the presynaptic potential.[1-3] Now it is possible to monitor calcium concentration ($[Ca^{2+}]_i$) changes at single presynaptic boutons during synaptic transmission, and to rapidly alter presynaptic $[Ca^{2+}]_i$ in nerve terminals with photolabile calcium chelators. We present here some of our recent observations on the relationship between presynaptic $[Ca^{2+}]_i$ and transmitter release.

LOCALIZATION OF CALCIUM ACCUMULATION AT PRESYNAPTIC NERVE TERMINALS

We have focused on transmitter release at motor nerve terminals at crayfish neuromuscular junctions. The claw opener muscle is innervated by a glutamate-releasing excitor motor neuron and a γ-aminobutyric acid (GABA)–releasing inhibitor motor neuron, which branch together and release transmitter from varicosities and boutons about 1–5 μm in diameter.[4,5] Changes in presynaptic $[Ca^{2+}]_i$ can be followed by injecting the calcium-sensitive dye fura-2[6] into the preterminal axon. Using a sensitive video camera, we form images of the fluorescence of fura when excited by 350 and 385 nm light.[7] Ratios of emitted light intensities at each pixel are color-coded to generate an image of the spatial distribution of $[Ca^{2+}]_i$. The time resolution of our measurements is on the order of 1 s, whereas the spatial resolution is limited by the wavelength of light and related optical properties. We are therefore unable to measure the local high submembrane $[Ca^{2+}]_i$ after single action potentials, but instead measure the average $[Ca^{2+}]_i$ in nerve processes during repetitive stimulation. Because equilibration of $[Ca^{2+}]_i$ across the small volume of the terminal occurs rapidly, measurements of residual $[Ca^{2+}]_i$ following electrical activity probably reflect accurately $[Ca^{2+}]_i$ at sites of transmitter release.

FIGURE 1 (on the color plate) shows two regions of presynaptic terminals of a single excitor motor neuron. During repetitive presynaptic stimulation, $[Ca^{2+}]_i$ rises

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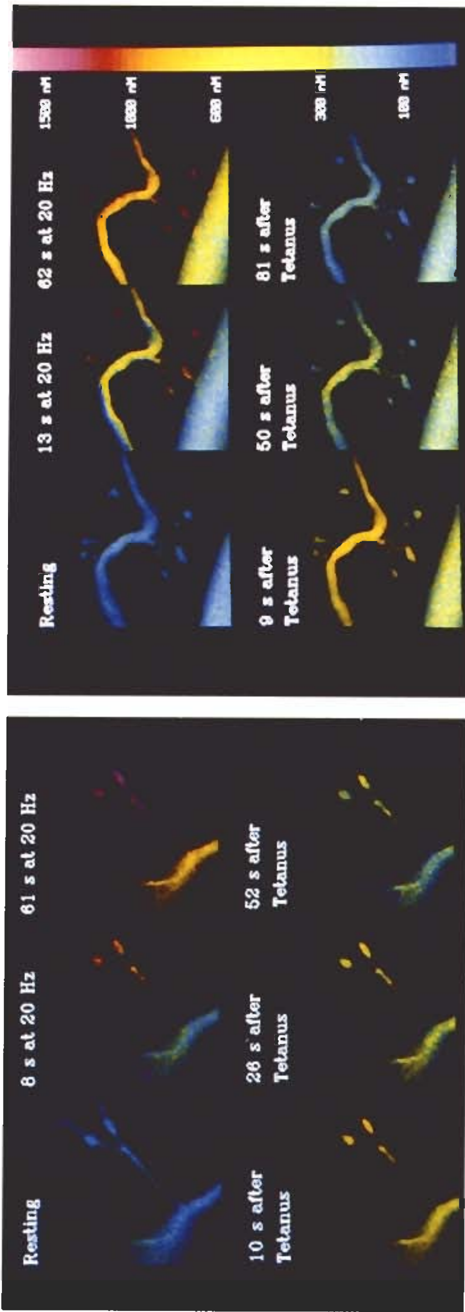
1
Resting
8 s at 20 Hz
61 s at 20 Hz
10 s after Tetanus
26 s after Tetanus
52 s after Tetanus
Resting
13 s at 20 Hz
62 s at 20 Hz
9 s after Tetanus
50 s after Tetanus
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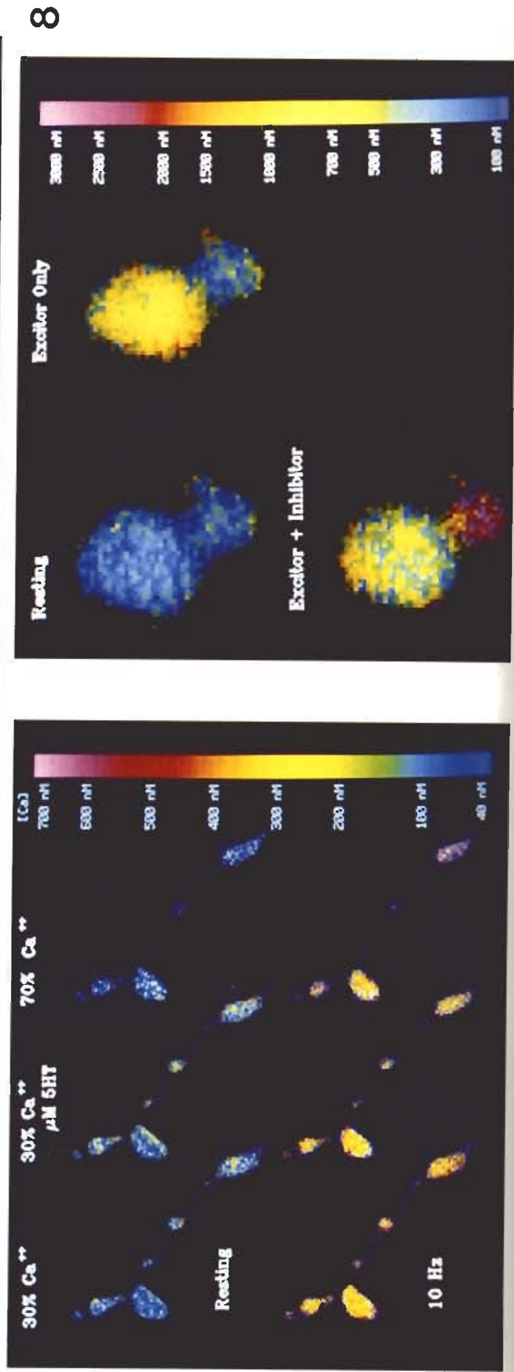
100 nM


8
Resting
Exciter Only
Exciter + Inhibitor
3000 nM
2500 nM
2000 nM
1500 nM
1000 nM
700 nM
500 nM
300 nM
100 nM
7
30% Ca++
30% Ca++ μM 5HT
70% Ca++
Resting
10 Hz
[Ca]
700 nM
600 nM
500 nM
400 nM
300 nM
200 nM
100 nM
40 nM

rapidly in boutons and small processes, and more slowly in major axonal branches. After cessation of stimulation, $[Ca^{2+}]_i$ drops more rapidly in the boutons and fine processes than in larger branches. This data is shown graphically in FIGURE 2.

