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CALCIUM RELEASED BY PHOTOLYSIS OF DM-NITROPHEN TRIGGERS TRANSMITTER RELEASE AT THE CRAYFISH NEUROMUSCULAR JUNCTION

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SUMMARY

1. Spontaneous and evoked transmitter release at the crayfish neuromuscular junction were potentiated in response to photolytic release of calcium from the 'caged' calcium compound DM-nitrophen, which had previously been injected into presynaptic terminals.

2. The amount of calcium released from DM-nitrophen photolysis depends on the concentration of DM-nitrophen, its photoproducts, Ca^{2+}, Mg^{2+}, H^+, ATP and the cell's native buffer. Since none of these are known in the crayfish terminal, the study was conducted in a qualitative fashion.

3. Photolytic release of calcium from DM-nitrophen increased excitatory junctional potentials (EJPs) by a range of 2–31 times over control values and the miniature excitatory junctional potential (MEJP) frequency increased from resting values of 1–10 quanta/s to 3000–11000 quanta/s.

4. Extracellular calcium was not required for the light-evoked asynchronous release of transmitter. Calcium-bound DM-nitrophen previously pressure injected into crayfish presynaptic terminals increased the MEJP frequency from resting values of 1–8 quanta/s to 800–10000 quanta/s during photolysis in a calcium-free cobalt Ringer solution.

5. Iontophoresis of calcium-free DM-nitrophen into presynaptic terminals released transmitter upon photolysis, but only in a calcium-containing Ringer solution. This suggests that DM-nitrophen is capable of binding calcium once injected into terminals, but this is dependent on the presence of external calcium.

6. Photolysis of DM-nitrophen at lower light intensities produced a slower rate of transmitter release.

7. Brief light exposures, i.e. those which photolysed 5–20% of the DM-nitrophen, resulted in a rapid decay of postsynaptic responses on extinguishing the light, due to rebinding of photolytically released calcium to unphotolysed DM-nitrophen. Longer light exposures which completely photolysed DM-nitrophen, leaving only the low affinity photoproducts, produced a slow decay of transmitter release after the light

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pulse, presumably due to the active extrusion of calcium from the presynaptic terminals.

8. During photolysis of DM-nitrophen, the time courses of changes in EJP amplitude and MEJP frequency were different, indicating that the two measures of transmitter release were not linearly related.

9. MEJP frequency and EJP amplitudes during DM-nitrophen photolysis were fitted to a 'non-linear summation model' in which photolytically released calcium sums with calcium entering during an action potential to evoke transmitter release with a calcium co-operativity of five.

INTRODUCTION

Intracellular calcium is known to be essential for the normal release of transmitter substances (Katz, 1969) but numerous questions remain as to the role of calcium in the transmitter release process. The invasion of an action potential into the presynaptic terminal briefly opens voltage-gated calcium channels, admitting calcium and leading to the phasic release of neurotransmitter (Llinás, Steinberg & Walton, 1981). Even in the absence of presynaptic spikes, transmitter is released spontaneously in the form of single quanta which produce miniature excitatory postsynaptic potentials (MEPSPs) in the postsynaptic cell.

The relationship between transmitter release and presynaptic calcium is thought to be highly non-linear. The experimental evidence in support of this hypothesis is as follows. (1) A non-linear dependence of transmitter release on external calcium concentration has been found at both the frog (Dodge & Rahamimoff, 1967) and the crayfish neuromuscular junction (NMJ; Dudel, 1981); (2) measurements of intracellular calcium concentration in crayfish presynaptic terminals (Zucker, Delaney, Mulkey & Tank, 1991) show a similar non-linear dependence of release on intracellular calcium; and (3) the relationship between synchronous and asynchronous release following trains of action potentials indicates a non-linearity somewhere in the release process. For example, if a train of action potentials is delivered, followed by a single impulse, this impulse will release more transmitter than if it occurred in isolation and the frequency of MEPSPs will also be greater. This increase in synchronous and asynchronous release is attributed to the residual calcium which accumulates during repetitive action potentials (Katz & Miledi, 1968). The residual calcium hypothesis, being the most prominent theory to explain this phenomenon, states the increase in the MEPSP frequency is a direct but non-linear reflection of residual calcium, whereas the increase in the excitatory postsynaptic potential (EPSP) amplitude is dependent on both the calcium which comes in through calcium channels plus the residual calcium which remains from previous activity. The EPSP facilitation should be less sensitive to residual calcium and therefore show smaller amplitude changes and decline more slowly than the changes in the MEPSP frequency. Such a correlation between MEPSP frequency and facilitation of EPSP amplitude has been observed at both the frog (Barrett & Stevens, 1972) and the crayfish NMJ (Zucker & Lara-Estrella, 1983), using paired pulses and brief tetani respectively to elevate intracellular calcium. Contrary to these experimental observations, Zengel & Magleby (1981) reported at the frog NMJ a larger percentage increase in miniature end-plate potential (MEPP) frequency than

in end-plate potential (EPP) amplitude, but a similar decay rate following the tetanus.

Various methods to raise intracellular calcium concentration ($[Ca^{2+}]_i$), other than tetanic stimulation, have been applied at presynaptic terminals to study the dependence of transmitter release on intracellular calcium. At the squid giant synapse, the injection of calcium ions is sufficient to facilitate transmitter release to a spike (Charlton, Smith & Zucker, 1982) and evoke asynchronous release of quanta (Miledi, 1973). At the frog NMJ, calcium-containing liposomes fused with the presynaptic membrane (Rahamimoff, Meiri, Erulkar & Barenholz, 1978), mitochondrial poisons which inhibit calcium sequestering mechanisms (Glagoleva, Liberman & Khashayev, 1970; Alnaes & Rahamimoff, 1975) and application of calcium ionophores (Kita & Van Der Kloot, 1974; Statham & Duncan, 1975) all increase evoked release to a spike and MEPP frequency. However, none of these techniques are able to rapidly elevate the $[Ca^{2+}]_i$ in the cell and do it in a controlled fashion. The availability of photolabile caged calcium compounds which can uniformly raise $[Ca^{2+}]_i$ to the micromolar range in a millisecond or less, can circumvent both of these problems (Kaplan & Ellis-Davies, 1988).

