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Calcium and transmitter release

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Summary — The mechanism of transmitter release by intracellular Ca has been explored by recording presynaptic Ca concentration ($[Ca^{2+}]_i$) with Ca-sensitive fluorescent dyes and by controlling $[Ca^{2+}]_i$ with photosensitive Ca chelators. $[Ca^{2+}]_i$ decays slowly (in seconds) after presynaptic action potentials, while transmitter release lasts only a few ms after each spike at fast synapses. Simulations of Ca diffusing from Ca channels opened during action potentials suggest that transmitter is released by brief, localized $[Ca^{2+}]_i$ reaching about 100 μM ('Ca domains'). Several indirect measures of $[Ca^{2+}]_i$ levels achieved at release sites are in agreement with this estimate. Synaptic facilitation is a short-term synaptic plasticity in which transmitter release is enhanced for up to 1 s following prior activity. This seems to be due to the residual effect of Ca bound to a different site from the multiple fast, low-affinity binding sites that Ca must occupy to trigger secretion. The release of transmitter by localized Ca domains explains the variable degree of apparent cooperativity of Ca action obtained when relating transmitter release to Ca influx. Increasing Ca influx by elevating extracellular $[Ca^{2+}]$ increases the $[Ca^{2+}]_i$ in each Ca domain, and release increases with a high-power dependence on Ca influx because of a high degree of Ca cooperativity. However, prolonging presynaptic spikes or using depolarizing pulses of increasing amplitude increases Ca influx by opening more Ca channels and increasing the number of Ca domains locally triggering release. Partial overlap of these domains results in a slightly greater than linear dependence of release on total Ca influx. Post-tetanic potentiation (PTP) is a minute-long form of synaptic plasticity that correlates with measures of residual presynaptic $[Ca^{2+}]_i$. The linear relationship between PTP and residual $[Ca^{2+}]_i$ suggests that, as in synaptic facilitation, Ca seems to act at a different site from those that directly trigger release. Presynaptic sodium accumulation also contributes to PTP, apparently by reducing the Na gradient across the presynaptic membrane and impeding the removal of presynaptic Ca accumulated in the tetanus by Na/Ca exchange. Transmitter release at crayfish motor nerve terminals can be reduced by presynaptic inhibition, which reduces the Ca influx into terminals. Serotonin enhances transmitter release without increasing either resting $[Ca^{2+}]_i$ or Ca influx during spikes, apparently operating at a site 'downstream' of Ca to modulate release. Spikes transiently accelerate transmitter release triggered by elevation of $[Ca^{2+}]_i$ using photosensitive chelators, even in low-$[Ca^{2+}]$ media that blocked detectable transmitter release. This was believed to show that the depolarization of an action potential can directly trigger phasic transmitter release when presynaptic $[Ca^{2+}]_i$ is raised. However, measurements of presynaptic $[Ca^{2+}]_i$ in these solutions showed that $[Ca^{2+}]_i$ influx had not been fully blocked, and increasing the stimulus frequency revealed clear postsynaptic responses. When Ca influx was effectively blocked using external Ca chelators, action potentials were ineffective in triggering release, even when it was activated by photolytic release of Ca from presynaptically injected caged Ca chelators. Flash photolysis of the slowly binding photosensitive Ca chelator DM-nitrophen leads to an intense, brief presynaptic $[Ca^{2+}]_i$ spike that triggers phasic transmitter release, producing a postsynaptic response resembling an EPSP. Secretion from chromaffin cells can be monitored as a membrane capacitance increase while raising $[Ca^{2+}]_i$ with photosensitive chelators. Exposure to a conditioning, modest rise in $[Ca^{2+}]_i$ lasting 30 s increases the maximum rate of secretion triggered by a subsequent step rise in $[Ca^{2+}]_i$. Ca therefore not only triggers exocytosis, but seems to 'prime' the process, perhaps by mobilizing secretory granules to docking sites at the membrane. Large $[Ca^{2+}]_i$ steps sometimes trigger a sudden reduction in membrane capacitance which may reflect a Ca-activated recovery of vesicular membrane by endocytosis. Slow synapses release transmitter from vesicles not clustered near the membrane, and not subject to the high $[Ca^{2+}]_i$ levels in Ca domains. LHRH release from preganglionic sympathetic terminals appears to be rate-limited by a different Ca-sensitive step from that operating at fast synapses, since release is linearly related to $[Ca^{2+}]_i$ above a threshold level of about 200 nM.

calcium / transmitter release / synaptic transmission / secretion / hormone release

Introduction

The study of transmitter release has been advanced by the use of two recently developed techniques: 1) the measurement of intracellular free Ca concentration ($[Ca^{2+}]_i$) in nerve terminals during synaptic transmission with presynaptically injected indicator dyes; and 2) the control of presynaptic $[Ca^{2+}]_i$ by use of injected photolabile Ca chelators. This review will summarize recent progress on the physiological mechanisms involved in transmitter release by application of these techniques to a number of synaptic preparations, as well as to hormonal secretion by endocrine cells.

Transmitter release at 'calcium domains' in fast synapses

Fura-2 can be injected into crustacean motor axons near their terminals, filling synaptic boutons on the surface of the muscle (Delaney *et al*, 1989). Then changes in $[Ca^{2+}]_i$ can be measured in the boutons while stimulating the motor neuron and recording postsynaptic potentials. Single spikes have barely detectable effects on $[Ca^{2+}]_i$, raising it perhaps 10 nM; nevertheless, each spike can evoke substantial transmitter release during a brief interval following the spike. Conversely, tetanic stimulation causes the measurable $[Ca^{2+}]_i$ to rise gradually in the boutons during the tetanus, while transmitter release is still phasic, and occurs only for a few ms after each spike. Clearly, the rise in $[Ca^{2+}]_i$ measured with fura-2 is poorly correlated temporally with phasic transmitter release.

