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Ca^{2+} Cooperativity in Neurosecretion Measured Using Photolabile Ca^{2+} Chelators

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SUMMARY AND CONCLUSIONS

1. The photolabile Ca^{2+} chelator DM-nitrophen was injected into crayfish motor neuron terminals and photolyzed with light flashes of different intensity to determine the cooperativity of Ca^{2+} action in releasing neurotransmitter.

2. Each flash elicited a phasic postsynaptic response resembling an excitatory junctional potential, apparently due to a presynaptic "spike" in intracellular calcium concentration ($[Ca^{2+}]_i$).

3. When postsynaptic currents were measured under voltage clamp, a Ca^{2+} cooperativity of ~3–4 was inferred from a supralinear dependence of responses on changes in peak $[Ca^{2+}]_i$ caused by flashes differing in intensity by 32–46%.

4. A similar Ca^{2+} cooperativity was inferred from postsynaptic potentials in response to flashes of varying intensity.

5. The time course of transmitter release indicated by flash responses had slightly slower rising and falling phases than excitatory postsynaptic potentials. There was also a slow tail of transmitter release lasting for ~200 ms after a flash.

6. This time course was explained quantitatively by simulations of DM-nitrophen photolysis and binding reactions and a model of Ca^{2+} activation of transmitter release.

INTRODUCTION

An understanding of the molecular basis of neurosecretion requires knowledge of the dependence of the rate of transmitter release on intracellular calcium concentration ($[Ca^{2+}]_i$). This was initially inferred from the dependence of excitatory postsynaptic potential (EPSP) amplitude on extracellular $[Ca^{2+}]$ (Dodge and Rahamimoff 1967; Dudel 1981; Katz and Miledi 1970), where a highly nonlinear (4th power) relationship was attributed to cooperativity of Ca^{2+} ions acting intracellularly at release sites, although cooperative interactions among Ca^{2+} ions in permeating Ca^{2+} channels from outside are also consistent with such measurements.

More recently, voltage clamp of the presynaptic terminal of the squid giant synapse permitted transmitter release to be related to Ca^{2+} influx, measured as a current carried by Ca^{2+} ions. Again, when Ca^{2+} influx was altered by varying external $[Ca^{2+}]$ a highly nonlinear dependence of EPSP on Ca^{2+} current was found, approximating the 3rd or 4th power, suggesting a high positive Ca^{2+} cooperativity. However, somewhat lower degrees of nonlinearity, ranging from 1 to 4, were found when different numbers of Ca^{2+} channels were opened by varying the amplitude or duration of presynaptic depolarizations (Augustine 1990; Augustine and Charlton 1986; Augustine et al. 1985; Charlton et al. 1982; Llinás et al. 1981).

These discrepancies have been attributed to differences in the effects of the two procedures on presynaptic $[Ca^{2+}]_i$ (Augustine 1990; Zucker et al. 1991): raising external $[Ca^{2+}]$ should elevate Ca^{2+} influx through each Ca^{2+} channel and increase the local $[Ca^{2+}]_i$ near channel mouths (although not necessarily exactly linearly), whereas larger or longer depolarizations open more Ca^{2+} channels but with less influx per channel (as the Ca^{2+} equilibrium potential is approached). Although transmitter release will occur from more "Ca^{2+} domains," the locally effective $[Ca^{2+}]_i$ might be lower or higher depending on the closeness of open Ca^{2+} channels and the degree of overlap of neighboring Ca^{2+} domains (Simon and Llinás 1985; Zucker and Fogelson 1986).

In addition, many other factors confound estimates of cooperativity based on measures of presynaptic Ca^{2+} current or external $[Ca^{2+}]$. The apparent cooperativity can be depressed by *1*) saturation of transmitter release (Augustine and Charlton 1986), *2*) saturation of postsynaptic potential as the synaptic equilibrium potential is approached (Augustine et al. 1985), *3*) synaptic depression at high $[Ca^{2+}]$ levels (Augustine and Charlton 1986), *4*) problems of presynaptic spatial nonuniformity at giant squid synapses (Augustine et al. 1985), *5*) ignoring the contributions of Ca^{2+} tail currents to transmitter release measured after the ends of depolarizing pulses (Zucker and Fogelson 1986), or *6*) choosing incorrect values for the appropriate delay to use in measuring transmitter release triggered by prior Ca^{2+} current (Augustine et al. 1985). On the other hand, the apparent cooperativity can be enhanced by saturation of presynaptic cytoplasmic Ca^{2+} buffers (Nachshen and Drapeau 1982) or by the local depletion of releasable vesicles during prolonged release (Simon and Llinás 1985).

