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CALCIUM AND SHORT-TERM SYNAPTIC PLASTICITY

by

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ABSTRACT

The sites of presynaptic action of calcium ions in triggering exocytosis and in activating various forms of short-term enhancement of synaptic transmission are discussed. A detailed presentation of methods and results is left to original publications. Instead, an attempt is made to collate a variety of findings and synthesize a picture of how Ca^{2+} operates in nerve terminals to trigger release and enhance evoked release following electrical activity. It is concluded that Ca^{2+} triggers neurosecretion by acting very near Ca^{2+} channel mouths, at high concentration, with high stoichiometry, to activate low affinity binding sites with fast kenetics. Facilitation, augmentation, and potentiation are consequences of actions of residual presynaptic Ca^{2+} remaining after prior electrical activity. Facili6tation is caused by Ca^{2+} acting with fast kinetics, but probably with moderately high affinity at a site distinct from the secretory trigger. Augmentation and potentiation are caused by residual Ca^{2+} acting at yet another site, probably of high affinity, and with rate constants of about 1s. Post-tetanic potentiation lasts so long because nerve terminals cannot remove residual Ca^{2+} quickly after prolonged stimulation. Processes similar to augmentation and potentiation apear to occur at some hormonal cells as well as in neurons. The molecular receptors for Ca^{2+} in short-term synaptic plasticity have yet to be identified, but Ca^{2+}/calmodulin protein kinase II is not a likely candidate.

KEY WORDS: synapse, calcium, plasticity, transmitter, release, facilitation, augmentation, potentiation, post-tetanic potentiation, DM-nitrophen, calmodulin.

CALCIUM AND TRANSMITTER RELEASE

That Ca^{2+} is required for synaptic transmission has been recognized since the work of RINGER (1883). KATZ & MILEDI (1967a) showed that extracellular Ca^{2+} must be present during the presynaptic action potential, and their discovery of an 'off-EPSP' to large depolarizations (KATZ & MILEDI, 1967b, c) implicated an influx of Ca^{2+} to act at an intracellular site. LLINÁS *et al.* (1981) found a close correlation between presynaptic Ca^{2+} current and transmitter release to different depolarizing pulses at the squid giant synapse. These observations established the role of Ca^{2+} acting presynaptically to trigger transmitter release, while other studies (ZUCKER & LANDÒ, 1986; ZUCKER *et al.*, 1986; LANDÒ *et al.*, 1986; ZUCKER, 1987; ZUCKER & HAYDON, 1988; DELANEY & ZUCKER, 1990; MULKEY & ZUCKER, 1991; ZUCKER *et al.*, 1991) demonstrated that presynaptic depolarization acts only to open

Ca^{2+} channels and admit Ca^{2+} to release sites in nerve terminals. When flash photolysis of the photo-sensitive Ca^{2+} chelator DM-nitrophen is used to produce a brief 'spike' in presynaptic Ca^{2+} concentration ($[Ca^{2+}]_i$), transmitter release occurs in the total absence of presynaptic potential changes with an amplitude and time-course that generate a postsynaptic response resembling a normal EPSP (DELANEY & ZUCKER, 1990; ZUCKER *et al.*, 1991; ZUCKER, 1993; LANDÒ & ZUCKER, 1994).

CALCIUM AND SHORT-TERM SYNAPTIC PLASTICITY

That Ca^{2+} is involved in short-term synaptic plasticity has also long been known. Repeated presynaptic action potentials often evoke increased transmitter release to successive spikes (reviewed in ZUCKER, 1989 and DELANEY *et al.*, 1989). When a pair of presynaptic action potentials occur within an interval of 1 second or less, the second often releases more transmitter than the first. This 'facilitation' accumulates in a train of action potentials, reaching steady-state in about a second, and the effect decays after activity at a similar rate. Facilitation usually displays rapid (tens of ms) and slower (hundreds of ms) kinetic components. A train of presynaptic action potentials lasting tens of seconds is often accompanied by a more slowly accumulating increase in transmitter release to successive action potentials, which is usually called 'augmentation'. More prolonged stimulation is accompanied by an even slower process of increasing transmitter release per impulse, growing over minutes and termed 'potentiation', or post-tetanic potentiation (PTP). At some synapses, prolonged stimulation can activate a more permanent increase in synaptic efficay, termed long-term facilitation (LTF), lasting tens of minutes, while a high frequency train can increase transmitter release to test impulses for hours, a process called long-term potentiation (LTP), but not to be confused with processes occurring at cortical synapses bearing the same name.

