J. Physiol. (1985), **369**, *pp.* 145–159
With 8 text-figures
Printed in Great Britain

INHIBITORS OF CALCIUM BUFFERING DEPRESS EVOKED TRANSMITTER RELEASE AT THE SQUID GIANT SYNAPSE

BY D. J. ADAMS*, K. TAKEDA† AND J. A. UMBACH‡

*From the Laboratory of the Marine Biological Association, Plymouth PL1 2PB, the *Department of Pharmacology, University College London, London WC1E 6BT, the †Laboratorie de Pharmacodynamie, Université Louis Pasteur, 67048 Strasbourg Cedex, France, and the ‡Department of Pharmacology, U.C.L.A. School of Medicine, Los Angeles, CA 90024, U.S.A.*

(*Received 29 March 1985*)

SUMMARY

1. Evoked release of transmitter at the squid giant synapse was examined under conditions where the calcium ion concentration in the presynaptic terminal was manipulated by inhibitors of calcium sequestration.

2. Simultaneous intracellular recordings of presynaptic and post-synaptic resting and action potentials were made during bath application of one of the following metabolic inhibitors: sodium cyanide (NaCN), carbonyl cyanide-*p*-trifluoro-methoxyphenyl hydrazone (FCCP); ruthenium red (RuR) and sodium-free (lithium) sea water.

3. Cyanide and lithium sea water reversibly depressed the post-synaptic potential (p.s.p.) whilst RuR and FCCP blocked the evoked post-synaptic response irreversibly. The progressive reduction of p.s.p. amplitude was accompanied by a reversible increase in synaptic delay.

4. The time course of block of the p.s.p. was similar for different agents and dependent on the rate of presynaptic activity (30–40 min at 0·01 Hz). Recovery of the post-synaptic action potential following block by cyanide and lithium sea water was obtained within 40 min and 5 min respectively.

5. Synaptic depression by the metabolic inhibitors does not result from changes in presynaptic resting or action potentials, nor from a change in post-synaptic receptor sensitivity. The post-synaptic response to the local ionophoresis of L-glutamate was unchanged following inhibition of evoked release of transmitter by cyanide.

6. Injections of EGTA into presynaptic terminals poisoned by cyanide produced transient increases in p.s.p. amplitude, suggesting that cyanide is having its effect through raising intracellular calcium rather than lowering ATP. Control experiments injecting EGTA into unpoisoned nerve terminals showed no apparent effect on evoked transmitter release.

* Present address: Department of Pharmacology, University of Miami School of Medicine, P.O. Box 016189, Miami, FL 33101, U.S.A.

INTRODUCTION

Calcium ions play a fundamental role in the process of excitation–secretion coupling in synaptic transmission (Katz, 1969). The dependence of transmitter release on the external calcium ion concentration has been extensively investigated at the squid giant synapse (Katz & Miledi, 1967, 1969; Kusano, 1970; Llinas, Steinberg & Walton, 1981*b*; Charlton, Smith & Zucker, 1982; Augustine & Eckert, 1984); however, the relationship between the free (ionized) calcium concentration in the presynaptic terminal and transmitter release is ill defined. Manoeuvres to alter cytosolic calcium levels, either indirectly, using ion-containing lipid vesicles (liposomes) at the frog neuromuscular junction (Rahamimoff, Meiri, Erulkar & Barenholz, 1978; Kharasch, Mellow & Silinsky, 1981) or directly, via intraterminal calcium injection at the squid giant synapse (Miledi, 1973; Miledi & Parker, 1981; Charlton *et al.* 1982) can increase the amount of transmitter released. Paradoxically, large and prolonged injections of calcium ions into the presynaptic terminal have been reported to depress evoked release of transmitter (Miledi & Slater, 1966; Kusano, 1970).

Calcium homeostasis in presynaptic nerve terminals is largely dependent on intracellular organelles, such as mitochondria and endoplasmic reticulum, which sequester calcium by energy-linked processes (Alnaes & Rahamimoff, 1975; Blaustein, Ratzlaff & Kendrick, 1978; Martin & Miledi, 1978). One possible way in which to manipulate the level of ionized calcium in the presynaptic terminal is to interfere with sequestration of calcium with metabolic inhibitors (cyanide, carbonyl cyanide-*p*-trifluoromethoxyphenyl hydrazone), ruthenium red or inhibitors of sodium–calcium exchange (lithium sea water). Therefore, we have investigated the action of these agents on evoked transmitter release at the squid giant synapse. Our results are consistent with the hypothesis that an increased intracellular level of ionized calcium in the presynaptic terminal can also act to block synaptic transmission. A preliminary report of these results has been presented to the Physiological Society (Adams, Takeda & Umbach, 1983).

METHODS

Experiments were made on the distal (giant) synapse of the isolated stellate ganglion of adult *Alloteuthis subulata* (see Gillespie, 1979). Small live squid (mantle lengths 5–10 cm) were used within 48 h of capture. The ganglia were dissected free of the mantle and the remaining lengths (1–2 cm) of the preganglionic nerves and the 5th stellar (giant) axon were tied off at their extremities with fine silk thread. The bulk of the surrounding muscular and connective tissues was removed, as was the large blood vessel on the 'muscle' surface (Miledi, 1967). Both ganglia were pinned out on a Sylgard base in a small Perspex bath. Using a combination of oblique transmitted and direct light, it was possible to visualize clearly the pre- and post-synaptic elements in the majority of preparations.

The bath was perfused continuously, and as measured by dye dilution, the extracellular solution was completely exchanged within 5–7 min. The temperature was kept constant at 12–16 °C as monitored by a thermistor located within 2 mm of the ganglion. Artificial sea water (ASW) contained (mM): NaCl, 470; KCl, 10; $CaCl_2$, 11; $MgCl_2$, 55; TRIS buffer, 10, pH 7·8. In one series of experiments, high-calcium sea water of the following composition (mM) was used: NaCl, 400; KCl, 10; $CaCl_2$, 112; TRIS buffer, 10, pH 7·8. Sodium cyanide (NaCN; Sigma) titrated to pH 7·8, carbonyl cyanide-*p*-trifluoromethoxyphenyl hydrazone (FCCP; Sigma) or ruthenium red (RuR;

contained 20% dye; Sigma) were added to ASW to give the final concentrations indicated. Sodium-free (lithium) sea water was made by equimolar substitution of LiCl for NaCl. Cadmium chloride was added to ASW at a concentration of 0·1–1 mM. Hydrogen peroxide was added to all solutions to a concentration of 0·002% (v/v) as a source of molecular oxygen (Llinas, Steinberg & Walton, 1981*a*; Walton & Fulton, 1983). Although the giant synapse of other species of squid has been found to be very sensitive to oxygen (e.g. Bryant, 1958; Katz & Miledi, 1967), *Alloteuthis* is relatively insensitive to anoxia (our observations and J. I. Gillespie, personal communication). Resting and action potentials (and synaptic transmission) could be maintained at initial levels for periods of at least 4 h.