In an effort to circumvent the difficulties of interpretation and conflicts among results using voltage clamp to measure Ca^{2+} influx, Zucker et al. (1991) related EPSP amplitude to measures of presynaptic $[Ca^{2+}]_i$ during tetanic stimulation and again found a high degree of cooperativity (~4) when external $[Ca^{2+}]$ was changed and a lower cooperativity (~2) when presynaptic spikes were broadened pharmacologically. However, Ca^{2+} indicators measure volume-average $[Ca^{2+}]_i$, not the local peaks of $[Ca^{2+}]_i$ that trigger transmitter release at active zones (Augustine et al. 1987; Smith and Augustine 1988). Interpretation of Ca^{2+}-indicator measurements requires an assumption of a linear relationship between the local peaks of $[Ca^{2+}]_i$ that trigger neurosecretion and the average $[Ca^{2+}]_i$ measured in a tetanus when external $[Ca^{2+}]$ is varied.

These considerations prompted us to seek a different and more direct method to measure the Ca^{2+} dependence of transmitter release. We turned to the photolabile Ca^{2+} chelator DM-nitrophen (Kaplan and Ellis-Davies 1988) as a

means of controlling presynaptic $[Ca^{2+}]_i$. This substance releases Ca^{2+} by photolysis of the chelator to produce nonbinding photoproducts on exposure to ultraviolet (UV) light. We injected it into crayfish motor neuron preterminal axons and permitted it to diffuse into synaptic endings. Exposure to flashes of UV light results in partial photolysis of DM-nitrophen, leading to a spatially uniform but intense "spike" in presynaptic $[Ca^{2+}]_i$ followed by a small increase in the plateau level of $[Ca^{2+}]_i$ in presynaptic boutons. The Ca^{2+} spike results from the delay in rebinding of Ca^{2+} to unphotolyzed chelator (Zucker 1993) and has a similar duration to the formation and collapse of Ca^{2+} domains in active zones during an action potential, as deduced from computer simulations of Ca^{2+} diffusion in nerve terminals (Fogelson and Zucker 1985; Simon and Llinás 1985; Yamada and Zucker 1992). This brief Ca^{2+} transient evokes transmitter release, and consequently a postsynaptic response, with a time course similar to that of a normal EPSP (Delaney and Zucker 1990).

For small amounts of photolysis the peak $[Ca^{2+}]_i$ elicited is a linear function of flash intensity. Thus relating postsynaptic response to flash intensity provides a measure of presynaptic Ca^{2+} cooperativity. We have found that neurosecretion shows a positive Ca^{2+} cooperativity of ≥ 3 in most preparations. We have also been able to account for the time course of postsynaptic responses to flash photolysis of presynaptic DM-nitrophen from simulations of the kinetics of the reactions of DM-nitrophen in producing Ca^{2+} (Zucker 1993) and a model of Ca^{2+} action in triggering secretion (Yamada and Zucker 1992).

METHODS

All experiments used the dactyl opener muscle of the first walking leg of crayfish (*Procambarus clarkii* Girard). Small specimens (5–7 cm long) were obtained locally (Western Scientific Supply, West Sacramento, CA), kept in shallow trays whose fresh water was changed weekly, and fed Purina trout chow. Legs were removed by the autotomy reflex, which promotes healing and survival. The ventral surface of the propodite carapace was cut away, the closer removed, and the sheath covering the motor nerve and ventral surface of the opener muscle removed. The excitatory axon was dissected in the meropodite and stimulated with a suction electrode. Physiological saline contained (in mM) 195 NaCl, 13.5 $CaCl_2$, 5.4 KCl, 2.6 $MgCl_2$, and 10 Na-*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid (NaHEPES), pH 7.3. Temperature was held at 18°C by a Peltier cooling system.