Facilitation occurs to repeated constant presynaptic depolarizations that admit invariant amounts of Ca^{2+} and cause invariant increments in presynaptic $[Ca^{2+}]_i$ (CHARLTON *et al.*, 1982). Extracellular Ca^{2+} must be present not only for a spike to evoke transmitter release, but also for it to facilitate subsequent release (KATZ & MILEDI, 1968). Together these properties suggest that facilitation is due to Ca^{2+} acting at some presynaptic site, rather than to changes in the presynaptic electrical signal, or magnitude of Ca^{2+} influx, or change in $[Ca^{2+}]_i$. Augmentation and potentiation are also dependent on Ca^{2+} influx (reviewed in ZUCKER, 1989). Although long-term facilitation does not require Ca^{2+} entry, it and LTP might involve changes in presynaptic $[Ca^{2+}]_i$ arising

from another source. Thus most of these forms of synaptic enhancement appear to involve Ca^{2+} ions acting presynaptically in some way to increase synaptic strength.

THE SINGLE-SITE/NONLINEAR-SUMMATION/RESIDUAL-Ca^{2+} HYPOTHESIS

An early hypothesis for facilitation, PTP, and related processes proposed that they are the consequence of 'residual calcium' left over in nerve terminals following electrical activity. This residual Ca^{2+} could add to the incremental change in $[Ca^{2+}]_i$ caused by an action potential to yield an increased peak $[Ca^{2+}]_i$ at sites of transmitter release. Transmitter release varies as the fourth power of extracellular Ca^{2+} concentration ($[Ca^{2+}]_e$), while Ca^{2+} influx is a linear function of $[Ca^{2+}]_e$ (DODGE & RAHAMIMOFF, 1967; DUDEL, 1981; AUGUSTINE & CHARLTON, 1986), suggesting that multiple Ca^{2+} ions must bind stoichiometrically to the release trigger to activate neurosecretion. In such a highly nonlinear process, even substantial amounts of residual Ca^{2+} would activate little transmitter release, probably increasing the frequency of miniature postsynaptic potentials (MPSPs), while a small increase in peak $[Ca^{2+}]_i$ would dramatically increase evoked phasic release to an action potential (KATZ & MILEDI, 1968; MILEDI & THIES, 1971). The post-tetanic correlation between changes in MPSP frequency and facilitation (BARRETT & STEVENS, 1972; ZUCKER & LARA-ESTRELLA, 1983) are generally consistent with this hypothesis. The idea, then, is that all these forms of synaptic enhancement following presynaptic activity are due to the effects of residual Ca^{2+} summating with Ca^{2+} entering during an action potential and the nonlinear dependence of transmitter release on $[Ca^{2+}]_i$ at release sites. We'll call this the 'single-site/nonlinear-summation/residual-Ca' hypothesis of synaptic enhancement.

The different time courses of facilitation, augmentation, potentiation, LTF, and LTP could simply reflect the different time courses for removal of residual Ca^{2+} after different regimens of stimulation; the latter could arise from differentially saturated buffers, pumps, and organelles following different stimulus regimens, or from different times needed to remove Ca^{2+} by diffusion from near release sites after a few spikes, from whole boutons after short tetani, and from larger terminal arborizations after longer tetani. Early simulations of Ca^{2+} diffusing away from the plasma membrane or from arrays of Ca^{2+} channels at release sites, with binding to intracellular buffers, uptake into organelles, and removal by Ca^{2+} extrusion pumps, and with residual Ca^{2+} adding to entering Ca^{2+} to occupy one class of binding

sites triggering exocytosis, were able to reproduce many of the kinetic properties of facilitation, augmentation, and potentiation (ZUCKER & STOCKBRIDGE, 1983; FOGELSON & ZUCKER, 1985).

A role for residual Ca^{2+} in synaptic enhancement also received experimental support from measurements of $[Ca^{2+}]_i$ in nerve terminals following repetitive activity, using the Ca-dependent potassium current (KRETZ *et al.*, 1982), the Ca-sensitive metallochromic indicator arsenazo III (CONNOR *et al.*, 1986), or the fluorescent indicator dye fura-2 (DELANEY *et al.*, 1989; REGEHR *et al.*, 1994; DELANEY & TANK, 1994). Residual $[Ca^{2+}]_i$ decayed with the same time courses as augmentation and potentation even when manipulations like temperature changes and injecting Ca^{2+} buffers were used to alter $[Ca^{2+}]_i$ decay kinetics. However, the magnitude of measured residual $[Ca^{2+}]_i$ did not match that predicted from the single-site/nonlinear-summation/residual-Ca^{2+} model and the magnitudes of PTP and augmentation (DELANEY *et al.*, 1989; DELANEY & TANK, 1994). This led to the suggestion that Ca^{2+} acts at a site distinct from that triggering transmitter release in augmentation and potentiation.

