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TRANSMITTER RELEASE: RUTHENIUM RED USED TO DEMONSTRATE A POSSIBLE ROLE OF SIALIC ACID CONTAINING SUBSTRATES

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SUMMARY

1. The possible function of sialic acid-containing substrates (SACS) in synaptic terminals of *Aplysia* was studied by intracellular injection of ruthenium red and of neuraminidase.

2. Ruthenium red, a dye known to have sialic acid as a molecular target, blocked transmission irreversibly in both cholinergic (buccal ganglion) and non-cholinergic (cerebral ganglion) synapses.

3. An intracellular site of action is likely because much less ruthenium red was necessary to block transmission when it was injected intracellularly than when it was presented by bath perfusion.

4. Ca^{2+} spikes recorded in the presence of tetrodotoxin or in Na^{+}-free solution were not modified by ruthenium red or neuraminidase injections or perfusions. It is therefore improbable that these substances blocked transmission by blocking voltage-dependent Ca^{2+} influx.

5. Strong electrotonic depolarization of a pre-synaptic interneurone in the presence of 10^{-4} M-tetrodotoxin caused a sustained post-synaptic response, which was abolished by ruthenium red. This result eliminates axonal conduction block as the principal mechanism of ruthenium red action.

6. Post-synaptic responses to ionophoretically applied acetylcholine (ACh) were not modified by bath perfusion of 2×10^{-2} M-ruthenium red.

7. Biochemical analysis of pools of [^{3}H]ACh was performed after injection of a precursor, [^{3}H]acetate, into an identified interneurone. Ruthenium red appeared to increase significantly the 'free' (cytoplasmic) ACh pool without any change of 'bound' (vesicular) [^{3}H]ACh-pool.

8. A model is proposed in which SACS act as intracellular Ca^{2+} receptors involved in transmitter release.

INTRODUCTION

Gangliosides and sialoglycoproteins are two classes of molecules characterized principally by their content of N-acetylneuraminic acid. From a quantitative point of view, sialoglycoproteins are a minor component of membranes, but they may perform important specialized functions, e.g. glycophorin in red blood cells (Rothstein, Cabantchik & Knauf, 1976).

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Gangliosides are present in many tissues but are found in highest concentration in the nervous system, especially in the plasma membranes of neurones (Warren, 1976). The highest levels of both sialoglycoproteins and gangliosides are found in synaptosomal membranes (Brunngraber, Dekirmenjian & Brown, 1967; Lapetina, Soto & De Robertis, 1967, 1968; Dekirmenjian & Brunngraber, 1969). It has been suggested that gangliosides comprise the bulk of negative surface charges in various cell types and that they consititute membrane receptors, e.g., for tetanus toxin and serotonin (Van Heyningen, 1977; Fishman & Brady, 1976). In addition, change in distribution of surface sialic acids were suggested to play a major role in cell recognition (Barondes, 1970; Roth, 1973).

In neurones the functions of such molecules remain unknown. Perturbation in the catabolism of gangliosides are responsible for severe nervous system illnesses characterized by accumulation of gangliosides, such as Tay Sachs disease and G_{M1} gangliosidosis (Fishman & Brady, 1976).

A neurophysiological approach to establishing the role of sialic acid-containing substrates (SACS) in neuronal activity has been to use enzymatic microdissection of the neuronal membrane by specific enzymes introduced inside the cell bodies (Tauc & Hinzen, 1974; Tauc, Hoffmann, Tsuji, Hinzen & Faille, 1974; Hinzen & Tauc, 1977). Neuraminidase injected into presynaptic interneurones was shown to block synaptic transmission (Tauc & Hinzen, 1974), presumably by cleaving intracellular SACS involved in transmitter release. Histological identification of SACS has been made by various methods. One of them was selective application of ruthenium red, a polycationic dye (Luft, 1971*a*, *b*; Bayer, Skutelsky, Wynne & Wilchek, 1976; Skutelsky, Danon, Wilchek & Bayer, 1978; Kahane, Polliack, Rachmilewitz, Bayer & Skutelsky, 1978) which binds to negative charges of sialic acid.

As the molecular target for both neuraminidase and ruthenium red appears to be the same, this latter substance was used by us in a similar way to study synaptic transmission. The data presented below indicate that both neuraminidase and ruthenium red act on SACS situated on the inner surface of the pre-synaptic terminal and that these substrates are involved in transmitter release. Brief reports of some of these data have appeared elsewhere (Tauc, Baux & Simonneau, 1977; Baux, Simonneau & Tauc, 1978; Simonneau, Baux & Tauc, 1978).