Electrodes 1.2 mm OD containing a glass fiber were used for presynaptic recording and injection. They were backfilled by capillarity with 0.5–1.0 μl of a solution containing (in mM) 50 DM-nitrophen (Calbiochem, San Diego, CA), 30 $CaCl_2$, 10 fluorescein (Molecular Probes, Eugene, OR), and 25 KHEPES (pH 7.3), beveled gently to a resistance of 60–80 MΩ in saline, and then backfilled by injection with 3 M KCl. The excitor motor neuron was penetrated at a steep angle at or just distal to the first major Y junction by use of a hydraulic manipulator. DM-nitrophen was injected by applying 50- to 100-ms pulses of 5- to 15-psi pressure to the pipette until the axon fluoresced brightly. The presynaptic nerve action potential was continuously monitored with 1/3-Hz stimulation and injection was halted if membrane potential or spike waveform changed. Filling of nearby terminals on proximal-central muscle fibers occurred within a few minutes and was monitored by observing fluorescein fluorescence with a Makler blue fluorescence objective adapter (VeeGee Scientific, Kirkland, WA) on an upright Nikon microscope with a ×10, 5.2-mm working distance objective. Injection was stopped when the fluorescence of terminal boutons suggested a final DM-nitrophen concentration of ~5–10 mM. At this point the excitatory junctional potentials (EJPs) were usually ~50% larger than before injection started. Injection of fluorescein alone had no effect on EJP amplitude, so the effect of DM-nitrophen is probably due to an elevation of resting $[Ca^{2+}]_i$ (Mulkey and Zucker 1993).

Postsynaptic potentials were recorded from surface proximal-central fibers with clearly filled boutons using 4- to 10-MΩ electrodes filled with 3 M KCl. Because gigaohm seals cannot be formed onto intact muscle fibers (Franke and Dudel 1987), whole-cell voltage clamp with patch electrodes was not appropriate. A fast, high-voltage (150 V) two-electrode voltage-clamp circuit with separate ground clamp to eliminate bath lead polarization potentials was employed. Muscle fibers were penetrated centrally with two electrodes that were similar to those used for voltage recording experiments but were shielded with silver paint insulated from the bath and connected to ground (current electrode) or a driven shield (voltage electrode). The clamp gain was often reduced to attenuate current noise and minimize ringing in response to light flashes used for photolysis, but clamp settling time remained <1 ms. The membrane potential was held near the resting potential (0-current level) throughout experiments. Only fibers with resting potentials between −60 and −80 mV were used. Both current and voltage recordings were filtered at 1 kHz.

Photolysis of DM-nitrophen was achieved with a Chadwick-Helmuth (El Monte, CA) flashlamp modified as described in Landò and Zucker (1989) and Zucker (1993). The 50-J electrical discharges used in these experiments produced 1-ms, 180-mJ flashes at 360 nm (measured with an IL1700 radiometer, International Light, Newburyport, MA) and photolyzed 21% of calcium-free DM-nitrophen, measured as described in Zucker (1993). Far UV and infrared were removed with a liquid filter (Tsien and Zucker 1986). Flash intensities were further reduced with neutral density filters whose UV transmittance was measured with a spectrophotometer. The light was focused with an elliptical reflector to a nearly uniform spot of ~4 mm diam that was aimed at the opener muscle. The carapace of the propodite and carpopodite were cut down to the level of the muscle and electrodes were placed to avoid casting shadows on the penetrated fiber.

RESULTS

Postsynaptic conductance as a measure of transmitter release

We needed a measure of the magnitude and time course of quantal transmitter release [$R(t)$]. Because quanta are released independently at distinct locations at neuromuscular junctions (Barrett and Stevens 1972a; Hartzell et al. 1975), their conductance effects sum linearly and so peak conductance is a good measure of the number of quanta released nearly synchronously by an action potential or photolysis flash. We also used the time course of postsynaptic conductance change [$G(t)$], as a measure of $R(t)$, but this is only approximately correct. A better measure of $R(t)$ is based on the fluctuations in minimum synaptic delay (Baldo et al. 1986; Barrett and Stevens 1972b). This method requires collecting many responses to identical stimuli and so is not applicable to flash photolysis experiments in which only one or two comparable responses are obtained.

$R(t)$ can also be calculated by convolution of excitatory junctional current (EJC) and miniature EJC (MEJC) time courses (Van der Kloot 1988). However, this measure requires accurate measurements of MEJCs. These are difficult to obtain in crayfish muscles, where large cell size with