Several objections had previously been raised to the idea of a unitary site of Ca^{2+} action. If facilitation, augmentation and potentiation are all due to the removal of residual Ca^{2+} from the site triggering exocytosis, then all these stimulation-dependent processes, or perhaps the fourth root of them, should add linearly when they occur in combination; however, more complex interactions between the processes were observed than predicted by this simple model (ZUCKER, 1974; MAGLEBY & ZENGEL, 1982). The relationship between MPSP frequency and evoked release, either following a tetanus (ZENGEL & MAGLEBY, 1981; BAIN & QUASTEL, 1992) or during elevation of $[Ca^{2+}]_i$ by DM-nitrophen photolysis (MULKEY & ZUCKER, 1993), did not always fit predictions of this single-site/nonlinear-summation/residual-Ca^{2+} model; nor did the growth of facilitation in a tetanus (ZUCKER, 1974). Differential effects of Ba^{2+} and Sr^{2+} on augmentation and the slow component of facilitation respectively (ZENGEL & MAGLEBY, 1980) also suggest separate sites of Ca^{2+} action, although they might reflect ion-specific effects on the processes involved in clearing residual divalent cations from nerve terminals. Recent simulations of Ca^{2+} diffusing from Ca^{2+} channels (YAMADA & ZUCKER, 1992) failed to generate as much facilitation by the simple unitary-site/nonlinear-summation/residual-Ca^{2+} model as is observed experimentally. Moreover, at temperatures near 0°C, facilitation rises to a peak 50 ms after evoked transmitter release (VAN DER KLOOT, 1994). These results suggest that Ca^{2+} acts at more than one site to trigger transmitter release and activate fast and slow facilitation, augmentation, potentiation, and

perhaps LTF and LTP, although not necessarily at a different site for each process. This makes it easier to understand why different synapses show such different magnitudes of facilitation, augmentation, and potentiation.

In the last few years, attempts have been made to test the idea that residual Ca^{2+} generates enhancement of synaptic transmission by increasing the presynaptic concentration of exogenous buffers that might obstruct the accumulation of residual Ca^{2+}. In some studies, presynaptic EGTA or BAPTA reduced a component of facilitation (DELANEY *et al.*, 1991; HOCHNER *et al.*, 1991; ROBITAILLE & CHARLTON, 1991; BAIN & QUASTEL, 1992; TANABE & KIJIMA, 1992; VAN DER KLOOT & MOLGÓ, 1993) or augmentation (SWANDULLA *et al.*, 1991); in other studies (TANABE & KIJIMA, 1989; WINSLOW *et al.*, 1994), it did not. Negative results were obtained when presynaptic terminals were loaded with the acetoxymethyl ester of BAPTA, relying on endogenous esterases to trap the chelator, which may also enter intracellular organelles, or bind to cytoplasmic proteins as fura-2 sometimes does (BAYLOR & HOLLINGWORTH, 1988). This procedure results in weak buffer concentrations that are nearly saturated at resting $[Ca^{2+}]_i$ levels and certainly after repetitive activity. We have observed that BAPTA-AM loading can have only very small effects on accumulation of residual $[Ca^{2+}]_i$ on repetitive stimulation (unpublished observations). Variable results might arise from use of nearly saturated buffers that are able to capture some of the entering Ca^{2+} in a single action potential to reduce transmission, but then quickly become saturated and cannot prevent the accumulation of residual Ca^{2+}, and also permit larger transient $[Ca^{2+}]_i$ on subsequent action potentials. Even positive results are open to alternative interpretations: Buffer present during conditioning stimulation could just as well prevent Ca^{2+} from reaching sites to which it remains bound after residual Ca^{2+} has dissipated as prevent the accumulation of residual Ca^{2+}. These ambiguities and the inconsistent nature of the results of these studies do not permit any clear conclusions to be drawn from them.