At rapidly transmitting synapses, such as the glutaminergic neuromuscular junction of the crayfish claw opener muscle or the squid giant synapse, transmission begins in a fraction of a millisecond after the peak of the presynaptic spike (Dudel and Kuffler, 1961), and even less time after the presynaptic Ca current starts to flow (Llinás *et al*, 1981). In this short time, Ca has had time to diffuse only a few tens of nanometers from Ca channel mouths. Solution of the diffusion equation from arrays of Ca channels clustered into active zones provides an estimate of the temporal and spatial $[Ca^{2+}]_i$ profiles caused by action potentials. Our most recent calculations consider diffusion with rapid but non-instantaneous binding to a slowly diffusible buffer whose parameters match those measured experimentally (Yamada and Zucker, 1992). The number of Ca channels per active zone opened by a spike, the single channel current, the disposition of active zones, and removal of cytoplasmic Ca by active transport were all based on experimental measurements (Fogelson and Zucker, 1985). Since vesicular fusion occurs on average about 50 nm from Ca channels (Heuser *et al*, 1979) segregated into active zones (Robitaille *et al*, 1990; Cohen *et al*, 1991), it is most interesting to look at this distance from Ca channels that open in an active zone during an action potential. Our simulations show $[Ca^{2+}]_i$ at this critical locus rising to a peak level of about 100 μM, and dropping rapidly (1/2 ms half width) to a residual $[Ca^{2+}]_i$ of less than 10 μM in 5 ms. After tens of milliseconds, $[Ca^{2+}]_i$ has diffusionally equilibrated and is of the order of 10 nM above its initial level.

These calculations suggest that transmitter release is triggered by very brief, localized, and high Ca concentrations in active zones. Present optical methods are unable to resolve such $[Ca^{2+}]_i$ transients. However, several experimental results suggest they do exist: 1) the weak effects of injected exogenous fast buffers on transmitter release are consistent only with such high $[Ca^{2+}]_i$ levels acting at release sites (Adler *et al*, 1991); 2) estimates of localized $[Ca^{2+}]_i$ based on the magnitude of Ca-dependent potassium current triggered by spikes in hair cells are also very high (Roberts *et al*, 1990); and 3) cumulative measurements of repeated presynaptic stimulation using the relatively insensitive Ca indicator *n*-aequorin-J reveal a spatial pattern of local $[Ca^{2+}]_i$ exceeding 100 μM resembling the disposition of active zones (Llinás *et al*, 1992).

Synaptic facilitation

A simple model of transmitter release involves Ca ions binding rapidly to a low affinity secretory trigger molecule, which in turn triggers a slower exocytotic process when fully occupied with five Ca ions. Ca binds cooperatively to reflect the highly non-linear dependence of release on extracellular $[Ca^{2+}]$ (Dodge and Rahamimoff, 1967; Dudel, 1981; Augustine *et al*, 1985). Binding to the secretory trigger must be fast, for it is little affected by slow buffers like EGTA and only modestly affected by rapid buffers like BAPTA (Adler *et al*, 1991; Swandulla *et al*, 1991). The Ca affinity of the binding sites must be low because transmission is still not saturated at normal levels

of Ca influx (normal levels of external $[Ca^{2+}]$), even for multiple action potentials at high frequency. In such a process the small residual $[Ca^{2+}]_i$, present for tens to hundreds of milliseconds following activity before diffusional equilibration is complete, would correspond to some trigger sites still being occupied with Ca ions. A subsequent spike would result in a higher proportion of Ca binding site occupancy on the trigger molecules, and hence a facilitated release of transmitter. However, simulations of such a process significantly underpredict facilitation (Yamada and Zucker, 1992), suggesting that it does not arise simply from the cooperativity of Ca ions binding to identical sites on a trigger molecule and the residual Ca still bound to such sites. A model with Ca binding to a second site, with higher affinity and slower kinetics, was able to simulate observed levels of facilitation (several hundred percent within a few milliseconds of a single action potential). At present, this remains our working hypothesis of how short-term synaptic facilitation works.

Consequences of calcium domains

Recognizing that local transient high $[Ca^{2+}]_i$ triggers secretion accounts for numerous otherwise anomalous results. When $[Ca^{2+}]$ in the medium is increased, a highly non-linear relationship (at least fourth power) between Ca influx per spike and transmitter release per spike is seen (fig 1), when Ca influx is assessed from the changes in $[Ca^{2+}]_i$ during a train of action potentials in small motor nerve terminals (Zucker *et al*, 1991) or from measures of Ca current at the squid giant synapse (Augustine *et al*, 1985). This would be expected, since the increased influx is due to an increase in single Ca channel currents, so that the $[Ca^{2+}]_i$ in the neighborhood of Ca channels in active zones increases approximately proportionately with increased macroscopic Ca influx (fig 2). On the contrary, increasing Ca influx by prolonging action potentials or using larger depolarizations has a less dramatic effect on transmitter release. Release per spike increases with something like a first to third power dependence on measured or inferred Ca influx (Llinás *et al*, 1981; Charlton *et al*, 1982; Augustine *et al*, 1985; Zucker *et al*, 1991; see fig 1). These maneuvers do not increase the Ca influx per channel; rather they increase the number of channels that open. If few channels open, this would result