METHODS

Preparation

Experiments were performed on buccal, abdominal, and cerebral ganglia of *Aplysia californica* obtained from Dr R. Fay, Pacific Bio-Marine Supply Co., Venice, California, U.S.A. The isolated ganglia were pinned on the bottom of a plastic chamber containing a thin layer of Sylgard (Dow Corning), and most of the connective tissue covering them was removed with fine forceps. The buccal ganglia each contain a pair of presynaptic contacts with a number of post-synaptic cells situated close by in the same ganglion (Gardner, 1971; Fiore & Meunier, 1975; Gardner, 1977*a*; Gardner & Kandel, 1977). Synapses between identified neurones in the cerebral ganglia were also used (Fredman & Jahan-Parvar, 1975; Hinzen & Davies, 1978). The somata of the cells used were 200–250 μm in diameter in the buccal ganglia, smaller in the cerebral ganglia. Their recorded resting potentials were between -50 and -60 mV. None of the cells showed rythmic spike

activity. Studies on Ca^{2+} spikes were performed on the abdominal ganglion cells designated L_1 to L_6 by Frazier, Kuppfermann, Coggeshall, Kandel & Waziri (1967). These cells had a diameter of about 400 μm.

Solutions

The ganglia were continuously perfused at rates varying from 1·0 to 2 ml./min with artificial sea water (ASW) (NaCl 460 mM; KCl 10 mM; $MgCl_2$ 25 mM; $MgSO_4$ 28 mM; Tris-tris HCl pH 7·8, 10 mM; $CaCl_2$ 11 mM). For Na-free solution, Na was substituted by Tris-Tris HCl. A chamber volume of 0·9 ml. permitted rapid exchange of bathing solutions.

The following drugs and chemical were used: acetylcholine chloride (ACh); lanthanum chloride (La^{3+}), neuraminidase (N-acetylneuraminate glycohydrolase, E.C.3.2.1.18) from *Clostridium perfringens*, (Sigma), ruthenium red (Merck no. 12139, $Ru_2(OH)_2Cl_4 7NH_3 3H_2O$), [^{3}H]Na acetate (Amersham, 3·8 c/n-mole) was used in solutions containing 1 mc/μl.

Electrical recordings and intracellular micro-injection technique

Single or double-barrelled glass micro-electrodes were pulled on a de Fonbrune microforge and filled with 3 M-KCl and had a resistance of 5–20 MΩ. One of the barrels served for recording, the second for passing current to polarize the membrane. Pipettes for ionophoresis contained ACh (1 M in distilled water) or ruthenium red (2 mM in distilled water). Conventional ionophoretic injection techniques were used (Tauc & Gerschenfeld, 1961). We used voltage-clamp techniques similar to those described in Hinzen & Tauc (1977).

The quantity of ruthenium red injected was estimated by the formation of a faint rose halo around the injection tip. This was obtained with different durations of injection (injection current was a constant 30 nA) usually on the order of 1 min for the cells of the buccal ganglion and less for those in the cerebral ganglion. The variability of injection time is principally attributable to differing, and low, efficiencies of the injection electrodes. We estimated that the ruthenium red concentration in the soma was maximally 10^{-4} M; only a much smaller concentration can have reached the synapses.

Neuraminidase was used as a 5 % solution in 200 mM-KCl and injected into the cells by an air pressure system (Tauc *et al.* 1974). Simultaneous opening and closing of two solenoid valves (Danfoss type EVJ3) permitted an injection duration as short as 50 msec. The injection time could be controlled by a square current pulse delivered by a stimulator, but most of the injections were hand-initiated with a microswitch device.

Biochemical analysis

Presynaptic interneurones were loaded with [^{3}H]ACh precursors from micro-electrodes by passage of a 30 nA ionophoretic current for 60 min. Different precursors have been tested: choline, pyruvate and acetate. The best precursor is undoubtedly [^{3}H]choline (Koike, Kandel & Schwartz, 1974; Eisenstadt & Schwartz, 1975), but quantification of the ACh synthetized from it requires chromatographic analysis. Under our experimental conditions, only small amounts of [^{3}H]pyruvate were incorporated into ACh. Incorporation of [^{3}H]acetate was more efficient, but 500–1000 injected molecules of [^{3}H]acetate were apparently needed to label one molecule of ACh. This ratio is high enough to suggest that in *Aplysia* [^{3}H]acetate can be a precursor of ACh, as it was shown to be in the electric organ of Torpedo (Israel & Tucek, 1974) and the frog neuromuscular junction (Dreyfus, 1975). In our experiments, we preferred [^{3}H]acetate to [^{3}H]choline, as it permitted us to avoid the chromatographic step. Two hours after the injection of the precursor, when migration, synthesis, and storage of [^{3}H]ACh had presumably reached a steady state, ruthenium red was ionophoretically injected. Ninety minutes later, synaptic transmission being then completely blocked, ganglia were homogenized in 0·5 ml. microhomogenizer. In control experiments the same timing was respected, excluding the ruthenium red injection.

