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CALCIUM RELEASED BY PHOTOLYSIS OF DM-NITROPHEN STIMULATES TRANSMITTER RELEASE AT SQUID GIANT SYNAPSE

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SUMMARY

1. Transmitter release at the squid giant synapse was stimulated by photolytic release of Ca^{2+} from the 'caged' Ca^{2+} compound DM-nitrophen (Kaplan & Ellis-Davies, 1988) inserted into presynaptic terminals.

2. Competing binding reactions cause the amount of Ca^{2+} released by DM-nitrophen photolysis to depend on the concentrations of DM-nitrophen, total Ca^{2+}, Mg^{+}, ATP and native cytoplasmic Ca^{2+} buffer. Measurements of presynaptic [Ca^{2+}] changes by co-injection of the fluorescent indicator dye Fura-2 show that DM-nitrophen photolysis causes a transient rise in Ca^{2+} followed by decay within about 150 ms to an increased steady-state level.

3. Rapid photolysis of Ca^{2+}-loaded nitrophen within the presynaptic terminal was followed in less than a millisecond by depolarization of the postsynaptic membrane. As with action potential-evoked excitatory postsynaptic potentials (EPSPs), the light-evoked response was partially and reversibly blocked by 1–3 mM-kainic acid which desensitizes postsynaptic glutamate receptors.

4. Release was similar in magnitude and rate to normal action potential-mediated EPSPs.

5. The release of transmitter by photolysis of Ca^{2+}-loaded DM-nitrophen was not affected by removal of Ca^{2+} from the saline or addition of tetrodotoxin. Photolysis of DM-nitrophen injected into presynaptic terminals without added Ca^{2+} did not stimulate release of transmitter nor did it interfere with normal action potential-mediated release.

6. Stimulation of presynaptic action potentials in Ca^{2+}-free saline during the light-evoked response did not elicit increased release of transmitter if the ganglion was bathed in Ca^{2+}-free saline, i.e. in the absence of Ca^{2+} influx. Increasing the intensity of the light or stimulating presynaptic action potentials in Ca^{2+}-containing saline increased the release of transmitter. Therefore the failure of presynaptic voltage change to increase transmitter release resulting from release of caged Ca^{2+} was not due to saturation or inhibition of the release mechanism by light-released Ca^{2+}.

7. Decreasing the temperature of the preparation increased the delay to onset of

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the light-evoked response and reduced its amplitude and rate of rise to an extent similar to that observed for action potential-evoked EPSPs.

INTRODUCTION

Calcium, entering the presynaptic terminal through voltage-gated Ca^{2+} channels, is essential for the normal release of chemical transmitter substances into the synaptic cleft (Katz, 1969). Numerous questions remain about the process by which elevation of intracellular Ca^{2+} leads to release of neurotransmitter (Augustine, Charlton & Smith, 1987; Smith & Augustine, 1988) including whether the sudden large increase in submembrane Ca^{2+} concentration acts in concert with the change in the voltage across the membrane that occurs during invasion of the terminal by the action potential to promote release (Zucker, Landò & Fogelson, 1986; Dudel, 1988). Rapid flash-photolysis of caged Ca^{2+} compounds provides a new and powerful technique for examining many aspects of the transmitter release process.

Manipulation of submembrane Ca^{2+} concentration independent of voltage within the synaptic terminal is difficult because it is normally necessary to depolarize the membrane in order to generate Ca^{2+} influx through voltage-gated Ca^{2+} channels. In the squid giant synapse, direct injection of Ca^{2+} or Ca^{2+}-buffered solutions (Miledi, 1973; Charlton, Smith & Zucker, 1982; Llinás, Sugimori & Walton, 1987) indicates that increased intracellular Ca^{2+} is by itself sufficient to greatly accelerate non-synchronous release of transmitter quanta. Direct injections of high concentrations of Ca^{2+} produce highly variable responses apparently dependent upon the position of the pipette relative to release sites and the restricted diffusion of Ca^{2+} within the terminal as a result of cytoplasmic Ca^{2+} buffers and uptake into organelles. These limitations reduce the utility of this technique for quantitative or kinetic studies. Elevation of intracellular Ca^{2+} by fusion of Ca^{2+}-loaded vesicles, or by application of Ca^{2+} ionophores, also suggests that increasing presynaptic Ca^{2+} concentration increases the rate of release of transmitter without requiring a change in transmembrane voltage (Statham & Duncan, 1975; Rahamimoff, Meiri, Erulkar & Barenholz, 1978; Zucker & Landò, 1986). None of these techniques permits widespread elevation of $[Ca^{2+}]$ in the presynaptic terminal within a millisecond and so do not mimic the time course of magnitude of the submembrane $[Ca^{2+}]$ transient which occurs after invasion of the terminal by an action potential. We have used the ability to uniformly elevate the submembrane $[Ca^{2+}]$ from resting level 1–10 μM with temporal control of a millisecond or less with and without concurrent changes in transmembrane voltage to test directly the hypothesis that voltage and high Ca^{2+} act together to produce normal release of transmitter (Dudel, Parnas & Parnas, 1983; Dudel, 1984*a*, *b*, 1988; Parnas & Segal, 1984; Parnas & Parnas, 1986; Parnas, Dudel & Parnas, 1986; Zucker & Landò, 1986; Zucker *et al.* 1986; Zucker & Haydon, 1988).

The release of transmitter is a biochemical process with an unknown number of steps. The initial steps involve the activation, opening and closing of voltage-dependent Ca^{2+} channels to produce the Ca^{2+} influx. Elegant voltage-clamp studies have revealed to some extent how the kinetics of the Ca^{2+} channels and therefore the Ca^{2+} current participate in the timing and kinetics of the release process (Llinás, Steinberg & Walton, 1981; Augustine *et al.* 1985). However, the kinetics of processes

which are activated by the Ca^{2+} influx are temporally coincident with much of the Ca^{2+} channel activity making it difficult to manipulate and examine them in isolation. We have used rapid photolytic release of caged Ca^{2+} to study the modulation of Ca^{2+}-activated transmitter release processes by temperature without the complication of effects on Ca^{2+} current.

METHODS

All experiments were performed on squid (*Loligo opalescens*) of approximately 12–18 cm mantle length obtained by hand collection near Hopkins Marine Station, Monterey, CA, USA. Squid were transported to Berkeley where they were maintained in a 600 gallon recirculating artificial seawater system. Squid were fed small goldfish daily and survival was approximately 30–60% during two weeks. Females were used for almost all experiments. Stellate ganglia were dissected and prepared for intracellular recording using previously published protocols (Charlton *et al.* 1982). Presynaptic action potentials were elicited using a suction electrode placed on the pallial nerve. Presynaptic spikes and membrane potential were monitored with the DM-nitrophen injection electrode and postsynaptic responses were recorded with electrodes filled with 5 M-CsCl. In a few experiments, postsynaptic current was recorded using a conventional two-electrode voltage-clamp configuration.

All electrophysiological data were obtained and analysed using a digital oscilloscope (Nicolet, Madison, WI, USA, Model 4094). Ganglia were constantly perfused with oxygenated (99·5% O_2:0·5% CO_2) saline. Normal saline contained 466 mM-NaCl, 10 mM-KCl, 11 mM-$CaCl_2$, 54 mM-$MgCl_2$, 3 mM-$NaHCO_3$ and 10 mM-Na-HEPES buffered to pH 7·2. Carbon dioxide and bicarbonate served to maintain intracellular pH buffering. Low-Ca^{2+} saline contained 0·5 mM-$CaCl_2$, 57 mM-$MgCl_2$ and 7 mM-$MnCl_2$. Zero-Ca^{2+} saline contained 456 mM-NaCl, 10 mM-KCl, 0 mM-$CaCl_2$, 65 mM-$MgCl_2$, 3 mM-$NaHCO_3$, 10 mM-Na-HEPES and 10 mM-Na-EGTA. In some experiments 2 μM-tetrodotoxin (TTX, Calbiochem, La Jolla, CA, USA) was added to the saline. The temperature of the preparation was controlled between 6 and 25 °C with a peltier device. Most experiments were performed at 19 ± 2 °C.

A Ca^{2+}-activated K^+ current sensitive to tetraethylammonium (TEA) is observed in squid giant presynaptic terminals (Augustine & Eckert, 1982). A hyperpolarization of the presynaptic terminal follows photolysis of DM-nitrophen which is blocked by 20 mM-TEA. This amount of TEA has no effect on the delayed rectifier current (Tasaki & Hagiwara, 1957), and we observed no effect on presynaptic action potential duration. TEA (20 mM, Sigma, St Louis, MO, USA) was added to the saline in most experiments.

Solutions containing 72 mM-DM-nitrophen (Kaplan & Ellis-Davies, 1988) in distilled water were pressure injected into the presynaptic terminal through double-bevelled 1·2 mm outer diameter standard wall microelectrodes. This double bevelling technique provided a wide-tip opening with a much sharper point than was obtained with a single bevel and contributed significantly to the success of the experiments by permitting clean impalement of the presynaptic terminal and rapid filling of the terminal with DM-nitrophen. Pressure was applied with brief pulses, usually less than 100 ms duration using a stimulator-controlled solenoid valve (Clippard Instrument Lab., Cincinnati, OH, USA). DM-nitrophen solutions were injected which contained either no Ca^{2+} (zero-Ca^{2+}) or a mixture of 32% Ca^{2+} and DM-nitrophen (32%-Ca^{2+}- and 68%-Ca^{2+}-free DM-nitrophen). DM-nitrophen was the generous gift of Drs J. H. Kaplan and G. Ellis-Davies.

Arsenazo III (Sigma, St. Louis, MO, USA) was added at 1·8 mM to the DM-nitrophen solutions to monitor the injection and to provide a rough estimate of the concentration of DM-nitrophen in the terminal after the injection. After injection final concentrations of Arsenazo III never exceeded 0·4 mM. An estimate of the concentration of DM-nitrophen in the presynaptic terminal was obtained by visually comparing the colour of the synapse after injection of DM-nitrophen mixed with arsenazo III with the colour of 0·1–0·5 mM-Arsenazo III solutions in 20–50 μm pathlength micro-cuvettes (Vitro Dynamics, Rockaway, NY, USA). Since the ratio of Arsenazo III to DM-nitrophen in the injection solution was kept constant at 1:40 we were able to estimate that we had DM-nitrophen concentrations in presynaptic terminals of at least 2 mM when we could detect a change in colour and that we did not exceed 15–20 mM when we finished injecting.