Conventional electrophysiological techniques were used for intracellular recordings with 3 M-KCl-filled micro-electrodes (resistances *ca.* 15 MΩ). The preganglionic nerve was stimulated extracellularly at a frequency of 0·01 Hz using fine platinum electrodes and short-duration pulses (200 μs). Usually responses were stored on the oscilloscope screen every 5 min and were photographed directly using Polaroid film. Some experiments were recorded on FM tape (Racal Store 4, band width d.c. to 20 kHz) for later analysis.

Simultaneous recordings from the presynaptic terminal and the post-synaptic axon were made in most cases. The pre-synaptic electrode was inserted (usually first) in the last (most distal) preterminal digit, which often overlay or was adjacent to the proximal termination of the giant axon (see Fig. 1). It was possible to elicit a presynaptic action potential and normal synaptic transmission by passing current (with a bridge circuit) through the pre-synaptic electrode (Fig. 1*B*). The aim was to insert the post-synaptic electrode close to the pre-synaptic electrode (< 200 μm away): the rising phase of the post-synaptic potential (p.s.p.) was rapid, almost merging completely with the post-synaptic potential and the synaptic delay was short, between about 0·5 and 1·2 ms (Fig. 1*A*).

Electrodes for glutamate ionophoresis contained 1 M-sodium glutamate, pH 7·2 and had resistances of > 30 MΩ. Current was passed using a constant-current source; a few nA of braking current prevented leakage. Ethyleneglycol-bis-(aminoethylether)-*N*,*N*-tetraacetic acid (EGTA, Sigma) injection electrodes (5–10 MΩ) were filled with 0·5 M-K_2 EGTA, pH 7·2. A bridge circuit was used when injecting current presynaptically.

RESULTS

In all experiments the post-synaptic response was evoked either by stimulating the preganglionic nerve with extracellular platinum electrodes (Fig. 1*A*) or by injecting current through a micro-electrode located in the presynaptic terminal (Fig. 1*B*). The mean values obtained for the pre- and post-synaptic resting potentials were $-59{\cdot}5 \pm 5{\cdot}1$ mV (± 1 S.D., $n = 25$) and $-64{\cdot}1 \pm 4{\cdot}6$ mV ($n = 32$), respectively. Action potentials recorded from the presynaptic terminal and post-synaptic axon were typically 90–110 mV in amplitude, although the amplitude of the preterminal spike was consistently lower than that of the post-synaptic axon (see Gillespie, 1979).

Block of evoked release of transmitter by pharmacological agents which inhibit the calcium influx during the presynaptic action potential provides a sensitive indicator of the equilibration time of the extracellular solution with the preparation. Cadmium inhibits synaptic transmission by blocking ion movement through open presynaptic terminal calcium channels (Llinas *et al.* 1981*a*; Augustine & Eckert, 1984). Evoked release of transmitter as determined from p.s.p. amplitude was inhibited within 5–7 min (essentially the bath exchange time) in sea water containing 1 mM-$CdCl_2$ (Fig. 2). Recovery from the block of synaptic transmission followed a similar time course on the wash-out of cadmium. The rapid onset and reversal of this cadmium blockade shows that the bath exchange in the experimental chamber was relatively efficient and that there were few diffusional barriers for the access of

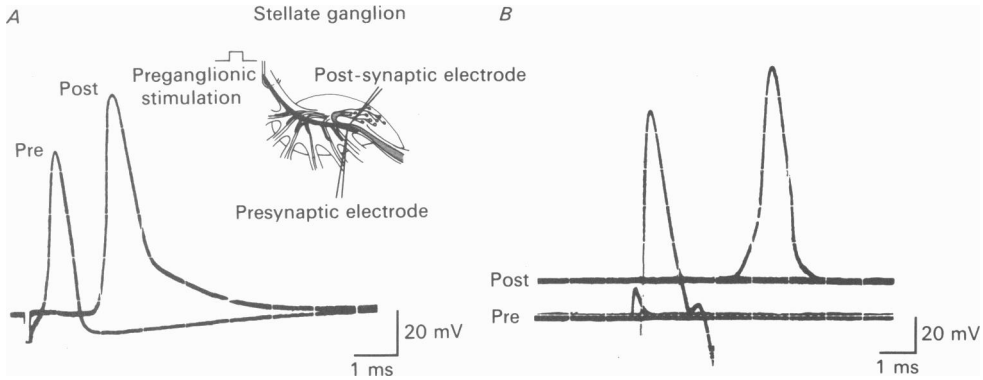

Fig. 1. *A*, simultaneous intracellular recording of presynaptic and post-synaptic potentials in response to stimulation of the preganglionic nerve. Resting membrane potentials: −53 mV presynaptic; −60 mV post-synaptic. Temperature 14 °C. Note that the post-synaptic action potential is superimposed on the p.s.p. Inset: schematic diagram of the squid stellate ganglion and location of intracellular micro-electrodes in the presynaptic terminal and post-synaptic axon. *B*, presynaptic and post-synaptic action potentials elicited in response to current injection via the micro-electrode located in the presynaptic terminal. The brief (2 ms) depolarizing current pulse was superimposed on a constant current hyperpolarizing the presynaptic membrane to −65 mV. Temperature 12 °C.