The caged calcium compound DM-nitrophen (Kaplan & Ellis-Davies, 1988) was used at the squid giant synapse to raise calcium quickly upon photolysis and produce transient release of transmitter and facilitation of evoked release to a spike (Delaney & Zucker, 1990). However, at the squid giant synapse MEPSP frequency is not measurable, due to the high input conductance of the postsynaptic axon, so a relationship between intracellular calcium and asynchronous transmitter release could not be studied. The crayfish NMJ is better suited for observing miniature excitatory junctional potential (MEJP) frequency and evoked release to a spike, with little or no depression of transmitter release occurring at high frequency stimulation (Hatt & Smith, 1976). We chose to raise intracellular calcium in the crayfish presynaptic boutons using the caged calcium compound DM-nitrophen and record MEJP frequency and evoked release, relating their frequency and amplitude changes respectively to a non-linear summation model with a high degree of calcium co-operativity.

METHODS

Dye injection

All experiments were performed on the first walking leg of the crayfish, *Procambarus clarkii*, using animals measuring 5–6·5 cm. Two experimental set-ups were used for this study. One set-up contained a standard stereomicroscope with incident light (1 mm plastic fibre optic positioned with a manipulator) to illuminate the preparation. The excitatory axon of the opener muscle in the propodite segment was impaled near the Y-branch using a motor drive to advance the microelectrode (AB Transvertex, Stockholm, Sweden) and DM-nitrophen was pressure injected. Pressure injection electrodes were bevelled using 1·2 mm outer wall diameter standard microelectrodes and filled with 72 mM DM-nitrophen, 21·6 mM $CaCl_2$ (30% Ca^{2+}) and 1·8 mM Arsenazo III (Sigma, St Louis, MO, USA). DM-nitrophen was the generous gift of Drs J. H. Kaplan and G. Ellis-Davies. Arsenazo III was used to monitor the injection and provide a rough estimate of how much DM-nitrophen was injected into the axon and diffused to secondary and tertiary branches. Brief pressure pulses were applied, usually less than 50 ms duration, using a stimulator-controlled solenoid valve (Clipper Instrument Lab., Cincinnati, OH, USA).

The second experimental set-up used an upright epifluorescence microscope (Nikon Optiphot, Garden City, NY, USA). The excitatory axon was impaled with the DM-nitrophen electrode using a stage-mounted hydraulic manipulator (Narishige, Greenvale, NY, USA; Model MO-203M) while

viewing the preparation through a Nikon CF-E 10 × long working distance air objective (5·2 mm). DM-nitrophen was either pressure injected or iontophoresed into the axon. Bevelled pressure injection electrodes were filled with 60 mM DM-nitrophen, 18 or 36 mM $CaCl_2$ (30 or 60%) and 10 mM sulphorhodamine B (Molecular Probes, Eugene, OR, USA) in 0·5 M K-Hepes, pH 7·3. By including 10 mM sulphorhodamine B in the electrode, we could monitor the filling of the terminals with a rhodamine epifluorescence cube (Nikon) without photolysing the DM-nitrophen. Pressure pulses were applied with similar duration to that mentioned previously using a WPI Pneumatic Picopump (New Haven, CT, USA).

Iontophoresis of DM-nitrophen into the excitatory axon was performed using a 1·2 mm outer wall diameter standard microelectrode filled with 60 mM DM-nitrophen, 150 mM KCl, 10 mM rhodamine in 0·5 M K-Hepes, pH 7·3. Continuous hyperpolarizing current (10–20 nA) was applied for 30–40 min, or until terminals were brightly filled with rhodamine.

In both set-ups, general illumination passed through a UV-blocking fibre optic light guide which caused no photolysis of DM-nitrophen while viewing the preparation.

UV photolysis

The light source in the first experimental set-up was a 150 W collimated xenon lamp (Cermax, ILC Technology, Sunnyvale, CA, USA) fitted with a UniBlitz shutter (Vincent Assoc., Rochester, NY, USA). The light source was calibrated following procedures described in Delaney & Zucker (1990). A 2 s exposure of the xenon lamp at 15 A photolysed approximately 20% of the DM-nitrophen. A 100 W mercury epifluorescence illuminator fitted with a UniBlitz shutter was focused onto terminals through the 10 × objective to photolyse the DM-nitrophen in the second experimental set-up. The light source was calibrated using the calcium indicator dye fura-2 to estimate the increase in calcium upon DM-nitrophen photolysis. Fifty micrometre path length cuvettes (Vitro Dynamics, Rockaway, NJ, USA) were filled with a solution to mimic the intracellular ionic environment containing 100 μM fura-2, 1 mM DM-nitrophen, 0·5 mM $MgCl_2$, 300 μM $CaCl_2$, and 300 mM KCl. Using a 25% transmission neutral density filter, 20% of the DM-nitrophen was photolysed in less than 500 ms, as calculated using the procedures discussed in Delaney & Zucker (1990). In some experiments a second, 25% neutral density filter was used to slow DM-nitrophen photolysis.

The concentration of DM-nitrophen was estimated by visually comparing the brightness of the rhodamine fluorescence or Arsenazo III colour in the terminals and fine branches following injection of DM-nitrophen mixed with rhodamine or Arsenazo III, to a known concentration of these dyes in the tips of microelectrodes with similar diameters to the terminals. With this method, the DM-nitrophen concentration in the terminals was estimated to be between 2 and 10 mM.

Electrophysiology

The DM-nitrophen electrode monitored the presynaptic action potential and a postsynaptic electrode filled with 3 M KCl (10 MΩ) was placed in a muscle fibre in the vicinity of filled terminals seen using rhodamine fluorescence or placed near tertiary axonal branches filled with Arsenazo III. The excitatory axon was dissected from the meropodite and stimulated with a plastic suction electrode. Electrophysiological recordings of muscle EJPs and MEJPs were stored on FM tape and a Gould pen recorder for later analysis. The shutter timing pulse was also stored on FM tape to indicate the light exposure. The temperature of the preparation was kept at 18 ± 2 °C with a stage-mounted Peltier device. A gravity perfusion system with inflow above the opener muscle provided continuous perfusion with the appropriate solution.

Solutions

Normal Van Harreveld's solution (NVH) contained (in mM): 195 NaCl, 13·5 $CaCl_2$, 5·4 KCl, 2·6 $MgCl_2$, and 10 Na-Hepes at pH 7·4. EGTA Ringer solution contained 185 NaCl, 5·4 KCl, 30 $MgCl_2$, 2 Na_2EGTA, and 10 Na-Hepes at pH 7·4. Cobalt Ringer solution contained $CoCl_2$ substituted for $CaCl_2$ (13·5) along with 175 NaCl, 5·4 KCl, 30 $MgCl_2$, and 10 Na-Hepes at pH 7·4.