Photolysis of DM-nitrophen was accomplished using either brief, intense discharges from a 75 W

xenon arc lamp (Chadwick–Helmuth, El Monte, CA, USA) or a lower intensity collimated light beam from a continuously run xenon lamp (150 W Cermax, ILC Technology, Sunnyvale, CA, USA) fitted with a Uniblitz shutter (Model 225LOAOW5, Vincent Asso., Rochester, NY, USA). The Chadwick–Helmuth lamp and power supply were modified as described in Landò & Zucker (1989) to permit delivery of light flashes from a capacitive discharge of between 20 and 200 J electrical energy, with 75% of the light delivered within 400 μs.

In some experiments, the DM-nitrophen pipette contained 1 mM-Fura-2 (pentapotassium salt, Molecular Probes, Eugene, OR, USA) in order to measure change in presynaptic [Ca^{2+}] caused by photolysis of DM-nitrophen. Elevation of Ca^{2+} was detected by measuring the decrease in Fura-2 fluorescence above 510 nm. Low levels of 385 nm illumination were used to excite Fura-2, to limit photolysis of DM-nitrophen to < 1% during the measurement of Ca^{2+} following each flash. We used a photomultiplier tube (Type 9789, EMI Gencom, Plainview, NY, USA) attached to an upright epifluorescent microscope (Optiphot, Nikon, Garden City, NY, USA) equipped with a Nikon CF-E 10X long working distance (5·2 mm) air objective. An electronic shutter opening within 2 ms (Uniblitz Model 225LOAOT5, A. W. Vincent, Rochester, NY, USA) protected the photomultiplier tube from damage during the flash used for DM-nitrophen photolysis.

We calibrated our light sources in terms of their DM-nitrophen photolysing efficiencies by filling 100 μm pathlength cuvettes (Vitro Dynamics, Rockaway, NJ, USA) with a solution designed to mimic the intracellular ionic environment, comprised of 13 mM-DM-nitrophen, 200 mM-KCl, 3 mM-ATP, 6·5 mM-$MgCl_2$, 4·2 mM-$CaCl_2$ and 50 mM-K^+-HEPES, pH 7·3. The effect of light exposures on the absorbance spectrum of DM-nitrophen (measured with a Lambda Array 3840 spectrophotometer, Perkin-Elmer, Norwalk, CT, USA) was used to determine the average photolysis of DM-nitrophen in the cuvettes, following procedures described in Tsien & Zucker (1986). After correction for the effect of absorbance by DM-nitrophen on the average light intensity in the cuvette we calculate that a 200 J discharge of the Chadwick–Helmuth flash lamp or a 2 s exposure of the Cermax light source run at 15 A photolyses approximately 20% of the DM-nitrophen at the surface of the cuvette facing the light.

RESULTS

Photolysis of Ca^{2+}-loaded DM-nitrophen in artificial squid cytoplasm

In order to interpret synaptic responses to flash photolysis of presynaptically injected DM-nitrophen, we need to have some idea of the effect of such photolysis on presynaptic Ca^{2+} levels. The photochemistry of DM-nitrophen is rather complex. Upon exposure to ultraviolet light, DM-nitrophen is converted from a high-affinity Ca^{2+} buffer to an iminodiacetic acid and nitrosoacetophenone iminodiacetic acid with very weak Ca^{2+} affinities. The photolysis of DM-nitrophen may be summarized by the following reaction:

$$H^+ + (HNPh)^{3-} + h\nu \rightarrow NIA^- + IA^-,$$

where NPh represents DM-nitrophen, NIA represents nitrosoacetophenone-substituted iminodiacetic acid, IA represents iminodiacetic acid and $h\nu$ denotes the absorption of a photon of light. This photolysis proceeds with a quantum efficiency of about 0·18 for 350 nm light, and is accompanied by some change in protonation of the amine groups. Analogous reactions occur when DM-nitrophen is bound to divalent cations.

In a cell containing partially photolysed DM-nitrophen, the concentration of free Ca^{2+} will depend on the affinities of DM-nitrophen and its photoproducts for Ca^{2+}. DM-nitrophen binds Ca^{2+} with a dissociation constant of 5 nM at 33 °C, pH 7·1, and 135 mM ionic strength (Kaplan & Ellis-Davies, 1988). The iminodiacetic photoproducts bind Ca^{2+} only very weakly, with dissociation constants of about 3 mM. However, DM-nitrophen also binds Mg^{2+} at the same site as Ca^{2+}, with a dissociation

constant of 2·5 μM under the above conditions, and the photoproducts bind Mg^{2+} about as weakly as they do Ca^{2+} (Goldman & Kaplan, 1988). Since Mg^{2+} will tend to displace Ca^{2+} from DM-nitrophen, the free Ca^{2+} concentration in a mixture containing DM-nitrophen will depend on both the total Mg^{2+} and Ca^{2+} concentrations, as well as the concentrations of DM-nitrophen and its photoproducts.

The total concentration of Mg^{2+} in squid cytoplasm is about 6·5 mM, about 3·5 mM of which is free and the remainder is bound to anions like ATP (Baker & Crawford, 1972; Brinley & Scarpa, 1975), with a dissociation constant of about 700 μM (Brinley, Tiffert, Scarpa & Mullins, 1977). Since Mg^{2+} binding to ATP is weaker than its binding to DM-nitrophen, some Mg^{2+} will dissociate from ATP and bind to DM-nitrophen when the latter is added to cytoplasm. The Ca^{2+} released by photolysis of DM-nitrophen will be strongly bound by unphotolysed DM-nitrophen as long as the latter remains in excess. However, once sufficient photolysis occurs that the Ca^{2+} injected with the DM-nitrophen exceeds the remaining DM-nitrophen, the 'uncaged Ca^{2+}' will bind to native cytoplasmic Ca^{2+} buffers which have Ca^{2+} dissociation constants (K_Ds) of 0·1–50 μM (Baker & DiPolo, 1984). Thus any attempt to estimate the free Ca^{2+} concentration in a cell injected with DM-nitrophen must take account of all of the following reactions:

$$Ca^{2+} + NPh^{4-} \rightleftharpoons CaNPh^{2-} \quad K_D = 10^{-8}\ \text{M}$$
$$Ca^{2+} + NIA^{-} \rightleftharpoons CaNIA^{+} \quad K_D = 0{\cdot}006\ \text{M}$$
$$Ca^{2+} + B^{2-} \rightleftharpoons CaB \quad K_D = 2{\cdot}5 \times 10^{-5}\ \text{M}$$
$$Mg^{2+} + NPh^{4-} \rightleftharpoons MgNPh^{2-} \quad K_D = 5 \times 10^{-6}\ \text{M}$$
$$Mg^{2+} + NIA^{-} \rightleftharpoons MgNIA^{+} \quad K_D = 0{\cdot}006\ \text{M}$$
$$Mg^{2+} + ATP^{2-} \rightleftharpoons MgATP \quad K_D = 10^{-4}\ \text{M}.$$

In the above list, the dissociation constants for Mg^{2+} and Ca^{2+} binding to DM-nitrophen and nitrosoacetophenone iminodiacetic acid have been adjusted upwards by a factor of two from their measured values at low ionic strength to account for the likely effects of the ionic strength of marine cytoplasm (Harrison & Bers, 1987). Even these equations do not represent fully the chemistry of DM-nitrophen in cells, since the separate weak binding of Ca^{2+} and Mg^{2+} by IA^{-} have not been included, and the different photoproducts formed by photolysis of bound and free DM-nitrophen (Kaplan & Ellis-Davies, 1988) have not been considered.

The reaction rates for Ca^{2+} and Mg^{2+} binding to DM-nitrophen have not been measured. However, by analogy with the parent EDTA compound, we expect Ca^{2+} to equilibrate rapidly while Mg^{2+} should equilibrate more slowly (Eigen & Hammes, 1963). Rapid DM-nitrophen photolysis should suddenly release Ca^{2+} and Mg^{2+}, which then re-equilibrate with available buffers in two phases. In the first phase, released Ca^{2+} equilibrates rapidly with any remaining DM-nitrophen, its photoproducts, or native buffer not already bound to Ca^{2+} or Mg^{2+} (see Charlton *et al.* 1982 for evidence that Ca^{2+} equilibrates rapidly with native cytoplasmic buffers in squid), while released Mg^{2+} equilibrates rapidly with ATP. This phase results in a rapid rise in free Ca^{2+} with little rebinding to DM-nitrophen. In the second phase, released Mg^{2+} binds more slowly to any remaining DM-nitrophen, while released Ca^{2+} slowly displaces bound Mg^{2+} from unphotolysed DM-nitrophen. This second phase of re-equilibration

