Journal of Physiology (1993), **465**, *pp.* 1–8
With 3 figures
Printed in Great Britain

POSTSYNAPTIC ACTIVATION AT THE SQUID GIANT SYNAPSE BY PHOTOLYTIC RELEASE OF L-GLUTAMATE FROM A 'CAGED' L-GLUTAMATE

By J. E. T. CORRIE, A. DeSANTIS†, Y. KATAYAMA‡, K. KHODAKHAH§, J. B. MESSENGER†, D. C. OGDEN* and D. R. TRENTHAM

From the National Institute for Medical Research, Mill Hill, London NW7 1AA and the Laboratory of the Marine Biological Association, Plymouth PL1 2PB

(*Received 5 January 1993*)

SUMMARY

1. Pharmacological evidence suggests L-glutamate is a strong candidate as a transmitter at the giant synapse of the squid. Postsynaptic activation at the giant synapse cannot be effected by conventional application of putative neurotransmitters by iontophoresis or perfusion, apparently because the complex structure of the synapse prevents a sufficiently rapid change in concentration at the postsynaptic membrane. Flash photolytic release of L-glutamate from a pharmacologically inert 'caged' L-glutamate pre-equilibrated in the stellate ganglion of *Alloteuthis* or *Loligo* was used to determine whether L-glutamate can produce postsynaptic activation when released rapidly in the synaptic clefts.

2. The preparation, reaction mechanism and properties of the caged L-glutamate, *N*-1-(2-nitrophenyl)ethoxycarbonyl-L-glutamate, are described. The product quantum yield on photolysis was 0·65 (±0·05). On flash photolysis glutamate release followed a single exponential timecourse in the pH range 5·5–7·8. The rate constant was proportional to $[H^+]$ and was 93 s^{-1} at pH 5·5 and 16 °C in artificial sea water (ionic strength, $I = 0{\cdot}68$ M).

3. At pH 7·8 flash photolysis of caged glutamate pre-equilibrated in the synapse caused only a slow depolarization. A second photolytic release of L-glutamate or transynaptic activation produced no further depolarization, suggesting desensitization and inactivation of postsynaptic mechanisms by the initial pulse of L-glutamate.

4. Synaptic transmission in the giant synapse was normal at pH 5·5. Flash photolysis at pH 5·5 caused rapid production of L-glutamate within the synaptic cleft and a fast postsynaptic depolarization which generated postsynaptic action potentials.

* To whom correspondence should be sent at the National Institute for Medical Research.
† Permanent address: Stazione Zoologica, Naples 80121, Italy.
‡ Permanent address: Dojindo Laboratories, Kumamoto Techno Research Park, Tabaru 2025-5, Mashiki-machi, Kamimashiki-gun, Kumamoto 861-22, Japan.
§ Permanent address: Department of Physiology, University of Pennsylvania, Philadelphia 19104 6085, USA.

MS 2008

5. These results, together with appropriate controls, provide direct evidence that L-glutamate is a neurotransmitter in the squid giant synapse.

INTRODUCTION

The identity of the neurotransmitter at the squid giant synapse is not known with certainty (Miledi, 1969; Llinás, Joyner & Nicholson, 1974). L-Glutamate is the strongest candidate, since transmission at the synapse is reversibly inhibited by several specific blockers of glutamate receptors in other systems (DeSantis & Messenger, 1989). However, attempts to mimic transmission by close postsynaptic iontophoresis or bath application of glutamate or glutamate receptor agonists fail to discharge postsynaptic action potentials and only result in slow, weak depolarizations (Miledi, 1967; DeSantis, Eusebi & Miledi, 1975; Adams & Gillespie, 1988; DeSantis & Messenger, 1989). The giant synapse is a complex structure (Young, 1973) with numerous postsynaptic processes penetrating a tight sheath to make about 15000 functional contacts with the presynaptic fibre (Martin & Miledi, 1986). The synaptic clefts are only 12 nm wide (Martin & Miledi, 1986) and this, together with the tortuous access paths, must mean that diffusion of externally applied agents into the synapse is slow. The consequent slow concentration changes lead to desensitization of the postsynaptic receptors and a postsynaptic potential change that rises too slowly to discharge action potentials. If physiological transmitter release could be mimicked by applying a putative transmitter at a large number of synaptic contacts on a millisecond time scale it should be possible to generate propagating action potentials in the postsynaptic fibre. This was tested directly by flash photolysis of 'caged' glutamate pre-equilibrated in the synaptic cleft. The results show that L-glutamate can elicit action potential discharge in the postsynaptic fibre, providing strong evidence that this amino acid is a neurotransmitter at the squid giant synapse.