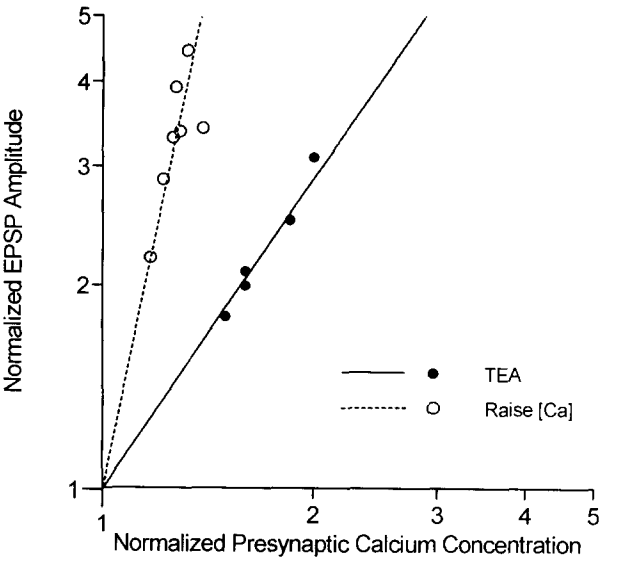

Fig 1. Dependence of transmitter release on Ca influx in crayfish nerve terminals (Zucker *et al*, 1991). Transmitter release was monitored as excitatory postsynaptic potential (EPSP) amplitude, and relative Ca influx was determined from the steady state increase in presynaptic $[Ca^{2+}]_i$ after 20 s of 8–10 Hz stimulation. Open circles indicate experiments in which Ca influx was altered by raising $[Ca^{2+}]$ in the medium from 50 to 100% normal (13.5 mM) or from 30 to 70% normal. EPSP amplitude and $[Ca^{2+}]_i$ are normalized to the responses in the lower external $[Ca^{2+}]$. Filled circles refer to experiments in which presynaptic action potentials were prolonged in 5 mM tetraethylammonium chloride (TEA). EPSP amplitude and $[Ca^{2+}]_i$ are normalized to the responses before adding TEA. The results are plotted on logarithmic coordinates and the lines are regression fits to the data, indicating power relationships of 5.0 when $[Ca^{2+}]$ is changed and 1.5 when spikes are prolonged.

in recruitment of release from vesicles within reach of the additional open Ca channel domains, and release would increase nearly linearly with macroscopic Ca influx (fig 2). If many channels open, the $[Ca^{2+}]_i$ from neighboring domains may overlap at a releasable vesicle, local $[Ca^{2+}]_i$ will summate from these multiple channel openings, and some of the cooperativity of Ca action in exocytosis will be expressed, leading to a more than linear but less than fully non-linear relationship between release and macroscopic Ca current. A similar explanation accounts for the observation (Llinás *et al*, 1981; Smith *et al*, 1985) that a given Ca current at large potentials, with many open channels causing overlapping channel $[Ca^{2+}]_i$ domains at vesicles, causes more release than the same Ca current at low potentials, with few channels opening far from each other, albeit with in-