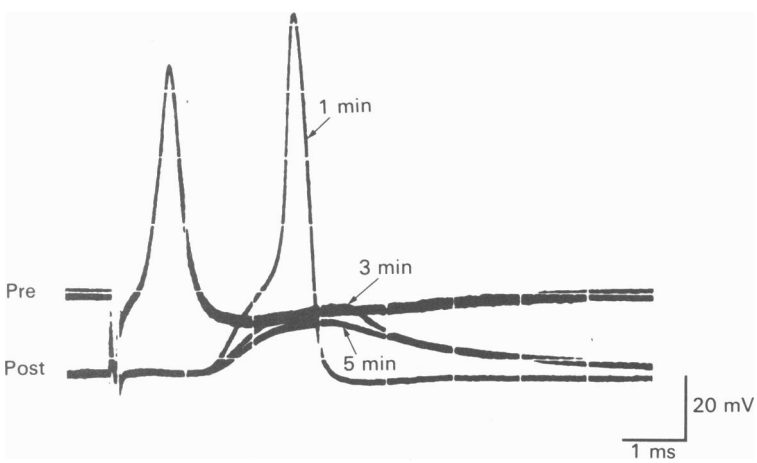

Fig. 2. Time course of inhibition of evoked release by cadmium. Superimposed records of presynaptic and post-synaptic potentials obtained following 1, 3 and 5 min exposure of the synapse to ASW containing 1 mM-$CdCl_2$. Resting membrane potentials: −56 mV presynaptic; −64 mV post-synaptic. Temperature 16 °C.

cadmium to its site of action at the presynaptic terminal. In three experiments, there was no change in the amplitude of either resting or presynaptic action potentials in the presence of cadmium (see Fig. 2).

Cyanide depresses evoked release of transmitter

Synaptic transmission at the squid giant synapse was studied in the presence of metabolic inhibitors which have been shown to increase the axoplasmic calcium ion

concentration in the squid (see Baker, 1978; Requena & Mullins, 1979). The effect of cyanide on evoked release of transmitter was monitored by recording pre- and post-synaptic potentials simultaneously during bath perfusion with sea water containing 2 mM-NaCN. Cyanide sea water produced a progressive decline in the amplitude of the evoked post-synaptic response: after approximately 10 min exposure to 2 mM-NaCN the p.s.p. failed to reach threshold for generation of the

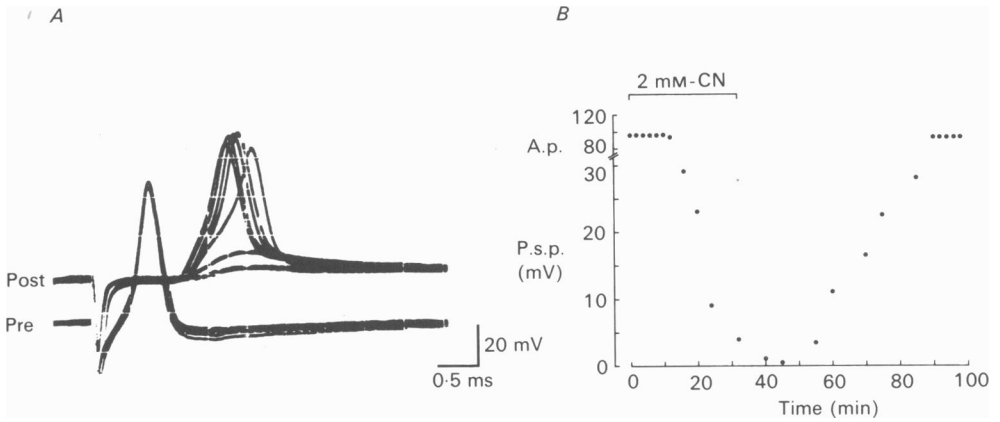

Fig. 3. *A*, depression of evoked release of transmitter during exposure to 2 mM-NaCN. Superimposed traces of the pre- and post-synaptic potentials (stimulation at 0·01 Hz). The post-synaptic potential failed to reach threshold for generation of an action potential (a.p.) after approximately 10 min exposure to cyanide sea water. Resting membrane potentials: −54 mV presynaptic; −55 mV post-synaptic. Temperature 16·5 °C. *B*, time course of onset and recovery from block by 2 mM-NaCN. The post-synaptic potential amplitude (mV) is plotted during (32 min) and following exposure to cyanide. Resting membrane potentials: −63 mV presynaptic; −64 mV post-synaptic. Temperature 16 °C.

action potential and complete depression of the p.s.p. occurred after 30–45 min (Fig. 3*A*). Failure of synaptic transmission was not due to a change in either the presynaptic resting and action potentials or the post-synaptic resting potential. Recovery of the post-synaptic action potential was observed following 30–45 min wash out. The time course of onset and recovery of the p.s.p. from block by cyanide is described in Fig. 3*B*. The onset of depression of the post-synaptic response was dependent on the frequency of depolarization of the presynaptic terminal. Increasing the rate of stimulation to 0·1 Hz in the presence of 2 mM-NaCN accelerated the rate of block of the evoked p.s.p. approximately 2·5–3-fold. In eight experiments the depression of evoked release of transmitter observed in cyanide sea water was completely reversible upon wash-out. Similarly, the increase in intracellular ionized calcium levels measured in cyanide-poisoned axons has been shown to be completely reversible upon wash-out or by injecting ATP (Blaustein & Hodgkin, 1969; Baker, Hodgkin & Ridgeway, 1971).

The progressive reduction of the p.s.p. amplitude in the presence of cyanide was accompanied by an increase in the synaptic delay or latency of transmitter release (Katz, 1969). The synaptic delay was increased by approximately 50% when the evoked p.s.p. had been reduced to less than 10% of its initial value. The presynaptic

action potential amplitude and duration were invariant during the progressive reduction of the post-synaptic response, suggesting that the site of action of cyanide is either at the post-synaptic membrane or at the presynaptic locus for excitation–secretion coupling. Since the post-synaptic action potential initiated by direct stimulation of the axon is unaffected by cyanide poisoning (not shown), it is likely that the site of action of cyanide is of presynaptic origin. Lowering the extracellular calcium concentration from 11 to 2 mM depressed the p.s.p. to less than 2 mV amplitude over a period of 30 min, a similar time course to that observed for the effect of cyanide. However, the reduction of the post-synaptic response recorded in 2 mM-calcium sea water was not accompanied by a corresponding increase in synaptic delay, consistent with the lack of effect of extracellular calcium on the time course of phasic release of acetylcholine reported at the mammalian neuromuscular junction (Datyner & Gage, 1980).