Data analysis

EJP amplitudes and MEJP frequencies were digitized from chart recordings using a Numonics (Montgomeryville, PA, USA) 2200 Digitizing Pad, an IBM 386 computer and a program kindly supplied by Russell English.

Whenever an EJP amplitude exceeded 3 mV, it was corrected for the non-linear relation between

postsynaptic conductance changes and EJP amplitude (Martin, 1955): $v = v'/(1-v'/C)$, where v' is the observed EJP amplitude, v is the corrected amplitude and C is the difference between the membrane potential at the beginning of the EJP and the equilibrium potential for the EJP. The latter was taken as 0 mV (Taraskevich, 1971). The membrane potential at the beginning of the EJP was not necessarily the resting potential since the increase in MEJP frequency resulted in a depolarization of the membrane. Significant increases in MEJP frequency resulted in the summation of single quanta and depolarization of the membrane. This increase in membrane potential was measured (digitized) and converted into quanta per second using the formula presented by Katz & Miledi (1970):

$$f = V/(\tau q), \tag{1}$$

where V is the average voltage change, τ is the time constant of an MEJP, measured as 20 ms, q is the quantal amplitude (taken to be 50 μV; Bittner & Kennedy, 1970) and f is the frequency of MEJPs that it would take to give the measured voltage deflection. The total number of MEJP quanta released during the light exposure was computed by calculating the area of the depolarization due to MEJP summation.

Predictions were calculated from a model in which transmitter release is related by a power function to intracellular calcium concentration, reflecting a high positive co-operativity in the action of calcium on the secretory process. The model is similar to those described earlier (Zengel & Magleby, 1981; Zucker & Lara-Estrella, 1983). The Martin-corrected EJP amplitude in millivolts, v, can be approximately re-expressed in units of rate of transmitter release, R, that are the same as those used for MEJP frequency (quanta/s) by the equation

$$v = qTR, \tag{2}$$

where T is the duration of transmitter release at 18 °C, taken to be 2 ms (Parnas, Hovav & Parnas, 1989), and q is the amplitude of a quantum of transmitter (typically 50 μV). The frequency of MEJP release (f) is a non-linear power function of intracellular calcium:

$$f = K(Ca_R^{2+})^n, \tag{3}$$

where Ca_R^{2+} is the resting calcium prior to an action potential and K is a proportionality constant whose value depends on the units chosen for Ca_R^{2+}.

In the 'non-linear summation model' of synaptic facilitation (Zengel & Magleby, 1981; Zucker & Lara-Estrella, 1983), resting calcium may be augmented as a consequence of prior activity. This elevation in resting calcium represents a residuum of presynaptic calcium that entered the terminals during conditioning activity. It sums with the rise in presynaptic calcium at release sites during a test stimulus, Ca_E^{2+}, and the resulting total calcium generates a facilitated EJP according to the non-linear or power-law dependence of transmitter release on calcium concentration,

$$v/qT + f = K(Ca_E^{2+} + Ca_R^{2+})^n = KCa_E^{2+}(1 + Ca_R^{2+}/Ca_E^{2+})^n, \tag{4}$$

where the total rate of transmitter release is the sum of pre-existing frequency of MEJPs, f, and the incremental release during an EJP of amplitude v expressed in terms of frequency, v/qT. We assume that elevation of resting calcium by photolysis of DM-nitrophen has a similar effect on MEJP frequency and EJP amplitude to that of the residual calcium following conditioning activity in the non-linear summation model of synaptic facilitation. Since any units may be used for Ca_E^{2+} and Ca_R^{2+}, these were scaled by assigning the value of one to Ca_E^{2+}. Then resting calcium is expressed as a fraction of the calcium concentration increase caused by an action potential.

Combining eqns (3) and (4),

$$v/qT + f = K(1 + (f/K)\{1/n\})^n. \tag{5}$$

This equation relates MEJP frequency, f, to Martin-corrected EJP amplitude, v, in the absence of any explicit information about the levels of intracellular calcium during or preceding action potentials. Equation (5) was solved for the constant K for each given EJP amplitude and MEJP frequency using an n of 5. The exponent 5 provided the best fit in four out of eight experiments, with an exponent of at least three required for reasonable fits. Thus, 5 was chosen as the exponent for the non-linear summation model. In some preparations the value for K decreased during the light exposure. An average value of K was substituted into eqn (5) and EJP amplitude during light exposure was predicted and compared to the observed EJP values. This indicated whether a non-linear summation model could be used to describe the relationship between EJP amplitude and MEJP frequency.

RESULTS

Properties of DM-nitrophen photolysis

DM-nitrophen is a photolabile chelator which selectively binds calcium and magnesium ions. It is important to understand the chemistry of this compound in order to interpret the synaptic responses to illumination in terminals injected with DM-nitrophen. Upon exposure to UV light, DM-nitrophen is converted from a high affinity calcium buffer with a dissociation constant (K_D) of 10 nM at 22 °C, pH 7·1 and 280 mM ionic strength, to weakly calcium-binding photoproducts with K_D values of about 6 mM. The dissociation constants were doubled from the reported constants (Kaplan & Ellis-Davies, 1988) to compensate for the higher ionic strength of crayfish cytoplasm (Wallin, 1967; Harrison & Bers, 1987). The photorelease of calcium from the DM-nitrophen–calcium complex is summarized by the following reaction.

$$2H^+ + (CaNPh)^{2-} + h\nu \rightarrow NIA^- + IA^- + Ca^{2+},$$

where CaNPh represents calcium bound to DM-nitrophen, NIA^- represents nitrosoacetophenone-substituted iminodiacetic acid and IA^- represents iminodiacetic acid. The absorption of a photon of light is denoted by $h\nu$ and the quantum efficiency for calcium release from DM-nitrophen is 0·18 at pH 7·1 and 20 °C using 350 nm light.

DM-nitrophen also binds magnesium at the same site as calcium with a dissociation constant of 5 μM under the above conditions and the photoproducts bind magnesium with a similar affinity as they bind calcium. Therefore, upon injection of calcium-bound DM-nitrophen, magnesium will tend to displace calcium when the concentration of DM-nitrophen is lower than the concentration of free magnesium. It is important to achieve a higher concentration of DM-nitrophen in the cell than the free intracellular magnesium concentration. For crayfish presynaptic terminals, the free magnesium concentration is unknown, but is thought to be of the order of 1 mM or more in neurons (Alvarez-Leefmans, Gamiño & Rink, 1984).