The spatial distribution of the tetanic rise in $[Ca^{2+}]_i$ shows clearly that calcium accumulation occurs locally into boutons and small processes where synaptic vesicles and synaptic "active zones" are located.[5] The rapid posttetanic drop in $[Ca^{2+}]_i$ in boutons and small branches suggests that calcium is locally removed from cytoplasm in these regions as well. We may ask whether these rapid localized $[Ca^{2+}]_i$ changes reflect a high density of calcium channels and removal equipment locally, or simply the larger surface-to-volume ratio of small processes. One indication that calcium channel density may be higher at release sites comes from panel B of FIGURE 2. Point A measures a bouton of similar dimensions to a rather narrow region of an axonal branch marked point C. The faster $[Ca^{2+}]_i$ changes at point A suggest a real concentration of calcium influx and of sequestering or extruding capabilities near synaptic release sites. Differences in cytoplasmic calcium buffering, however, (greater in axonal branches) might also account for our observations.

INCREASING CALCIUM INFLUX NONLINEARLY INCREASES TRANSMITTER RELEASE

Doubling external $[Ca^{2+}]$ increases $[Ca^{2+}]_i$ accumulation about 30%, by increasing the calcium influx through each channel opened by an action potential. The small increase in calcium accumulation is probably due to saturation of influx through channels as external $[Ca^{2+}]$ is raised[8] and to local extracellular buffering of $[Ca^{2+}]$.[9] This modest rise in calcium influx increases transmission about four times (FIG. 3). If we suppose that the 30% increase in calcium accumulation reflects a 30% increase in calcium influx per channel, this suggests a highly nonlinear relationship between $[Ca^{2+}]_i$ at release sites and transmitter release.

At 5 mM, the potassium channel blocker tetraethylammonium (TEA) prolongs action potentials[10] and increases tetanic $[Ca^{2+}]_i$ accumulation about 70 percent. But this larger rise in calcium influx (compared to what we got by increasing external $[Ca^{2+}]_i$) is correlated with increased transmission only about twofold (FIG. 3). Thus

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FIGURE 1. Ratiometric fluorescent images of two regions of crayfish excitor motor nerve terminals filled with fura-2. Colors correspond to $[Ca^{2+}]_i$ levels indicated by the color bar on the right. The motor neuron was stimulated at 20 Hz for 70 s. The time before, during, or after stimulation is marked on each panel. Scale bar (see FIG. 8) corresponds to 4 μm.

FIGURE 7. Effect of 5 μM 5HT on presynaptic $[Ca^{2+}]_i$ in excitor terminals. The top three images show resting $[Ca^{2+}]_i$, and the bottom images show $[Ca^{2+}]_i$ during 10 Hz stimulation. The left column shows controls, the middle column shows effects of 5 μM 5HT, and the right column shows effects of increasing extracellular $[Ca^{2+}]$ from 30% to 70% of its normal level of 13.5 mM. The average resting $[Ca^{2+}]_i$ in the large central bouton was 98 nM in low $[Ca^{2+}]$, 112 nM in 5 μM 5HT, and 98 nM in high $[Ca^{2+}]$. During stimulation, $[Ca^{2+}]_i$ rose to 305 nM, 295 nM, and 376 nM in these solutions, respectively. Scale bar (see FIG. 8) corresponds to 12 μm.

FIGURE 8. Effect of inhibitor stimulation on tetanic $[Ca^{2+}]_i$ accumulation in excitor terminals. Both motor neurons were injected with fura-2; the excitor terminal is the larger circle on the upper left. The images show resting $[Ca^{2+}]_i$, stimulation of the excitor alone at 10 Hz, and concurrent stimulation of the excitor and inhibitor, with each excitor stimulus preceded by 4 inhibitor stimuli. Scale bar corresponds to 3 μm.

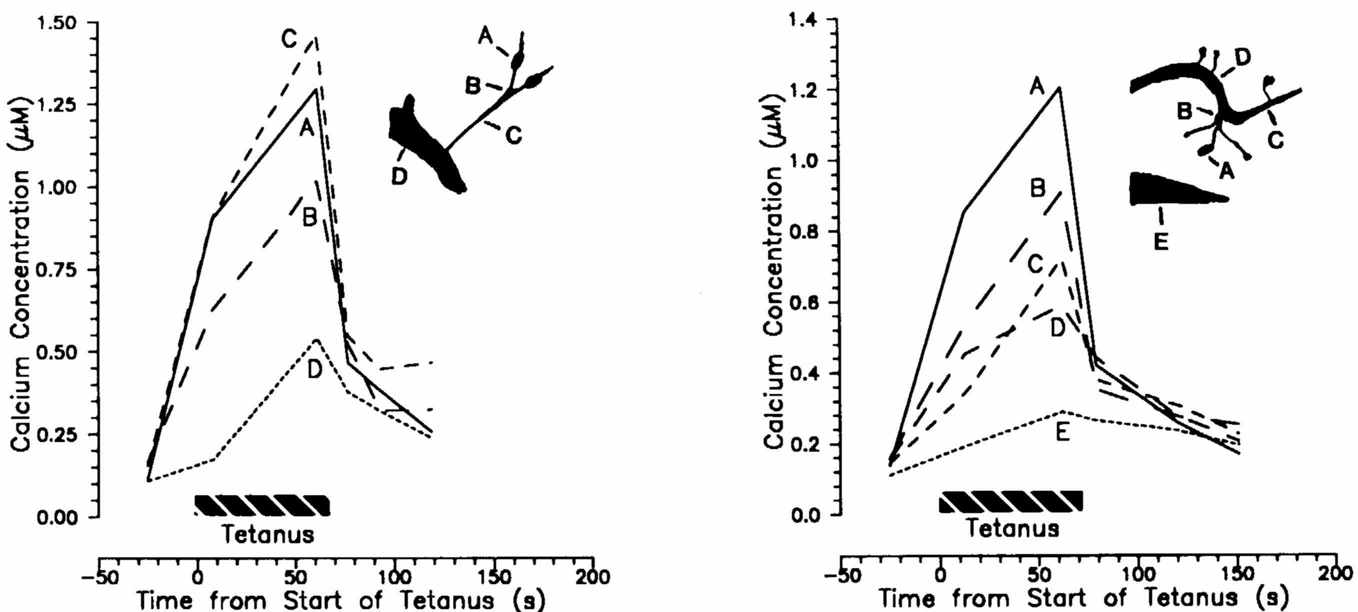

FIGURE 2. Changes in $[Ca^{2+}]_i$ in the terminals illustrated in FIG. 1. Each trace follows the evolution of $[Ca^{2+}]_i$ in a spot shown on the inset.

increasing calcium influx by prolonging action potentials is less efficient in enhancing transmitter release than is increasing calcium influx by raising $[Ca^{2+}]_i$ in the medium.