Irreversible inhibition of synaptic transmission by RuR and FCCP

FCCP is a potent mitochondrial uncoupler and has been shown to increase ionized calcium levels in squid axoplasm irreversibly (Brinley, Tiffert, Scarpa & Mullins, 1977). Bath perfusion with sea water containing 10–20 μM-FCCP produced a progressive reduction in the evoked p.s.p., as illustrated in Fig. 4*A*. Complete inhibition of the evoked post-synaptic response occurred after 35–40 min. The block of synaptic transmission in the presence of FCCP was not due to a failure of the presynaptic action potential, as shown in Fig. 4*B*. Both the pre- and post-synaptic resting potentials were unchanged in sea water containing 20 μM-FCCP. The rate of inhibition of the p.s.p. was also dependent on the frequency of stimulation of the presynaptic nerve, increasing with higher rates of stimulation. The effect of FCCP on synaptic transmission was irreversible: after 65 min wash-out there was no recovery of the post-synaptic response, as shown in Fig. 4*C*. In four experiments, the inhibition of evoked transmitter release by FCCP was not reversed even following wash-out periods of up to 120 min.

RuR, which interferes with mitochondrial uptake of calcium, dramatically increases spontaneous transmitter release and inhibits evoked release at neuromuscular and neuronal synapses, presumably by raising intracellular calcium levels (Alnaes & Rahamimoff, 1975; Baux, Simmoneau & Tauc, 1979). The effects of RuR on evoked release were investigated at the squid giant synapse in four experiments. RuR at a concentration of 10 μM produced no change in synaptic transmission at 0·1 Hz stimulation during 40 min continuous perfusion. However, raising the RuR concentration to 100 μM depressed the p.s.p. and eventually abolished it without any significant change (< 5 mV) in the pre- and post-synaptic resting potentials or in the presynaptic action potential amplitude or duration (Fig. 5). RuR block of evoked release followed a similar time course to that of FCCP (at the same rate of presynaptic activation) and was irreversible, even following prolonged periods of wash-out.

If the irreversible failure of synaptic transmission produced by RuR and FCCP was due to reduced calcium influx during the presynaptic action potential, then raising the extracellular calcium concentration may overcome the inhibition of evoked release. In two experiments, the synapse was bathed with high-calcium

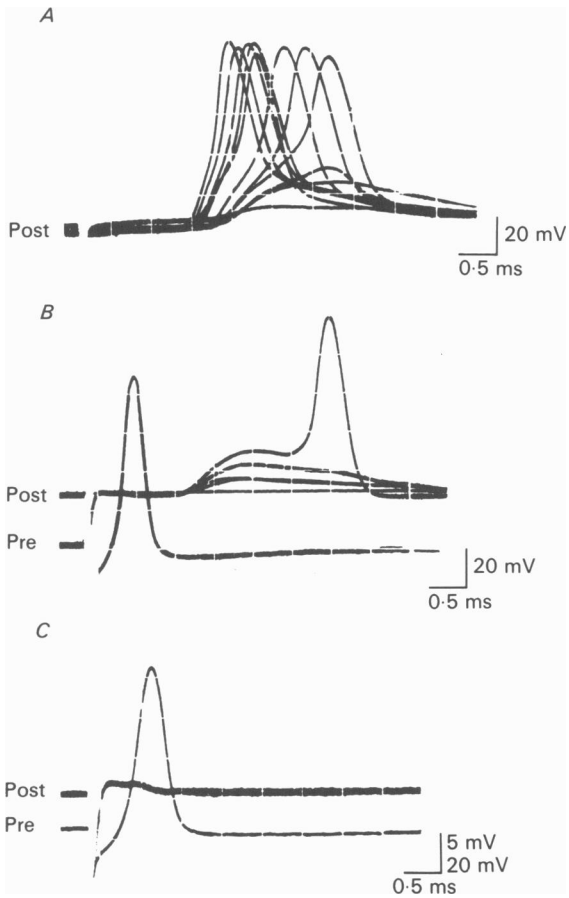

Fig. 4. Irreversible inhibition of synaptic transmission following bath application of FCCP. *A*, post-synaptic potentials recorded in response to preganglionic nerve stimulation at 0·01 Hz in the presence of 20 μM-FCCP. Post-synaptic resting potential −65 mV. Temperature 16 °C. *B*, superimposed traces of pre- and post-synaptic potentials in the presence of 20 μM-FCCP. Resting membrane potentials: −67 mV presynaptic; −59 mV post-synaptic. Temperature 16 °C. *C*, failure of recovery of synaptic transmission after 65 min wash-out following block of the evoked post-synaptic response by FCCP.

(112 mM) sea water following block of the p.s.p. The elevated extracellular calcium increased the threshold for initiation of the presynaptic action potential but no recovery of the evoked post-synaptic response was observed after 30 min.

The decrease in evoked release by FCCP and RuR was also accompanied by an increase in synaptic delay (see Figs. 4 and 5) similar to that observed in the presence of cyanide. The synaptic delay has been proposed to consist of two components: (a) the time dependence of calcium-channel opening and (b) the time required for secretion of transmitter following the entry of calcium (Llinas *et al.* 1981*b*; Augustine, Charlton & Smith, 1985). An increase in the latter component is most likely to account for the effect of the metabolic inhibitors on the synaptic delay,

rather than a change in the kinetics of the presynaptic calcium current (cf. Stimers & Byerly, 1982).

Effects of LiCl on synaptic transmission

Substitution of extracellular sodium with lithium is known to increase intracellular ionized calcium, but does so without disrupting energy-yielding processes in mitochondria. An established action of lithium is to interfere with the active extrusion

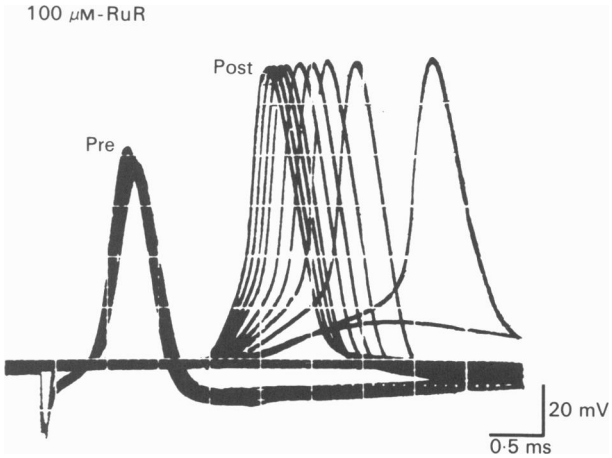

Fig. 5. Irreversible inhibition of evoked transmitter release following bath application of 100 μM-RuR. Superimposed traces of pre- and post-synaptic potentials obtained during 40 min exposure to RuR. Resting membrane potentials: −56 mV presynaptic; −66 mV post-synaptic. Temperature 15 °C.