METHODS

The stellate ganglion from small specimens of the squid *Alloteuthis subulata* was mounted in a bath of total volume 500 μl containing oxygenated artificial sea water buffered with 50 mM Hepes to pH 7·8. In some experiments small specimens of *Loligo forbesii* were used. Experiments at acid pH were made in artificial sea water buffered with 50 mM 2-phosphoglycerate (pK_a 6·2) or Mes (2-(*N*-morpholino)ethane sulphonic acid). Presynaptic stimulation of the pallial nerve (containing the second-order giant fibre) was by a suction electrode at 10 × threshold voltage with 0·1 ms pulses and postsynaptic potential was recorded with an intracellular microelectrode inserted close to the synapse in the largest (i.e. medial) third-order giant fibre. Temperature was maintained at 16 °C. The postsynaptic action potential was monitored at 5 min intervals during a 20–40 min incubation allowing for diffusion of the caged glutamate into the synaptic region. Synaptic transmission was stable in the pH range 7·8–5·0 and was not affected by the presence of caged glutamate. In one set of experiments the synapse was observed with a binocular microscope and photolysis was with a xenon arc flashlamp focused from above onto the synaptic area to a spot of 4 mm diameter (see Ogden, Capiod, Walker & Trentham, 1990). In other experiments a more elaborate procedure was followed to permit a better calibration of photolysis (see below for calibration). The synaptic region was mounted above a quartz window set in the base of the bath. The preparation was viewed with a compound microscope at 40 × magnification, and photolysis was with a 1 ms pulse at 300–350 nm (Schott UG 11 filter) from the arc of a xenon flashlamp focused into the synapse via a silica condenser, illuminating a spot of about 500 μm diameter. Caged glutamate was added to make a final concentration of 5–40 mM at the required pH. Solution volume and pH were measured at the end of the experiment.