The concentration of ATP is also important in the cell, as the dissociation constant for ATP binding magnesium is 700 μM, as calculated in the squid axon (Brinley, Tiffert, Scarpa & Mullins, 1977) and is much lower than that of DM-nitrophen for magnesium. Therefore, some magnesium will be displaced from the ATP to bind to the DM-nitrophen when the DM-nitrophen is injected into the cell.

To calculate accurately the free calcium concentration in the cell, it is necessary to know the concentration of all these compounds: DM-nitrophen, its photoproducts, calcium, magnesium, ATP and the cell's native buffer. The effective dissociation constants of DM-nitrophen with calcium and magnesium are also pH dependent. All of these values are presently unknown in the crayfish terminal. Therefore, we chose to analyse the data in a qualitative fashion.

Partial photolysis of DM-nitrophen should suddenly release calcium and magnesium and both are free to rebind to the unphotolysed DM-nitrophen. Since DM-nitrophen is based on the parent compound, EDTA, we expect calcium to re-equilibrate within a few milliseconds (Smith, Liesegang, Berger, Czerlinski & Podolsky, 1984) and magnesium more slowly (Eigen & Hammes, 1963; Delaney & Zucker, 1990). With sufficient photolysis of DM-nitrophen calcium will be released in excess of unphotolysed DM-nitrophen, and liberated calcium will be free to bind to

native buffers not already occupied by calcium. Active extrusion of calcium through pumps or exchangers will be important to reduce the $[Ca^{2+}]_i$ once the native buffers are saturated.

Potentiation of evoked and spontaneous transmitter release by photolysis of caged calcium

Calcium, elevated in presynaptic terminals with DM-nitrophen photolysis, produced potentiation of evoked and spontaneous transmitter release. The

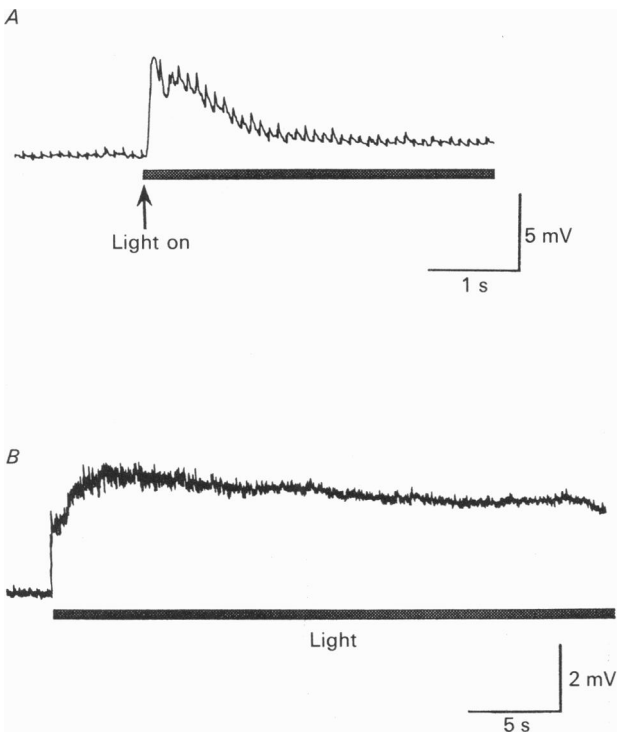

Fig. 1. DM-nitrophen photolysis in normal Ringer solution enhances both evoked and spontaneous transmitter release. Calcium-bound DM-nitrophen was pressure injected into the excitatory axon in both preparations shown in panels *A* and *B*. *A*, photolysis with a 10 s exposure to UV light, beginning at the arrow, elevated presynaptic $[Ca^{2+}]_i$ and increased MEJP frequency to about 5500 quanta/s while increasing the EJPs, evoked by 10 Hz stimulation, by a factor of 7·5. *B*, DM-nitrophen photolysis in normal Ringer solution without nerve stimulation. Photolysis of DM-nitrophen during a 30 s exposure to UV light (duration indicated by the bar) elevated presynaptic $[Ca^{2+}]_i$ and increased MEJP frequency to about 4000 quanta/s. The total number of quanta released during photolysis was approximately 83000.

preparation was bathed in calcium-containing medium which produced evoked release upon nerve stimulation. After filling terminals with DM-nitrophen, control EJPs and MEJPs were recorded. In thirteen out of twenty preparations, evoked release and MEJP frequency increased following pressure injection of calcium-bound DM-nitrophen (30–60%) and could be attributed to injection damage or dis-

placement of bound calcium from DM-nitrophen by magnesium. This post-injection potentiation of EJPs was taken as the new control value.

Figure 1*A* is typical of a response seen in ten experiments during DM-nitrophen photolysis and nerve stimulation. In this particular experiment, DM-nitrophen (30% calcium loaded) was pressure injected and photolysed using the mercury light, and a 25% transmission filter. The light was left on for 10 s. The EJPs increased by a factor of 7·5 over control values with a range of 2–31 recorded in the ten experiments. The MEJP frequency increased from 1 quantum/s to around 5500 quanta/s in this experiment with a MEJP frequency increase ranging from resting levels of 0·5–10 quanta/s to peak levels of 3000–11 000 quanta/s after DM-nitrophen photolysis in the full ten experiments. The time to peak for the effects on EJP amplitude and MEJP frequency varied from 110–500 ms for eight preparations using the mercury light source and 1·7 and 1·9 s for two preparations using the weaker xenon lamp.

The duration of light exposure varied in these experiments from 1 to 10 s and affected the total number of quanta released. Approximately 6000 quanta were released in Fig. 1*A* during the time after the light onset assuming a single MEJP amplitude is equal to 50 μV and has a decay time constant of 20 ms. There was a large range of quanta released, from 1100 to 29000 quanta; the latter was for a 10 s light exposure.

In six other preparations a smaller response to the light exposure was seen, probably attributable to low levels of DM-nitrophen in the terminals. In three of these preparations there was no measurable increase in MEJP frequency in the first second of light while the peak EJP amplitude increased by a factor of 2·6, 2·9 and 4·1 over control values. In the remaining three preparations, the peak EJP amplitude increased by a factor of 2·5, 2·6 and 4·7 over control values and the MEJP frequency increased by 55 ± 19 quanta/s (mean $\pm$ S.E.M.).