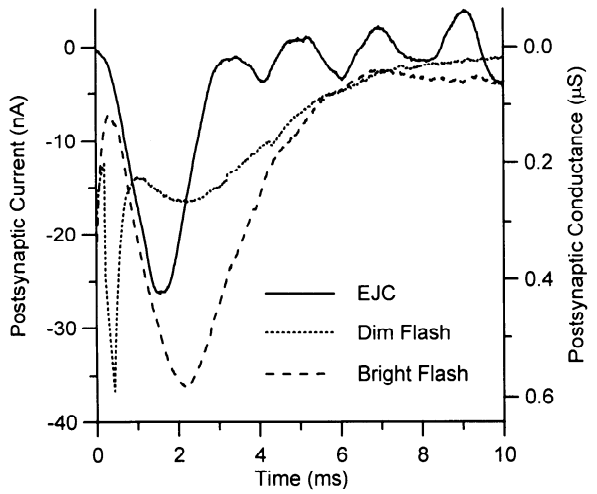

FIG. 1. Postsynaptic currents after presynaptic action potential or flash photolysis of presynaptic DM-nitrophen. A crayfish leg opener muscle fiber was voltage clamped at the resting potential and currents recorded to stimulation of the exciter motor neuron [excitatory junctional current (EJC)], or photolysis of DM-nitrophen that had been injected presynaptically with a dim flash (· · ·) or a 32% brighter flash (– – –). *Left ordinate*: current. *Right ordinate* converts this to postsynaptic conductance change. Flash responses are slower than EJCs and the slightly brighter flash evoked a much larger response. Noisy records are due to low clamp gain needed to minimize flash artifacts and high input conductance and capacitance of muscle fibers.

large capacitance and low input resistance results in noisy current records under voltage clamp in which single quanta of ~2 nA (Dudel et al. 1990) are not well resolved. Crayfish muscle MEJCs have usually been recorded extracellularly using macropatch electrodes, where they show a time course similar to that of evoked EJCs (cf. Dudel and Franke 1987 and Dudel et al. 1990 with Fig. 1 below). When EJC and MEJC time courses are similar, $R(t)$ must be substantially shorter than EJC duration; consequently the EJC is a poor measure of $R(t)$ (Van der Kloot 1988). However, flash responses were significantly longer both in rising and falling phases than EJCs. Therefore, unlike EJCs, flash-evoked $G(t)$ is not entirely limited by the time course of a single quantum and provides a more meaningful estimate of $R(t)$.

The most direct measure of conductance is to measure synaptic current under voltage clamp. This requires that the cell under study resemble electrically a single intracellular compartment. We estimated the muscle fiber length constant (λ) by use of the equation for input resistance (R_{in}) of a finite cable (of radius a and length l) measured at its center

$$R_{in} = \frac{\sqrt{R_{ax}R_m/2\pi^2a^2}}{2\tanh\left[1/2\sqrt{2R_{ax}/aR_m}\right]}$$

This equation may be solved numerically for R_m [membrane resistivity], given R_{ax} [axoplasmic resistivity] = 125 Ω/cm^2 (Fatt and Ginsborg 1958), a = 0.00229 ± 0.00093 (SD) cm (n = 8), l = 0.0986 ± 0.018 cm, and R_{in} = 6.91 ± 5.7 MΩ (n = 14), yielding $R_m = 9.7 \times 10^3$ Ω/cm^2. From this value λ is estimated as 0.30 cm from

$$\lambda = \sqrt{aR_m/2R_{ax}}$$

A centrally located electrode is then only 0.17 λ from either end of the fiber, which is therefore nearly isopotential. Even during the peak synaptic conductance of our largest flash responses, which reached ~1 μS, the length constant dropped to 0.11 cm and the electrode remained <1/2 λ from either end, so that a distributed synaptic conductance would not be very distorted by the measurement. Most responses in this study were much smaller than this.

Responses to bright and dim flashes and Ca^{2+} stoichiometry

To estimate the stoichiometric relationship between $[Ca^{2+}]_i$ and rate of transmitter release we injected motor nerve terminals with DM-nitrophen partially loaded with Ca^{2+} and recorded the postsynaptic current in response to flashes of different intensity. We began with a series of neutral density filters in front of the flash lamp and removed them one at a time until a small response was observed. Then another filter was removed and the response was recorded to a flash whose intensity increased by either 32% or 46%. Finally, to assure stability we reinserted the filter and repeated the weaker flash. Only the first two responses from fibers in which the dim flash responses agreed within 20% were used for analysis. In most experiments the bright flash photolyzed ~5% of DM-nitrophen bound to Ca^{2+}.