THE CALCIUM BINDING SITE TRIGGERING EXOCYTOSIS

Recent experiments have begun to shed light on the characteristics of the Ca^{2+} binding sites responsible for neurosecretion and short-term synaptic plasticity. Perhaps the most striking property of synaptic transmission at fast synapses is its speed. Transmitter release begins in only a few hundred μs after the start of presynaptic Ca^{2+} influx (LLINÁS *et al.*, 1981), or after the elevation of $[Ca^{2+}]_i$ by photolysis of DM-nitrophen (DELANEY & ZUCKER, 1990). This short synaptic delay allows

sufficient time for Ca^{2+} to diffuse only tens of nanometers from points of entry at Ca^{2+} channel mouths, and implies a very fast on-rate for Ca^{2+} binding to the secretory trigger.

Considerations of the geometrical relationships between Ca^{2+} channels and sites of transmitter release provide evidence that $[Ca^{2+}]_i$ reaches high levels at release sites. Ca^{2+} channels and synaptic vesicles containing quanta of transmitter are colocalized in active zones at patches of presynaptic membrane that are about 0.25 μm^2 in area (Robitaille *et al.*, 1990; Cohen *et al.*, 1991). Dividing the presynaptic Ca^{2+} current during an action potential (Llinás *et al.*, 1982) by the single channel current provides an estimate of the number of Ca^{2+} channels open during a spike at the squid giant synapse; dividing this by the measured number of active zones (Pumplin *et al.*, 1981) provides an average number of open Ca^{2+} channels per active zone. Assuming these form a regular array, diffusion simulations provide an estimate of the $[Ca^{2+}]_i$ in active zones during synaptic transmission (Fogelson & Zucker, 1985; Simon & Llinás, 1985; Yamada & Zucker, 1992). Even at points in active zones most distant from Ca^{2+} channels (about 50 nm for regular arrays of open channels), $[Ca^{2+}]_i$ should reach about 100 μM.

It is noteworthy that fast freezing and freeze fracture of the presynaptic terminal at neuromuscular junctions (Heuser *et al.*, 1979) shows that vesicle exocytosis occurs on average 50 nm from intramembranous particles thought to represent Ca^{2+} channels. Nonuniform clustering of Ca^{2+} channels, randomness in the disposition of open channels, and possible covalent interactions between Ca^{2+} channels and docked vesicles (Bennett & Scheller, 1994) would all put vesicles nearer than 50 nm to some Ca^{2+} channels, and predict higher levels of $[Ca^{2+}]_i$ triggering exocytosis of these vesicles. The high stoichiometry of Ca^{2+} action in exocytosis assures that only vesicles exposed to the highest $[Ca^{2+}]_i$ levels will be released.

$[Ca^{2+}]_i$ levels of 200-300 μM have been measured in active zones of the squid giant synapse with the low-affinity Ca-indicating photoprotein *n*-aequorin-J (Llinás *et al.*, 1992), although these levels might occur near Ca^{2+} channel mouths rather than at vesicles docked at release sites. In hair cells, $[Ca^{2+}]_i$ levels as high as 1 mM have been inferred using Ca-dependent K current (Roberts *et al.*, 1990), confirming the existence of local high presynaptic $[Ca^{2+}]_i$ levels. The relative effects of different concentrations of fast exogenous buffers of varying Ca^{2+} affinity on transmission also imply a total $[Ca^{2+}]$ of at least 100 μM at release sites (Adler *et al.*, 1991; Augustine *et al.*, 1991), although some of this Ca^{2+} would be bound by endogenous buffers before binding to the release trigger.

Additional evidence that secretion is triggered by locally high $[Ca^{2+}]_i$, or 'calcium domains,' comes from experiments on the dependence of transmitter release on magnitude of Ca^{2+} influx. When Ca^{2+} influx is increased by elevating $[Ca^{2+}]_e$, release varies with approximately the fourth power of $[Ca^{2+}]$ (DODGE & RAHAMIMOFF, 1967; DUDEL, 1981; AUGUSTINE & CHARLTON, 1986; ZUCKER *et al.*, 1991), reflecting the binding of multiple Ca^{2+} ions in triggering exocytosis. However, when Ca^{2+} influx is increased by prolonging action potentials or using larger depolarizations (LLINÁS *et al.*, 1981; CHARLTON *et al.*, 1982; AUGUSTINE *et al.*, 1985; AUGUSTINE, 1990; ZUCKER *et al.*, 1991), the Ca-dependence of transmitter release is not as steep. This is because these maneuvers do not increase Ca^{2+} influx through Ca^{2+} channels; instead they increase the total number of channels that open, either simultaneously with bigger depolarizations, or at different times with spike prolongation. Completely non-overlapping 'calcium domains' would then act only to recruit release from new release sites, and release would increase linearly with Ca^{2+} influx. Partially overlapping domains will also increase the $[Ca^{2+}]_i$ somewhat at vesicles within reach of more than one 'domain', and release should depend on Ca^{2+} influx with a power somewhere between one and the actual stoichiometry of Ca^{2+} action (ZUCKER & FOGELSON, 1986).