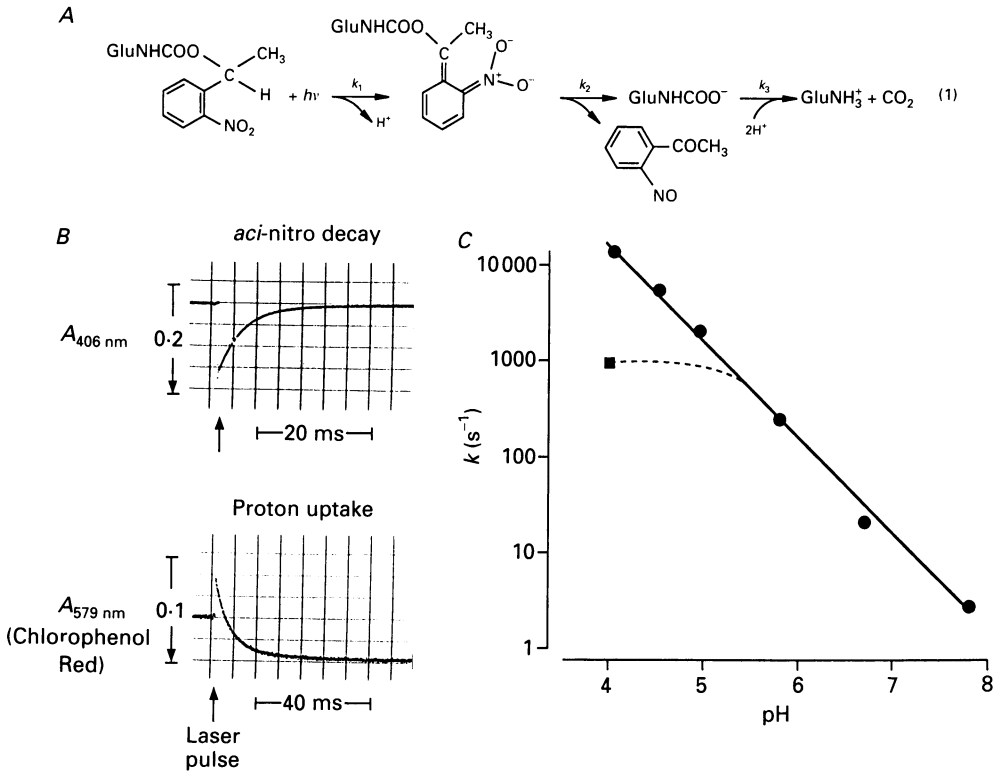

Fig. 1. Rate of glutamate ($gluNH_3^+$) formation on photolysis of caged glutamate. *A*, eqn (1) shows a minimal mechanism of caged glutamate photolysis in the pH range 5·5–8. At pH < 5·5 carboxylate groups are partially protonated, altering the H^+ stoichiometry. Spectral changes at 406 nm are associated with the formation and decay of the *aci*-nitro intermediate (steps 1 and 2). Spectral changes in the presence of a proton indicator are associated with H^+ release and H^+ uptake (steps 1 and 3). *B*, the upper trace is a spectrophotometric record of absorbance against time at pH 5·8 showing the *aci*-nitro intermediate formation and decay, from which rate constant $k_1 > 10^5\ s^{-1}$ (i.e. faster than the time resolution of the equipment) and $k_2 = 180\ s^{-1}$. The lower trace shows a proton indicator spectrophotometric record also at pH 5·8. Note the H^+ stoichiometry of one H^+ release to two H^+ uptake in accordance with eqn (1). (CO_2 hydration and HCO_3^- formation occur much more slowly; Ho & Sturtevant, 1963; Wang, Bishop & Himoe, 1972). Since the rate constants of the slower phases in the two records are similar, $k_3 > k_2$. Reaction conditions are described in the text. The arrows mark the time of the laser pulse that initiates photolysis. *C*, rate constant *k* (log scale) describing the exponential rate of decay of the *aci*-nitro intermediate as a function of pH (●). The data points lie on a line with a slope of −1, showing that *k* is proportional to $[H^+]$. Above pH 5 the rate constant of H^+ uptake closely matched that of the *aci*-nitro intermediate decay as in *B* so that $k_3 > k_2$. However, below pH 5 H^+ uptake was biphasic. The faster phase matched that of *aci*-nitro intermediate decay and was ascribed to H^+ uptake in step 2 because the carbamic acid was partially protonated. The slow phase (■) was associated with step 3, so that $k_3 < k_2$ at pH 4.

'Caged' L-glutamate

N-2-Nitrobenzyloxycarbonyl and related derivatives of amino acids were first described by Patchornik, Amit & Woodward (1970) as photolabile precursors for peptide synthesis, although