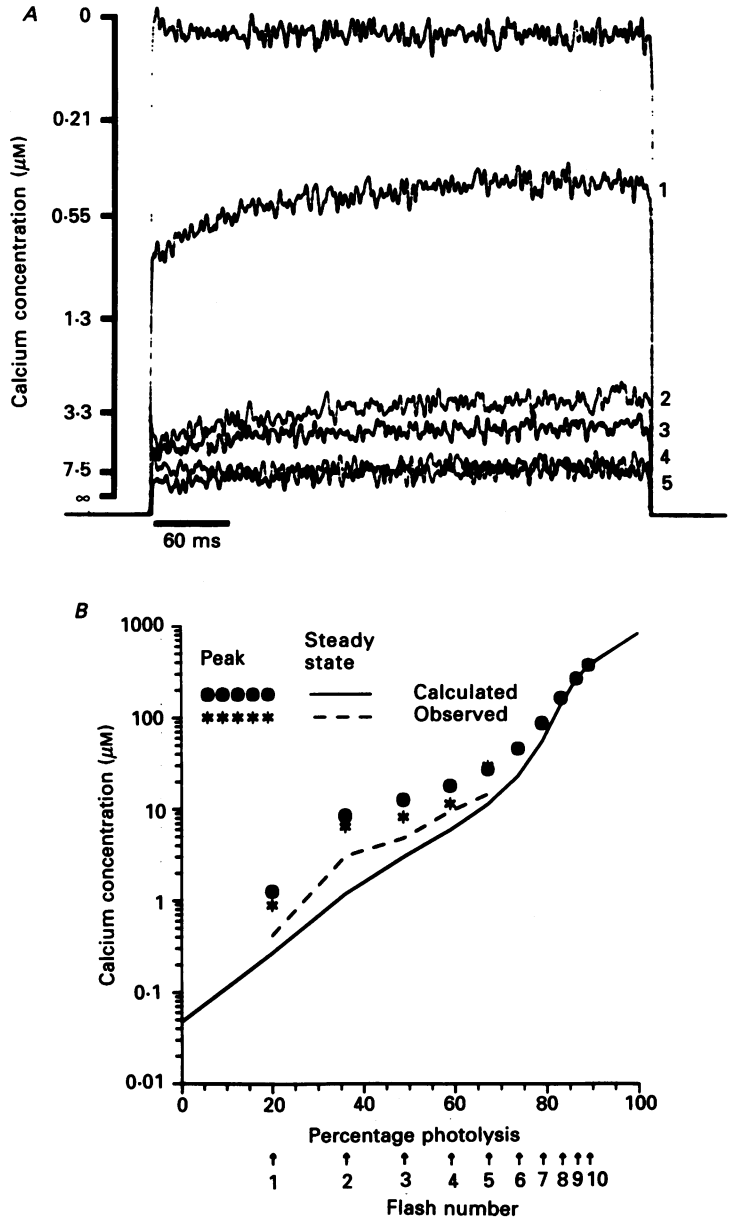

Fig. 1. *A*, Fura-2 fluorescence changes, excited at 385 nm, to 200 J light flashes, from a giant presynaptic terminal filled with a mixture of Fura-2 and 32%-Ca^{2+} DM-nitrophen. Individual traces are labelled with the flash number. *B*, predicted and observed effects of a series of 200 J flashes on [Ca^{2+}] in the same presynaptic terminal. Continuous lines show equilibrium responses to ten flashes, predicted from the simultaneous equilibration of binding reactions of Ca^{2+} and Mg^{2+} with photolysed and unphotolysed DM-nitrophen, ATP and cytoplasmic Ca^{2+} buffer. Dashed lines plot measurements from part *A*. Filled circles show calculated peak [Ca^{2+}] transients, assuming that the binding of Mg^{2+} to DM-nitrophen is slow, while asterisks represent measured [Ca^{2+}] transients.

should be dominated by the slow displacement of bound Mg^{2+} for Ca^{2+}, with a consequent sag in the concentration of free Ca^{2+}.

Measurements of Ca^{2+} photoreleased from DM-nitrophen in presynaptic terminals.

In order to confirm this theoretical description of DM-nitrophen's behaviour, we compared predictions of calculations of the simultaneous equilibration of the six reactions listed above, solved using MathCad (MathSoft, Cambridge, MA, USA), to measurements of Ca^{2+} released from DM-nitrophen in presynaptic terminals. Two terminals were injected with a mixture of 32 % Ca^{2+}-loaded DM-nitrophen and Fura-2 to final concentrations of about 10 mM and 100 μM respectively. We measured the fluorescence emitted above 510 nm excited at 385 nm in response to 200 J flashes of the Chadwick–Helmuth flash lamp. Fluorescence change was expressed as a fraction of the total loss of fluorescence when Fura-2 was saturated with Ca^{2+} by photolysis of most of the DM-nitrophen with several flashes. Assuming a low resting $[Ca^{2+}]$ before photolysis, we convert this to $[Ca^{2+}]$ using the data of Fig. 7 and the single wavelength formula (eqn (6)) of Grynkiewcz, Poenie & Tsien (1985), with a Ca^{2+}–Fura-2 dissociation constant of 775 nM appropriate for marine cytoplasm (Poenie, Alderton, Tsien & Steinhardt, 1985). This conversion provides as accurate an estimate of $[Ca^{2+}]$ as the two-wavelength ratiometric method, so long as the unbound Fura-2 fluorescence is known, pathlength and Fura-2 concentration remain constant, and Fura-2 bleaching is minimal.

Figure 1*A* shows a series of fluorescence changes in one of these experiments. A shutter in front of the photomultiplier tube was opened within 3–5 ms after each flash, and by this time the $[Ca^{2+}]$ had already risen from its resting level. The top trace shows $[Ca^{2+}]$ before the first flash, and responses to five sequential flashes repeated every 45 s are superimposed. Each trace ends with the closing of the photomultiplier shutter.

The first flash elicited a transient Ca^{2+} rise to at least 1 μM, indicated by a decline in the fluorescence excited at 385 nm, which decayed with a time constant of about 65 ms to about 0·4 μM. Reopening the shutter in the interval between flashes showed little additional change in fluorescence beyond that shown in Fig. 1*A*. Subsequent flashes excited increasing peak and steady-state Ca^{2+} elevations, with a similar relaxation rate, until the Fura-2 was fully saturated.

These experimental results are also plotted in Fig. 1*B*, with peak $[Ca^{2+}]$ transients represented by asterisks and steady-state levels by the dashed line. These results may be compared to the expected behaviour of DM-nitrophen in cytoplasm. To predict the initial Ca^{2+} level after a flash, we solved the system of reactions excluding the equation for the binding of Mg^{2+} to DM-nitrophen and assuming that $[MgNPh^{2-}]$ remained at its previous steady-state value, i.e. assuming no re-equilibration of Mg^{2+} with DM-nitrophen. Predicted initial Ca^{2+} concentrations are shown as filled circles in Fig. 1*B*. The continuous line shows steady-state $[Ca^{2+}]$ calculated by solving simultaneously all of the equilibrium equations, thus allowing $MgNPh^{2-}$ to form or dissociate. In solving these equations, we assumed a total $[Mg^{2+}]$ of 6·5 mM and a total [ATP] of 3·0 mM. We represented cytoplasmic Ca^{2+} binding with 1·25 mM of a buffer (Krinks, Klee, Pant & Gainer, 1988) with an average Ca^{2+} affinity of 12·5 μM. A total $[Ca^{2+}]$ equal to 32 % of the DM-nitrophen concentration was used. We selected a value of 9 mM for the latter, in the middle of the estimated range of

intracellular DM-nitrophen concentration after injection, and adjusted to give a good fit to the data of Fig. 1*B*. Calculations were made for a series of flashes each photolysing 20% of the remaining DM-nitrophen.

The predictions provide a good qualitative, albeit quantitatively imperfect, description of the measured Ca^{2+} transients, suggesting that our representation of the chemistry of DM-nitrophen may be a reasonably good first approximation.

Release of transmitter by photolysis of caged Ca^{2+}

Our first set of physiological experiments was designed to characterize the response of the postsynaptic membrane to photolysis of DM-nitrophen in the presynaptic terminal. With the synapse bathed in normal Ca^{2+}-containing saline, the action potential-evoked EPSP persisted after injection of unloaded and Ca^{2+}-loaded DM-nitrophen (Fig. 2*B*). At least a 20% reduction in the amplitude of the EPSP or a decrease in the rate of rise of the EPSP was seen after injection of 5–15 mM-32% Ca^{2+}-loaded DM-nitrophen.

If the EPSP disappeared or became much smaller during or after the injection of DM-nitrophen then no response or a much smaller than normal response to photolysis was seen. Since Arsenazo III was co-injected with DM-nitrophen we were able to detect whether the injection of DM-nitrophen was associated with a large rise in concentration of Ca^{2+} or Mg^{2+}. Whenever the Arsenazo III turned blue within the synapse during injection (indicating an increase in divalent cations) the EPSP rapidly decremented and disappeared, independent of changes in the action potential, and no response to photolysis of DM-nitrophen was observed. The sensitivity of this synapse to damage and the depleting effect of elevated resting Ca^{2+} have been previously documented (Miledi, 1973; Charlton *et al.* 1982; Adams, Takeda & Umbach, 1985; Llinás *et al.* 1987).

In view of the affinity of DM-nitrophen for Mg^{2+} (approximately 0·002 times that for Ca^{2+}) and the millimolar concentrations of free or weakly bound Mg^{2+} present in squid axoplasm (Baker & Crawford, 1972; Brinley & Scarpa, 1975), it was expected that Mg^{2+} would displace Ca^{2+} from DM-nitrophen when the concentration of DM-nitrophen within the cytoplasm was less than that of the cytoplasmic Mg^{2+}, i.e. below a few millimolar. Consistent with this prediction, injection of only a small amount of DM-nitrophen into the terminal resulted in a change in colour of the Arsenazo III from purple to blue, indicating a rise in $[Ca^{2+}]$, especially at the diffusion front. Because of this effect of Mg^{2+} it was necessary to inject quickly and fill most of the terminal within about 30 s to minimize the transient rise in $[Ca^{2+}]_i$ which would rapidly deplete the synapse of transmitter.

Figure 2A is typical of the responses seen in six experiments which employed a shutter-gated light source to photolyse DM-nitrophen. Rapid depolarization of the postsynaptic membrane followed onset of the light and peaked at about 1 mV (range 0·3–1·1 mV) within 200 ms and gradually decayed despite continued illumination. Approximately $5{\cdot}0 \times 10^5$ quanta were released during the period after the onset of the light in Fig. 2*A* assuming a single quantum of transmitter results in a PSP with a peak amplitude of 10 μV and a decay time constant of 2 ms (Miledi, 1973) or an approximate area of 20 μV ms. This corresponds to 300–500 action potential-evoked EPSPs. Since the postsynaptic potential is still decaying at the end of the record shown in Fig. 3*A* we have underestimated the total release with our measurement. It is possible that postsynaptic receptor desensitization occurs during the prolonged release of transmitter. This would also cause underestimation of the total amount of transmitter released. Five minutes after the two 5 s periods of illumination in the experiment of Fig. 2*A*, the membrane potential had returned to pre-light levels and a third 5 s illumination did not produce any further release. Two other experiments

using 10 s of illumination yielded total release estimates of $2{\cdot}3 \times 10^5$ and $2{\cdot}6 \times 10^5$ quanta during the 15 s after the onset of illumination. Membrane potentials returned to pre-photolysis levels in about 30 s and additional illumination produced no further transmitter release.