Evidence for fast Ca-binding kinetics at the site triggering release comes from the effects of injecting exogenous buffers into the squid giant presynaptic terminal. The slow buffer EGTA left transmission practically unaffected (ADLER *et al.*, 1991); only fast buffers based on BAPTA with on-rates of about $5 \times 10^8\ M^{-1}\ s^{-1}$ (NEHER, 1986) effectively block transmitter release. This suggests that the on-rate for Ca^{2+} binding to the release trigger is on the order of $10^8\ M^{-1}\ s^{-1}$. Transmitter release terminates rapidly after Ca^{2+} influx ceases in an action potential ($\tau \approx 0.25$ ms; KATZ & MILEDI, 1965; PARNAS *et al.*, 1989). For a Ca^{2+} stoichiometry of 4 at release sites, this implies an off-rate of $10^3\ s^{-1}$. The high temperature dependence of the time course of release suggests that a process subsequent to Ca^{2+} binding is rate-limiting in exocytosis (YAMADA & ZUCKER, 1992), in which case the off-rate must be faster than $10^3\ s^{-1}$. This in turn implies a low Ca^{2+} affinity of the secretory trigger, with dissociation constant $K_D \geq 10\ \mu M$.

For $[Ca^{2+}]_i$ peaks of 100 μM, Ca^{2+} would equilibrate with a binding site with the above binding rates in 100 μs or less. This time fits comfortably within the minimum synaptic delay and still allows time for Ca^{2+} to diffuse tens of nanometers from channel mouths to docked vesicles. The time course of the $[Ca^{2+}]_i$ change during an action potential in active zones is complicated by the rapidly rising single channel current as the action potential repolarizes, and by closure of

Ca^{2+} channels shortly thereafter. Simulations (YAMADA & ZUCKER, 1992) suggest the presence of a '[Ca] spike' with half-width of about 0.5 ms, in which case Ca^{2+} does not quite have time to equilibrate with its binding site during an action potential. Then equilibrium calculations do not strictly apply, but roughly speaking, equilibrium will be approached during action potentials. Since small changes in $[Ca^{2+}]_e$ dramatically affect evoked release, the Ca^{2+} binding sites triggering release are not saturated by the peak $[Ca^{2+}]_i$ reached. This suggests that they have a quite low affinity, with $K_D \geq 100$ μM. Thus, at fast synapses, secretion is triggered by about 4 (or possibly more; PARNAS *et al.*, 1982; BARTON *et al.*, 1983) Ca^{2+} ions binding simultaneously to release molecules.

A more direct measurement of the properties of the release trigger for exocytosis has been obtained in recent experiments on bovine chromaffin cells (HEINEMANN *et al.*, 1994). We used flash photolysis of DM-nitrophen to rapidly increase $[Ca^{2+}]_i$, and recorded the effect on secretion measured as an increase in cell membrane capacitance. Secretion initially increased with a sigmoidal time course reflecting the binding of multiple (probably 3 or 4) Ca^{2+} ions to trigger release. The maximum rate of release saturated at $Ca^{2+} \approx 1$ mM, implying a final rate limiting exocytotic step of about 1000 s^{-1}. The association and dissociation rates for Ca^{2+} binding to the exocytosis trigger were estimated as 15×10^6 M^{-1} s^{-1} and 130 s^{-1} by adjusting these values to optimize a fit of simulated release to observed release. Such binding sites have a Ca^{2+} affinity of about 10 μM. A similar affinity has been estimated from flash photolysis studies of secretion from pituitary melanotrophs (THOMAS *et al.*, 1993).

These kinetics are somewhat slower, and the affinity somewhat higher, than we expect for fast neuronal synapses. But chromaffin cell and melanotroph secretory granules are dense-core vesicles that are not docked at active zones, and release by depolarizing pulses is slower than at fast synapses (CHOW *et al.*, 1992). Recently, similar experiments on retinal bipolar cells (HEIDELBERGER *et al.*, 1994) suggest that at these neurons exocytosis is triggered by Ca^{2+} binding with faster kinetics and lower affinity than from chromaffin cells and melanotrophs, similar to what is expected as described above.