We explain these results by recognizing that transmitter release occurs in the immediate neighborhood of calcium channels opened by action potentials, before the sharp gradients of $[Ca^{2+}]_i$ near channel mouths have had time to collapse.[11-13] Increasing calcium influx per channel by raising extracellular $[Ca^{2+}]$ simply scales up the highly nonuniform submembrane distribution of $[Ca^{2+}]_i$ and captures much of the stoichiometry of the nonlinear activation of exocytosis by $[Ca^{2+}]_i$. By contrast, TEA prolongs action potentials and allows for the desynchronized opening of calcium

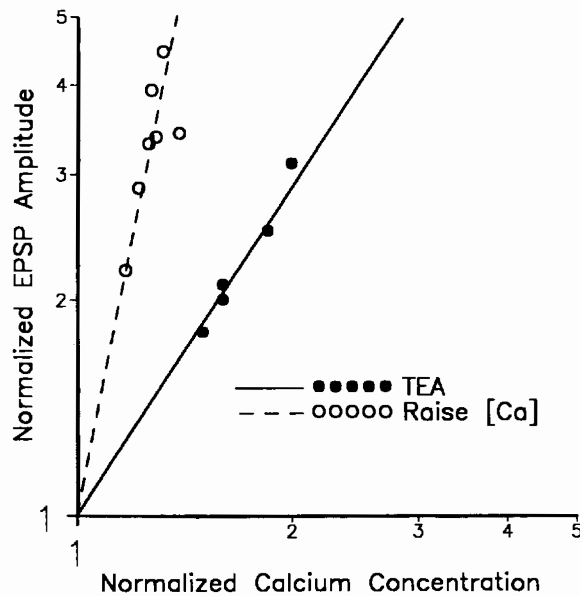

FIGURE 3. Relationship between changes in EPSP amplitude and changes in $[Ca^{2+}]_i$. Each point represents a different experiment. In each case, the excitor was stimulated at 8–10 Hz for at least 20 s before the EPSP and $[Ca^{2+}]_i$ values were recorded in control solution to provide the normalizing levels. In some experiments, 5 mM TEA was added, whereas in others, external $[Ca^{2+}]$ was raised (from 50% to 100% normal or from 30% to 70% normal); then the tetanus was repeated to obtain changes under the experimental condition. The results are plotted on double logarithmic coordinates, and lines are drawn with the average slope for each type of experiment.

channels early and late in the spike. This leads to shifting clusters of open calcium channels and shifting domains of high $[Ca^{2+}]_i$ that trigger transmitter release. In an extreme case, an action potential of doubled duration might evoke transmitter release from two nonoverlapping sets of high $[Ca^{2+}]_i$ domains occurring early and late in the action potential. Then both calcium influx (and $[Ca^{2+}]_i$ accumulation) and transmitter release would double, and the change in the two would be linearly related. Because the observed relationship between the increase in transmission and increase in $[Ca^{2+}]_i$ accumulation has an apparent power of about 2, this suggests that some overlap occurs between $[Ca^{2+}]_i$ domains early and late in a TEA-prolonged

action potential, which is hardly surprising. A low power dependence of transmitter release on calculated calcium influx in TEA-prolonged action potentials was recently observed at the squid giant synapse and explained in a similar manner.[14]

ROLE OF RESIDUAL CALCIUM IN POSTTETANIC POTENTIATION

Stimulation of the excitor motor neuron at 20–35 Hz for 10–20 min is accompanied by a rise in transmitter released per impulse that takes several minutes to develop and that lasts for 10–50 min after the end of the tetanus.[15] This phase of potentiated release depends on calcium ions. Blocking calcium influx by stimulation in a calcium-free medium results in substantially reduced posttetanic potentiation (PTP).[5,16]

We have followed the time course of $[Ca^{2+}]_i$ accumulation and removal during the development and decay of excitatory postsynaptic potential (EPSP) potentiation and have found them to be similar (FIG. 4). The posttetanic decay of $[Ca^{2+}]_i$ matched closely the decay of PTP,[7] suggesting that the PTP was directly caused by the elevated residual $[Ca^{2+}]_i$. Transmission during a tetanus was greater for a given level of $[Ca^{2+}]_i$ than after the tetanus, partly because a tetanus $[Ca^{2+}]_i$ does not have enough time to fully equilibrate. Then the local submembrane $[Ca^{2+}]_i$ will be slightly higher than the volume average $[Ca^{2+}]_i$ that we measure and should more effectively potentiate release. During the tetanus, additional short-term facilitatory processes add to longer-lasting potentiation. These decay within a few seconds of the offset of stimulation and so do not contribute to transmitter release when comparing posttetanic potentiation to posttetanic changes in $[Ca^{2+}]_i$.

How does increased $[Ca^{2+}]_i$ potentiate release? PTP could be a direct consequence of the nonlinear relationship between $[Ca^{2+}]_i$ and transmitter release.[17] Transmitter release would be very sensitive to a small increase in the peak $[Ca^{2+}]_i$ achieved near calcium channels during action potentials in the presence of increased residual $[Ca^{2+}]_i$ from prior activity. Calculations of such a model, however, predict a significantly more rapid decay of PTP than observed experimentally (FIG. 4). Therefore, the linear relationship between EPSP PTP and $[Ca^{2+}]_i$ suggests that $[Ca^{2+}]_i$ operates in some other way to potentiate release. Possibilities include mobilizing vesicles into docking positions at release sites and sensitizing the release apparatus to sudden large changes in $[Ca^{2+}]_i$.

ROLE OF SODIUM ACCUMULATION IN POSTTETANIC POTENTIATION

PTP is reduced when $[Na^+]_i$ accumulation is prevented, and it is enhanced when $[Na^+]_i$ accumulation is boosted with ouabain to block sodium extrusion by sodium/potassium exchange.[5,15,16] Sodium might act internally to directly potentiate transmitter release. Alternatively, sodium could affect the amount of $[Ca^{2+}]_i$ accumulation in a tetanus and thereby influence PTP.