Five other preparations were bathed in normal calcium medium but the nerve was not stimulated. The increase in MEJP frequency was measured during light exposure. In all five preparations, the xenon light was used and the range of light exposure varied from multiple light responses in the same muscle fibre as brief as 300 ms to a 30 s light exposure, shown in Fig. 1*B*. The MEJP frequency increased from a resting MEJP frequency of 1–10 quanta/s to 420–12500 quanta/s with the total number of quanta ranging from 300 (1 s exposure) to 83000 (30 s light exposure, Fig. 1*B*).

Extracellular calcium is not required for the light-evoked asynchronous release of transmitter

The preparation was bathed in a cobalt Ringer solution in which $CoCl_2$ replaced $CaCl_2$; magnesium was increased to 30 mM to prevent depolarization of the membrane which occurs in this preparation when calcium is removed. Calcium released through photolysis of pressure-injected calcium-bound DM-nitrophen produced asynchronous transmitter release. Figure 2 shows a typical result from a total of seven, in which the MEJP frequency increased from 7 to 10000 quanta/s. MEJP frequency increased from resting values of 0·5–8 quanta/s to 800–10000 quanta/s in these experiments. The total number of quanta released in Fig. 2 was 17000 during a 10 s exposure; this number ranged from 900 to 34000 with light exposures between 2 and 10 s.

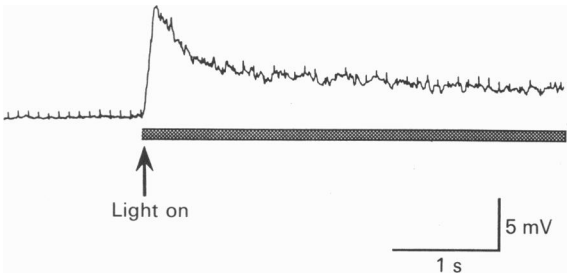

Fig. 2. DM-nitrophen photolysis in a calcium-blocking solution still triggers spontaneous transmitter release. The preparation was bathed in a cobalt Ringer solution, where 13·5 mM cobalt was substituted for calcium and the magnesium was increased to 30 mM. A 10 s photolysis of previously injected calcium-loaded DM-nitrophen elevated the MEJP frequency from a resting value of 7 quanta/s to 10000 quanta/s with the total number of quanta released approximately equal to 17000.

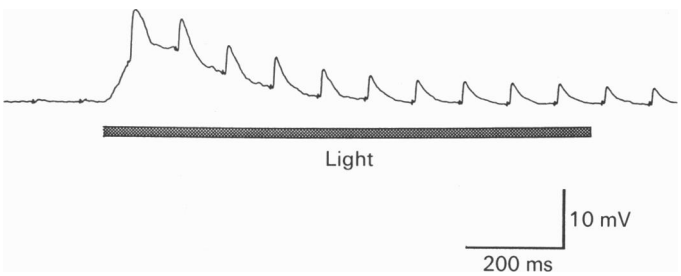

Fig. 3. Photolysis of DM-nitrophen previously injected without calcium is effective in triggering transmitter release. Photolysis with a 1 s exposure of UV light increased EJP amplitude from 0·5 to 13·5 mV and the MEJP frequency increased from 1 to 11000 quanta/s. The total number of quanta released during the 1 s light exposure was 2600. This effect of photolysing unloaded DM-nitrophen was seen only in a calcium-containing medium.

Iontophoresis of calcium-free DM-nitrophen releases transmitter upon photolysis, but only in a calcium-containing medium

To inject DM-nitrophen loaded with a certain percentage of calcium ions, it is necessary to pressure inject the compound into the axon. Iontophoresis, using hyperpolarizing current, will inject only DM-nitrophen without calcium ions. Surprisingly, iontophoresis of DM-nitrophen without calcium ions was effective in causing transmitter release upon photolysis, but only in a calcium-containing medium. As shown in Fig. 3, the Martin-corrected EJP amplitude increased from 0·5 to 13·5 mV along with an increase in MEJP frequency from 1 to 11000 quanta/s, as large an effect as in those experiments in which we injected calcium-loaded DM-nitrophen. The total number of quanta released in the experiment shown in Fig. 3 was 2600 during a 1 s light exposure. However, in a zero-calcium medium, either a cobalt Ringer solution ($n = 4$) or an EGTA Ringer solution, in which 2 mM EGTA replaced the calcium and the magnesium was at 30 mM ($n = 3$), photolysis of injected calcium-free DM-nitrophen did not produce transmitter release. Apparently, photolysis of DM-nitrophen, which has been injected without calcium, produces transmitter release *only* when external calcium is present. This is in contrast to

photolysis in a cobalt Ringer solution of DM-nitrophen which has been pressure injected with calcium (e.g. Fig. 2). This suggests that unloaded DM-nitrophen is only able to absorb significant amounts of calcium if the preparation is bathed in a calcium-containing solution. This result also shows that responses to DM-nitrophen