these authors did not describe the glutamate derivative. The caged glutamate used in these experiments, *N*-1-(2-nitrophenyl)ethoxycarbonyl-L-glutamate Na_2, is shown in Fig. 1*A*, together with the probable reaction mechanism of its photolytic decay to L-glutamate and by-products, 2-nitrosoacetophenone and CO_2. Caged glutamate was prepared and characterized as follows. A suspension of di-*tert*-butyl L-glutamate hydrochloride (557 mg, 1·89 mmol) (from Sigma, Poole, Dorset) in toluene (11 ml) was cooled in an ice-bath and treated with 1 M aqueous sodium hydroxide (3·95 ml) and water (3·6 ml) and treated dropwise over 5 min with a toluene solution (5 ml) of crude 1-(2-nitrophenyl)ethyl chloroformate (479 mg) (prepared by treatment of trichloromethyl chloroformate (Fluka, Gillingham, Dorset) with 1(2-nitrophenyl)ethanol; Kurita, Matsuma & Iwakura, 1976; Corrie, Reid, Trentham, Hursthouse & Mazid, 1992). The two-phase mixture was stirred in the ice-bath for 30 min, then diluted with ether and the organic phase washed with water, dilute hydrochloric acid and aqueous $NaHCO_3$, dried over Na_2SO_4 and evaporated. The residue was purified by flash chromatography in ethyl acetate–hexane (15:85) to give di-*tert*-butyl *N*-1-(2-nitrophenyl)ethoxycarbonyl-L-glutamate as a pale oil (0·71 g, 83%), which was dissolved in trifluoroacetic acid (5 ml) and kept at room temperature for 1 h. The trifluoroacetic acid was evaporated under reduced pressure and the residue was mixed with water (25 ml) and neutralized with 1 M sodium hydroxide. One-half of this solution was diluted to a conductivity $< 800\ \mu S$ and chromatographed on DEAE-cellulose (2 × 40 cm column) with a linear gradient formed from 10 and 200 mM triethylammonium bicarbonate (each 1000 ml). Fractions recovered contained 0·65 mmol caged glutamate and were freed from buffer salts by evaporation at 1 mmHg, followed by repeated evaporation with methanol. One-quarter of this material was chromatographed on Sephadex LH20 (2 × 40 cm column), eluting with water, and the peak recovered was concentrated under reduced pressure and redissolved in water (1·05 ml). Quantitative UV ($\epsilon = 4700\ M^{-1}\ cm^{-1}$ at 263 nm) showed the concentration of caged glutamate to be 94·7 mM. Amino acid analysis of the unpurified product and of the recovered material from ion exchange and LH20 chromatography showed free glutamate contamination levels of 0·17, 0·05 and 0·016% respectively.

Caged L-glutamate was characterized by HPLC (high-performance liquid chromatography), ^1H NMR and its negative ion fast atom bombardment (FAB) mass spectrum. Analytical HPLC (Whatman SAX column, mobile phase 0·15 M ammonium phosphate, pH 4·9 plus 5% methanol (v/v), flow rate 1·5 ml min^{-1}) showed caged glutamate as a double peak, corresponding to the two diastereoisomers which arise from the combination of an asymmetric centre in the amino acid and a racemic centre in the 'cage' moiety. The two peaks had a retention time t_R 2·7 and 2·85 min. The ^1H NMR spectrum also showed the presence of two diastereoisomers: δ (chemical shift, D_2O, sodium salt) 7·47–8·11 (4 H, m, ArH), 6·06 and 6·08 (1 H, 2 × q, J = 6·5 Hz, ArC*H*), 3·56–4·18 (1 H, m NHC*H*), 1·76–2·41 (4 H, m, CH_2CH_2), 1·60 and 1·62 (3 H, 2 × d, $CHCH_3$): where m is a multiplet; q, a quartet; J is a coupling constant and d, a doublet. FAB mass spectrum: found M^- 339; $C_{14}H_{14}N_2O_8 + H$ requires M^- 339.

Attempts at synthesis by direct acylation of free glutamate yielded a mixture of caged glutamate and caged pyroglutamate (the 1-[1-(2-nitrophenyl)ethyl] ester of 5-oxopyrrolidine-1,2-dicarboxylate) in approximately equal proportions.