There are two ways that $[Na^+]_i$ could elevate $[Ca^{2+}]_i$: by release of calcium from internal stores, or by entry from the external medium through Na/Ca exchange. To test these possibilities, we looked at the effect of elevating $[Na^+]_i$ on the level of $[Ca^{2+}]_i$ (FIG. 5). $[Na^+]_i$ was raised either by injection from a micropipette placed in an excitor branch on the surface of the claw opener muscle, or by exposure to veratridine, a drug that shifts the activation curve for sodium channels so that they are partially open at resting membrane potential. In either case, we found that $[Ca^{2+}]_i$

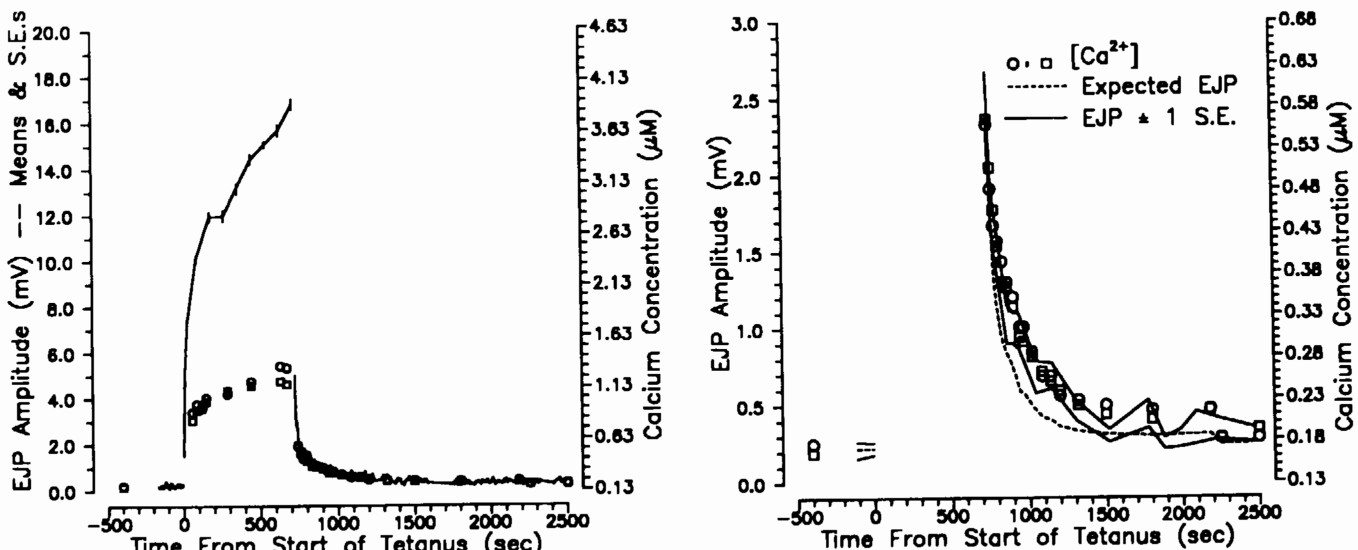

FIGURE 4. EPSP from the muscle fiber (solid lines) and $[Ca^{2+}]_i$ in two presynaptic boutons (symbols) before, during, and after a 12 min 20 Hz tetanus. The vertical bars on the left show SEs for mean EPSPs, whereas the two solid lines on the right represent the mean EPSPs ± 1 SE. EPSP means were calculated using a running average of 10 responses to smooth EPSP fluctuations. The dotted line was calculated from $V = K (P + R)$,[5] where V represents EPSP amplitude, R is the corresponding value of $[Ca^{2+}]_i$, and P is the peak rise in $[Ca^{2+}]_i$ at active zones caused by an action potential. P and K were determined from V and R at rest and shortly after the tetanus, and these were used to predict the posttetanic EPSP decay from the observed decay in $[Ca^{2+}]_i$.

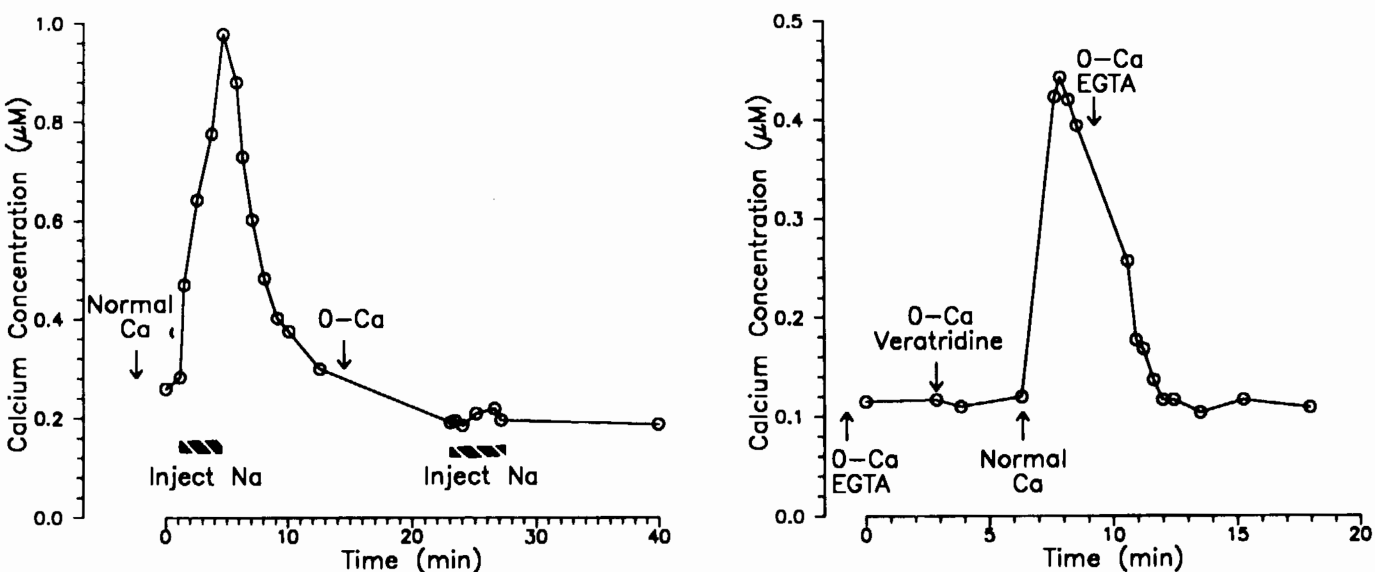

FIGURE 5. The left panel monitors changes in $[Ca^{2+}]_i$ during iontophoretic injection of Na^+ (10 nA) into a major branch of the excitor motor neuron, in normal Ringer and with 13.5 mM Co^{2+} substituted for the calcium. The right panel shows effects of 0.5 μM veratridine on $[Ca^{2+}]_i$ in a calcium-free medium and after changing to normal Ringer.

increased, but only when calcium was present in the external medium. This suggests that increased $[Na^+]_i$ can cause an elevation of $[Ca^{2+}]_i$, but only by way of changes in Na/Ca exchange, and not by release from intracellular stores.