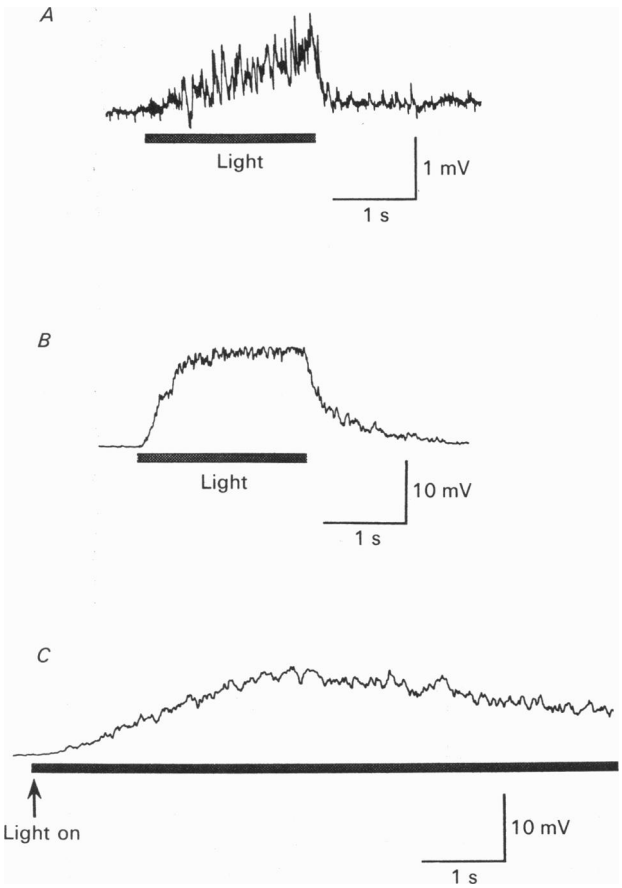

Fig. 4. Photolysis of DM-nitrophen using lower light intensities slows the rise time to peak for spontaneous release of transmitter. *A*, the postsynaptic response to DM-nitrophen photolysis using a 2 s mercury light exposure and two 25% transmission filters which reduced the light transmission to 6%. The rise time to peak for the MEJP frequency was 1·7 s and the MEJP frequency peak response was 1200 quanta/s. *B*, recording from the same muscle fibre as shown in panel *A*, photolysis of DM-nitrophen was performed with a 2 s mercury light exposure and only one 25% transmission filter. The rise time to peak was faster than in *A*, measured at 0·9 s. The peak response for MEJP frequency was about 16000 quanta/s. *C*, the xenon lamp, which photolyses a smaller percentage of DM-nitrophen than the mercury lamp, was used to photolyse DM-nitrophen for 20 s in a different preparation than in panel *A* or *B*. The rise time to peak for the MEJP frequency was approximately 3 s, with a peak response of 13500 quanta/s.

photolysis are due neither to magnesium released nor DM-nitrophen's photoproducts, for then they should occur in both normal and zero-calcium media whether or not DM-nitrophen was loaded with calcium.

Photolysis of caged calcium at lower light intensities produces a slower rate of rise of transmitter release

A lower light intensity, either by using neutral density filters or the xenon light source (which photolyses a smaller percentage of the DM-nitrophen than the mercury

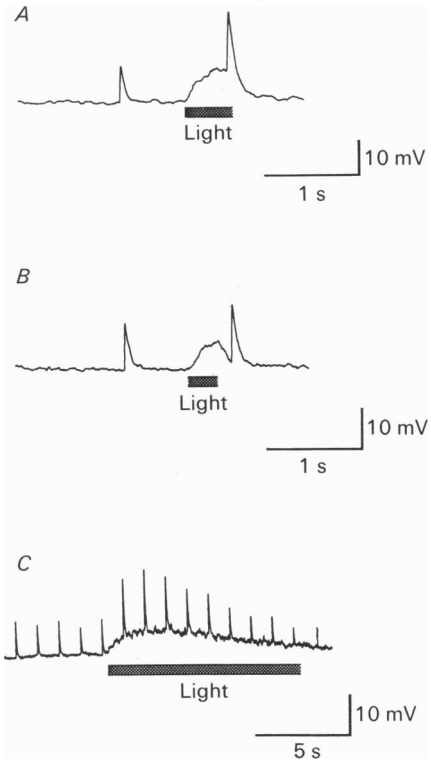

Fig. 5. The time course for the decay of transmitter release which occurs due to DM-nitrophen photolysis is dependent on the length of light exposure. *A*, a 500 ms UV light exposure, indicated by the bar, resulted in a rapid decline of spontaneous transmitter release from about 7000 quanta/s to pre-light levels following shutter closure. EJPs were evoked at approximately 0·9 Hz. Due to short light exposure and low frequency stimulation, only one EJP occurred during photolysis, and it increased by 70%. *B*, a second 300 ms light exposure in the same muscle fibre as in *A* also gave a rapid decline of spontaneous transmitter release from approximately 5000 quanta/s to pre-light values upon shutter closure. The EJP was increased by 30% during the short light exposure. *C*, a third light exposure for 10 s in the same muscle fibre as shown in *A* and *B* produced a slow decline of transmitter release lasting many seconds. The MEJP frequency increased to 4500 quanta/s during the light while the EJP amplitude increased to about twice the prior amplitude.

lamp) produced a slower rate of transmitter release to photolysis. This is similar to the results of Goldman & Kaplan (1988) who reported decreased levels of skeletal muscle tension and onset rates in response to decreased light energy. Shown in Fig. 4*A* is the postsynaptic response to a 2 s exposure using the mercury light and two 25% transmission filters, reducing the light transmission to 6%. The rise time to

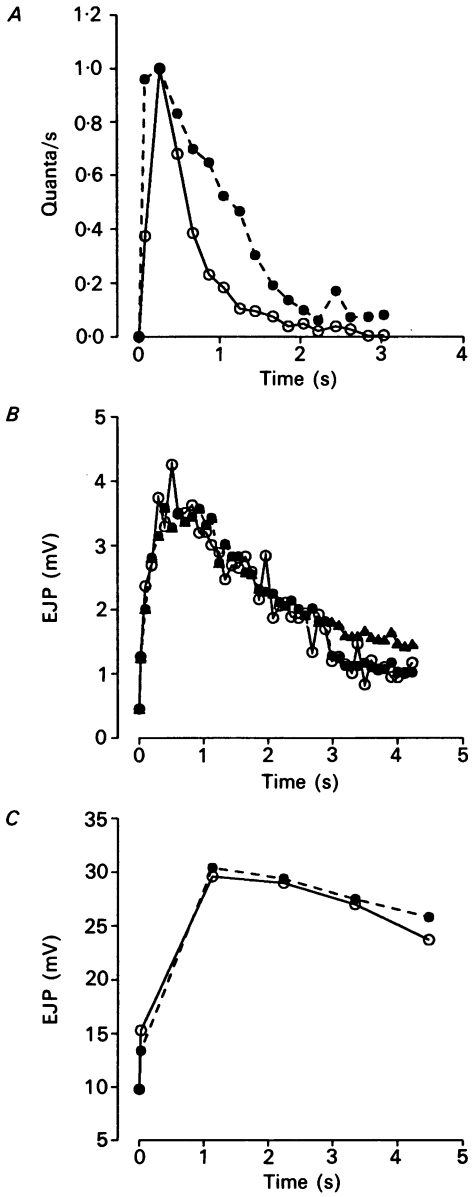

Fig. 6. *A*, rise and fall of MEJP frequency (○) and EJP amplitude (●) over time during DM-nitrophen photolysis. Photolysis of DM-nitrophen elevated presynaptic $[Ca^{2+}]_i$ and produced different rates for the rise and fall of MEJP frequency *versus* the EJP amplitude. Both EJP and MEJP frequency were measured in quanta per second with their largest response normalized to one. The EJP amplitude rose faster than the MEJP frequency in response to elevation of $[Ca^{2+}]_i$, but declined to pre-light levels more slowly than the MEJP frequency. The change in MEJP frequency *versus* EJP amplitude during light photolysis was not proportional and reflects a non-linearity in the release process. *B* and *C*, predictions of a non-linear summation model for transmitter release. *B*, measured values for EJP amplitudes and MEJP frequency during light photolysis and elevation of $[Ca^{2+}]_i$ were fitted to a non-linear calcium model for transmitter release using a calcium co-operativity of five. The predicted values for EJP amplitudes using one value for K (▲),

maximal response for the MEJP frequency was 1·7 s compared to the same muscle fibre illuminated again for 2 s using only one 25% transmission filter (Fig. 4*B*), where the time to maximal response was 0·9 s. In comparison an experiment in which the xenon lamp was used is shown in Fig. 4*C*: the rise time for the MEJP frequency to peak was approximately 3 s.