The product quantum yield of caged glutamate, measured by comparison with the P^3-1-(2-nitrophenyl)ethyl ester of ATP (caged ATP) (Walker, Reid & Trentham, 1989), was 0·65 (±0·05, estimated limit of error). The kinetics of photolysis were measured in an absorption spectrophotometer linked to a frequency doubled ruby laser (347 nm) (Walker, Reid, McCray & Trentham, 1988). For measurements of the *aci*-nitro anion intermediate at 406 nm the solution (Fig. 1*B*, upper trace) at 20 °C contained (mM): 0·1 caged glutamate, 50 KCl, 2 dithiothreitol, 100 Mes, adjusted to pH 5·8. For H^+ measurements at 579 nm the solution (Fig. 1*B*, lower trace) at 20 °C contained (mM): 0·2 caged glutamate, 100 KCl, 1 dithiothreitol, Chlorophenol Red (concentration defined by A = 0·75, 4 mm path cell), 1 Mes adjusted to pH 5·8. For other measurements in Fig. 1*C* solutions were the same but with standard H^+ indicators and buffers appropriate to the pH.

The extent of photolysis was calibrated in the experimental microscope by spectrofluorimetry of the proton indicator 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF) to measure the protons released stoichiometrically with MgATP during photolysis of caged ATP (Walker *et al.* 1988). To determine the loss of light intensity in the ganglion the synapse was located with a × 40 water immersion objective (total magnification × 600) and the depth from the bottom of the chamber measured. The photolysis light at the synapse was corrected for attenuation in the

ganglion with the extinction coefficient of 10 cm^{-1} reported for *Aplysia* ganglia with near UV light (Lando & Zucker, 1989).

The interaction of *N*-1-(2-nitrophenyl)ethoxycarbonyl-L-glutamate with glutamate receptors was tested in rat cerebellar granule neurones and dorsal root ganglion neurones in tissue culture. No activation of NMDA or non-NMDA glutamate channels was detected at 1 mM (potencies $< 1/1000$ and $< 1/30$ that of L-glutamate), nor was the response to low concentrations of L-glutamate inhibited. Interference of the byproducts of photolysis with glutamate action was tested by photolysis of 1-(2-nitrophenyl)ethyl phosphate, which releases phosphate and the nitrosoketone via a similar reaction mechanism (Walker *et al.* 1988). In control experiments at the squid giant synapse (described here), incubation with 50 mM *N*-1-(2-nitrophenyl)ethoxycarbonyl-β-alanine (the corresponding 'caged' β-alanine) followed by photolysis neither depolarized the postsynaptic fibre nor prevented normal synaptic transmission.

RESULTS

At pH 7 the 1-(2-nitrophenyl)ethoxycarbonyl cage group has the disadvantage of a slow decay of intermediates formed following the light pulse, releasing free glutamate with a half-time of about 50 ms. Synaptic events occur on a millisecond timescale and the photolysis reaction at neutral pH would be rate limiting. However, the rate of decay was found to increase in proportion to $[H^+]$ down to pH 5·5 (Fig. 1*B* and *C*). In the experiments described here this property, and the ability of the giant synapse to tolerate pH 5·5, were exploited to release glutamate quickly in the synaptic cleft.

The results of an experiment in normal sea water at pH 7·8 are shown in Fig. 2. After 40 min equilibration in 20 mM caged glutamate release of about 5 mM free glutamate produced a 20 mV depolarization of the postsynaptic cell with slow onset (half-time 60 ms) and long duration, 1·6 s (Fig. 2*A*). In spite of a large depolarization postsynaptic action potentials were not triggered, perhaps because the slow change of potential favoured inactivation of the Na^+ conductance. Normal transynaptic action potentials were generated after photolysis once the depolarization had subsided (Fig. 2*B*). Two tests were made at the plateau of the glutamate-evoked depolarization; a second photolytic release of glutamate produced no further depolarization even though about 75% of the caged glutamate was still available for photolysis, providing evidence for desensitization of the postsynaptic receptors, and transynaptic activation was also suppressed during the glutamate depolarization, by receptor desensitization or inactivation of the action potential. The slow depolarization observed with glutamate release from caged glutamate at pH 7·8 is similar to that seen with iontophoretic application of L-glutamate and other glutamate receptor agonists to the synapse, although iontophoresis from a microelectrode has the additional problem of not uniformly activating contacts over the whole 1 mm length of the synapse.