Next we asked, What does a physiological sodium load do to $[Ca^{2+}]_i$? FIGURE 6A shows that stimulating the motor neuron tetanically in a calcium-free medium had no effect on $[Ca^{2+}]_i$. Following the tetanus, the bathing solution was changed to one containing normal $[Ca^{2+}]$; there was still no rise in $[Ca^{2+}]_i$. Apparently sodium accumulation in a tetanus neither releases calcium from intracellular stores nor admits calcium from outside by significantly altering the function of Na/Ca exchange. There is a third way in which $[Na^+]_i$ and $[Ca^{2+}]_i$ might interact in producing PTP. During a normal tetanus, presynaptic terminals would be loaded with both sodium and calcium. Perhaps the concomitant rise in $[Na^+]_i$ slows the extrusion of the increased $[Ca^{2+}]_i$ by reducing the sodium gradient that drives Ca extrusion by the Na/Ca pump.

To test this idea, we asked what effect a sodium load has on the extrusion of a calcium load. Preparations were depolarized with high potassium to admit both sodium and calcium through voltage-dependent channels while monitoring the rise in $[Ca^{2+}]_i$. Then normal potassium was restored, and the decay of $[Ca^{2+}]_i$ was followed. This provided a measure of the rate of removal of extra internal calcium in the presence of an elevated sodium load. The preparation was again depolarized by high potassium with choline in place of sodium. Again removing excess potassium, the decay of $[Ca^{2+}]_i$ was followed when no sodium load had developed. We found (FIG. 6B) that an intracellular sodium load slows the removal of calcium, consistent with the idea that $[Na^+]_i$ operates to reduce the extrusion of $[Ca^{2+}]_i$ and therefore increases and prolongs PTP. In other experiments, we used ouabain to increase sodium loading during a tetanus by blocking the active extrusion of sodium, and therefore to increase the sodium load during a tetanus. FIGURE 6C shows that this treatment increases the amount of EPSP potentiation and $[Ca^{2+}]_i$ accumulation during a tetanus and prolongs the posttetanic decay of both. These results all suggest that presynaptic sodium loading augments PTP by slowing the extrusion of calcium by Na/Ca exchange.

SEROTONIN-ACTIVATED ENHANCEMENT OF TRANSMITTER RELEASE

Serotonin (5-hydroxytryptamine, 5HT) is a naturally occurring hormone in crayfish, which increases the amount of transmitter release to action potentials at neuromuscular junctions.[18] Because the frequency of spontaneously released quanta, or miniature EPSPs, is also increased, it seemed possible that 5HT's effects were mediated by a rise in $[Ca^{2+}]_i$. This is especially possible because 5HT depolarizes presynaptic axons by up to 10 mV, which might open some calcium channels.[19] We found (FIG. 7, on the color plate) that even 5 μM 5HT, however, which augments EPSPs approximately fourfold, has little or no effect on resting $[Ca^{2+}]_i$ levels in presynaptic boutons. We doubt that the small increases in nerve terminals, typically under 25 nM, could be responsible for 5HT's augmentation of transmitter release, because a similar amount of PTP requires a $[Ca^{2+}]_i$ increase of about 100 nM.[7] Apparently the presynaptic depolarization caused by 5HT is insufficient to open many calcium channels; motor nerve terminals had to be depolarized by at least 15 mV (using elevated potassium) before a detectable increase in $[Ca^{2+}]_i$ could be observed. Such a depolarization decreases transmitter release slightly,[20] so it is unlikely to be involved in the augmentation of transmitter release by 5HT.

Instead, 5HT might increase voltage-dependent calcium current in crayfish

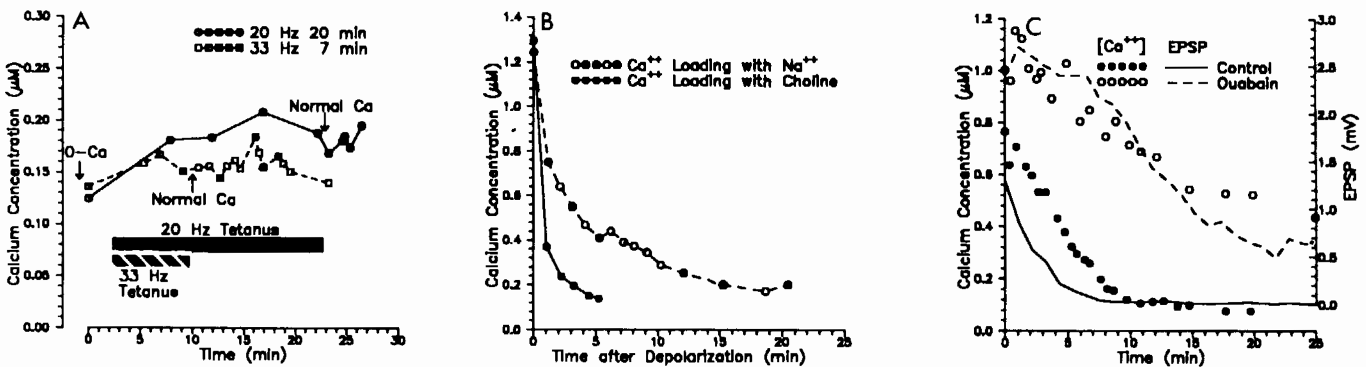

FIGURE 6. **A:** The excitor was stimulated tetanically in calcium-free Ringer in two experiments; normal calcium was restored at the end of each tetanus. **B:** Excitor nerve terminals were calcium-loaded two times by depolarizing the terminals in a solution with $[K^+]$ increased by 35 mM. In one case, they were also sodium-loaded with normal $[Na^+]$; in the other, sodium was replaced by choline. **C:** The excitor was stimulated with two tetani (33 Hz for 5 min): once in normal Ringer and once in the presence of 0.5 mM ouabain to block sodium extrusion.

motor terminals, as it does in molluscan neurons.[21] 5HT, however, caused no increase in tetanic $[Ca^{2+}]_i$ accumulation; on the contrary, we usually saw about a 10% decrease in $[Ca^{2+}]_i$ accumulation in the presence of 5 μM 5HT (FIG. 7). Nevertheless, the EPSPs were increased about fourfold. We contrast this result with the effect of increasing calcium influx by either raising extracellular $[Ca^{2+}]$ or prolonging action potentials: a fourfold increase in transmitter release is accompanied by a 30%–70% increase in $[Ca^{2+}]_i$, depending upon how the calcium influx is increased (FIGURES 3 and 7). We conclude that serotonin does not enhance transmitter release by modulating the calcium influx through calcium channels or by increasing the number of open channels, the way raising extracellular $[Ca^{2+}]$ or adding TEA does. Rather, our results suggest an effect independent of presynaptic $[Ca^{2+}]_i$ action. Perhaps transmitter stores are mobilized, or the secretory machinery is made more sensitive to $[Ca^{2+}]_i$.