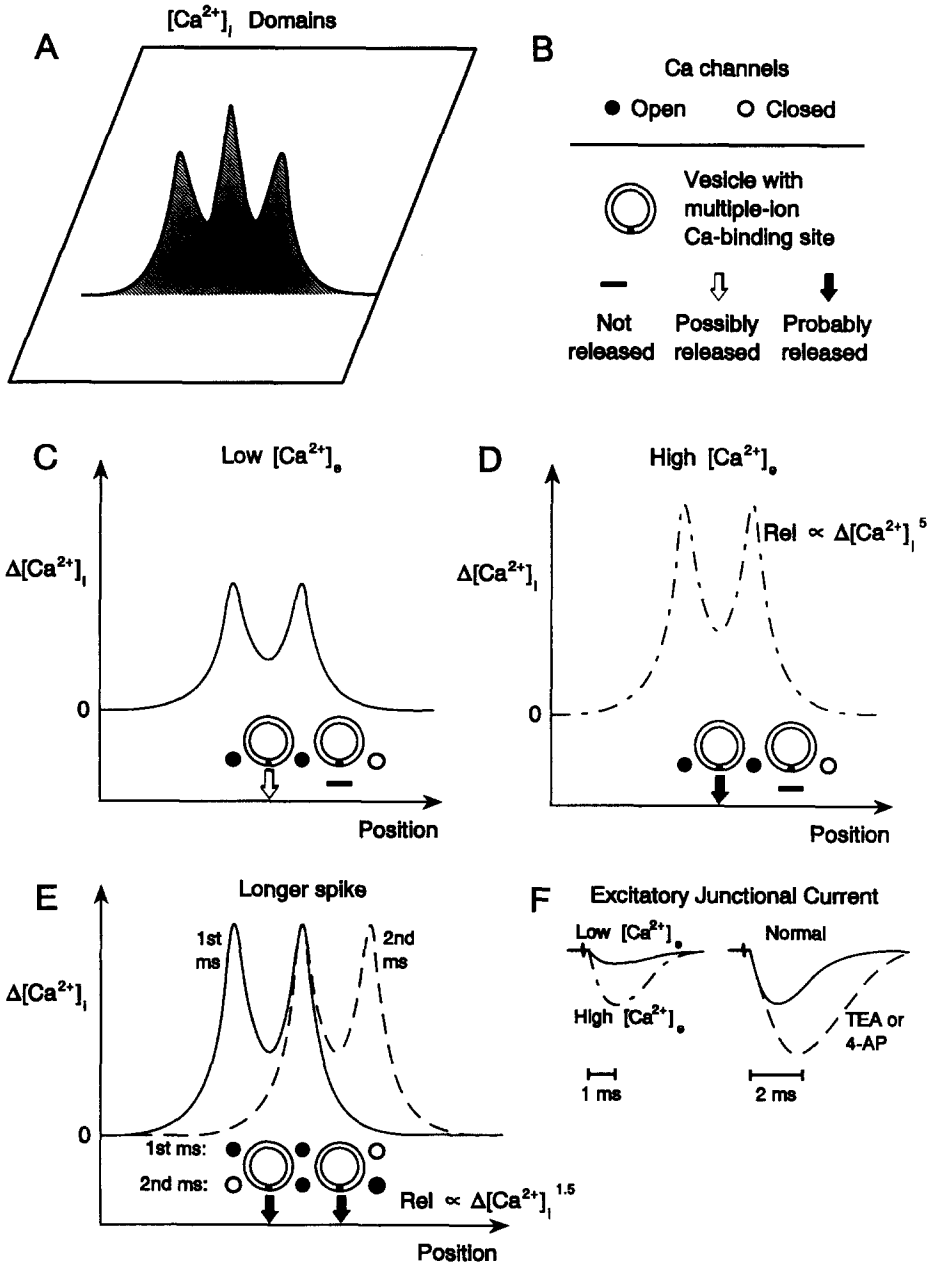

Fig 2. Schematic explanation of the different apparent Ca cooperativities of transmitter release when external $[Ca^{2+}]$ is altered and when spikes are prolonged (fig 1). **A.** Transmitter release occurs from vesicles within clouds of Ca ions ('Ca domains') near open Ca channels. **B.** Symbols used to represent vesicles, open and closed Ca channels, and probability of release of a vesicle depending on local $[Ca^{2+}]_i$. **C.** Local $[Ca^{2+}]_i$ rises near open Ca channels during an action potential, and vesicles surrounded by such channels may be released, even in relatively low external $[Ca^{2+}]$. Vesicles near only one or no open Ca channels are not likely to be released. **D.** Elevating $[Ca^{2+}]_i$ does not change the spatial or temporal pattern of Ca channels which open in an action potential, but increases the Ca influx through open Ca channels and so increases the magnitude of the submembrane $[Ca^{2+}]_i$ profile near open channels. Vesicles near such channels are much more likely to be released, because of the high degree of Ca cooperativity, requiring at least 5 Ca ions acting at a vesicle (or plasma membrane) binding site to trigger release. **E.** Prolonging action potentials with TEA results in the opening of additional Ca channels later in the spike. Some of these will be channels opened earlier; some will not. Increased release will occur largely from vesicles near the new late opening Ca channels. To the extent that later release is from entirely different vesicles unaffected by the Ca channels opened early in a spike, this recruitment of additional release sites with similar $[Ca^{2+}]_i$ will increase release linearly with the increase in total Ca influx. However, some vesicles will see an increase in local $[Ca^{2+}]_i$ as new Ca channels open near other Ca channels that are already open; these vesicles will be released with higher probability than before, so that some non-linearity between transmitter release and total Ca influx is expected when release is increased by prolonging spikes. **F.** Postsynaptic excitatory currents are increased when $[Ca^{2+}]$ is elevated, because release probability of vesicles near open Ca channels is increased. When spikes are prolonged, the main effect is prolongation of the postsynaptic current, as release is increased primarily by the creation of additional Ca domains at later times.

creased single channel current (Simon and Llinás, 1985; Zucker and Fogelson, 1986).

Calcium and post-tetanic potentiation (PTP)

The above considerations make clear that measurements of $[Ca^{2+}]_i$ should not bear a simple relationship to the level of transmitter release evoked by spikes, since it is not possible to measure the relevant presynaptic $[Ca^{2+}]_i$ in space and time. However, indicator dyes do provide accurate measures of $[Ca^{2+}]_i$ at release sites following electrical activity, when the sharp $[Ca^{2+}]_i$ gradients near Ca channels have subsided. Measurements of post-tetanic $[Ca^{2+}]_i$ correlate well with post-tetanic potentiation (PTP) of transmitter release (Delaney *et al*, 1989), suggesting that this residual $[Ca^{2+}]_i$ directly causes PTP, or is in equilibrium with Ca bound to some site which causes PTP. This is supported by the finding that chelation of residual $[Ca^{2+}]_i$ with a slow buffer (EGTA) that has no effect on transmitter release to single spikes, blocks both the measured accumulation of residual Ca in a tetanus and potentiation caused by that tetanus (Swandulla *et al*, 1991). However, like synaptic facilitation, PTP cannot be explained simply by supposing that residual free $[Ca^{2+}]_i$ sums with peak local $[Ca^{2+}]_i$ in active zones during action potentials to generate higher local $[Ca^{2+}]_i$ peaks and potentiate release. Such a model requires a much lower peak-to-residual $[Ca^{2+}]_i$ ratio in active zones than is expected from the considerations of the previous section (fig 3), and predicts a faster decay of PTP of transmitter release than of $[Ca^{2+}]_i$, contrary to observation (Delaney *et al*, 1989). Rather, it appears that residual Ca acts presynaptically at a separate site to linearly potentiate release, perhaps by a calmodulin-dependent phosphorylation of synapsin I to liberate vesicles from cytoskeletal binding sites and permit them to dock at release sites (Llinás *et al*, 1991).

Sodium and PTP

Besides Ca, Na accumulation in nerve terminals has also been implicated in PTP (Rahamimoff *et al*, 1980; Wojtowicz and Atwood, 1985), and recent optical measurements of $[Na^+]_i$ show rises in

A

V = size of EPSP
P = peak $[Ca^{2+}]_i$
K = proportionality constant
R = residual $[Ca^{2+}]_i$
n = cooperativity of Ca action

$$V = K(P + R)^n$$

$$\frac{V_2}{V_1} = \left[\frac{P + R_2}{P + R_1}\right]^n = \text{EPSP potentiation}$$

R_1 = 170 nM at rest
R_2 = 560 nM 1 min after tetanus,

n = 5, and V_2/V_1 = 9.09 → P = 412 nM

$$\frac{V_x}{V_1} = \left[\frac{P + R_x}{P + R_1}\right]^n \rightarrow V_x \text{ for any } R_x$$

B

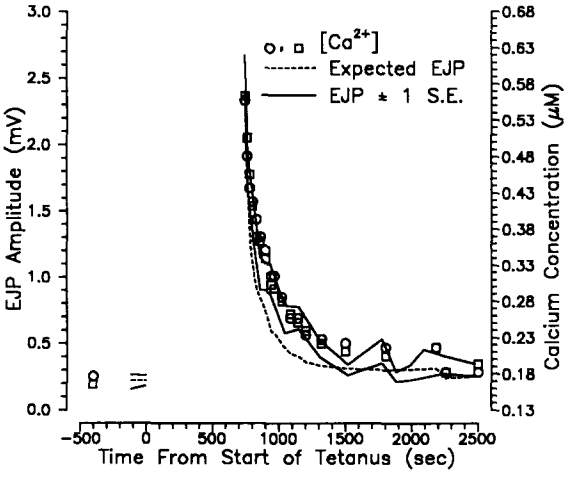

Fig 3. Predictions of a model of post-tetanic potentiation (PTP) based on the summation of residual presynaptic $[Ca^{2+}]_i$ summing with peak $[Ca^{2+}]_i$ in Ca domains to trigger release cooperatively. **A.** The model may be used to calculate the required peak $[Ca^{2+}]_i$ reached in active zones during an action potential from measurements of EPSP magnitudes and $[Ca^{2+}]_i$ before and after induction of PTP in crayfish neuromuscular junctions (Delaney *et al*, 1989). The inferred peak $[Ca^{2+}]_i$ is much lower than expected from other experimental and theoretical data. **B.** From the measured post-tetanic decay of $[Ca^{2+}]_i$ in two nerve terminals (symbols), the model predicts a time course of post-tetanic EPSP decay (dotted line) that is significantly faster than that observed experimentally at the same synapse (solid lines showing average excitatory junctional current, EJP, plus and minus one standard error).