Figure 1 shows synaptic currents to bright and dim flashes and the EJC to nerve stimulation. Responses are also expressed as conductances [$G(t)$] by dividing the current by the driving force, or the difference between resting potential and the equilibrium potential, which for these synapses is about +6 mV (Dekin 1983). The figure shows several characteristics that were typical of our results. *1*) Flash responses were slower than EJCs, both in rising and falling phases. *2*) A small (32%) increase in flash intensity evoked a much larger (127%) peak conductance. Because the peak $[Ca^{2+}]_i$ reached is proportional to the flash intensity, the apparent cooperativity n of Ca^{2+} action can be estimated from

$$\text{Ratio of Response Peaks} = (\text{Ratio of Flash Intensities})^n$$

In seven experiments the average value of n was 3.33 ± 0.74 (SD) with a range of 1.84–4.02; the geometric mean was 3.24.

The small size of the synaptic currents compared with the noise of the voltage clamp limited the duration of meaningful current measurements to ~10 ms. A clearer indication of long-lasting synaptic current was obtained by measuring the membrane potential responses. Figure 2 shows an EJP and flash response in a different preparation. In all such experiments the flash response showed a slower rise time and falling phase than the EJP and a very low rate of transmitter release appeared to extend for ~200 ms after the flash.

For an isopotential cell the time course of synaptic conductance $G(t)$ can be calculated from the membrane potential $V(t)$, the synaptic equilibrium potential E, the input conductance G_{in}, and the membrane capacitance C, using

$$G(t) = \frac{1}{E - V(t)}\left[C\frac{dV(t)}{dt} + G_{in}V(t)\right]$$

Figure 3 shows this transformation of the synaptic and flash responses of Fig. 2. G_{in} and C were determined from the voltage responses of muscle cells to measured current pulses causing ~10-mV, 100-ms hyperpolarizations. As in the voltage clamp experiments the flash-evoked $G(t)$ outlasts the synaptic $G(t)$ and persists clearly for ≥20 ms. Beyond this time fluctuations in the voltage recording in-

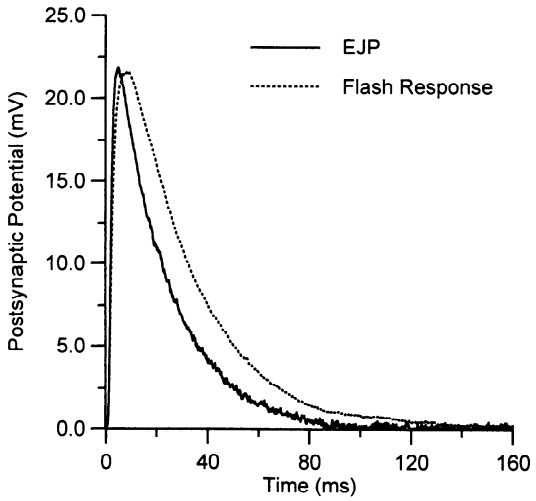

FIG. 2. Postsynaptic potentials after presynaptic action potential or flash photolysis of presynaptic DM-nitrophen. Postsynaptic potentials were recorded from an unclamped muscle fiber. Flash response (· · ·) has slower rising and falling phases than excitatory junction potential (EJP, ——). Lower noise reveals a late component of flash response, decaying with a 35-ms time constant.

troduce noise through the derivative term, which obscures the small persistent $G(t)$ lasting >100 ms that is evident in $V(t)$ in Fig. 2.

In nine similar experiments $G(t)$ was calculated in this fashion for responses to dim and bright flashes. The average apparent Ca^{2+} cooperativity measured from such data was 2.89 ± 1.13 with a range of 1.80 to 5.05; the geometric mean was 2.71.

Time course of the flash responses

To try to understand the origin of the slower time course of the flash responses compared with the synaptic responses, we turned to previously developed models of DM-nitrophen photolysis and transmitter release. Delaney and Zucker (1990) and Zucker (1993) presented a model of DM-nitrophen chemistry that considered the reactions of Ca^{2+} and Mg^{2+} with unphotolyzed nitrophen, its photoproducts, ATP, and native cytoplasmic Ca^{2+} buffer. We used this model with the following ON and OFF rates (in $mM^{-1}ms^{-1}$ and ms^{-1}, respectively): Ca-DM-Nitrophen—0.75 and 7.5×10^{-6} (from Zucker 1993, adjusted for crayfish ionic strength); Mg-DM-Nitrophen—0.15 and 0.0003 (based on Delaney and Zucker 1990); Ca-photoproduct—0.75 and 0.375 (based on Neher and Zucker 1993, adjusted for ionic strength); Mg-photoproduct—0.15 and 0.9 (from Kaplan and Ellis-Davies 1988, modified for ionic strength); Mg-ATP—50 and 5 (see Delaney and Zucker 1990); cytoplasmic Ca^{2+} buffer—2 and 0.12. The native buffer in crayfish cytoplasm has not been characterized kinetically; the values chosen correspond to a Ca^{2+} affinity of 60 μM. The time course of the light flash was based on an earlier measurement (Zucker 1993), whereas the intensity was adjusted to produce 5.25% photolysis of Ca-DM-Nitrophen to match our measurement for the bright flash in the experiment of Figs. 2 and 3. The concentrations of DM-nitrophen, Mg^{2+}, ATP, and cytoplasmic buffer were assumed to be 10, 2, 1, and 2 mM, respectively. The nitrophen was assumed to become 77% loaded with Ca^{2+} in cytoplasm to produce a slightly elevated free resting $[Ca^{2+}]_i$ of 200 nM, as discussed in Mulkey and Zucker (1993).