Short light exposure results in rapid decay of the postsynaptic response versus long light exposures whose responses decline over seconds

If the amount of calcium released from DM-nitrophen does not exceed the remaining unphotolysed DM-nitrophen that is not already bound to calcium, then the released calcium can quickly rebind to the unphotolysed DM-nitrophen (Kaplan, 1990). If the amount of calcium release exceeds the amount of still unphotolysed and unbound DM-nitrophen, then the released calcium will bind to native buffers, be taken up into intracellular organelles, or be extruded out of the terminal through pumps and exchangers known to exist in these terminals (Zucker *et al.* 1991). A short light exposure, i.e. 300–500 ms, photolyses approximately 3–5% (xenon lamp) or 15–25% (mercury lamp) of the DM-nitrophen and following shutter closure, recently released calcium is free to rebind to unphotolysed DM-nitrophen. If the rebinding of calcium takes about 3 ms, as it does for EGTA (Smith *et al.* 1984), the calcium released by photolysis within the last 3 ms would remain free. This concentration is about 3 μM for 5 mM DM-nitrophen, 50% loaded with calcium. Therefore, the free calcium will be approximately 3 μM until the shutter closes, but will rapidly re-equilibrate with the unphotolysed DM-nitrophen approaching a steady-state level of less than 1 μM. This is demonstrated by the rapid decline in transmitter release shown in Fig. 5*A* and *B* following 500 and 300 ms xenon light exposures respectively in the same muscle fibre. A third illumination of light in the same muscle fibre for 10 s produced a slow decline of transmitter (Fig. 5*C*) probably due to the decay of free calcium by active extrusion and buffering, after complete photolysis of DM-nitrophen. Another example of rapid transmitter decay following shutter closure, due to rebinding of free calcium to unphotolysed DM-nitrophen, is shown in Fig. 4*A* and *B*, after 2 s illumination at 6% transmission and 25% transmission respectively, using the mercury lamp. Apparently the dim light in Fig. 4*A* photolysed little DM-nitrophen, as with the brief exposure of Fig. 5*A* and *B*. The bright light of Fig. 4*B* caused an intermediate rate of MEJP frequency decay after shutter closure, perhaps due to the displacement of magnesium by released calcium when the remaining unphotolysed DM-nitrophen is all bound to magnesium (Delaney & Zucker, 1990).

Increase in intracellular calcium through a caged calcium compound supports a non-linear calcium-release hypothesis

In eight experiments, EJP amplitude and MEJP frequency were measured continuously during the photolysis of DM-nitrophen. EJP amplitude and MEJP frequency rose for up to 500 ms (mercury lamp) or 2 s (xenon lamp), and then

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or with K changing at 3·0 s (●) were plotted against the observed values (○). *C*, in a different preparation from *B*, the observed values for EJP amplitudes (○) matched predicted values (●) derived from MEJP frequency during DM-nitrophen photolysis with a single value of K.

declined during continued illumination. However, the changes in these two indices of transmitter release were not proportional (see Fig. 6*A*), indicating a non-linearity somewhere in the release process. We used eqn (5) to determine how well the data fitted a non-linear calcium model for transmitter release. We substituted into eqn (5) values for MEJP frequency and EJP amplitudes during light photolysis and elevated intracellular calcium and calculated the value for K from these potentiated values. In five of the eight preparations, K appeared to decrease over the course of the light exposure. The peak value of K ranged from 402 to 22852. Figure 6*B* shows the predicted EJP values with the observed EJP values plotted *versus* time using two calculated values of K. In three preparations the value for K remained constant throughout the light exposure and plots of the predicted EJPs *verus* time resembled the experimental values (see Fig. 6*C* for an example).

DISCUSSION

Recent objections to the non-linear calcium-release model

A linear dependence of EJP amplitude on presynaptic calcium concentration (as measured in crayfish) was shown recently for post-tetanic potentiation (PTP) (Delaney, Zucker & Tank, 1989) and a slow component of facilitation called augmentation (Delaney, Llinás & Tank, 1990) after a long tetanus, although in the first few seconds following the tetanus, the EJP amplitude departed from a linear dependence on intracellular calcium. In contrast, our study suggests a non-linear relationship between EJP amplitude and $[Ca^{2+}]_i$, when $[Ca^{2+}]_i$ was elevated for 0·3–20 s. Our experiments dealt with shorter time periods, more like a brief tetanic train, where $[Ca^{2+}]_i$ is elevated for seconds, whereas the PTP study elevated $[Ca^{2+}]_i$ for minutes. A possible explanation for the linear dependence of transmitter release on intracellular calcium (Delaney *et al.* 1989) is that there is a secondary process (affected by longer tetani) which adds to the facilitation that would be expected from a non-linear summation model, making the EJP appear more nearly linearly related to the intracellular calcium.

Limitations of caged compounds in studying the calcium dependence of spontaneous and evoked release

We have explored a new method in which calcium can be elevated to the micromolar range in presynaptic terminals for periods of seconds during which MEJP frequency and evoked release can be measured. There are limitations to this technique which may prevent an accurate support and prediction of a non-linear calcium hypothesis. One is that the caged calcium compound, DM-nitrophen, as applied in this study can only be used as a qualitative tool. We have not been able to quantify how much calcium is being released from the DM-nitrophen upon photolysis. Only if a calcium indicator were co-injected with DM-nitrophen into the terminals, would approximate $[Ca^{2+}]_i$ be known following light exposure. Therefore, since we could not directly measure the $[Ca^{2+}]_i$ in these experiments, we could only relate the EJP amplitude to the MEJP frequency.