CALCIUM BINDING SITES IN SHORT-TERM SYNAPTIC ENHANCEMENT

In order to characterize the sites of Ca^{2+} action in processes of short-term synaptic plasticity, we have used photolabile Ca^{2+} chelators to raise or lower $[Ca^{2+}]_i$ in nerve terminals and observe effects on facilitation, augmentation, and potentiation at excitatory neuromuscular

junctions of the crayfish claw opener muscle (KAMIYA & ZUCKER, 1994). Our procedure was to establish facilitation, augmentation, or PTP in isolation, and then look at the effects of suddenly increasing the Ca^{2+} buffer power of cytoplasm by photolyzing diazo-2 that had been injected into the presynaptic terminals. Before photolysis, the diazo-2 had little effect on single EPSPs, or on facilitation, augmentation, or PTP.

A train of 10 spikes at 50 Hz generates substantial facilitation, with EPSPs at the end of the train over 10 times their unfacilitated amplitude at the beginning of the train. A test impulse 50 or 200 ms later still shows EPSPs that are about 650% their initial value, due mainly to the more slowly decaying second component of facilitation. When diazo-2 was photolyzed to produce about 200 μM of high affinity photoproduct just 10 ms before the test EPSP, facilitation was markedly reduced, so that the test EPSP was only 300% as big as its unfacilitated level. This remaining facilitation decayed normally, and the final unfacilitated EPSP was unaffected by diazo-2 photolysis, as expected for the small amount of buffer produced (ADLER *et al.*, 1991). But 200 μM of diazo-2 photoproduct ought to be sufficient to strongly buffer the residual $[Ca^{2+}]_i$ in the micromolar range expected after such a train (FOGELSON & ZUCKER, 1985). This result shows that most of facilitation, especially its second component, is dependent upon residual $[Ca^{2+}]_i$, acting at a site that equilibrates rapidly, within less than 10 ms. The alternative possibility, that facilitation is due to $[Ca^{2+}]_i$ remaining bound to some site after free $[Ca^{2+}]_i$ has decayed to baseline (BALNAVE & GAGE, 1974; STANLEY, 1986; YAMADA & ZUCKER, 1992), is inconsistent with our results. The fact that a few μM $[Ca^{2+}]_i$ can cause so much facilitation, and the reasons given above for believing facilitation acts at least in part at a site distinct from triggering secretion, suggest that facilitation is caused by Ca^{2+} acting with fast kinetics at a site with high affinity, with K_D perhaps in the high micromolar range. To keep this site from being saturated by a single action potential, it must either have kinetics substantially slower than the submillisecond $[Ca^{2+}]_i$ spike in 'Ca^{2+} domains' at release sites, or be located more distant from Ca^{2+} channels than the release trigger. It is also still possible that some fraction of facilitation is due to the nonlinear summation of residual Ca^{2+} with peak transient $[Ca^{2+}]_i$ changes at the release trigger.

The same methods were used to study augmentation and potentiation. Augmentation is generated by a 4 s, 50 Hz train, and testing 2 s after the train to allow facilitation to dissipate. When diazo-2 was photolyzed immediately before the test EPSP, the test response was hardly affected. As the interval between photolysis flash and test EPSP

increased, augmentation was almost entirely eliminated. The block of augmentation developed with a time constant of a few hundred ms. Potentiation is generated by stimulating 5 min at 50 Hz, and waiting 1 min for facilitation and augmentation to dissipate. Like augmentation, PTP was eliminated by diazo-2 photolysis, but the block took a few hundred ms to develop. These results indicate that augmentation and PTP are caused by actions of residual Ca^{2+} that have much slower kinetics than secretion or facilitation. A recent study of hippocampal mossy fiber synapses (REGEHR *et al.*, 1994) reported a delay between changes in $[Ca^{2+}]_i$ and augmentation of several seconds, apparently reflecting similar (but somewhat slower) kinetics of Ca^{2+} action on augmentation in that preparation.

We observed one difference in the effects of diazo-2 photolysis on augmentation and potentiation. After photolysis eliminated PTP, potentiation recovered slowly (in about 30 s), while augmentation did not. It may be that the prolonged stimulation needed to establish potentiation loads presynaptic organelles with Ca^{2+}, and that leakage from these organelles after reducing $[Ca^{2+}]_i$ by photolyzing diazo-2 eventually saturated the photoproduct and permitted free $[Ca^{2+}]_i$ to rise again.