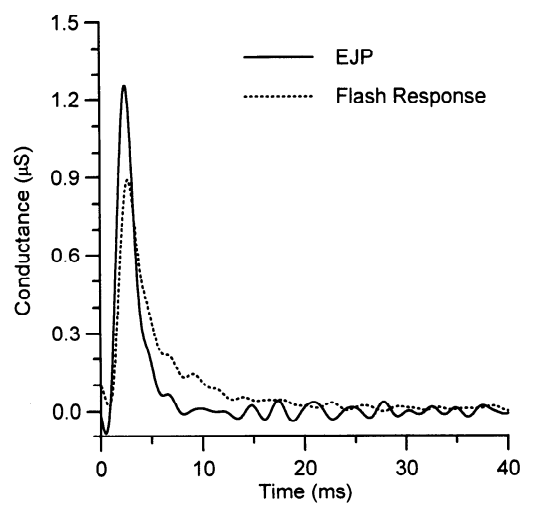

FIG. 3. Postsynaptic conductance changes computed from potential recordings after presynaptic action potential or flash photolysis of presynaptic DM-nitrophen. Conductances were calculated from the traces of Fig. 2, and have been digitally filtered at 500 Hz. Flash response (· · ·) outlasts EJP (——).

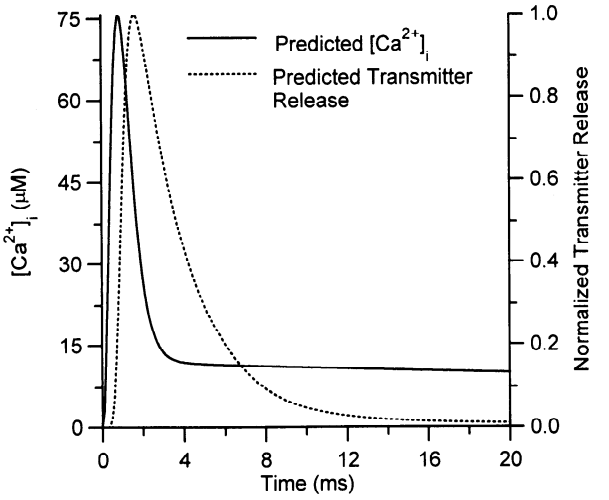

FIG. 4. Predicted presynaptic $[Ca^{2+}]_i$ and normalized rate of transmitter release in response to photolysis of presynaptic DM-nitrophen. A kinetic model (Delaney and Zucker 1990; Zucker 1993) of DM-nitrophen reactions and photolysis was used to simulate changes in $[Ca^{2+}]_i$ (——), whereas a model of transmitter release reactions (Yamada and Zucker 1992) produced the simulated time course of transmitter release (· · ·). The DM-nitrophen reactions delay the rise in $[Ca^{2+}]_i$ and transmitter release reactions delay release further. The duration of the flash causes the $[Ca^{2+}]_i$ "spike" and phasic release to last longer than responses to action potentials. The slow decay of the $[Ca^{2+}]_i$ tail is due to slow Mg-nitrophen and Ca-buffer equilibration and is reflected in a somewhat faster decay of late transmission due to the cooperativity of Ca^{2+} action in triggering secretion.

Figure 4 shows the simulated effect of a bright flash on presynaptic $[Ca^{2+}]_i$. A peak $[Ca^{2+}]_i$ of 76 μM is predicted. A plateau of $[Ca^{2+}]_i$ also appears after the peak and on the time scale of the figure decays very slowly. In fact, simulations for longer times show that the $[Ca^{2+}]_i$ falls slowly to 617 nM, with an apparent time constant of ~175 ms fitted from 20 to 150 ms.