PRESYNAPTIC INHIBITION REDUCES TETANIC $[CA^{2+}]_i$ ACCUMULATION

At the claw opener neuromuscular junction, GABA released from inhibitory nerve terminals reduces the number of quanta released by excitor nerve impulses while simultaneously shunting EPSPs postsynaptically. We have now observed changes in $[Ca^{2+}]_i$ accumulation in excitor nerve terminals during presynaptic inhibition. We injected both excitor and inhibitor nerves with fura-2. By stimulating either neuron alone and looking for increases in $[Ca^{2+}]_i$, we identified excitatory and inhibitory nerve terminals. FIGURE 8 (on the color plate) shows a case of an excitatory and inhibitory bouton making contact with each other on the muscle surface. When the excitor was stimulated at 10 Hz, $[Ca^{2+}]_i$ rose in this excitor terminal by 475 nM. When each excitatory action potential was preceded by 4 inhibitory spikes at 50 Hz, with the last one preceding the excitatory action potential by the optimal interval for presynaptic inhibition[22] (2.3 ms at this synapse), then $[Ca^{2+}]_i$ in the excitor terminal rose by only 270 nM. If this reflects a similar reduction (to 57%) in calcium influx per excitatory action potential, this would have a profound effect on the amount of transmitter released by this particular bouton. Such a reduction in the number of calcium channels opened by an action potential might be expected to reduce transmitter release to about 18% at this particular synapse,[23] well within the range of presynaptic inhibition observed physiologically.[22]

ROLE OF PRESYNAPTIC POTENTIAL IN TRANSMITTER RELEASE

It has been suggested that presynaptic depolarization might play a direct role in neurosecretion, beyond simply opening calcium channels. In recent experiments by Hochner *et al.*,[24] supporting this hypothesis, the excitor axon was injected with the photolabile caged calcium chelator nitr-5. When the medium contained little calcium (12.5 mM magnesium with no added calcium), action potentials (10 Hz pairs separated by 20 ms) evoked no detectable transmitter release. Photolysis of nitr-5 increased the spontaneous release of transmitter fourfold, presumably by release of $[Ca^{2+}]_i$. Now, action potentials caused a slight increase in transmitter release within 5 ms of the spikes. In other experiments, calcium influx was blocked by 2 mM manganese with 0.2 mM calcium, and mitochondrial inhibitors were used to elevate $[Ca^{2+}]_i$. Again, action potentials evoked phasic release of transmitter.

These results were taken to show that when $[Ca^{2+}]_i$ is already high, membrane

depolarization evokes neurotransmitter release in the absence of calcium entry. The solutions used, however, did not include chelators to remove extracellular calcium, which is known to leak out of muscles and collect in the bath,[17] and was already present at 0.2 mM in the manganese solution. We used fura-2 to check whether these solutions really block presynaptic calcium entry. FIGURE 9 shows that $[Ca^{2+}]_i$ more than doubled in these solutions when the excitor was stimulated with 10 Hz pairs. This small calcium influx was apparently insufficient to release transmitter at detectable levels, not surprising in view of the nonlinear dependence of release on calcium. Stimulation at 100 Hz revealed massive calcium influx and very large EPSPs in these solutions.[25] Such experiments, therefore, allow no conclusion about a direct effect of membrane depolarization on release.

We found that solutions in which all of the calcium is replaced by either cobalt or manganese, or in which calcium is chelated with 2 mM EGTA, show absolutely no rise in $[Ca^{2+}]_i$ to stimulation with 10 Hz pairs, and only 10–20 nM increase at 100 Hz. No transmitter release was observed at all in these solutions. These solutions may be used to test for an effect of presynaptic potential on transmitter release. Instead of nitr-5 we used DM-nitrophen,[26] a photolabile EDTA analogue that causes a much larger rise in $[Ca^{2+}]_i$ and displays none of the toxic effects of nitr-5 on neurosecretion.[27]

FIGURE 10 illustrates our results. Action potentials elicited normal EPSPs in normal Ringer. Photolysis of nitrophen caused a massive increase in transmitter release (the miniature EPSP frequency increased from 1 s^{-1} to over 3,000 s^{-1}), and the EPSPs were facilitated about 10-fold. After a few seconds, transmitter release subsided to a lower but nearly constant level (probably due to depletion of the most readily releasable store of quanta), and spikes still evoked strongly facilitated EPSPs. We performed similar experiments on preparations bathed in a solution containing cobalt instead of calcium. This blocked spike-evoked release. Nitrophen photolysis still accelerated miniature EPSP frequency, but now action potentials caused no additional release of transmitter. A nerve terminal field potential recorded from the muscle fiber indicated that the motor axon still conducted action potentials. When

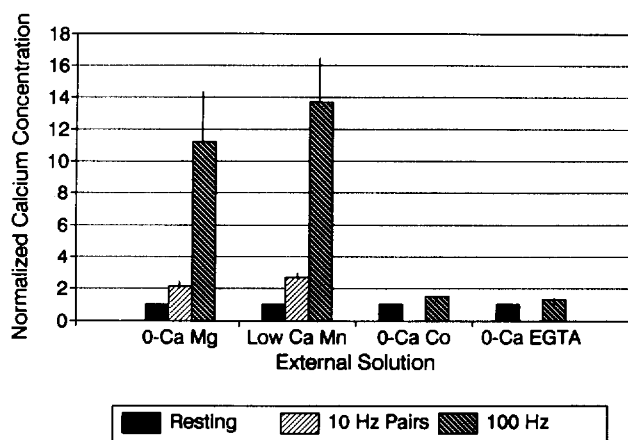

FIGURE 9. Effects of various nominally calcium-free solutions on $[Ca^{2+}]_i$ accumulation in presynaptic boutons, measured at rest, after stimulation with 10 Hz pairs separated by 20 ms, or stimulation at 100 Hz.