of intracellular calcium ions across the plasma membrane (see Baker, 1972). Since both metabolic inhibitors and lithium can raise intracellular calcium, but do so through different mechanisms, the effect of (sodium-free) lithium sea water on synaptic transmission was investigated. Changing the external solution to lithium sea water reduced the resting potential of both the presynaptic terminal and post-synaptic axon by 5–8 mV; this effect was completely reversible on return to normal (sodium) sea water (Fig. 6). Lithium sea water also progressively depressed the evoked post-synaptic response elicited at 0·01 Hz to less than 2 mV within 30–40 min. The failure of synaptic transmission cannot be attributed to a loss of the presynaptic spike since the action potential amplitude and duration were not significantly changed during exposure to lithium sea water (Fig. 6*A*). This observation contradicts the explanation proposed by Crawford (1975) for the block of synaptic transmission by lithium at the frog neuromuscular junction. Return to normal (sodium) sea water resulted in recovery of the post-synaptic action potential (Fig. 6*B*) within 3–5 min (the time required to exchange the external solutions).

Metabolic inhibition and post-synaptic receptor channels

To determine the site of action of the metabolic inhibitors and lithium on evoked release, the response of the post-synaptic membrane was tested by application of

a putative neurotransmitter before and during block of synaptic transmission. The post-synaptic response to L-glutamate was examined since L-glutamate has been proposed as a possible transmitter at the squid giant synapse (Miledi, 1967; Kelly & Gage, 1969; Kawai, Yamagishi, Saito & Furuya, 1983). Local ionophoresis of L-glutamate was used to elicit a post-synaptic response since bath application of 10

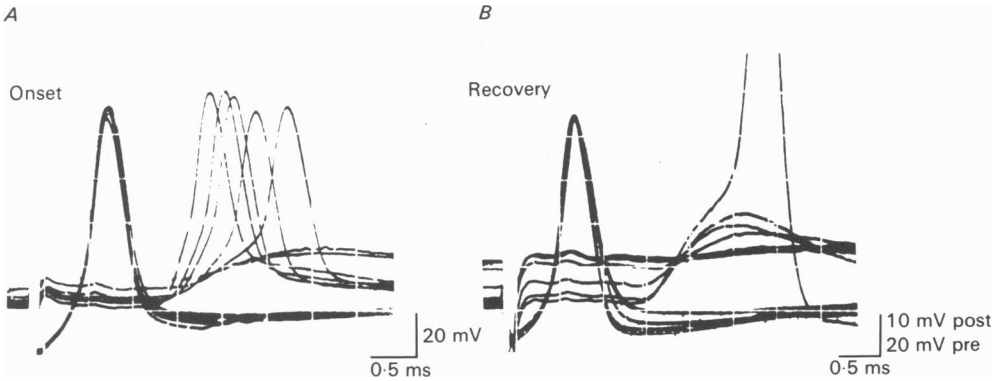

Fig. 6. Reversible block of synaptic transmission in (sodium-free) lithium sea water. Pre- and post-synaptic potentials recorded in response to preganglionic nerve stimulation at 0·01 Hz. *A*, onset of depression of the post-synaptic response during bath perfusion of the giant synapse with lithium sea water. Temperature 16·5 °C. *B*, recovery from block of evoked transmitter release by lithium sea water. Pre- and post-synaptic potentials recorded during 15 min period following return to normal (sodium) sea water. Note that in the presence of lithium sea water there is a reversible depolarization (5–8 mV) of the resting membrane potentials but no change in the amplitude of the presynaptic action potential.

mM-sodium glutamate did not produce a detectable change (< 3 mV) in the post-synaptic resting potential. However, exposure to 10 mM-glutamate for 40 min did cause a reversible decrease in the amplitude of the p.s.p. elicited at 1 Hz stimulation. The post-synaptic response to the local ionophoresis of L-glutamate in an unpoisoned synapse is shown in Fig. 7*A*. The glutamate-induced p.s.p. had a typical rise time of less than 500 ms and an amplitude of 10–12 mV, but these parameters were critically dependent on location of the ionophoretic electrode with respect to the post-synaptic chemosensitive sites. The post-synaptic response to L-glutamate following inhibition of evoked release of transmitter by 2 mM-NaCN was unchanged, as shown in Fig. 7*B* where the glutamate-induced p.s.p. was recorded at the same site as the control response. The post-synaptic sensitivity to L-glutamate ionophoresis was also unchanged in another experiment where the p.s.p. was inhibited irreversibly by 10 μM-FCCP. These results indicate that synaptic depression produced by metabolic poisons is unlikely to be a consequence of desensitization or pharmacological block of the post-synaptic receptors.

Presynaptic injection of EGTA

To test the hypothesis that the depression of evoked release by the metabolic inhibitors and lithium is a consequence of a rise in intraterminal calcium levels, the

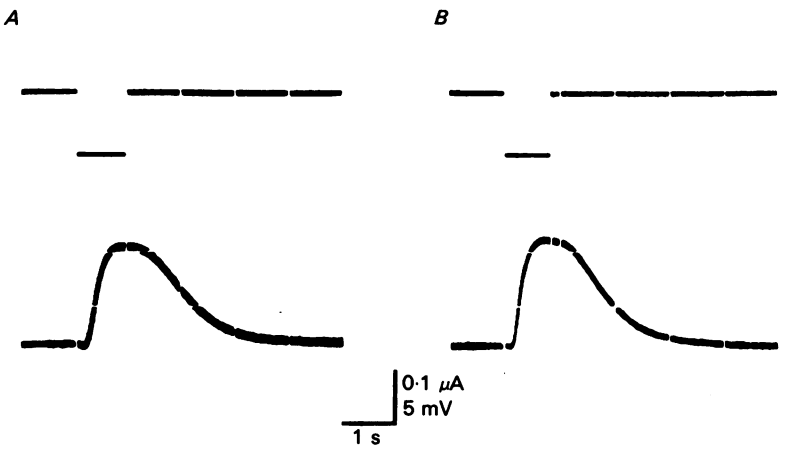

Fig. 7. Post-synaptic response to local ionophoresis of L-glutamate before (*A*) and following block of synaptic transmission by 2 mM-NaCN (*B*). Upper traces: current pulse (−0·12 μA; 800 ms) passed through a glutamate-filled extracellular micro-electrode. A 5–10 nA braking current was maintained on the ionophoretic electrode to avoid leakage of L-glutamate. Lower traces: post-synaptic potentials produced in response to ionophoresis of L-glutamate in ASW (*A*) and following inhibition of evoked release after 35 min exposure to cyanide (*B*). Post-synaptic resting potential −70 mV. Temperature 14 °C.