the order of 10 mM in nerve terminals during tetani causing PTP (Delaney and D Tank, personal communication). It has been suggested that elevated $[Na^+]_i$ operates by releasing Ca from internal stores, but a tetanus in Ca-free medium still loads terminals with Na but does not result in any rise in $[Ca^{2+}]_i$ (Mulkey and Zucker, 1992). A reversal of Na/Ca exchange leading to a leak of Ca into Na-loaded terminals has also been proposed, but no rise in $[Ca^{2+}]_i$ could be detected in terminals following a Na load when Ca was washed back into the medium (Mulkey and Zucker, 1992). Instead, three lines of evidence indicate that Na accumulation reduces the rate of active extrusion of Ca during and after a tetanus, slowing the removal of Ca that has entered through Ca channels during action potentials: 1) increasing the Na load with ouabain to block Na extrusion enhances and prolongs PTP in proportion to an increase in the accumulation of Ca in a tetanus and slowing of its post-tetanic decay; 2) reducing the Na load (by depolarizing in a Na-free medium) results in a more rapid extrusion of a Ca load; and 3) blocking Na/Ca exchange with lithium slows post-tetanic Ca removal and increases its tetanic accumulation. Apparently Na/Ca exchange participates in Ca removal from terminals, and diminishing this process by reducing the transmembrane Na gradient enhances and prolongs the elevation in $[Ca^{2+}]_i$ that causes PTP (fig 4).

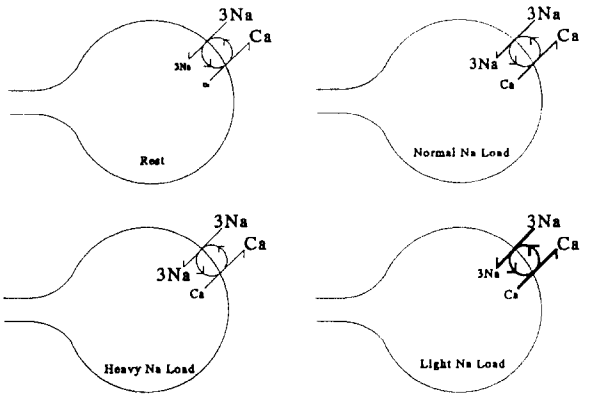

Fig 4. Role of Na/Ca exchange in PTP (Mulkey and Zucker, 1992). Na/Ca exchange helps keep the $[Ca^{2+}]_i$ very low at rest. During a tetanus in normal medium, both Ca and Na accumulate presynaptically; the normal Na load results in accumulated Ca being pumped out at a moderate rate (normal line thickness for exchanger) with the help of Na/Ca exchange. If Na extrusion is blocked with ouabain, $[Na^+]_i$ rises more, and this heavy Na load interferes with the removal of Ca by Na/Ca exchange (thin lines for exchanger); Ca accumulates more during a tetanus and is extruded more slowly, enhancing and prolonging PTP. Blocking Na/Ca exchange with Li has a similar effect on presynaptic $[Ca^{2+}]_i$ in a tetanus. If Na accumulation is blocked by preventing Na influx in a Na-free medium, Na/Ca exchange operates more effectively due to the light Na load and larger Na gradient (thick lines for exchanger); Ca is removed more rapidly.

Modulation of transmission

We have studied two processes that alter synaptic transmission at the crayfish neuromuscular junction: presynaptic inhibition and serotonergic modulation. GABAergic synapses form between inhibitory motor nerve terminals and excitatory terminals, as well as onto muscle cells. By filling both the inhibitor and exciter with fura-2, the terminals of each can be identified by stimulating either alone and observing the selective $[Ca^{2+}]_i$ rise. Stimulating both simultaneously results in a reduction of Ca accumulation in excitatory terminals (Zucker *et al*, 1991), as GABA opens presynaptic chloride channels (Takeuchi and Takeuchi, 1966) and shunts action potentials (Baxter and Bittner, 1991), reducing the number of Ca channels opened by a spike in exciter terminals.

Serotonin enhances both spontaneous and evoked glutamate release from exciter terminals by a cAMP- and phospholipase C-dependent process (Dixon and Atwood, 1989). Since neither resting $[Ca^{2+}]_i$ nor Ca influx during action potentials is enhanced (Delaney *et al*, 1991), serotonin must work 'downstream' of Ca in the secretory process, either by sensitizing the receptor to $[Ca^{2+}]_i$ or by mobilizing vesicles to release sites and increasing the store of releasable transmitter.

Effect of the voltage change during a spike on transmission

A number of properties of synaptic transmission have suggested that the time course of transmitter release is determined primarily by the time course of the presynaptic membrane potential acting directly on the exocytotic machinery, in concert with a rise in $[Ca^{2+}]_i$. Examples of such properties are the difference in time course of transmitter release and presynaptic $[Ca^{2+}]_i$ measured after an action potential, and the fact that large depolari-

zations evoke greater release than small depolarizations leading to the same macroscopic Ca influx. As indicated above, these properties could also be consequences of the release of transmitter by localized $[Ca^{2+}]_i$ not measurable with present indicators and the different spatial distributions of submembrane $[Ca^{2+}]_i$ at different membrane potentials. A number of other experiments on neuromuscular junctions were originally interpreted as evidence that presynaptic membrane potential directly triggers transmitter release (Parnas and Parnas, 1986), but these were all shown to arise from procedural artefacts (Zucker *et al*, 1986). Recent simulation studies (Parnas *et al*, 1989) also purport to show that the collapse of Ca domains in active zones could not explain the time course of transmitter release. In particular, its relative constancy at different levels of release (when changing extracellular $[Ca^{2+}]$ or following prior stimulation) and its temperature sensitivity were thought to be inconsistent with theoretical predictions. However, when the parameters of simulations were adjusted to better fit experimental procedures and exocytosis was represented as a slow temperature-sensitive process following binding of Ca to trigger molecules (Yamada and Zucker, 1992), the simulations matched experimental results.