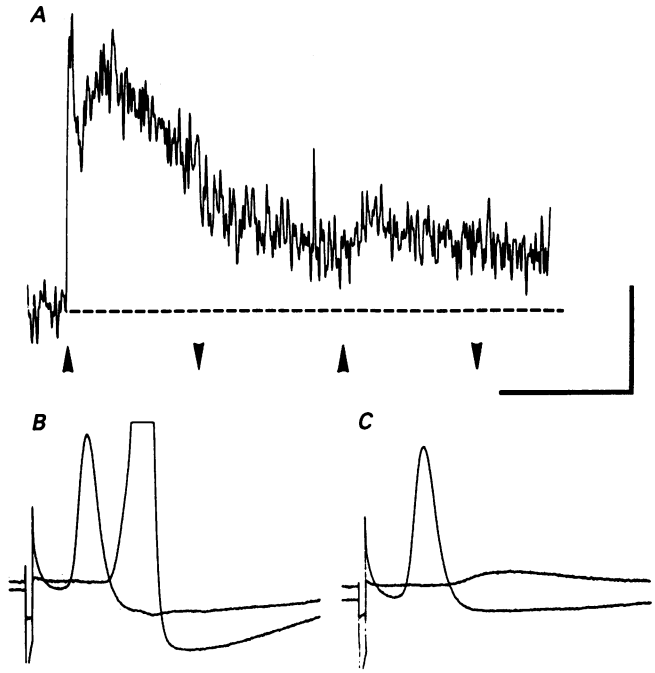

Fig. 2. Postsynaptic response to photolysis of DM-nitrophen with continuous low-intensity illumination. The preparation was bathed in normal saline. *A* shows the postsynaptic response resulting from two 5 s periods of 15 A illumination with a xenon arc lamp. The dashed line indicates average pre-light membrane potential. The duration of illumination was controlled with a shutter as indicated by the arrow-heads. *B* shows the suprathreshold postsynaptic response following a presynaptic action potential after injection of the terminal with 32%-Ca^{2+}-loaded DM-nitrophen. *C* shows the reduced postsynaptic response to a presynaptic action potential following photolysis of DM-nitrophen in the presynaptic terminal, measured 3 min after the offset of the light. Scale bars, 300 μV and 5 s for *A*, and 50 mV presynaptic, 12 mV postsynaptic, 3·8 ms for *B* and *C*.

Decline of the response to photolysis of DM-nitrophen and release of Ca^{2+} were always paralleled by a decrease in the size of the spike-evoked EPSP (Fig. 2*C*). Photolysis and release of Ca^{2+} did not appear to affect the amplitude or duration of the presynaptic action potential for up to 30 min after photolysis. However, no recovery of the action potential-evoked EPSP was observed up to 30 min after photolysis. In a few preparations a small, 1 mV or less, spike-evoked EPSP persisted after no further light-evoked responses could be elicited. This partial EPSP eventually disappeared within a few minutes after photolysis and probably reflects release from a part of the synapse which was not filled with DM-nitrophen at the time of exposure to light. The geometry of the presynaptic element of the giant synapse is complex and variable between preparations (Martin & Miledi, 1986) so it is likely

that a small finger of presynaptic terminal could have remained unfilled since light was delivered within 2–3 min of completing the injection.

Although the total postsynaptic response evoked by photolysis of DM-nitrophen with the low-intensity continuous light was equivalent to that of many hundreds of spike-evoked EPSPs, the peak voltage response and the initial rate of depolarization were much smaller than a normal EPSP. Depression of synaptic transmission at the squid giant synapse is normally substantial, so that repeated stimulation produces a rapid decline in EPSP amplitude unless release per action potential is greatly reduced by lowering Ca^{2+} influx (Kusano & Landau, 1975; Charlton & Bittner, 1978). Transmitter release equivalent to several action potential-mediated EPSPs occurs by the time of the peak postsynaptic response, i.e. within 200–300 ms of the start of photolysis. Release equivalent to more than 100 action potential-mediated EPSPs occurs by the time even higher Ca^{2+} concentrations are attained after several seconds of photolysis. Therefore, it is likely that the failure of the postsynaptic response to continue to increase despite increasing $[Ca^{2+}]$ reflects depletion of the readily releasable pool of transmitter, although a component of postsynaptic receptor desensitization or a direct inhibitory effect of sustained high $[Ca^{2+}]$ on release cannot be excluded (Adams *et al.* 1985). Finally, some of the reduction of the action potential-evoked EPSP may result from inactivation of the presynaptic Ca^{2+} current by high $[Ca^{2+}]$ (Augustine & Eckert, 1984).

To raise $[Ca^{2+}]$ more quickly, high-intensity, short-duration light flashes were used for the rest of the experiments presented in this paper. At 200 J the Chadwick–Helmuth flash lamp converts approximately the same amount of DM-nitrophen in 400–500 μs as the continuous light converts in 2 s at 15 A.

Flash-evoked postsynaptic responses are transient

Flash-photolysis of DM-nitrophen produces a large postsynaptic depolarization similar to or greater in size and rate of rise than that elicited by invasion of the presynaptic terminal by action potentials (Fig. 3). After injection of Ca^{2+}-loaded DM-nitrophen the action potential-evoked EPSP was usually subthreshold while 150–200 J flash-evoked postsynaptic responses were suprathreshold. In saline which did not contain TTX the first several 200 J flashes usually resulted in suprathreshold postsynaptic responses. Decline of successive flash-evoked responses following several flashes was paralleled by a decline in the amplitude of the spike-evoked EPSP, just as with photolysis using the continuous light.

The postsynaptic depolarization following a flash is transient, peaking within 1 ms and then decaying with rapid (15–20 ms) and slow (about 3–5 s) time constants. This is clearly seen in Fig. 4*A* and *B* where the postsynaptic action potential was blocked with TTX. Several possible explanations for the transient nature of the response exist, including depletion of transmitter, desensitization of postsynaptic receptors or a transient rise in Ca^{2+} concentration. It is not due to activation of delayed rectification since the time course of the flash-evoked postsynaptic current under voltage clamp is similarly transient (data not shown).

A transient increase in intracellular Ca^{2+} following photolysis of a fraction of the DM-nitrophen in the presynaptic terminal is consistent with the fact that several flash-evoked responses could be obtained from the preparation despite the propensity

for this synapse to show depression of transmitter release in the face of sustained increases in intracellular Ca^{2+} (Fig. 2 and Miledi, 1973; Adams *et al.* 1985; Llinás *et al.* 1987).

A series of flash-evoked responses appears to release a number of quanta similar to the total number of presynaptic vesicles (Martin & Miledi, 1986). Summing the area

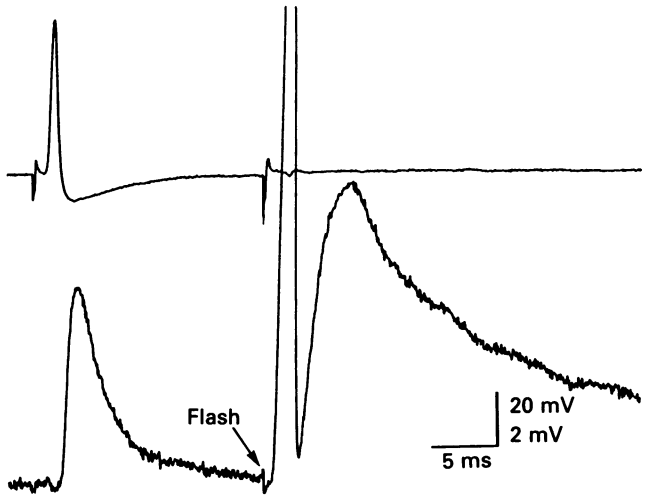

Fig. 3. Flash-evoked transmitter release often exceeds spike-evoked release. After injection of presynaptic terminal with DM-nitrophen a presynaptic action potential (upper trace) produces a subthreshold EPSP (lower trace). A short-duration, high-intensity (200 J) flash of light releases a large amount of transmitter producing a postsynaptic potential sufficient to produce an action potential at short latency and a persistent depolarization of the postsynaptic membrane which outlasts the spike by tens of milliseconds.

under the responses from the first eleven flashes of the experiment illustrated in Fig. 4 we find approximately 2×10^5 quanta released, with the first few flashes each releasing approximately $2{\cdot}4 \times 10^4$ quanta, or the equivalent of ten to twenty action potential-evoked EPSPs. Again we are underestimating the total release because (1) the records terminate well before the voltage fully recovers, (2) we have not accounted for postsynaptic desensitization, and (3) we have not corrected the peak voltages for the non-linear summation of postsynaptic responses (Martin, 1955). In another flash experiment in which data were sampled more slowly so that the slow decay to rest was measured more fully, although the transient peak response was imperfectly resolved (Fig. 4*D*), the total number of quanta released by ten successive flashes was approximately $1{\cdot}8 \times 10^6$.

Figure 4*B* also shows that later responses decay more rapidly. If successive flashes raise presynaptic $[Ca^{2+}]$ to ever higher levels, later flashes ought to release larger fractions of the remaining store of transmitter. If depletion contributes to the decay of the postsynaptic potential, then later responses ought to decay more rapidly as exhaustion proceeds more rapidly while $[Ca^{2+}]$ remains high. The small slow component of response decay may be a further consequence of transmitter depletion occurring during the maintained phase of $[Ca^{2+}]$ elevation, although diffusional

equilibration of Ca^{2+} along the longitudinal axis of the terminal, active extrusion of Ca^{2+}, or postsynaptic receptor desensitization may also contribute to this relaxation.