In acid conditions glutamate release from caged glutamate is faster (Fig. 1*C*) and is 93 s^{-1} at 16 °C in artificial sea water (ionic strength, $I = 0{\cdot}68$ M). The rate is much slower than that shown at pH 5·5 in Fig. 1*C* because of the increased ionic strength and lower temperature (McCray & Trentham, 1989). In strongly buffered sea water transynaptic activation of the ganglion was well maintained at pH 5·5. Figure 3*A* shows postsynaptic action potentials elicited by presynaptic stimulation 19 min after replacing the solution around the ganglion with sea water containing 33 mM caged

glutamate at pH 5·5. After 20 min in caged glutamate, a 1 ms pulse of near UV light was given at an intensity that released an estimated 9 mM L-glutamate in the synapse. The postsynaptic fibre depolarized with a half-time of 10 ms and generated a train of five action potentials, shown on a fast time scale in Fig. 3*B*. The first action

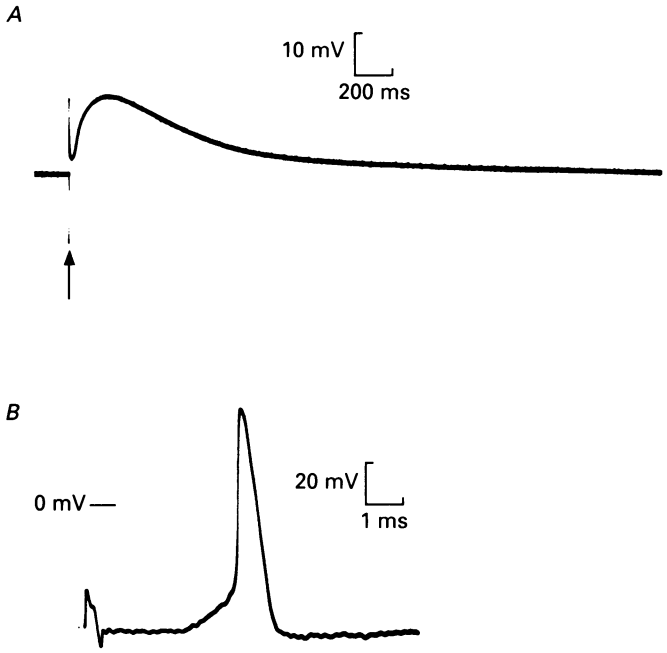

Fig. 2. Postsynaptic action of photolytically released L-glutamate at pH 7·8. *A*, depolarization of postsynaptic fibre by photolysis of 20 mM caged glutamate with 1 ms 300–350 nm light pulse at the time indicated by the arrow. Ganglion pre-equilibrated 40 min with caged glutamate at pH 7·8. Resting potential, −61 mV. *B*, action potential in the postsynaptic fibre elicited by transynaptic stimulation about 1 min after photolysis trial shown in *A*. Experimental details are given in text.

potential was identical in amplitude to single spikes elicited transynaptically. Subsequent amplitudes declined as the depolarization was maintained and the action potential mechanism accommodated, and failed after the fifth spike. Once the fibre had repolarized a full amplitude transynaptic action potential could be elicited (Fig. 3*C*). Figure 3*D* shows the depolarization after a weaker flash, releasing about 3 mM L-glutamate, when the rate of change of concentration was too small to elicit an action potential, though a subsequent full intensity flash did.

DISCUSSION

The results presented here demonstrate that L-glutamate released quickly at the giant synapse is able to generate a large depolarization fast enough to elicit action potentials in the postsynaptic fibre.