Recently, experiments have been described using the caged Ca chelator nitr-5, presynaptically injected into crayfish motor nerve terminals, to probe the separate roles of $[Ca^{2+}]_i$ and membrane potential in evoking transmitter release (Hochner *et al*, 1989). When nitr-5 partially loaded with Ca is exposed to ultraviolet illumination, it is converted to a Ca buffer with somewhat lower Ca affinity, resulting in a higher equilibrium level of free $[Ca^{2+}]_i$ (Adams *et al*, 1988). Only a modest elevation of $[Ca^{2+}]_i$ is possible, from a resting level of about 150 nM to about 1 μM. This results in a modest increase (quadrupling) of the frequency of miniature excitatory postsynaptic potentials (MEPSPs), representing the spontaneous release of transmitter quanta. Pairs of action potentials repeatedly delivered in a low $[Ca^{2+}]$ medium (0.5 mM) with 2 mM of the Ca channel blocker Mn^{2+}, or in a solution containing no added Ca^{2+} and somewhat elevated Mg^{2+}, failed to evoke detectable EPSPs before photolysis of nitr-5, but after photolysis led to a brief increase in the probability of appearance of an MEPSP immediately following a spike. Since spikes evoked no EPSPs before UV light exposure, it was assumed that $[Ca^{2+}]_i$ influx had been effectively blocked in the solutions used, and that membrane potential was therefore acting directly on the release mechanism that was 'sensitized' to voltage by the elevation in $[Ca^{2+}]_i$.

When we tested the efficacy of these solutions in blocking Ca influx in terminals filled with fura-2, we found that the same stimulus pattern used in the earlier study was accompanied by a readily detectable rise in presynaptic $[Ca^{2+}]_i$ (Mulkey and Zucker, 1991). Higher frequencies (100 Hz) also evoked large EPSPs, but these were not detectable at the lower frequencies used in the previous study. Apparently, the Ca influx was insufficient to evoke detectable transmitter release at these frequencies in low-$[Ca^{2+}]$ medium, which is not surprising given the high degree of Ca cooperativity in triggering exocytosis and the large facilitation that these synapses display. We found that Ca influx could only be effectively prevented by adding a Ca chelator like EGTA to the extracellular solution in sufficient concentration to overcome the native buffering of Ca in the

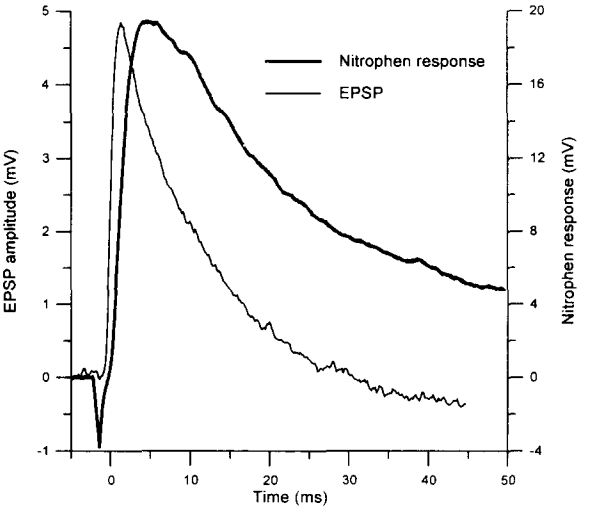

Fig 5. Comparison of an EPSP to a postsynaptic response to flash photolysis of presynaptically injected DM-nitrophen at the crayfish neuromuscular junction. If Ca-loaded nitrophen is suddenly partially photolyzed, Ca released from discharged chelator remains relatively free for about 1 ms before binding to unphotolyzed nitrophen, a derivative of the slow Ca buffer EDTA. This presynaptic 'Ca spike' triggers a brief intense release of transmitter similar to that evoked by an action potential. Since the equilibrium level of $[Ca^{2+}]_i$ following photolysis is higher than before, a sustained phase of transmitter release accompanies nitrophen photolysis, unlike spike-evoked release.

extracellular space and especially in the synaptic cleft (Ginsburg and Rahamimoff, 1983).

We used this solution to block Ca influx during spikes, and asked what effect they had on transmitter release triggered by an elevation in presynaptic $[Ca^{2+}]_i$. With nitr-5, we again found only a small elevation in MEPSP frequency upon photolysis, but now this was unaffected by action potentials. We also tried a different caged Ca chelator, DM-nitrophen (Kaplan and Ellis-Davies, 1988). This compound is photolyzed to products that hardly bind Ca^{2+} at all, permitting a higher elevation of $[Ca^{2+}]_i$, probably to about 5 μM and perhaps even higher. Photolysis of nitrophen elevated transmitter release to high rates (up to about 10000 quanta/s), nearly approaching the rate of release achieved by an action potential in normal medium (Mulkey and Zucker, 1993). However, this activation of transmission was also unaffected by spikes when Ca influx was blocked with external EGTA (Mulkey and Zucker, 1991). Similar experiments in the squid giant synapse (Delaney and Zucker, 1990) also failed to show any effect of the potential change during an action potential on transmitter release evoked by release of Ca from presynaptically injected nitrophen, when Ca influx was blocked with external EGTA.