Augmentation and PTP are linearly related to post-tetanic residual $[Ca^{2+}]_i$, with sensitivities of about a 10-fold increase in transmitter release per μM increase in $[Ca^{2+}]_i$ for augmentation (DELANEY & TANK, 1994) and a 20-fold increase per μM increase in $[Ca^{2+}]_i$ for PTP (DELANEY *et al.*, 1989). When the effect of a concurrent LTF that doubled transmission, present in the study of DELANEY *et al.* (1989), is factored in, PTP alone shows a 10-fold increase per μM $[Ca^{2+}]_i$. Since augmentation and potentiation have similar Ca^{2+} sensitivities, and respond to changes in $[Ca^{2+}]_i$ with similar kinetics, they are likely to be manifestations of the same process. The normal durations of augmentation and potentiation (seconds and minutes respectively) are not characteristics of the process itself, but rather reflect the time course for removal of $[Ca^{2+}]_i$ after short tetani causing augmentation and longer tetani causing potentiation. The slow removal of $[Ca^{2+}]_i$ in PTP is partly a consequence of a reduction in Ca^{2+} extrusion by Na^+/Ca^{2+} exhange due to Na^+ accumulation in nerve terminals (MULKEY & ZUCKER, 1992). Another factor may be loading of presynaptic Ca-storing organelles during potentiating stimuli, and the need to unload these organelles to restore cytoplasmic $[Ca^{2+}]_i$ to low levels.

If residual $[Ca^{2+}]_i$ in the micromolar range can act at presynaptic sites to facilitate and augment or potentiate release, then elevating $[Ca^{2+}]_i$ by this amount should increase transmitter release to single action potentials to well above the unfacilitated level. We first saw this

by injecting Ca^{2+} ions into the presynaptic terminal of the squid giant synapse (CHARLTON *et al.*, 1982). In our latest experiments, we used steady photolysis of DM-nitrophen to elevate presynaptic $[Ca^{2+}]_i$ to about 1-2 μM, and found that this greatly increased MEPSP frequency (from 1 s^{-1} to over 1000 s^{-1}) and doubled or tripled spike-evoked phasic release or EPSP amplitude while the photolyzing light remained on. When the light was extinguished, $[Ca^{2+}]_i$ returned to near its original levels, because unphotolyzed DM-nitrophen still exceeded total $[Ca^{2+}]_i$ (ZUCKER, 1993; MULKEY & ZUCKER, 1993). When the light was turned off, test EPSPs dropped rapidly to about 1.5 times the control value. Test EPSPs given at various times after turning off the light showed that this lingering enhancement decayed with a time constant of a few hundred ms. Apparently, the elevation in $[Ca^{2+}]_i$ activated two processes, one (facilitation) which lasted only as long as $[Ca^{2+}]_i$ was elevated, and another (augmentation/potentiation) which decayed entirely after about 2 s.

These results indicate that facilitation is caused by $[Ca^{2+}]_i$ acting at presynaptic sites, probably including the release trigger plus another site having fast kinetics and perhaps micromolar affinity, while augmentation and potentiation act at a third site with slower kinetics and probably higher affinity. Little information is available regarding the mechanisms responsible for LTF and presynaptic LTP at fast synapses. LTF seems to occur in the absence of an elevated $[Ca^{2+}]_i$ (DELANEY *et al.*, 1989) and not to depend upon Ca influx (WOJTOWICZ & ATWOOD, 1988). LTP does seem to be accompanied by an increase in $[Ca^{2+}]_i$ (DELANEY *et al.*, 1989).

A number of quantitative problems remain. For example, residual changes in $[Ca^{2+}]_i$ of 1 μM following tetanic stimulation (DELANEY *et al.*, 1989; DELANEY & TANK, 1994) increased evoked transmitter release by about ten times; and such a $[Ca^{2+}]_i$ elevation increased MEPSP frequency by about 10 times. We calculate that a similar increase in $[Ca^{2+}]_i$ caused by photolysis of DM-nitrophen increased phasic release by only 2-3 times, while increasing MEPSP frequency about 1000-fold. This discrepancy in effects on MEPSP frequency and evoked release under the different experimental conditions is troubling. Another difficulty is that raising $[Ca^{2+}]_e$ increases transmitter release, augmentation, and potentiation (reviewed in ZUCKER, 1989), as would be expected if these are separate Ca-dependent processes. The effect of changing $[Ca^{2+}]$ on facilitation is much smaller, and can be quite complex—like changing its time course or showing a non-monotonic dependence of facilitation on $[Ca^{2+}]_i$ (RAHAMIMOFF, 1968; ZUCKER, 1974; DUDEL, 1989). These results could arise from effects of $[Ca^{2+}]_e$ on basal $[Ca^{2+}]_i$ level, and differential saturation of the secretory