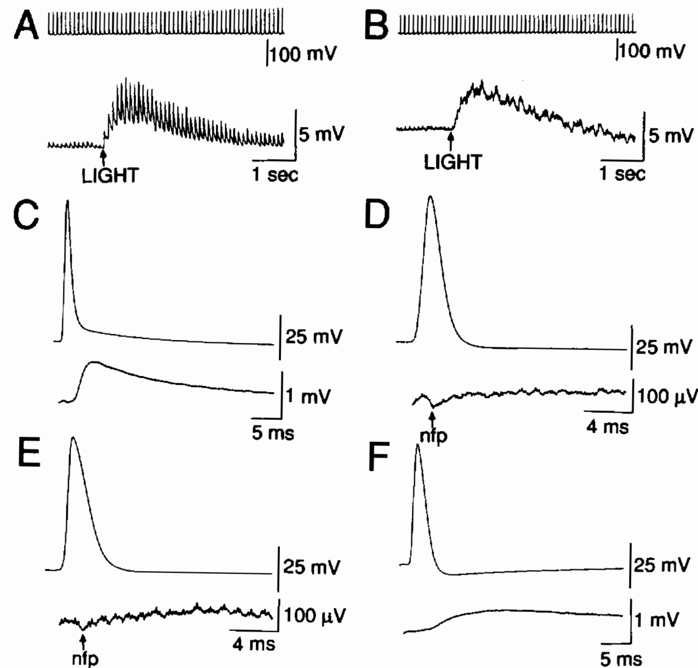

FIGURE 10. Action potentials evoke no transmitter release without calcium influx. The excitor axon was injected with DM-nitrophen 30% bound to calcium. In each panel, the top trace shows intracellularly recorded presynaptic action potentials, and the bottom trace shows intracellularly recorded postsynaptic potentials. Panel **A** shows results from an experiment in which nitrophen was photolyzed (with a 10 s exposure to UV light beginning at the arrow) while stimulating the motor nerve at 10 Hz in normal Ringer. Miniature EPSP frequency increased and evoked EPSPs were facilitated. The remaining panels are from a different experiment in which nitrophen was photolyzed while stimulating the nerve in a zero-calcium medium containing 13.5 mM Co^{2+}. Panel **B** shows that photolysis still increased miniature EPSP frequency, but that action potentials evoked no EPSPs either before or after the photolysis. The remaining panels show computer averages of 50 responses from the experiment of panel **B.** In panel **C,** spikes evoke EPSPs before injection of nitrophen while still in normal Ringer. In panel **D,** spikes evoke no EPSPs in the calcium-free cobalt Ringer after nitrophen injection. In panel **E,** spikes still evoke no EPSPs in the calcium-free Ringer even after photolysis increased the miniature EPSP frequency. Panel **F** shows the recovery of EPSPs after replacement of the solution with normal Ringer.

the medium was replaced with normal Ringer, spike-evoked transmission was restored. We think these results demonstrate that action potentials cannot evoke transmitter release in the absence of calcium influx, even when presynaptic $[Ca^{2+}]_i$ has been elevated to directly activate transmitter release at high levels.

We have also explored this question at the giant synapse in the stellate ganglion of the squid, a fast glutaminergic central nervous system synapse.[28] When the presynaptic terminal is injected with DM-nitrophen, action potentials evoke transmitter release with calcium present in the medium[27] (FIG. 11). Replacement of calcium with 10 mM EGTA completely blocked transmitter release to action potentials. Nitrophen photolysis by an intense light flash triggered transmitter release by

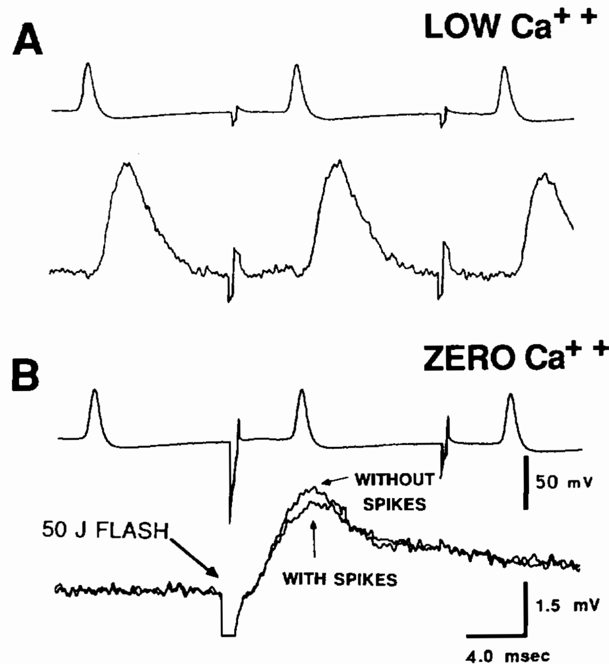

FIGURE 11. Presynaptic action potentials evoke no transmitter release in the absence of calcium entry at the squid giant synapse. The presynaptic terminal has been injected with DM-nitrophen 32% loaded with calcium. In each panel, the top trace shows the presynaptically recorded action potential, while the bottom trace shows postsynaptic potentials. In panel **A**, spikes evoke small EPSPs in low calcium medium (while replacing calcium with 10 mM EGTA). In panel **B**, extracellular $[Ca^{2+}]$ has been fully chelated, and spikes now evoke no transmitter release, even after flash photolysis of nitrophen elevates $[Ca^{2+}]_i$ to activate transmitter release directly. Two postsynaptic traces are superimposed showing responses to two flashes, but presynaptic action potentials were present during only one of the flashes.

elevating presynaptic $[Ca^{2+}]_i$, but action potentials failed to have any effect on this release. We conclude that at these synapses, as at crayfish neuromuscular junction, the only effect of the depolarization of an action potential is to open calcium channels and admit calcium ions, and that calcium alone triggers exocytosis at active zones.

A striking property of phasic spike-evoked release is that its time course is virtually unaffected by changes in extracellular $[Ca^{2+}]$, when comparing facilitated to unfacilitated release, or when release is triggered by pulses of different amplitude. A recent theoretical paper[29] used simulations of calcium diffusion from calcium channel mouths to nearby active zones to test the possibility that calcium could evoke transmitter release with a constant time-course under all these conditions. These simulations showed that, even when calcium was made to bind to a sensor that subsequently triggered exocytosis, changes in pulse amplitude, external $[Ca^{2+}]$, and state of facilitation all should affect the time-course of transmitter release. This was taken as proof that calcium entering through channel mouths and acting locally to

trigger release and then quickly diffusing away cannot explain the constancy of the time-course of release.