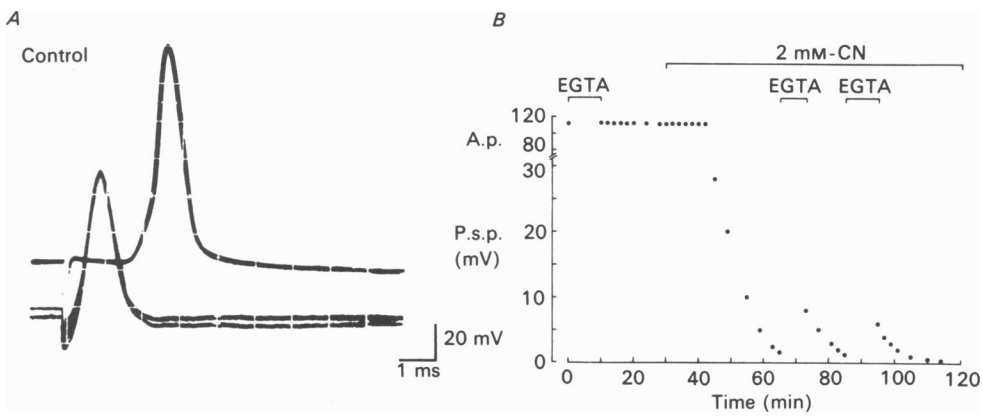

Fig. 8. EGTA injection into unpoisoned (*A*) and cyanide-poisoned (*B*) presynaptic nerve terminals. *A*, superimposed records of pre- and post-synaptic potentials obtained following prolonged (20 min) injection of EGTA. Resting potentials: −58 mV presynaptic; −69 mV post-synaptic. Temperature 12 °C. *B*, the effect of EGTA injection on post-synaptic potential amplitude before and during exposure to 2 mM-NaCN. Note the lack of effect of EGTA on the action potential. Following loss of the spike and depression of p.s.p. by CN, 10 min injections of EGTA transiently increased the p.s.p.

effect of EGTA injection to lower the intracellular calcium levels was investigated. Prolonged ionophoretic injections of EGTA (−50 to −100 nA for 10 min periods) had no apparent effect on synaptic transmission in unpoisoned terminals. Simultaneous intracellular recording of the presynaptic and post-synaptic resting and action potentials in response to 0·1 Hz stimulation following presynaptic injection of EGTA is shown in Fig. 8*A*.

Repeated injections of EGTA failed to inhibit the evoked post-synaptic response. This paradoxical insensitivity of calcium-dependent evoked release of transmitter to presynaptic EGTA injection might be accounted for by the difference in kinetics of calcium buffering by EGTA and that which normally follows the presynaptic action potential (see Thomas, 1982).

The effect of EGTA injection was also examined during block of synaptic transmission by metabolic inhibitors. In two experiments where the evoked post-synaptic response (0·01 Hz) was inhibited by exposure to 2 mM-NaCN, EGTA injected into the presynaptic terminal transiently and reproducibly increased the evoked p.s.p. amplitude (Fig. 8*B*). The transient recovery of the p.s.p. amplitude following EGTA injection indicates an increase in evoked release of transmitter. The decline in p.s.p. amplitude after initial recovery probably reflects continued calcium loading of the presynaptic terminal with maintained nerve stimulation in the presence of cyanide. The transient increase in p.s.p. amplitude following EGTA injection into metabolically poisoned terminals suggests that cyanide may inhibit evoked release by raising the intracellular calcium ion concentration, rather than by lowering ATP levels.

DISCUSSION

Cyanide, FCCP, RuR and lithium sea water depressed evoked transmitter release at the squid giant synapse. This effect was first seen as a loss in the post-synaptic action potential followed by a decrease in the p.s.p., which can be completely suppressed during long periods of drug application. Since these agents significantly increase intracellular free calcium in the squid axon (Baker, 1972, 1978; Requena & Mullins, 1979) a simple explanation for our observations is that a sustained rise in presynaptic calcium levels exerts an inhibitory effect on evoked transmitter release. This hypothesis, as well as alternative possibilities for the action of metabolic inhibitors and lithium, are considered below.

It could be argued that synaptic depression arises from a loss of membrane excitability during drug application. Indeed, Crawford (1975) has suggested that this is the mechanism by which lithium blocks synaptic transmission at the frog neuromuscular junction. However, we observed that resting potentials and presynaptic action potentials were essentially unchanged during transmission block by metabolic inhibitors and lithium. Also, our results are unlikely to result from pharmacological inhibition or desensitization of post-synaptic receptors: during transmission failure produced by either cyanide or FCCP, ionophoretic pulses of L-glutamate elicited the same post-synaptic response as they did under control conditions (Fig. 7). The continued sensitivity of the post-synaptic membrane to glutamate in the presence of cyanide and FCCP suggests that the block of transmission occurs presynaptically.