To test a putative transmitter more rigorously and obtain information on

mechanisms of postsynaptic processes, an ideal experiment would be one in which photolytic application of transmitter can mimic precisely the form of the synaptic potential. In the giant synapse the postsynaptic potential evoked by transynaptic stimulation rises in 1 ms and declines in a further 5 ms. In contrast the depolarization

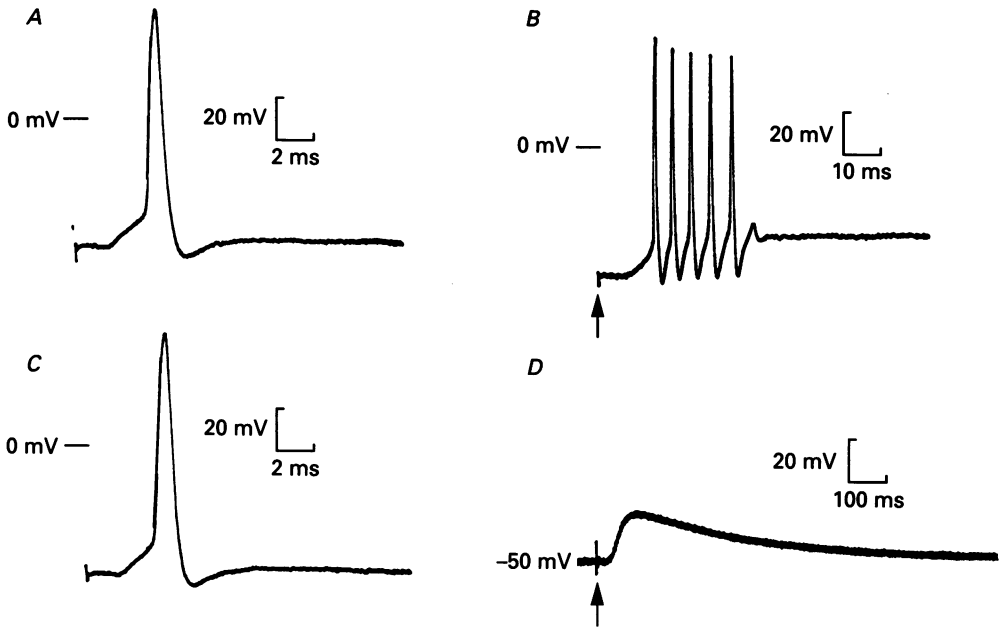

Fig. 3. Photolytic release of glutamate at pH 5·5. *A* and *C*, postsynaptic action potentials evoked transynaptically before and after photolysis shown in *B*. *B*, action potentials evoked on photolytic release of 9 mM L-glutamate from 33 mM caged glutamate equilibrated for 20 min. Arrow indicates time of flashlamp pulse. *D*, subthreshold depolarization elicited by a weaker flash releasing about 3 mM L-glutamate.

following photolytic release of glutamate at pH 5·5 rises in 10–20 ms and declines in about 1 s. The slow rise can be attributed to the rate of photolysis of the cage. After normal presynaptic release, transmitter is localized in the synaptic cleft and most likely removed by diffusion and uptake into surrounding neural and glial tissue. The slow decline when compared with the synaptic potential, and the firing of several rather than a single action potential, are probably due to the diffuse photolysis of caged glutamate within the ganglion, resulting in activation of receptors outside the area of synaptic contact, for example in the numerous postsynaptic giant fibre cell bodies, and diffusion of glutamate into the synapses from the immediate surroundings.

The generation of postsynaptic action potentials by rapid application of L-glutamate in the synaptic cleft is further evidence for a transmitter role at the squid giant synapse. It supports pharmacological evidence that transmission is blocked by antagonists active at mammalian non-NMDA receptors, and particularly by the spider toxins argiotoxin$_{636}$ and Joro toxin which block invertebrate glutamate receptors (DeSantis & Messenger, 1989; Messenger, DeSantis & Ogden, 1993). More

generally, the results show that flash photolysis of caged neurotransmitters can be applied to mimic transmitter action, to obtain evidence of the identity of neurotransmitters at inaccessible synapses and to elucidate postsynaptic mechanisms in transmission.

We thank the Director and Staff of the Marine Biological Association, Plymouth for use of their facilities. We are grateful to Mr S. Howell for the negative ion mass spectral data. K.K. was a Glaxo Scholar.

REFERENCES

ADAMS, D. J. & GILLESPIE, J. I. (1988). The actions of L-glutamate at the postsynaptic membrane of the squid giant synapse. *Journal of Experimental Biology* **140**, 535–548.