Extracellular Ca^{2+} is not required for the flash-evoked postsynaptic response

The flash-evoked postsynaptic response was not abolished by 20 min of perfusion of the ganglion with 0 mM-Ca^{2+}–10 mM-EGTA or low-Ca^{2+}, high-Mn^{2+}-containing

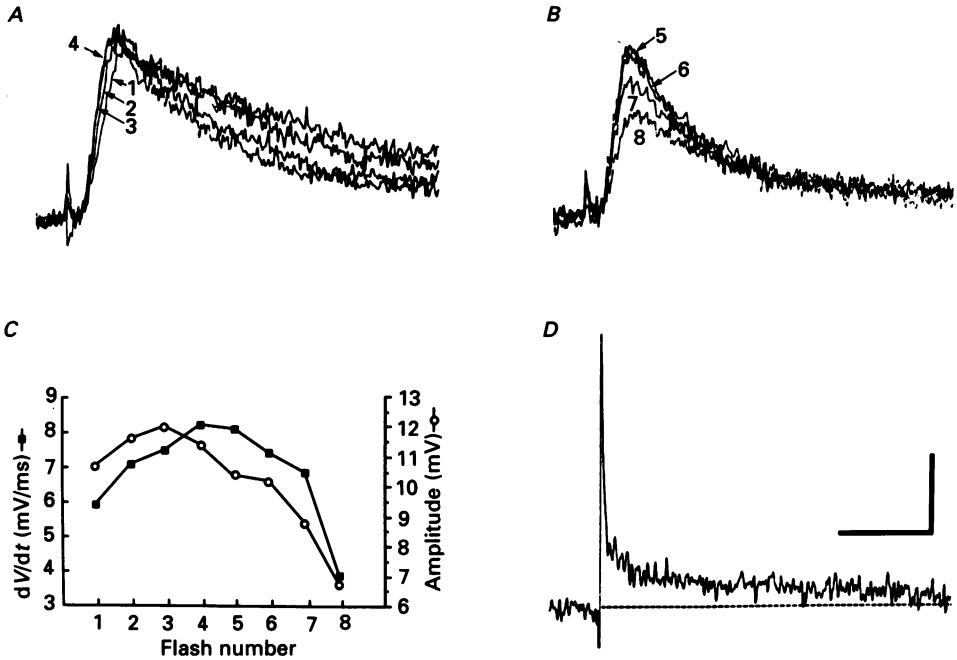

Fig. 4. TTX does not block flash-evoked release of transmitter. *A* and *B*, response of postsynaptic membrane to eight successive 200 J flashes delivered at 15 s intervals in normal Ca^{2+} Ringer solution containing 2 μM-TTX. Numbers near traces indicate flash number. *C*, rate of rise and peak amplitude of first eight 200 J flashes from *A* and *B*. *D*, response to 200 J flash recorded in another preparation at slow speed to illustrate late phase of recovery of membrane potential. Calibration bars indicate 5·0 mV, 6·5 ms in *A* and *B* and 0·6 mV, 1·25 s in *D*.

saline (0·5 mM-Ca^{2+}, 7·0 mM-Mn^{2+}), although spike-evoked EPSPs were completely blocked (e.g. Figs 6, 8 and 9).

The postsynaptic depolarization following flash photolysis is due to release of transmitter

To address whether the postsynaptic response to flash-released Ca^{2+} was due to release of transmitter we superfused the ganglion with kainic acid which has been shown to desensitize glutamate-mediated synaptic responses in squid (De Santis, Eusebi & Miledi, 1978). In two preparations superfused with 5 mM-kainic acid a depolarization of the postsynaptic membrane of 8–12 mV was observed accompanied by a total block of the spike-evoked EPSP. In each of these preparations no flash-evoked EPSP was seen.

To control for the possibility that injection had damaged the synapse or that

insufficient DM-nitrophen had been injected we attempted to show that the kainic acid block of flash-evoked EPSPs was reversible. Application of somewhat less kainic acid (1 or 3 mM) in three other preparations resulted in nearly total block of the action potential-evoked EPSP and nearly total abolition of the flash-evoked EPSP (Fig. 5). Flash-evoked responses were 0·6–2·0 mV during application of 1–3 mM-kainic acid. In two preparations rapid washing of the ganglion with normal saline resulted in partial reversal of the kainic acid block within 2 min. Both the spike-evoked EPSP and the flash-evoked EPSP increased as the kainic acid was removed (Fig. 5). The parallel sensitivity of the flash-evoked EPSP and the spike-evoked EPSP to kainic acid indicates that the flash-evoked response results from release of transmitter from the presynaptic terminal.

Photolysis of Ca^{2+}-free DM-nitrophen does not release transmitter

The light-evoked response was dependent upon photolysis of Ca^{2+}-loaded DM-nitrophen. Photolysis of unloaded DM-nitrophen using the steady light did not produce release of transmitter in two experiments although EPSP-evoked release remained nearly constant (Fig. 6). In one of four flash-lamp experiments in which the terminal was injected with at least 5 mM-zero-Ca^{2+} DM-nitrophen with the ganglion bathed in normal Ca^{2+}-containing saline, a small response was seen to the first six flashes (2·8, 3·5, 2·8, 2·0, 1·0 and 0·3 mV). No responses were seen in the other three preparations. Spike-evoked release remained nearly constant before and several minutes after at least ten 200 J flashes of unloaded DM-nitrophen in the latter three experiments. The small flash-evoked responses seen in one experiment were probably explained by either inadvertent loading of the DM-nitrophen in the tip of the electrode with Ca^{2+} from the saline prior to penetrating the synapse, by loading of DM-nitrophen from intracellular Ca^{2+} stores, or by Ca^{2+} leaking into the synapse at the injection site and loading the DM-nitrophen during injection. Consistent with this latter explanation the injection of DM-nitrophen was accompanied by loss of 70% of EPSP amplitude, suggesting the synapse was damaged or $[Ca^{2+}]$ was high before DM-nitrophen photolysis.

These results indicate that release of transmitter following photolysis of 32%-Ca^{2+} DM-nitrophen is not due to an effect of the photolysis of DM-nitrophen itself. In particular, release of Mg^{2+} or changes in pH do not evoke transmitter release. Also, these results confirm that photoproducts of non-Ca^{2+}-loaded DM-nitrophen do not interfere with the normal action potential-evoked release of transmitter for at least 5–10 min after photolysis.

Since fairly repeatable responses could be elicited by several successive flashes (e.g. Fig. 4) it proved possible to conduct experiments comparing responses to successive flashes before, during and after changing presynaptic voltage or temperature.

Effect of presynaptic depolarization during flash-evoked elevation of presynaptic Ca^{2+}

We stimulated the presynaptic axon with an extracellular electrode during flash-evoked elevation of Ca^{2+} in order to test whether invasion of the presynaptic terminal by action potentials has a modulatory effect on release of transmitter in the absence of Ca^{2+} influx. Short trains of action potentials were elicited at 70–90 Hz and timed to arrive before the flash and during the rising phase, the peak and the falling phase of the flash-evoked response. Perfusion of the ganglion with either zero-Ca^{2+} saline

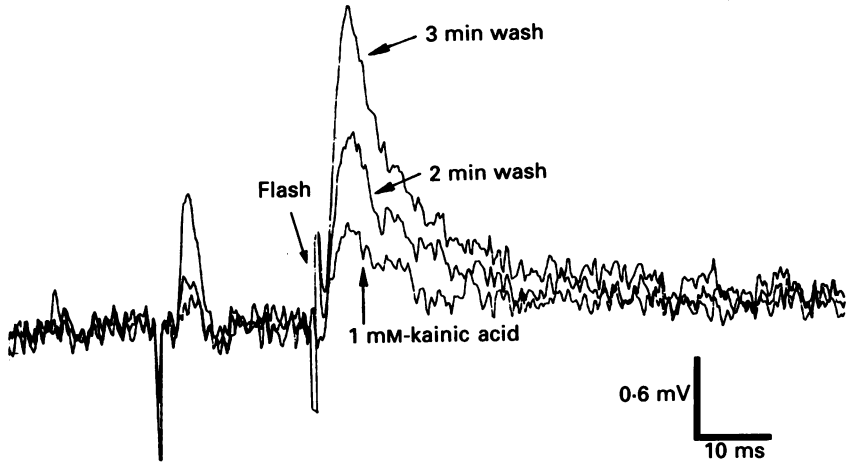

Fig. 5. Kainic acid blocks the spike-evoked and flash-evoked EPSPs equally. A small action potential-evoked EPSP and a small response to a 150 J flash are seen with the preparation bathed in 1 mM-kainic acid. Both the action potential-evoked and flash-evoked EPSPs increase in size as the kainic acid is washed off.

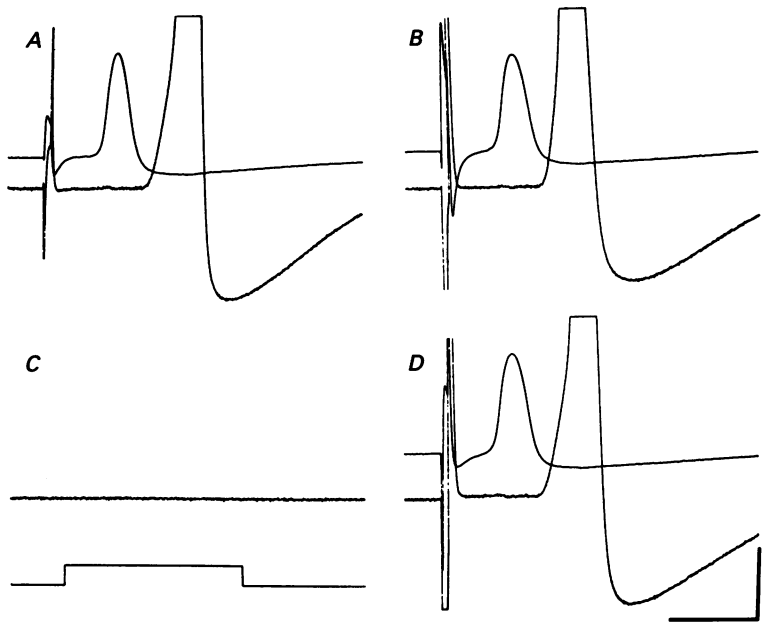

Fig. 6. Transmitter release requires Ca^{2+} release from DM-nitrophen. Presynaptic action potentials (upper traces in *A*, *B* and *D*) release substantial transmitter before and after 5 s of 15 A illumination while no release of transmitter occurs on photolysis of zero-Ca^{2+}-loaded DM-nitrophen. *A* shows EPSP before injection of zero-Ca^{2+}-loaded DM-nitrophen. *B* shows EPSP after injection of zero-Ca^{2+}-loaded DM-nitrophen. *C*, photolysis of DM-nitrophen during period indicated by bottom trace. The upper trace is postsynaptic membrane potential. *D* shows the spike-evoked EPSP 3 min after three 5 s periods of 15 A illumination. Scale bar is 50 mV presynaptic, 5 mV postsynaptic in all panels and 2·5 ms in *A*, *B* and *D* and 2·5 s in *C*. EPSPs in *A*, *B* and *D* are all suprathreshold.

containing 10 mM-EGTA or 0·5 mM-Ca^{2+}, 7 mM-Mn^{2+} saline blocked all signs of spike-evoked transmission within 10–20 min. Superposition of action potentials on flash-evoked responses did not result in any additional release of transmitter (Fig. 7; see also Fig. 9).