In the squid axon, cyanide poisoning begins to raise ionized calcium within 20–40 min of application (Baker *et al.* 1971) and the calcium concentration eventually equilibrates at or above the micromolar range (DiPolo, Rojas, Vergara, Lopez & Caputo, 1983; Baker & Umbach, 1983). The rate of rise and final level of ionized calcium can be enhanced by previous loading of calcium into the nerve and by

elevating extracellular calcium during poisoning. Cyanide blocks evoked synaptic transmission in 20–30 min and this rate of block is dependent on presynaptic nerve activity. However, in the axon, substantial recovery of the intracellular calcium concentration from cyanide poisoning usually occurs within 5 min of wash-out (Baker *et al.* 1971; DiPolo *et al.* 1983). The slow recovery of evoked transmission at the synapse may reflect the time required for the nerve terminal to recover from high levels of free calcium. In the squid axon, FCCP can also raise ionized calcium to reach the micromolar range and prolonged exposure results in irreversible increases of intracellular calcium (Brinley *et al.* 1977). At the synapse, FCCP and RuR irreversibly blocked evoked transmission, and by analogy with the axon it may be that increased intracellular calcium levels result in the inhibition of evoked transmitter release. Alnaes & Rahamimoff (1975) have shown that RuR depressed the evoked release of transmitter while it also increased the frequency and decreased the amplitude of miniature end-plate potentials at the frog neuromuscular junction. They suggested that this increase in spontaneous transmitter release was due to an increase in intracellular free calcium evoked by RuR. At the squid giant synapse, RuR irreversibly blocked synaptic transmission within 30–40 min. The time course of this effect most likely reflects a gradual rise of intracellular calcium following inhibition of mitochondrial calcium uptake processes.

Lithium sea water effectively depresses calcium buffering in squid axoplasm (Baker & Schlaepfer, 1978; P. F. Baker & J. Umbach, unpublished observation) and can increase (beyond 1 μM) the ionized calcium at the periphery of intact axons (Baker, Blaustein, Hodgkin & Steinhardt, 1969; Baker *et al.* 1971; DiPolo *et al.* 1983). On return to normal (sodium) sea water, the high level of ionized calcium is reduced to resting values within minutes. Similarly, at the squid synapse gradual depression of evoked transmission was observed in lithium sea water, which was rapidly reversed when sodium replaced lithium.

Each of the agents used are known to act at different sites, either to disrupt mitochondrial energy metabolism, block calcium uptake by mitochondria and endoplasmic reticulum or to interfere with calcium pumping across the membrane; yet all of them can cause a large increase in ionized calcium. All of our observations on the blocking of synaptic transmission by the metabolic inhibitors, RuR and lithium can be correlated with the data available for the effects of these agents on ionized calcium in the giant axon, and are consistent with the hypothesis that greatly elevated levels of ionized calcium in the presynaptic terminal block the evoked release of transmitter. The analogy developed here does not rule out other alternatives; however, the consequences of EGTA injections clearly implicate elevated calcium in the blockade of synaptic transmission caused by these substances.

Assuming that cyanide, FCCP, RuR and lithium all act to depress evoked synaptic transmission through elevating intracellular calcium, there are many sites in the release process that could be affected. High internal calcium, for example, could reduce the calcium influx by reducing the driving force and/or via calcium-dependent inactivation of presynaptic calcium channels (see Eckert & Chad, 1984). Alternatively, high levels of calcium could simply cause the depletion of transmitter by an increased steady leakage (prior to blockage). Again, however, the EGTA result and the rapid

recovery from lithium are difficult to resolve with this notion. Another possibility concerns the decrease in intracellular pH following exposure to metabolic inhibitors such as cyanide (Boron & De Weer, 1976). High internal proton concentrations directly block calcium current in *Paramecium* (Umbach, 1982) and probably facilitate the accumulation of intracellular calcium either by directly affecting the calcium–hydrogen exchange system or by displacement of calcium by hydrogen ions from internal sites (Moody, 1984). A final possibility is that high intracellular calcium could 'desensitize' some other step in the excitation–secretion process (see Fig. 18, Katz & Miledi, 1969) which normally requires the binding of calcium for it to be operational.

In conclusion, the most likely explanation for the observed depression of synaptic transmission by metabolic inhibitors and lithium sea water, is that a large rise in intraterminal calcium blocks the release of neurotransmitter. Indeed, it has been suggested previously that elevated presynaptic calcium levels (whether caused by prolonged absence of external sodium or by calcium injection) can depress evoked release at the squid giant synapse (Katz & Miledi, 1969; Kusano, 1970). Also, in other calcium-dependent systems a high intracellular level (5–100 μM) of ionized calcium can act as an inhibitor of exocytotic processes (Knight & Baker, 1982).

This project was supported by a Royal Society grant to D.J.A. A Beit Memorial Fellowship was held by D.J.A., a N.S.E.R.C. Fellowship (Canada) by K.T. and a Muscular Dystrophy Association Fellowship (U.S.A.) by J.A.U. We thank Professor P. F. Baker for helpful discussion and for the loan of equipment, Dr Karl Magleby for comments on a draft of the manuscript and the Director and Staff of the M.B.A. for laboratory space and facilities.

REFERENCES

ADAMS, D. J., TAKEDA, K. & UMBACH, J. A. (1983). Metabolic inhibitors and lithium depress synaptic transmission at the squid giant synapse. *Journal of Physiology* **345**, 147*P*.

ALNAES, E. & RAHAMIMOFF, R. (1975). On the role of mitochondria in transmitter release from motor nerve terminals. *Journal of Physiology* **248**, 285–306.

AUGUSTINE, G. J., CHARLTON, M. P. & SMITH, S. J. (1985). Calcium entry and transmitter release at voltage-clamped nerve terminals of squid. *Journal of Physiology* **367**, 163–181.

AUGUSTINE, G. J. & ECKERT, R. (1984). Divalent cations differentially support transmitter release at the squid giant synapse. *Journal of Physiology* **346**, 257–271.

BAKER, P. F. (1972). Transport and metabolism of calcium ions in nerve. *Progress in Biophysics and Molecular Biology* **24**, 177–233.

BAKER, P. F. (1978). The regulation of intracellular calcium in giant axons of *Loligo* and *Myxicola*. *Annals of the New York Academy of Sciences* **307**, 250–268.

BAKER, P. F., BLAUSTEIN, M. P., HODGKIN, A. L. & STEINHARDT, R. A. (1969). The influence of calcium on sodium efflux in squid axon. *Journal of Physiology* **200**, 431–458.

BAKER, P. F., HODGKIN, A. L. & RIDGEWAY, E. B. (1971). Depolarization and calcium entry in squid giant axons. *Journal of Phsiology* **218**, 709–755.

BAKER, P. F. & SCHLAEPFER, W. W. (1978). Uptake and binding of calcium by axoplasm isolated from giant axons of *Loligo* and *Myxicola*. *Journal of Physiology* **276**, 103–125.