We can estimate, however, the $[Ca^{2+}]_i$ in the presence of unphotolysed DM-nitrophen and during photolysis. We believe the $[Ca^{2+}]_i$ before photolysis to be

0·2–0·7 μM for the following reasons. (1) The normal resting $[Ca^{2+}]_i$ is approximately 0·1 μM, as measured in crayfish presynaptic terminals (Mulkey & Zucker, 1991). (2) Calculations of $[Ca^{2+}]_i$ following injection of 5 mM DM-nitrophen, 30% calcium-loaded, assuming an intracellular concentration of 2 mM Mg^{2+} and 1 mM MgATP (solved using MathCad, see Delaney & Zucker, 1990) would yield a free calcium concentration of 40 nM. (3) We know photolysis of calcium-free DM-nitrophen (injected by iontophoresis) increased MEJP frequency and EJP amplitude, so the DM-nitrophen must become loaded with calcium in the cytoplasm to a higher level than what is injected. This is probably true even when 30% calcium-loaded DM-nitrophen is injected. (4) Injection of 30 or 60% loaded DM-nitrophen often results in increased EJP amplitude and MEJP frequency, when MEJPs are detectable. Therefore, resting calcium before photolysis appears to be raised above its normal level of 0·1 μM by injection of 30 or 60% calcium-loaded DM-nitrophen. The loading of 30% DM-nitrophen would have to increase to only 50% for the free $[Ca^{2+}]_i$ to rise to 0·2 μM and at 60% calcium-loading, the free $[Ca^{2+}]_i$ would be around 0·7 μM. Thus we estimate the free $[Ca^{2+}]_i$, following pressure injection of 30 or 60% calcium-loaded DM-nitrophen, to be somewhere between 0·2 and 0·7 μM. Photolysis of 20% of the DM-nitrophen, loaded with 50% calcium, should raise intracellular calcium to the micromolar range.

Inferences about Ca_R^{2+}/Ca_E^{2+}

A non-linear summation model was used to relate MEJP frequency to EJP amplitude during light exposures that elevate presynaptic calcium. When the data were fitted to one formulation of this model (eqn (5)), values of Ca_R^{2+}/Ca_E^{2+} were calculated to be in the range of 0·68–1·53 in experiments in which MEJP frequency (calculated from eqn (1)) increased to 3000–11000 quanta/s.

This result is a little surprising. We estimate Ca_R^{2+} will rise to about 4 μM when DM-nitrophen is photolysed, while the peak $[Ca^{2+}]_i$ at release sites during action potentials probably reaches at least 10–30 μM (Fogelson & Zucker, 1985; Zucker & Fogelson, 1986), and perhaps closer to 100 μM (Adler, Augustine, Duffy & Charlton, 1991; Yamada & Zucker, 1992). We thus expect Ca_R^{2+}/Ca_E^{2+} to be about 0·1, in which case the EJPs should be larger than we observed or the MEJP frequency should have increased by less.

Several factors might contribute to this discrepancy. (1) DM-nitrophen acts uniformly at all release sites, while action potentials may evoke release from only those sites near clusters of calcium channels that open simultaneously in an action potential. In that case, we should reduce our measure of MEJP frequency by the fraction of release sites activated by calcium influx in action potentials, in order to compare release from the same release sites. However, even if f is reduced to 1% in eqn (5), Ca_R^{2+}/Ca_E^{2+} is still 0·4, and it seems unlikely that action potentials evoke release of less than 1% of the available quanta. (2) We may have underestimated $[Ca^{2+}]_i$ during light exposure, so it may actually be more than 10% of Ca_E^{2+}. (3) The calculation of Ca_R^{2+}/Ca_E^{2+} from eqn (5) is sensitive to the values of quantal size (q) and phasic release time course (T), and our estimates of these values may be in error. (4) Following DM-nitrophen photolysis, EJP amplitudes were often quite large (Figs 3 and 5). We may have undercorrected EJP amplitudes that were subject to saturation

of processes leading to secretion. (5) Calcium may reduce EJPs by activating presynaptic calcium-dependent potassium conductance (Sivaramakrishnan, Bittner & Broadwick, 1991), shunting presynaptic action potentials. (6) Our model may oversimplify the relationship relating transmitter release to $[Ca^{2+}]_i$ (Yamada & Zucker, 1992).

We also observed that in some preparations the parameter K fell during light exposure, i.e. EJP amplitudes dropped below what was predicted from MEJP frequency using the value of K derived from responses early during illumination. This could be due to an oversimplified formulation of the relation between calcium and transmitter release, to a progressive activation of calcium-dependent potassium current and consequent spike reduction, or to synaptic depression. Even though depression at this synapse is uncommon (Hatt & Smith, 1976), we are increasing, in some cases, MEJP frequency to a range equivalent to 50–100 action potentials per second. Despite these complications, we observe a non-linear relation between EJP amplitude and MEJP frequency during the early rising and subsequent falling phases of the responses to illumination. The results are generally consistent with a model relating EJP amplitude and MEJP frequency to changes in intracellular calcium. During the initial period of light exposure, calcium should be increasing continuously. Once DM-nitrophen is fully photolysed, calcium should decline as it is pumped out through Na^+–Ca^{2+} exchange (Zucker *et al.* 1991), or taken up into internal organelles.

Injection of unloaded DM-nitrophen evokes release in a calcium-containing Ringer solution

We originally thought iontophoresis of DM-nitrophen into presynaptic axons would not produce transmitter release upon photolysis, as the DM-nitrophen had no calcium bound to it. Kao, Harootunian & Tsien (1989) injected fibroblasts with unloaded nitr-5, a photolabile calcium chelator, and subsequently measured calcium release upon photolysis with fluo-3 (a fluorescent calcium indicator), proving that caged calcium chelators are capable of binding calcium upon introduction to the intracellular environment. In the squid giant synapse, injection of unloaded DM-nitrophen produced transmitter release to a flash in only one out of six preparations (Delaney & Zucker, 1990). This small response was attributed to the loading of DM-nitrophen, either in the tip of the electrode from calcium in the saline prior to penetrating the synapse, with calcium from intracellular stores, or by calcium leaking into the synapse due to injection damage. In contrast, we consistently observed robust responses to photolysis of unloaded DM-nitrophen. This effect at crayfish terminals could also be attributed to a calcium leak into the axon as a result of injection damage, as it was seen only when external calcium was present, or perhaps there is a difference between the handling of intracellular calcium in the squid *versus* the crayfish presynaptic terminal. It is possible crayfish are more susceptible than squid to a 'leak' of calcium into terminals from the external medium when intracellular calcium is abnormally low. This 'leak' could be due to Na^+–Ca^{2+} exchange operating in reverse (see Mulkey & Zucker, 1992). The higher surface to volume ratio of crayfish terminals might also facilitate the loading of injected DM-nitrophen by extracellular calcium.

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