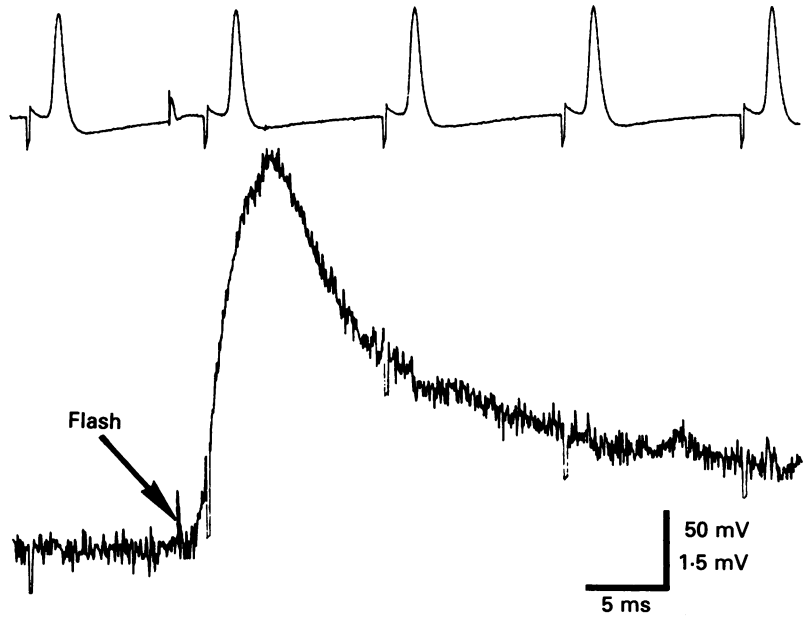

Fig. 7. Presynaptic action potentials (upper trace) do not increase flash-evoked transmitter release (lower trace) when Ca^{2+} influx is blocked. Synapse bathed in 0·5 mM-Ca^{2+}, 7 mM-$MnCl_2$ saline. 150 J flash delivered when indicated by arrow. Presynaptic action potentials were stimulated extracellularly at 90 Hz via the pallial nerve. Voltage scale is 50 mV for upper presynaptic trace and 1·5 mV for lower postsynaptic trace.

We were concerned that we might not detect an increase in release during the invasion of the terminal by the action potential if release were already saturated by the flash-evoked Ca^{2+} release. We controlled for this possibility in two ways. First we checked to see whether the influx of Ca^{2+} through voltage-gated Ca^{2+} channels could add to the flash-evoked release. In these experiments we partially blocked action potential-evoked transmission with 6·8 mM-Ca^{2+} and 3·5 mM-Mn^{2+} saline before releasing caged Ca^{2+} (Fig. 8). We used reduced-Ca^{2+} saline in order to prevent the release of too much transmitter by the action potential-mediated Ca^{2+} influx which otherwise produces postsynaptic action potentials. Figure 8 shows that additional release of transmitter is possible during even the maximal flash-evoked response if some extracellular Ca^{2+} is present when the action potential invades the terminal.

Our second approach was to use flash intensities that were sufficient to produce reasonably large but submaximal releases of transmitter so that we could be certain that if presynaptic voltage could modulate release it would not be limited by saturation of the release mechanism. To ensure that the release to the low-intensity flashes was really submaximal a higher intensity flash was delivered afterwards.

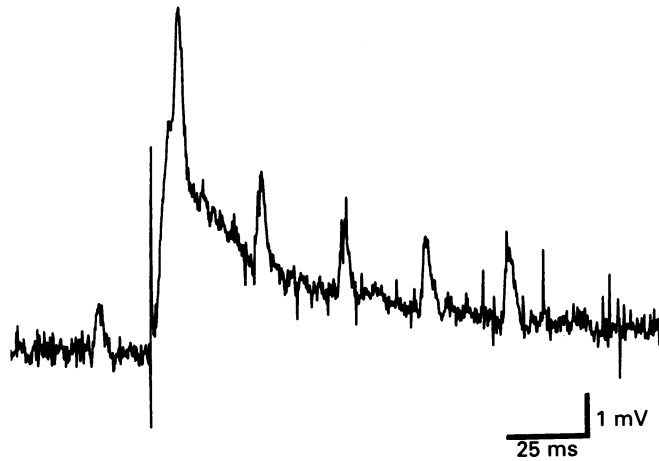

Fig. 8. Action potential-evoked EPSPs sum with flash-evoked EPSP in reduced-Ca^{2+} saline. This synapse was bathed in 6·5 mM-Ca^{2+}, 3·5 mM-$MnCl_2$ sea water. Action potential-mediated EPSPs are seen on top of the flash-evoked response. This is the 6th flash response; earlier responses were suprathreshold or significantly regenerative and so obscured the action potential-mediated EPSPs.

Figure 9*A* shows the effect of action potentials during rinsing with zero-Ca^{2+}, 10 mM-EGTA saline before total block of transmission had occurred. This figure is useful for noting the timing of normal EPSPs relative to the presynaptic action potential. In Fig. 9*B* two successive 50 J flashes are delivered. During the first, presynaptic action potentials were not elicited, while during the next 50 J flash a short train of action potentials was delivered. No additional effect of the action potentials on release is seen. However, increasing the intensity of the flash to 150 J, which increases the amount of Ca^{2+} released by the flash, results in a large increase in the release of transmitter (Fig. 9*C*), triggering a postsynaptic action potential. This experiment was repeated three times on this synapse and in another synapse with similar results, indicating that the failure to see any effect of presynaptic voltage on release was not due to saturation of the release process by either excess internal Ca^{2+} or depletion of readily releasable transmitter.

In a total of seven experiments in which complete block of action potential-mediated EPSPs was achieved by perfusing with low-Ca^{2+} or zero-Ca^{2+} saline, no additional effect of presynaptic action potentials on flash-evoked EPSPs was seen. Timing of action potentials was varied so that they occurred on the peak, on the rising phase and on the falling phase of the flash-evoked responses. In three other experiments where a small action potential-evoked EPSP was present before the flash, e.g. Fig. 8, an action potential-mediated EPSP was seen on top of the flash-evoked release. Apparently an action potential has no effect on transmitter release even while internal [Ca^{2+}] is high, unless the spike itself is accompanied by additional Ca^{2+} influx.

Effect of temperature on flash-evoked release of transmitter

As at many other synapses, transmitter release parameters such as latency to onset, magnitude of peak release, and rate of release have been shown to be temperature-sensitive at squid synapses: decreasing temperature results in an

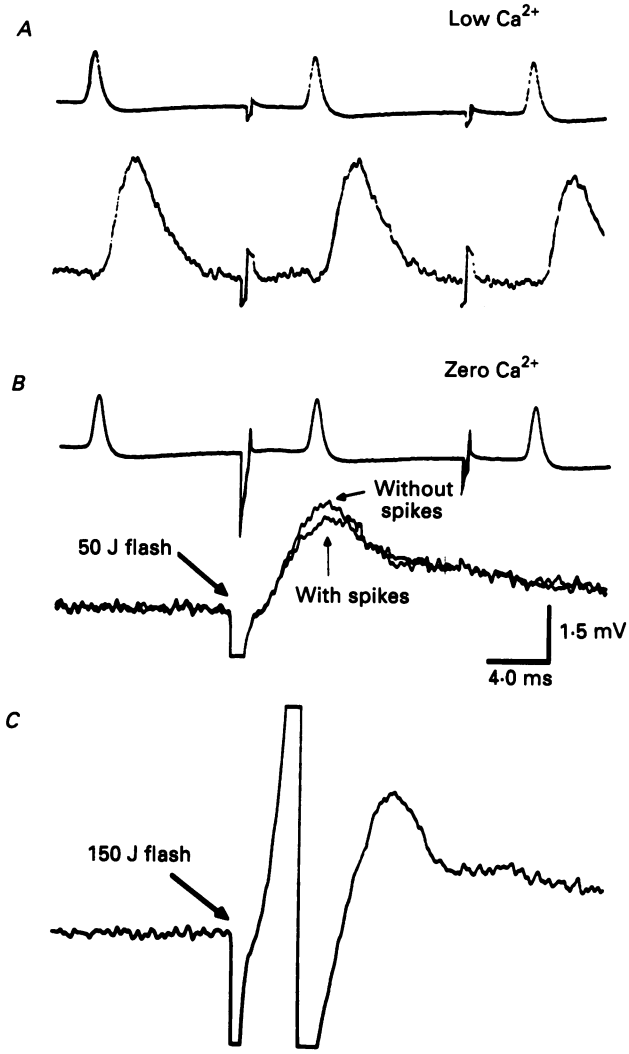

Fig. 9. Increasing flash intensity increases flash-evoked release while presynaptic action potentials produce no additional release in zero-Ca^{2+} saline. *A*, shows the partially blocked action potential-evoked EPSP while washing in zero-Ca^{2+}, 10 mM-EGTA saline. Note synaptic delay for comparison to *B*. In *B* responses to two successive 50 J flashes are shown. Presynaptic action potentials were added to the second 50 J flash as indicated. In *C* the response to a third flash of 150 J intensity is shown without presynaptic action potentials added. Note the much larger response and postsynaptic action potential (whose peak and after-hyperpolarization are clipped in the record) elicited by the higher intensity flash. The 1·5 mV calibration bar refers to postsynaptic responses (bottom traces); the bar corresponds to 50 mV presynaptically (top traces in *A* and *B*).

increased synaptic delay and a decrease in peak amplitude and rate of rise of the EPSP (Lester, 1970). Previous studies using voltage clamp of presynaptic terminals suggested that the increased delay to onset of Ca^{2+} current during depolarizing voltage steps at low temperature was insufficient to account for increased synaptic delay (Charlton & Atwood, 1979; Llinás *et al.* 1987). Additionally, presynaptic Ca^{2+}

current appears to be less sensitive to temperature than EPSP amplitude and rate of rise in some studies (Llinás *et al.* 1987), suggesting greater effects of temperature on the release processes which follow Ca^{2+} influx than on Ca^{2+} influx itself.

Here we have used flash-released Ca^{2+} to stimulate transmitter release and thereby circumvent effects of temperature on Ca^{2+} influx. As with previous studies (e.g.