Flash photolysis of caged Ca and phasic transmitter release

In many of these experiments, nitrophen was photolyzed by exposure to a bright (150 W) collimated xenon light source. However, rapid photolysis of nitrophen can be achieved using a focused xenon flash lamp. Rapid partial photolysis of nitrophen releases Ca which rebinds relatively slowly to unphotolyzed nitrophen, which is a derivative of the slow Ca buffer EDTA. This is different from the case of nitr-5, a derivative of the fast Ca buffer BAPTA. Therefore, partial flash photolysis of presynaptically injected nitrophen generates a brief spike of $[Ca^{2+}]_i$, up to about 100 μM in amplitude but lasting only about a millisecond before rebinding to unphotolyzed chelator and establishing a new modestly higher equilibrium $[Ca^{2+}]_i$ of about 1 μM. These consequences of nitrophen photolysis have been measured directly with the Ca indicator fluo-3 (Zucker, 1992, 1993). Rapid photolysis of nitrophen evokes an intense phase of transmitter release resembling a normal EPSP (Delaney and Zucker, 1990; Kamiya and Zucker, unpublished work; see fig 5), while flash photolysis of nitr-5 barely increases MEPSP frequency. These results also suggest that phasic transmitter release requires high $[Ca^{2+}]_i$ at release sites.

Multiple sites of Ca action in secretion from chromaffin cells

The simulations of $[Ca^{2+}]_i$ change in active zones during and following nerve activity suggest that short-term synaptic facilitation does not arise simply from residual $[Ca^{2+}]_i$ summing with peak $[Ca^{2+}]_i$ transients during action potentials and acting at a single class of binding site. The similar post-tetanic decays of $[Ca^{2+}]_i$ and EPSP amplitude also argue against a simple residual Ca summation model, and suggest a separate site of Ca action. Recent experiments on chromaffin cells provide additional evidence for such a separate site of Ca acting to influence secretion. We have recently triggered secretion in chromaffin cells by photolysis of Ca loaded nitrophen perfused into single cells, with $[Ca^{2+}]_i$ measured with the indicator furaptra and secretion due to exocytotic fusion of secretory granules monitored as an increase in cell membrane area measured as a membrane capacitance change (Neher and Zucker, 1993). Using bright photolytic flashes, we found that a large step rise in $[Ca^{2+}]_i$ triggered a brief, intense phase of secretion (about 1000 fF/s decaying in 2 s or less) followed by a much slower persistent phase of about 40 fF/s. Although Ca clearly triggered the rapid phase of secretion, it was unclear whether the subsequent slow phase was due to a Ca-dependent or Ca-independent mobilization of granules up to release sites, or to an independent parallel Ca-dependent pathway for secretion. One way to distinguish these possibilities was to manipulate a phenomenon we call the 'loading transient'. When a whole-cell configuration is established by breaking the membrane under the patch pipette, dilute nitrophen enters a cell containing millimolar $[Mg^{2+}]_i$ and initially this excess Mg competes with Ca for cationic binding sites on the nitrophen, displacing the bound Ca and elevating $[Ca^{2+}]_i$ to about 10 μM for about 30 s, until the nitrophen concentration in the cell exceeds the $[Mg^{2+}]_i$. This loading transient can be minimized by filling the tip of the perfusion pipette with a dilute EGTA solution, and backfilling with the Ca/nitrophen/furaptra mixture. This procedure almost eliminated both the transient modest rise in

$[Ca^{2+}]_i$ and secretion during filling. We wondered whether cells subjected to a loading transient were 'primed' in some way, mobilizing granules to 'docking' points at the membrane from which they could be more readily secreted. In fact, we found that a given $[Ca^{2+}]_i$ step evoked more intense transient secretion if it was preceded by this modest $[Ca^{2+}]_i$ elevation for 30 s, suggesting the existence of a Ca-dependent mobilization of secretory granules to release sites (fig 6).

In some cells, we observed that following massive secretion, an additional flash leading to another large $[Ca^{2+}]_i$ step often resulted in sudden reduction of membrane capacitance, which we think represents a Ca-dependent endocytosis or recovery of vesicular membrane from the cell surface. Thus there may be three sites of Ca action in secretory cells : triggering fast exocytosis, mobilizing granules to release sites, and activating a vesicular recovery process.

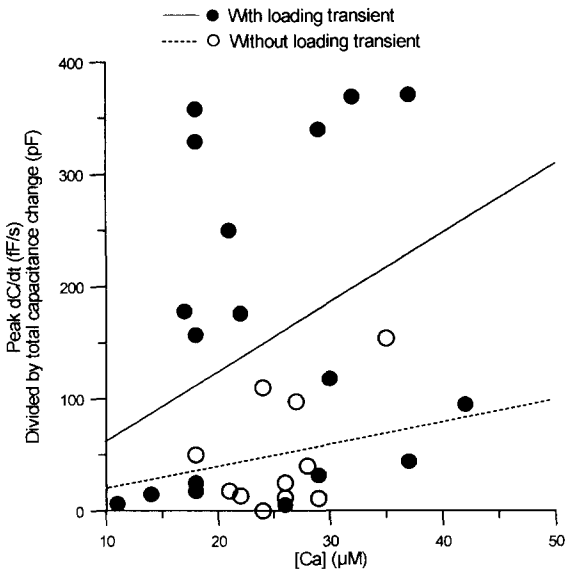

Fig 6. Effect of 'loading transient' on secretion evoked by flash photolysis of DM-nitrophen in bovine chromaffin cells (Neher and Zucker, 1993). When Ca-loaded nitrophen is perfused into chromaffin cells, the $[Mg^{2+}]_i$ initially exceeds the nitrophen concentration in the cytoplasm, and the excess Mg displaces Ca from its binding site on the chelator. This leads to a rise in $[Ca^{2+}]_i$ to up to 10 μM and a modest secretion of adrenaline lasting about 30 s. This 'loading transient' can be avoided by filling the tip of the patch pipette with a short column of dilute EGTA solution to act as a barrier to prevent mixing of the cytoplasmic Mg with nitrophen. The graph plots secretory responses to steps of $[Ca^{2+}]_i$ evoked by intense light flashes used to photolyse most of the nitrophen so that little is left for rebinding the released Ca. The maximum rate of secretion, monitored as the slope of membrane capacitance increase (dC/dt in fF/s) due to exocytosis of secretory granules, and normalized to the total secretory capacity of each cell (in pF), is plotted against the magnitude of the $[Ca^{2+}]_i$ step monitored with the Ca-sensitive dye furaptra. Cells subjected to a loading transient respond more vigorously to a step rise in $[Ca^{2+}]_i$. The solid line is a regression fit through the origin to responses in cells with a loading transient (filled circles), and the dashed line is a fit to responses in cells without a loading transient (open circles).