trigger, the facilitation site, endogenous Ca^{2+} buffers, and Ca^{2+} removal processes. A higher basal $[Ca^{2+}]_i$ can reduce facilitation because the effect of a given residual $[Ca^{2+}]_i$ is reduced. Increased Ca^{2+} influx can begin to saturate the secretory trigger, which reduces facilitation. Saturation of endogenous buffers should speed diffusion and the rate of decay of local residual $[Ca^{2+}]_i$, while saturation of active removal processes would slow its later phases. Nevertheless, a completely satisfactory resolution of all these issues has not been proposed.

Processes resembling augmentation and potentiation are observed in chromaffin cells. A sudden increase in $[Ca^{2+}]_i$ triggers a short-lived phasic release of hormone, followed by a slower secretion (NEHER & ZUCKER, 1993). This reflects release of a readily releasable pool of vesicles, followed by replenishment from reserve pools. The latter process occurs with time constants in the 1 s and 100 s ranges, and both steps appear to be Ca-dependent (HEINEMANN *et al.*, 1994). The slowest process is reflected by an increase in phasic release to a stimulus following elevation of $[Ca^{2+}]_i$ in the micromolar range for a minute (NEHER & ZUCKER, 1993), and in a faster recovery followed by a supernormal phase of stimulatable release following recovery of the releasable store when $[Ca^{2+}]_i$ is elevated moderately between two trains (VON RÜDEN & NEHER, 1993). The relative sizes of releasable and reserve hormone pools (1:10), and the Ca-dependence of the slow phase of secretion when cells are dialyzed with different $[Ca^{2+}]$, permits selection of the rate constants and their Ca^{2+}-dependence for refilling the releasable pool in a simple model of a process resembling augmentation/potentiation (HEINEMANN *et al.*, 1993, 1994). In this model, the slow process of refilling the releasable pool occurs with a Ca^{2+} affinity of 1.2 μM and maximum rate of 0.009 s^{-1}, and back rate of about 0.01 s^{-1}, rates generating relaxations similar to potentiation.

The slow kinetics of augmentation/potentiation allow time for secondary reactions or additional second messengers to play a role. A popular candidate for the augmentation/potentiation process is the Ca^{2+}/calmodulin dependent phosphorylation of synapsin I by Ca^{2+}/calmodulin dependent protein kinase II (CaM kinase II). Synapsin I impedes the movement of vesicles in axoplasm, apparently by cross-linking them to cytoskeletal elements (MCGUINESS *et al.*, 1989). This effect is lost when synapsin I is phorsphorylated by CaM kinase II. Presynaptic injection of dephosphorylated synapsin I inhibits synaptic transmission, while synapsin I phosphorylated by CaM Kinase II does not, and injection of CaM kinase II enhances spike-evoked release (LIN *et al.*, 1990; LLINÁS *et al.*, 1991). These results, however, do not impli-

cate these processes directly in any physiologically occurring enhancement of synaptic transmission.

There are conflicting reports regarding inhibitors of either calmodulin or CaM kinase II on short-term synaptic plasticity. Calmidazolium and KN62 have been reported to block PTP in hippocampal neurons (REYMANN *et al.*, 1988; ITO *et al.*, 1991), while sphingosine and H7 were found to leave PTP intact (MALINOW *et al.*, 1988). Mutant mice deficient in one form of CaM kinase II have normal cortical PTP but reduced facilitation (SILVA *et al.*, 1992). We examined effects of 50 μM of the inhibitor calmidazolium in 0.5% DMSO, and 1 μM of the CaM kinase II inhibitor KN62 (its solubility in 1% DMSO) on facilitation, augmentation, and PTP at crayfish neuromuscular junctions, and observed no consistent effects. Presynaptic injection of these substances, or a specific CaM kinase II inhibitor, calmodulin binding peptide (MALINOW *et al.*, 1989), also were without effect. Thus phosphorylation of proteins by this kinase does not seem to mediate short-term synaptic plasticity at these synapses, although a small reduction of facilitation by phosphatase inhibitors (SWAIN *et al.*, 1991: VAN DER KLOOT & MOLGÓ, 1993) suggests that phosphorylation may modulate the process of facilitation. Thus the identification of the molecular targets of Ca^{2+} in short-term synaptic plasticity awaits the development of more selective and effective pharmacological probes.

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