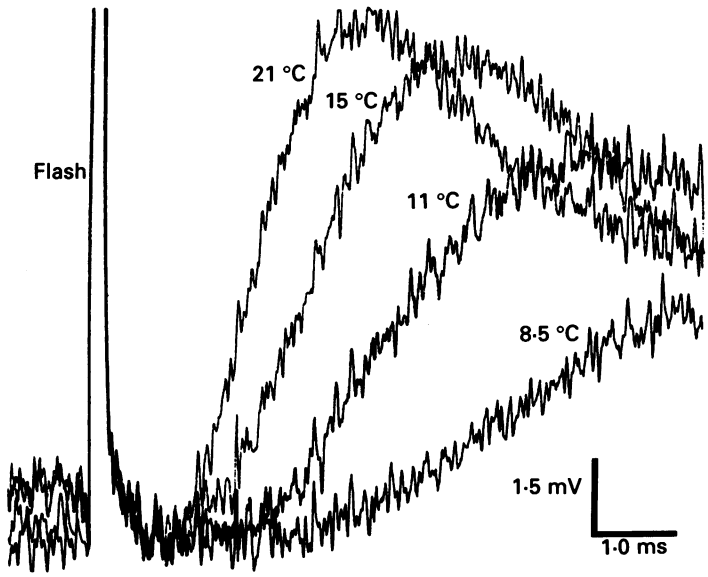

Fig. 10. Effect of temperature on flash-evoked release. Four successive 150 J flashes were delivered at different temperatures. The preparation was cooled to 8·5 °C prior to injection. After the first flash the temperature was raised rapidly by reversing the polarity of the peltier device. Approximately 4 min elapsed between the first flash at 8·5 °C and the fourth at 21 °C. 2 μM-TTX was added to normal saline in this experiment.

Lester, 1970; Charlton & Atwood, 1979), presynaptic action potential amplitude and duration were affected by temperature and lowering temperature increased synaptic delay and reduced EPSP amplitude. Similarly, flash-evoked EPSPs following injection of 32 %-Ca^{2+} DM-nitrophen were markedly affected by temperature. Figure 10 shows postsynaptic voltage responses to successive 150 J flashes at 8·5, 11, 15 and 21 °C. Compare these responses to Fig. 4 where successive flashes were delivered to another synapse at 19 °C. Figure 11 summarizes results from six experiments in which synaptic delay and rate of rise were determined for different temperatures.

Estimates for the rate of photolysis of DM-nitrophen at 25 °C in mammalian Ringer solution suggest a lower limit of 30 μs and an upper limit of 150 μs for photolysis after absorption of light (Fidler, Ellis-Davies, Kaplan & McCray, 1988). Assuming a similar dependence of rate of photolysis with temperature as that seen for caged ATP (Barabas & Keszthelyi, 1984) and a photolytic delay of 150 μs at 25 °C we estimate that the release of Ca^{2+} from DM-nitrophen would be slowed at most by a further 450 μs upon cooling from 25 to 5 °C. This change would serve to reduce the curvature in Fig. 11*A* but not eliminate it (Fig. 11*C*).

We have observed an effect of temperature on the delay between the onset of the

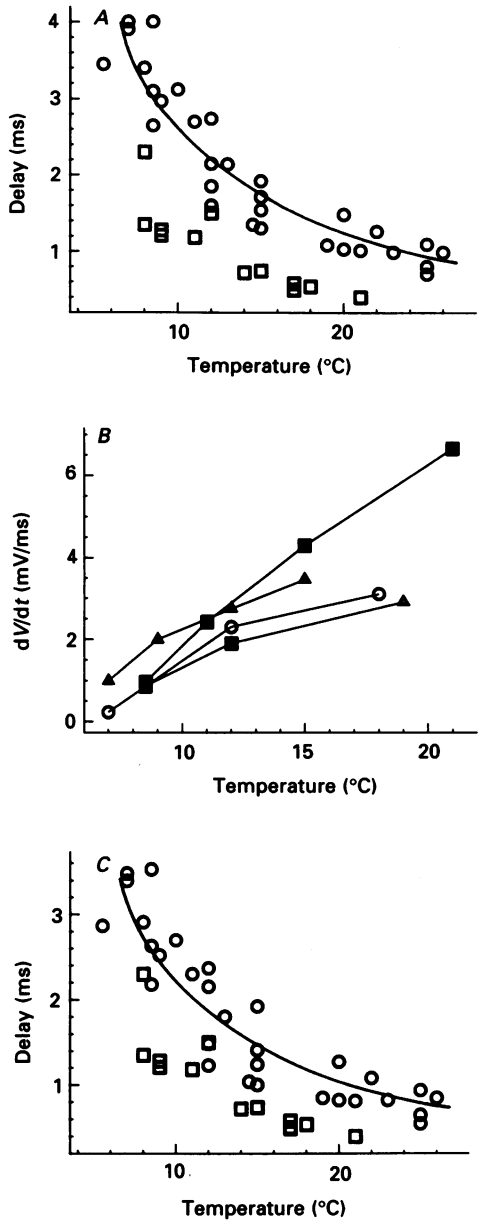

Fig. 11. *A*, delay to onset of postsynaptic depolarization plotted *vs.* temperature. ○, results of flash-evoked release from seven preparations, with delay measured from the start of the flash artifact to the start of the depolarization. □, delay from peak of presynaptic action potential to onset of postsynaptic response. *B*, maximum rate of rise of postsynaptic response from five of the seven preparations shown in *A*. *C* shows same data as in *A* with flash-evoked release delays corrected for expected effects of temperature on the time necessary for photolysis to begin to occur. Curves in *A* and *C* are least-squares fits to the logarithm of delay *vs.* temperature ($R^2 = 0{\cdot}88$ for *A*, 0·84 for *B*).

presynaptic action potential and the EPSP similar to that reported by Charlton & Atwood (1979) which is almost exactly the same as that for flash-evoked release (Fig. 11*A*).

The effect of temperature on maximum rate of release seen in different experiments was remarkably consistent despite using different flash intensities in different experiments (50–200 J) and different intracellular concentrations of DM-nitrophen. To minimize the effect of depletion of transmitter we delivered our first flashes at low temperatures where release was lowest and then raised the temperature prior to delivering the next flashes. We plot the data from the first two to four flashes in Fig. 11*B*. The increase in rate of release appears to be greatest between the first few flashes and less at higher temperatures, as would be expected if the release process were saturating or transmitter was depleted by earlier flashes. We therefore calculated a Q_{10} for the rate of release using only the first two or three points in each experiment – i.e. temperatures between 5 and 15 °C – and estimate a value of approximately 3·4 over this interval (range 2·9–5·1, $n = 6$).

Effects of changing flash intensity on release of transmitter

While it is difficult to quantify how much [Ca^{2+}] changes when different light intensities are used to photolyse DM-nitrophen, we were interested to see what effect changing the intensity would have on release parameters. Particularly, we were interested in determining whether the temperature-dependent changes in amplitude, latency and rate of rise of flash-evoked EPSPs might be explained by changes in the amount of Ca^{2+} released per flash. In most instances there was no detectable difference in delay between successive flashes of 100 and 200 J despite an approximate twofold difference in peak amplitude and up to a fivefold difference in maximum rate of rise. The largest effect we ever observed was a 200 μs increase in latency as flash intensity was reduced. Since the delay to postsynaptic response seems to be relatively independent of the amount of DM-nitrophen converted and the amount or rate of release we feel our observations regarding the effect of temperature on delay of flash-evoked release reflect the temperature dependence of the Ca^{2+}-dependent release process(es) rather than some aspect of DM-nitrophen chemistry.

DISCUSSION

Effect of DM-nitrophen photolysis on presynaptic Ca^{2+} and transmitter release

Our measurement of [Ca^{2+}] changes in nerve terminals revealed a rapid drop in Fura-2 fluorescence followed by an exponential sag to a level between the previous resting level and that reached immediately after the flash. We interpret these fluorescence changes as indicating a rapid rise in free [Ca^{2+}] followed by a drop to an intermediate level. This behaviour matches that predicted from a consideration of fast and slow steps in the equilibration of DM-nitrophen with Ca^{2+} and Mg^{2+} respectively.

We have considered the effects of two other processes on these transients: (1) Fura-2 kinetics might be slow enough to interfere with the detection of rapid Ca^{2+} changes. Kao & Tsien (1988) measured the equilibration of Fura-2 with heavily buffered Ca^{2+} at about 1 μM to have a time constant of < 2 ms. Baylor & Hollingworth (1988)

report that myoplasmic binding of Fura-2 can slow Fura-2 equilibration with Ca^{2+}, but Adler, Augustine, Duffy & Charlton (1990) detect no such binding of Fura-2 to squid axoplasm. Thus, the kinetics of Fura-2 are likely to mask the peak Ca^{2+} change measured immediately after the flash, but not the slow relaxation to steady-state afterwards. (2) The measured Ca^{2+} transients might reflect slow binding of Ca^{2+} to native Ca^{2+} buffers, but Charlton *et al.* (1982) found that Ca^{2+} entering nerve terminals during action potentials equilibrates with native buffers within 1 ms.

A number of other experimental problems limit the accuracy of our measurements of $[Ca^{2+}]$ changes: (1) The use of a shutter to protect the photomultiplier tube during the flash forces us to miss changes in $[Ca^{2+}]$ occurring within the first 3–5 ms after a flash. This certainly causes us to underestimate peak Ca^{2+} transients. (2) Later flashes appear to saturate Fura-2 with Ca^{2+}, just as they should do. This severely limits the accuracy of the measurement of Ca^{2+} increments to all but the first one or two flashes. (3) Photobleaching of Fura-2 can lead to underestimation of Ca^{2+} levels (Becker & Fay, 1987). In control experiments in which terminals were injected with Fura-2 but no DM-nitrophen, no fluorescence transients were observed, and each flash bleached about 5% of the dye. Therefore, the transients observed reflect a real change in $[Ca^{2+}]$ and Fura-2 bleaching should introduce little error for the first few flashes.

A number of factors also limit our ability to predict accurately the Ca^{2+} transients expected from photolysis of DM-nitrophen: (1) In solving the equilibrium equations for Ca^{2+} and Mg^{2+} binding to DM-nitrophen, its photoproducts, ATP and native Ca^{2+} buffers, we found that the magnitudes of the predicted Ca^{2+} peak and steady increment were extremely sensitive to small changes in DM-nitrophen and Mg^{2+} concentrations (a 20% difference in $[Mg^{2+}]$ can change Ca^{2+} levels by a factor of 2 to 4), and the peak is also rather sensitive to changes in concentrations of ATP and Ca^{2+} buffer. None of these values can be specified with even a 50% accuracy in nerve terminals. (2) The release of Ca^{2+} is also highly (non-linearly) dependent on the light intensity or extent of DM-nitrophen photolysis. Since the nerve terminal lies below the surface of the ganglion, absorbance by overlying neuropil will cut the incident light to perhaps half of its full intensity.