BAKER, P. F. & UMBACH, J. (1983). Calcium electrode determination of ionized Ca and Ca-buffering capacity of squid axoplasm. *Journal of Physiology* **341**, 61*P*.

BAUX, G., SIMMONEAU, M. & TAUC, L. (1979). Transmitter release: ruthenium red used to demonstrate a possible role of sialic acid containing substrates. *Journal of Physiology* **291**, 161–178.

BLAUSTEIN, M. P. & HODGKIN, A. L. (1969). The effect of cyanide on the efflux of calcium from squid axons. *Journal of Physiology* **200**, 497–527.

BLAUSTEIN, M. P., RATZLAFF, R. & KENDRICK, N. K. (1978). The regulation of intracellular calcium in presynaptic nerve terminals. *Annals of the New York Academy of Sciences* **307**, 195–211.

BORON, W. F. & DE WEER, P. (1976). Active proton transport stimulation by CO_2/HCO_3^-, blocked by cyanide. *Nature* **259**, 240–241.

BRINLEY JR, F. J., TIFFERT, T., SCARPA, A. & MULLINS, L. J. (1977). Intracellular calcium buffering capacity in isolated squid axons. *Journal of General Physiology* **70**, 355–384.

BRYANT, S. H. (1958). Transmission in squid giant synapses. The importance of oxygen supply and the effects of drugs. *Journal of General Physiology* **41**, 473–484.

CHARLTON, M. P., SMITH, S. J. & ZUCKER, R. S. (1982). Role of calcium ions and channels in synaptic facilitation and depression of the squid giant synapse. *Journal of Physiology* **323**, 173–193.

CRAWFORD, A. C. (1975). Lithium ions and the release of transmitter at the frog neuromuscular junction. *Journal of Physiology* **246**, 109–142.

DATYNER, N. & GAGE, P. W. (1980). Phasic secretion of acetylcholine at a mammalian neuromuscular junction. *Journal of Physiology* **303**, 299–314.

DIPOLO, R., ROJAS, H., VERGARA, J., LOPEZ, R. & CAPUTO, C. (1983). Measurements of intracellular ionized calcium in squid giant axon using calcium-selective electrodes. *Biochimica et biophysica acta* **728**, 311–318.

ECKERT, R. & CHAD, J. E. (1984). Inactivation of Ca channels. *Progress in Biophysics and Molecular Biology* **44**, 215–267.

GILLESPIE, J. I. (1979). The effect of repetitive stimulation on the passive electrical properties of the presynaptic terminal of the squid giant synapse. *Procedings of the Royal Society* B **206**, 293–306.

KHARASCH, E. D., MELLOW, A. M. & SILINSKY, E. M. (1981). Intracellular magnesium does not antagonize calcium dependent acetylcholine secretion. *Journal of Physiology* **314**, 255–263.

KATZ, B. (1969). *The Release of Neural Transmitter Substances*. Sherrington Lecture X. Liverpool: Liverpool University Press.

KATZ, B. & MILEDI, R. (1967). A study of synaptic transmission in the absence of nerve impulses. *Journal of Physiology* **192**, 407–436.

KATZ, B. & MILEDI, R. (1969). Tetrodotoxin-resistant electrical activity in presynaptic terminals. *Journal of Physiology* **203**, 459–487.

KAWAI, N., YAMAGISHI, S., SAITO, M. & FURUYA, K. (1983). Blockade of synaptic transmission in the giant squid synapse by a spider toxin (JSTX). *Brain Research* **278**, 346–349.

KELLY, J. S. & GAGE, P. W. (1969). L-glutamate blockade of transmission at the giant synapse of the squid stellate ganglion. *Journal of Neurobiology* **1**, 209–219.

KNIGHT, D. E. & BAKER, P. F. (1982). Calcium dependence of catecholamine release from bovine adrenal medullary cells after exposure to intense electric fields. *Journal of Membrane Biology* **68**, 107–140.

KUSANO, K. (1970). Influence of ionic environment on the relationship between pre- and postsynaptic potentials. *Journal of Neurobiology* **1**, 435–457.

LLINAS, R., STEINBERG, I. Z. & WALTON, K. (1981*a*). Presynaptic calcium current in squid giant synapse. *Biophysical Journal* **33**, 289–322.

LLINAS, R., STEINBERG, I. Z. & WALTON, K. (1981*b*). Relationship between presynaptic calcium current and postsynaptic potential in squid giant synapse. *Biophysical Journal* **33**, 323–352.

MARTIN, R. & MILEDI, R. (1978). A structural study of the squid synapse after ultra-axonal injection of calcium. *Proceedings of the Royal Society* B **201**, 317–333.

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

MILEDI, R. (1973). Transmitter release induced by injection of calcium ions into nerve terminals. *Proceedings of the Royal Society* B **183**, 421–425.

MILEDI, R. & PARKER, I. (1981). Calcium transients recorded with arsenazo III in the presynaptic terminal of the squid giant synapse. *Proceedings of the Royal Society* B **212**, 197–211.

MILEDI, R. & SLATER, C. R. (1966). The action of calcium in neuronal synapses in the squid. *Journal of Physiology* **184**, 473–498.

MOODY JR, W. (1984). Effects of intracellular H^+ on the electrical properties of excitable cells. *Annual Reviews of Neuroscience* **7**, 257–278.

RAHAMIMOFF, R., MEIRI, H., ERULKAR, S. D. & BARENHOLZ, Y. (1978). Changes in transmitter release by ion-containing liposomes. *Proceedings of the National Academy of Sciences of the U.S.A.* **75**, 5214–5216.

REQUENA, J. & MULLINS, L. J. (1979). Calcium movement in nerve fibres. *Quarterly Reviews of Biophysics* **12**, 371–460.

STIMERS, J. R. & BYERLY, L. (1982). Slowing of sodium current inactivation by ruthenium red in snail neurons. *Journal of General Physiology* **80**, 485–497.

THOMAS, M. V. (1982). *Techniques in Calcium Research.* Academic Press: London.

UMBACH, J. A. (1982). Changes in intracellular pH affect calcium currents in *Paramecium caudatum*. *Proceedings of the Royal Society* B **216**, 209–224.

WALTON, K. & FULTON, B. (1983). Hydrogen peroxide as a source of molecular oxygen for *in vitro* mammalian CNS preparations. *Brain Research* **278**, 387–393.