The value of the $[Ca^{2+}]_i$ peak should not be taken too seriously, because it depends strongly on the concentrations of DM-nitrophen and total Ca^{2+} and also on the total Mg^{2+} concentration. However, alterations in these values have

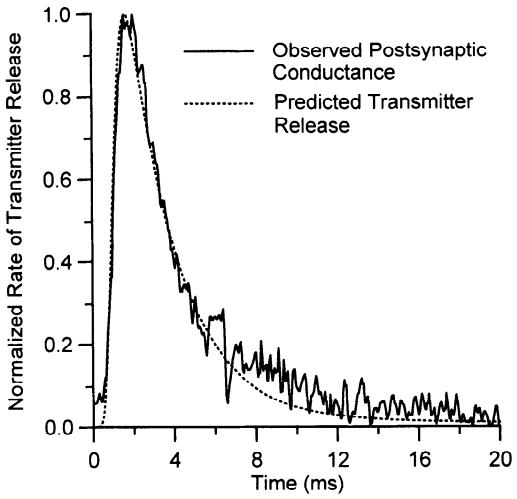

FIG. 5. Comparison of predicted rate of transmitter release to postsynaptic conductance change on photolysis of presynaptic DM-nitrophen. The conductance change calculated from the postsynaptic potential after a flash in Fig. 3 is plotted here (——, no digital filtering) along with the predicted time course of transmitter release from Fig. 4 (· · ·) to show their similarity.

little effect on the time course of the $[Ca^{2+}]_i$ simulation so long as the degree of Ca^{2+}-loading of nitrophen is adjusted to give a resting $[Ca^{2+}]_i$ of 200 μM, as suggested by Mulkey and Zucker (1993). The amount of ATP, its Mg^{2+} binding kinetics, and the Ca^{2+} and Mg^{2+} binding constants to the photoproducts also have almost no effect on the magnitude and time course of the computed $[Ca^{2+}]_i$, so that uncertainties in these values are inconsequential.

The undefined parameters with the largest effect on computed $[Ca^{2+}]_i$ time course are those associated with the native cytoplasmic buffer. Increasing the Ca^{2+}-binding ON rate or total buffer concentration depressed the peak $[Ca^{2+}]_i$, whereas increasing the OFF rate (decreasing the affinity) enhanced the shoulder of $[Ca^{2+}]_i$ after the peak. The values in the simulation illustrated were chosen to be consistent with measurements of buffer capacity in neurons (Smith and Zucker 1980) and to fall within the range of values for Ca^{2+}-binding proteins. They were then fine-tuned to provide a good fit between predicted and observed slow phase of transmitter release (see below).

The model of Yamada and Zucker (1992) was used to predict time course of transmitter release from the calculated time course of $[Ca^{2+}]_i$. This model proposes that four Ca^{2+} ions bind rapidly to one site (with ON rate of 500 $mM^{-1}ms^{-1}$ and OFF rate of 100 ms^{-1}) to trigger secretion at release sites and one ion binds slowly to another site (with ON rate of 5 $mM^{-1}ms^{-1}$ and OFF rate of 0.08 ms^{-1}) to generate a fast component of facilitation. The ON and OFF rates for exocytosis were 0.5 ms^{-1} per activated Ca^{2+} complex and 0.4 ms^{-1}. Figure 4 shows that transmitter release is delayed with respect to the $[Ca^{2+}]_i$ peak and that it has a much smaller and more rapidly decaying shoulder (due to the high Ca^{2+} cooperativity). Figure 5 compares this prediction with the measured postsynaptic $G(t)$ from Fig. 3. The agreement is striking.

Figure 2 revealed a small slow phase of transmitter release that decayed with a time constant of ~35 ms. As mentioned above, the $[Ca^{2+}]_i$ simulation of Fig. 4 showed a small tail of $[Ca^{2+}]_i$ decaying with a 175-ms time constant. The Yamada and Zucker (1992) model transformed this slow phase into a predicted tail of transmitter release having a 49-ms time constant, not too different from that observed.

We also ran simulations on the expected $[Ca^{2+}]_i$ peaks and peak rates of transmitter release for dim flashes 76% as bright as those used for Fig. 4. The Ca^{2+} peak was reduced from 76 to 58 μM and the peak release rate was reduced to 1/3. This is consistent with an apparent Ca^{2+} cooperativity of 4.0, similar to the values we observed.