Ca-dependence of secretion at slow synapses

Some synapses transmit effectively only to repeated stimulation, and the responses rise very slowly, reaching a peak seconds after stimulation ceases and decaying for tens of seconds afterwards. Peptidergic secretion often behaves this way, and we have recently studied such secretion at preganglionic terminals onto bullfrog sympathetic ganglion cells. Luteinizing hormone releasing hormone (LHRH) is released from dense core vesicles in these terminals with typical 'slow' characteristics (Jan and Jan, 1982). These vesicles do not cluster in active zones under the membrane, but are dispersed throughout cytoplasm and do not appear to be docked at or even near release sites (Taxi, 1976). Therefore, secretion of these vesicles cannot be triggered rapidly by local high $[Ca^{2+}]_i$ domains, since they must first be moved up to the membrane before exocytosis can occur, which probably accounts for the sluggish nature of release of LHRH from these terminals and the 'late, slow EPSP' that it evokes postsynaptically.

It is likely that the rate-limiting step in Ca-dependent secretion in such synapses is the movement of vesicles up to the membrane. This process would respond only to Ca that reaches vesicles distant from the membrane, and therefore should depend on much lower $[Ca^{2+}]_i$ levels than those achieved only near Ca channel mouths. In addition, this is the $[Ca^{2+}]_i$ that is detected by indicators such as fura-2, so it seemed interesting to relate release from such synapses to measurements of presynaptic $[Ca^{2+}]_i$ during stimulation.

The slow kinetics of the postsynaptic response are due partly to the long diffusion pathway of LHRH from release sites to postsynaptic targets (Jan and Jan, 1982). Thus no good measure of the rate of LHRH release is available, but the integral of the postsynaptic current provides a measure of total release. This was found to be linearly related to the integral of presynaptic

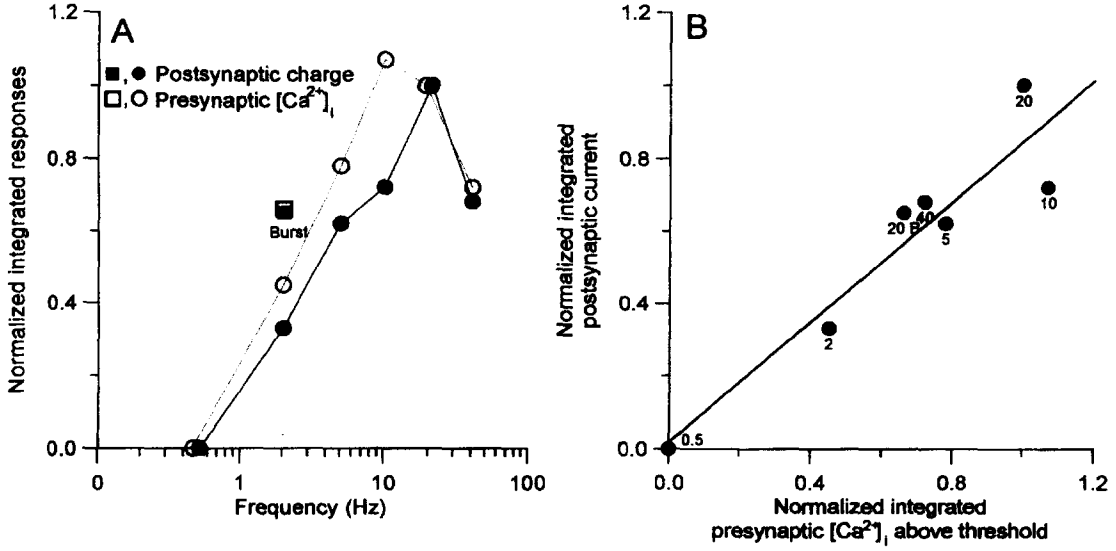

Fig 7. Linear relation between LHRH release and presynaptic $[Ca^{2+}]_i$ above a threshold level at bullfrog sympathetic ganglion synapses (Peng and Zucker, 1993). **A.** The integrated postsynaptic current (filled symbols) and presynaptic $[Ca^{2+}]_i$ change above 200 nM (open symbols) are plotted *vs* frequency of delivering 60 stimuli. $[Ca^{2+}]_i$ was measured with fura-2. Circles are for steady stimulation; squares for bursty stimulation (20 shocks at 20 Hz every 10 s). Responses are normalized to those at 20 Hz. **B.** Postsynaptic charge is plotted *vs* presynaptic $[Ca^{2+}]_i$ change above the apparent threshold level of 200 nM. The numbers indicate frequency of stimulation; 20 B refers to bursty stimulation.

changes in $[Ca^{2+}]_i$ above a threshold level of at least 200 nM (Peng and Zucker, 1993; see fig 7). LHRH release was also found to be a roughly linear function of external $[Ca^{2+}]$ (Peng and Horn, 1991). Thus the rate-limiting step at slow synapses may be a Ca-dependent mobilization of vesicles similar to that inferred for chromaffin cells, operating with little cooperativity and at much lower $[Ca^{2+}]_i$ levels than the Ca-sensitive trigger for exocytosis at fast synapses.

Future directions

The results on slow synaptic transmission and hormonal secretion suggest multiple sites of Ca action in the secretory process. To what extent are these processes also present at fast synapses? Are the 'priming' step in chromaffin cells and the mobilization of dense core vesicles in peptide secretion related to processes such as facilitation or potentiation at fast synapses? If mobilization of vesicles is the rate limiting step in LHRH secretion, must this still be followed by local high $[Ca^{2+}]_i$ levels near Ca channels to trigger exocytosis as in fast synapses? These are the sorts of questions we are pursuing now.

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