Despite these limitations, we feel that the qualitative match between the predicted and observed behaviour of $[Ca^{2+}]$ transients provides some support for our description of the reactions regulating Ca^{2+} in terminals, and gives some indication of the Ca^{2+} transients responsible for transmitter release. This transient rise in Ca^{2+} is almost certainly the main reason why transmitter release is also largely transient after each flash.

Both our Ca^{2+} measurements and our simulations indicate that after several flashes the steady-state level of Ca^{2+} exceeds the peak Ca^{2+} attained immediately after the first flash. However, transmitter release declines to nearly zero by about 10 s after each flash. We believe that transmitter release continues to decline after Ca^{2+} reaches its steady-state level because of depletion of the store of transmitter available for release. This depletion steadily reduces the ability of both action potentials or subsequent Ca^{2+} peaks in repeated flashes to release more transmitter (Fig. 2), and may also contribute to the more rapid decline of transmitter release in later flashes (Fig. 4). The depletion should be substantial, since one flash releases in

25 ms as many quanta as about ten action potentials, which would normally result in profound depression of release (Kusano & Landau, 1975). Besides depletion of transmitter stores and rebinding of Ca^{2+} to DM-nitrophen, other factors such as Ca^{2+} extrusion and postsynaptic desensitization may also influence the postsynaptic response to light flashes, especially the slow phase of decay.

In contrast to later responses, the postsynaptic response to the first flash of light in an experiment is little affected by transmitter depletion. We estimate that a single 200 J flash elevates presynaptic $[Ca^{2+}]$ to approximately 1 μM (Fig. 1*A*), although, as explained above, this estimate is extremely rough. This results in a postsynaptic response that often exceeds a spike-evoked EPSP (Fig. 3). This appears to contradict models of Ca^{2+} and transmitter release at squid terminals (Fogelson & Zucker, 1985; Simon & Llinás, 1985; Zucker & Fogelson, 1986). These models argue that spike-evoked EPSPs are triggered by Ca^{2+} concentrations as high as 10–50 μM within 10–50 nm of Ca^{2+} channel mouths. From this one might expect transmitter release evoked by a single flash to be much less than for a spike-evoked EPSP. However, the distribution of submembrane Ca^{2+} in spike-evoked release is markedly different from that for DM-nitrophen photolysis. In the former case, release occurs only in the vicinity of open Ca^{2+} channels, or perhaps near clusters of neighbouring channels that happen to open during an action potential. Thus, only a fraction of releasable quanta are exposed to high levels of Ca^{2+} sufficient to trigger release. When photolysis liberates Ca^{2+} from DM-nitrophen, it affects all releasable quanta equally. After a flash a relatively low $[Ca^{2+}]$ operates on many more releasable quanta than during an action potential. Apparently the uniformly modest $[Ca^{2+}]$ rise accompanying the first flash is roughly as effective as the highly non-uniform peaks of $[Ca^{2+}]$ caused by an action potential.

Effect of presynaptic potential on transmitter release

Sudden elevation of Ca^{2+} in the presynaptic terminal by flash-photolysis of the Ca^{2+} cage DM-nitrophen results in release of transmitter without requiring changes in presynaptic voltage or presynaptic Ca^{2+} current. Superposition of presynaptic voltage changes through invasion of the terminal by action potentials did not increase transmitter release over that induced by increased $[Ca^{2+}]$ alone. Failure to see an effect of presynaptic action potentials on release was not due to saturation of the release process by excess Ca^{2+} or depletion of a readily releasable pool of transmitter since higher intensity flashes released more transmitter and action potential-mediated Ca^{2+} influx could add to flash-evoked release.

Previous authors have suggested that long-lasting depolarizations of the presynaptic terminal facilitate release of transmitter when presynaptic $[Ca^{2+}]$ is increased by direct pressure injection of Ca^{2+} (Llinás *et al.* 1987). We have used action potentials to deliver brief, physiologically natural voltage changes to the presynaptic terminal and under these conditions see no evidence for a voltage-related effect. This result does not require a knowledge of $[Ca^{2+}]$ caused by the flash-evoked release of Ca^{2+}. The fact that flash-evoked responses showed delays to onset and rates of rise as well as peak amplitudes similar and in some instances greater than spike-evoked EPSPs suggests that voltage changes due to invasion of the presynaptic terminal by an action potential do not act synergistically with high intracellular $[Ca^{2+}]$ to directly

evoke transmitter release or shape its time course. This is similar to a recent conclusion based on rather different experiments on immature synapses formed between cell bodies of cultured neurones (Zucker & Haydon, 1988). The present experiments are important in that they were performed on a naturally occurring mature synapse and used action potentials rather than voltage ramps to test for an effect of potential on transmitter release.

Consequences of Mg^{2+} binding to DM-nitrophen

Although the significant binding of intracellular Mg^{2+} by DM-nitrophen and the photolytic release of both Ca^{2+} and Mg^{2+} from DM-nitrophen greatly complicate attempts to model changes in $[Ca^{2+}]$ resulting from photolysis, these features of the compound are an advantage in this system for two reasons. First they appear to result in a large transient increase in $[Ca^{2+}]$ following photolysis of a portion of the DM-nitrophen within the terminal. Thus, although $[Ca^{2+}]$ may be elevated more than an order of magnitude above rest and thereby stimulate an initially high rate of transmitter release, the slow re-binding of Ca^{2+} to unphotolysed Mg^{2+}-bound buffer following displacement of the Mg^{2+} (Eigen & Hammes, 1963) reduces the high level of transmitter release and prevents complete depletion of the releasable pool before the next flash. This permits Ca^{2+} to bind to transmitter release machinery immediately after photolysis rather than rebinding quickly to free unphotolysed DM-nitrophen, because there is little free DM-nitrophen present, most being bound to Ca^{2+} or Mg^{2+}. This aspect of DM-nitrophen chemistry provides a convenient period of about 20–30 ms of greatly elevated $[Ca^{2+}]$ in which brief presynaptic voltage changes can be applied.

Secondly, binding of cytoplasmic Mg^{2+} by DM-nitrophen probably slows the rate at which DM-nitrophen can buffer Ca^{2+} influxes associated with the opening of voltage-gated Ca^{2+} channels so that normal action potential-mediated synaptic transmission is not blocked. A similar delay in Ca^{2+} binding appears to be responsible for the persistence of transmitter release when the presynaptic terminal is filled with EGTA (Adler *et al.* 1990). Thus the ability of the synapse to sustain normal release of transmitter can be tested after injection, and manipulations which affect either flash-evoked or spike-evoked release can be compared in the same synapse.

Minimum 'synaptic delay' of transmitter release to photolytically released intracellular Ca^{2+}

Our measurements of the delay to the onset of transmitter release were typically 700 μs to 1 ms at 19 °C. The shortest delay observed in any experiment was 450 μs. Increasing the temperature to 23 or 25 °C did not shorten the delay further. This is in contrast to minimum synaptic delays following Ca^{2+} influx, determined using voltage clamp to be in the order of 200 μs (Llinás *et al.* 1981). The energy of the flash is not delivered instantaneously but peaks approximately 200 μs after the onset of the flash and requires 400 μs to deliver 75% of its energy. Some of the delay may therefore be explained by the time required to photolyse enough DM-nitrophen to achieve sufficiently high Ca^{2+} concentrations to drive detectable release. However, reducing the flash intensity and therefore shifting the time at which high concentrations of Ca^{2+} will be achieved more towards the peak or end of the flash

does not increase the delay more than 100–200 μs. In this respect, increasing flash intensity is similar to increasing the size of a presynaptic voltage clamp step in having little effect on minimum synaptic delay (Llinás *et al.* 1987). Estimates for the time required for DM-nitrophen to release Ca^{2+} indicate that the photolytic reaction will delay release of free Ca^{2+} for between 30 and 150 μs at 25 °C (Fidler *et al.* 1988). Perhaps this reaction proceeds more slowly *in vivo* than *in vitro*.

Another possible explanation for the longer delay of flash-evoked release may be that presynaptic depolarization associated with action potentials or voltage clamp which normally *precede* Ca^{2+} influx contribute to shortening the time required to initiate release. We would not have detected such an effect in our experiments since Ca^{2+} was raised by the flash *before* depolarization by the action potential. Alternatively, it is possible that DM-nitrophen cannot penetrate to the site where Ca^{2+} must act to stimulate release. After Ca^{2+} is released by the flash it might then have to diffuse to its site of action. We regard this explanation as unlikely because DM-nitrophen should release Ca^{2+} evenly beneath the presynaptic membrane. This contrasts with the influx of Ca^{2+} through discrete channels and the resultant highly non-uniform distribution of submembrane Ca^{2+} in voltage-clamp experiments (Simon & Llinás, 1985; Zucker & Fogelson, 1986). Ca^{2+} must subsequently diffuse to adjacent sites of transmitter release, and this ought to result in a briefer delay of release following DM-nitrophen photolysis than following Ca^{2+} influx.

The fact that photolysis of zero-Ca^{2+}-loaded DM-nitrophen (i.e. Mg^{2+}-loaded and free DM-nitrophen after injection) does not release transmitter or affect release evoked by action potentials indicates that Mg^{2+} does not support a phasic intense release of transmitter nor modulate significantly Ca^{2+}-evoked transmitter release. It also indicates that DM-nitrophen does not readily take up a lot of Ca^{2+} from cytoplasmic Ca^{2+}-binding proteins and organelles.

Our experiments examining flash-evoked release at different temperatures support some previous voltage-clamp studies in which it was concluded that changes in activation of presynaptic Ca^{2+} current and the magnitude of the current with temperature could not account for the changes in synaptic delay or EPSP amplitude (Charlton & Atwood, 1979). We estimate a Q_{10} for the delay of flash-evoked release of transmitter of 3·4 between 5 and 15 °C. The rate of release showed a Q_{10} of 3·4 between 5 and 15 °C, and 2·6 between 10 and 20 °C. These values are comparable to those obtained by Llinás *et al.* (1987) using voltage-clamped squid synapses.

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