DISCUSSION

We have exploited flash photolysis of photolabile Ca^{2+} chelators to measure the cooperativity of Ca^{2+} in triggering phasic transmitter release at crayfish neuromuscular junctions. Our procedures avoid many of the problems associated with other methods. In particular *1*) we avoid saturation of transmitter release by using flashes well below those that give a maximal response. *2*) We avoid saturation of postsynaptic potentials by measuring $G(t)$ under voltage clamp and keeping voltage responses well below the equilibrium potential for synaptic potentials. *3*) We choose a synapse that shows little or no synaptic depression or depletion of releasable vesicles. *4*) Photolysis generates a uniform rise in $[Ca^{2+}]_i$ in boutons. Because we were able to visualize filled terminals by coinjecting fluorescein, we made an effort to choose fibers where terminals were clustered primarily on the top or bottom muscle surface, so that all terminals would experience a similar flash intensity. *5*) We did not have to relate release to a changing Ca^{2+} that bears a complex relationship to the resulting spatial variation in presynaptic $[Ca^{2+}]_i$ profile. Instead $[Ca^{2+}]_i$ rises rapidly and uniformly to a transient peak and changes in this peak lead directly to changes in peak rates of transmitter release. *6*) We injected ~10 mM of DM-nitrophen into terminals and its photolysis generates a peak $[Ca^{2+}]_i$ that is linearly related to flash intensity. Simulations of DM-nitrophen reactions show that this linearity is little affected by even a few millimolar of a native buffer, which should be overwhelmed by the concentration of DM-nitrophen estimated from fluorescein fluorescence intensity in terminal processes. It is important that the linear relationship between flash intensity and peak $[Ca^{2+}]_i$ holds despite the uncertainty in the exact level of peak $[Ca^{2+}]_i$ achieved by flashes, so long as the photolysis rate is substantially <50%. Thus our procedures avoid many of the limitations inherent in previous measures of Ca^{2+} cooperativity (see INTRODUCTION).

In our most accurate procedure, using voltage clamp to measure postsynaptic $G(t)$, we found a cooperativity of ~3–4, similar to earlier measures that are based on changes in external $[Ca^{2+}]$ and higher than measures based on changes in Ca^{2+} influx altered by increasing or prolonging presynaptic depolarization (references listed in INTRODUCTION). This is consistent with the view that the latter measures underestimate the true Ca^{2+} cooperativity of exocytosis (Zucker et al. 1991). Our results are also consistent with predictions of a recent model of Ca^{2+} action in which four Ca^{2+} ions cooperate to trigger exocytosis and another binds at a different site to activate facilitation (Yamada and Zucker 1992).

Our measure of stoichiometry is subject to the limitation that small changes in $[Ca^{2+}]_i$ near the resting level, and any

changes in $[Ca^{2+}]_i$ above the affinity of the Ca^{2+}-binding site triggering exocytosis, are both liable to generate responses with an apparent stoichiometry that underestimates the true Ca^{2+} cooperativity (Barton et al. 1983). We found no clear evidence for such effects: the apparent stoichiometry did not correlate with the absolute size of our smallest or largest responses, which varied severalfold among preparations. Nevertheless our results should be interpreted as providing a minimal estimate of Ca^{2+} cooperativity in triggering neurosecretion.

Flash responses were somewhat slower than MEJCs, assuring that they were mainly determined by the time course of transmitter release. This time course was clearly slower than release evoked by action potentials. Our model of DM-nitrophen photolysis (Zucker 1993) predicts a $[Ca^{2+}]_i$ transient to flashes that is about twice as long as the $[Ca^{2+}]_i$ transient at release sites in an action potential inferred from simulations of Ca^{2+} diffusion (Yamada and Zucker 1992). This longer $[Ca^{2+}]_i$ spike, due largely to the duration of our flash, is the main reason for the broader peak in transmitter release, $G(t)$, or the rising phase of the voltage response, compared with the EJC or EJP evoked by action potentials. A slowly decaying plateau of $[Ca^{2+}]_i$ arises from the slow equilibration of DM-nitrophen with Mg^{2+} ions and the equilibration of released Ca^{2+} with unphotolyzed DM-nitrophen and native buffer. It is expressed as a late phase of decay of transmitter release.

We were thus able to account for temporal characteristics of flash responses by use of models of DM-nitrophen photolysis and Ca^{2+} action at nerve terminals (Yamada and Zucker 1992; Zucker 1993). The qualitative effects of varying parameters not known with certainty were listed in RESULTS. Our results do not prove all aspects or confirm the parameter choices of these models; they merely indicate that such models are able to account for our findings.

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