CORRIE, J. E. T., REID, G. P., TRENTHAM, D. R., HURSTHOUSE, M. B. & MAZID, M. A. (1992). Synthesis and absolute stereochemistry of the two diastereoisomers of P^3-1-(2-nitrophenyl)ethyl adenosine triphosphate ('caged' ATP). *Journal of the Chemical Society, Perkin Transactions* **I**, 1015–1019.

DESANTIS, A., EUSEBI, F. & MILEDI, R. (1975). Kainic acid and synaptic transmission in the stellate ganglion of the squid. *Proceedings of the Royal Society* B **202**, 527–532.

DESANTIS, A. & MESSENGER, J. B. (1989). New evidence that L-glutamate is a transmitter at the squid giant synapse. *Quarterly Journal of Experimental Physiology* **74**, 219–222.

HO, C. H. & STURTEVANT, J. M. (1963). The kinetics of the hydration of carbon dioxide at 25 °C. *Journal of Biological Chemistry* **238**, 3499–3501.

KURITA, K., MATSUMA, T. & IWAKURA, Y. (1976). Trichloromethyl chloroformate. Reaction with amines, amino acids and amino alcohols. *Journal of Organic Chemistry* **41**, 2070–2071.

LANDO, L. & ZUCKER, R. S. (1989). Caged calcium in *Aplysia* pacemaker neurones – characterisation of calcium activated potassium and non-specific cation currents. *Journal of General Physiology* **93**, 1017–1060.

LLINÁS, R., JOYNER, R. W. & NICHOLSON, C. (1974). Equilibrium potential for the postsynaptic response in the squid giant synapse. *Journal of General Physiology* **64**, 519–535.

MCCRAY, J. A. & TRENTHAM, D. R. (1989). Properties and uses of photoreactive caged compounds. *Annual Reviews of Biophysics and Biophysical Chemistry* **18**, 239–270.

MARTIN, R. & MILEDI, R. (1986). The form and dimension of the giant synapse of squids. *Philosophical Transactions of the Royal Society* B **312**, 355–377.

MESSENGER, J. B., DESANTIS, A. & OGDEN, D. C. (1993). Chemical transmission at the squid giant synapse. In *Cephalopod Neurobiology*, ed. ABBOTT, N. J. & WILLIAMSON, R. Oxford University Press, Oxford (in the Press).

MILEDI, R. (1967). Spontaneous synaptic potentials and quantal release of transmitter in the stellate ganglion of the squid. *Journal of Physiology* **192**, 379–406.

MILEDI, R. (1969). Transmitter action in the giant synapse of the squid. *Nature* **223**, 1284–1286.

OGDEN, D. C., CAPIOD, T., WALKER, J. W. & TRENTHAM, D. R. (1990). Kinetics of the conductance evoked by noradrenaline, inositol trisphosphate or Ca ions in guinea pig isolated hepatocytes. *Journal of Physiology* **422**, 585–602.

PATCHORNIK, A., AMIT, B. & WOODWARD, R. B. (1970). Photosensitive protecting groups. *Journal of the American Chemical Society* **92**, 6333–6335.

WALKER, J. W., REID, G. P., MCCRAY, J. A. & TRENTHAM, D. R. (1988). Photolabile 1-(2-nitrophenyl)ethyl phosphate esters of adenine nucleotide analogues. Synthesis and mechanism of photolysis. *Journal of the American Chemical Society* **110**, 7170–7177.

WALKER, J. W., REID, G. P. & TRENTHAM, D. R. (1989). Synthesis and properties of caged nucleotides. *Methods in Enzymology* **172**, 288–301.

WANG, T. T., BISHOP, S. H. & HIMOE, A. (1972). Detection of carbamate as a product of the carbamate kinase catalysed reaction by stopped flow spectrophotometry. *Journal of Biological Chemistry* **247**, 4437–4439.

YOUNG, J. Z. (1973). The giant fibre synapse of *Loligo*. *Brain Research* **57**, 457–460.