We have repeated and confirmed these simulations.[30] We discovered, however, that the parameters chosen would saturate the presynaptic calcium receptor even at low extracellular [Ca^{2+}], contrary to observation. Furthermore, the binding step was taken as being the rate-limiting step, rather than exocytosis. When binding constants (on and off rates) more similar to BAPTA were used for the calcium receptor, transmitter release became rate-limited by the exocytosis step, and its time-course was as invariant as shown by experimental result. Thus, this objection to the calcium hypothesis of transmitter release is easily overcome.

TRANSMITTER RELEASE BY SUDDEN ELEVATION OF PRESYNAPTIC [CA^{2+}]$_i$

FIGURES 11 and 12 illustrate a surprising property of transmitter release evoked by flash photolysis of DM-nitrophen at the squid giant synapse: the postsynaptic response is quite transient, dropping to half its peak in 5–10 milliseconds. A prolonged tail of release continues for several seconds. Part of this transience in the response arises from the chemical properties of DM-nitrophen. When this chelator is partially photolyzed by a light flash, the calcium released re-equilibrates with the remaining unphotolyzed nitrophen. Because nitrophen is an EDTA derivative, it also binds magnesium tightly, absorbing the several millimolar present in cytoplasm. Photolytically liberated calcium rebinds to unphotolyzed nitrophen only after displacing hydrogen and magnesium. But the unbinding of magnesium is slow, and this leads to a period during which the calcium liberated from photolyzed nitrophen remains free. This leads to a transient rise in [Ca^{2+}]$_i$ following partial flash photolysis of nitrophen. This behavior was confirmed by co-injecting fura-2 with nitrophen into squid giant synapses.[27] Measurement of [Ca^{2+}]$_i$ revealed a transient rise to the low micromolar range and a subsequent fall to about half this level with a time-constant of 65 milliseconds. A nonlinear dependence of transmitter release upon [Ca^{2+}]$_i$

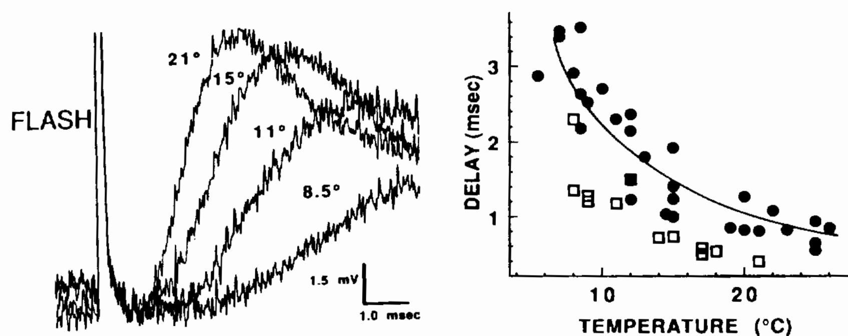

FIGURE 12. Effect of temperature on the postsynaptic responses to flash photolysis of DM-nitrophen injected into a squid presynaptic terminal. Sample responses are on the left, while the graph plots the delay to the beginning of postsynaptic potentials following nitrophen photolysis (filled circles) as well as synaptic delays from the peak of presynaptic spikes to the foot of the EPSP (open squares). The photolysis response delays have been corrected for the expected time between the flash and photolysis of nitrophen. The curve is a least squares fit of the logarithm of the delay from photolysis to changes in temperature.

would largely account for the rapidly decaying (15–20 ms time-constant) transient phase of transmitter release, followed by a smaller long-lasting tail. Depletion of releasable transmitter may also contribute to the decay of release.

Nitrophen photolysis also releases intracellular magnesium. We have found that a $[Mg^{2+}]_i$ jump alone evokes no transmitter release, because nitrophen injected without calcium evokes no transmitter release upon photolysis.

A measured rise of $[Ca^{2+}]_i$ to a few μM evokes transmitter release at a rate that resembles that caused by action potentials. Spikes are thought to release transmitter from very near calcium channels at concentrations well above 10 μM.[12–14] How, then, can a few μM $[Ca^{2+}]_i$ elicit such powerful transmitter release? Although we do not have a quantitative answer to this question, we believe that one factor is that calcium released by nitrophen acts uniformly on every single releasable vesicle. Action potentials open a small fraction of the available calcium channels and release transmitter quanta at only a few of the many release sites, so a higher local $[Ca^{2+}]_i$ is needed at these sites to release as many quanta as calcium released from nitrophen, acting globally.

Another factor may be that we have underestimated the peak calcium rise. Photolysis of 20% of the 10 or so mM of injected nitrophen 30% loaded with calcium releases 600 μM of calcium, most of which will be rapidly rebound by unphotolyzed free nitrophen. To protect our photomultiplier tube from damage, we could begin to measure $[Ca^{2+}]_i$ only 3–5 ms after the flash. Thus an initial intense peak in $[Ca^{2+}]_i$, lasting until the calcium binds to free nitrophen, would be missed in our measurements (and in any case would be blunted by the noninstantaneous association of calcium with fura-2). If the displacement of hydrogen from nitrophen takes longer than the binding of calcium to the receptor for releasing transmitter, then this undetected calcium spike could contribute significantly to the amount of transmitter released by a flash of light.

Postsynaptic responses following nitrophen photolysis show a "synaptic delay" consisting of the time to photolyze nitrophen (about 100 μs *in vitro*) plus the time for exocytosis to occur. The latter step is highly temperature-dependent, with a Q_{10} of about 3.5 between 5 and 15°C (FIG. 12). This is similar to the temperature sensitivity of the evoked synaptic delay following presynaptic impulses,[31] suggesting that some step in exocytosis following calcium entry has a high activation energy and is rate-limiting in determining the initial delay and subsequent time-course of transmitter release.

SUMMARY

This review gives some indication of the progress that has been made in understanding synaptic transmission by use of new methods for measuring and controlling presynaptic $[Ca^{2+}]_i$. Many unsolved problems remain. We still do not have a clear idea of the exact relationship between $[Ca^{2+}]_i$ and transmitter release and whether this relationship is the same under all circumstances. The apparently different $[Ca^{2+}]_i$-dependence of evoked transmitter release and of PTP suggest multiple molecular sites of calcium action that remain to be identified. A complete and comprehensive model of transmitter release has yet to be devised, and questions raised by our experiments may indicate that it is still too early to try to construct a precise model. We also do not know just how serotonin acts to modulate transmitter release, only that it does not appear to alter either resting or entering calcium. Some of these questions may be approachable with the techniques described here; others are not and require different methods for their resolution. The work continues.

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