Two different procedures were performed: (1) homogenization in 0·5 ml. 5 % trichloracetic acid (TCA) in ASW for measurement of total [^{3}H]ACh, and (2) homogenization in 0·45 ml. ASW for 4 min followed by addition of 0·05 ml. TCA 50 % in ASW to permit destruction of extravesicular ACh by endogenous esterases (Dunant, Gautron, Israel, Lesbats & Manaranche, 1972; Israel & Dunant, 1975). Extraction of [^{3}H]ACh was performed by the Kalignost-ethyl butyl

acetone method (Fonnum, 1969) [^{3}H]ACh was counted with an Intertechnique (SL 30) counter (liquid scintillator: instagel). If excess acetylcholinesterase was added at the beginning of the extraction procedure, no more radio-activity could be detected, showing that [^{3}H]acetate led to the formation of [^{3}H]ACh in *Aplysia* buccal ganglion. The counts were corrected for losses in volume during extraction. The results are given in counts per minute, since only their relative values are interpreted in this study. The perfusion liquid was collected during the whole experiment and the total radio-activity in the perfusate counted.

RESULTS

Action of ruthenium red on a cholinergic synapse

(1) *Intracellular ionophoretic injection.* Study of ruthenium red action on synaptic transmission is made possible by the presence in *Aplysia* buccal ganglion of cholinergic synapses between two identified interneurones and their identified follower cells. The two interneurones are connected by a non-rectifying electrical synapse (Gardner, 1971) (Fig. 1).

The monosynaptic and cholinergic nature of their connexions to their follower cells is well established by classical identification methods (Gardner, 1971; Gardner, 1977*a*; Gardner & Kandel, 1977). Moreover synaptic transmission to the follower cells was blocked when acetylcholinesterase was intracellularly injected into these interneurones (Tauc *et al.* 1974). The same cells were able to synthesize labelled acetylcholine when a ^{3}H precursor was injected into the soma (Baux & Tauc in Tauc, 1977). Both results points to ACh as the transmitter involved at these synapses.

The resting potentials of pre- and post-synaptic cells were about -55 mV. The post-synaptic cells produced Cl^- dependent inhibitory post-synaptic potentials (i.p.s.p.s). The reversal level of the i.p.s.p. was about -62 mV. Post-synaptic cells were hyperpolarized to -80 mV to facilitate measurement of p.s.p. amplitude changes. In spite of the use of KCl electrodes in the post-synaptic cell, the reversal level of the i.p.s.p. decreased only by a few millivolts and did not significantly impinge on the amplitude of p.s.p.s at -80 mV membrane potential. Moreover, in all experiments the i.p.s.p. produced by the injected interneurone was compared to the i.p.s.p. of the non-injected interneurone in order to exclude completely any interference of biophysical changes in the post-synaptic cell.

When ruthenium red was ionophoretically injected in the presynaptic neurone of the buccal ganglion, synaptic transmission was entirely blocked in 60–120 min (Fig. 1). The p.s.p. amplitude started to decline in 10–20 min (Fig. 1). The resistance of pre- and post-synaptic cells, measured by electrotonic pulses, did not vary; resting membrane potentials and the amplitude of pre-synaptic spike also remained without significant changes (Fig. 1). A blockade of p.s.p. was also obtained with air pressure injection of ruthenium red (250 mM in ASW), but the quantity of injected dye was more difficult to control. The time course of the blocking action of ruthenium red was variable and depended on the quantity injected. Repetitive stimulation at any time after ruthenium red introduction neither accelerated nor slowed the development of the block. No recovery occurred during 3–4 hr observation periods. The amplitude of the p.s.p. produced in the same post-synaptic cell by the non-injected (test) interneurone was not depressed (Fig. 1).

(2) *Bath application.* When applied in the bath ruthenium red, 5 mM or more,

also had a depressive action on synaptic transmission in the buccal ganglion (Fig. 2). The time course of depression appeared faster for higher concentrations. Ruthenium red (1 mM) did not affect transmission even when applied for 16 hr. Complete block of the p.s.p. was obtained after 120 min with 15 mM, after 40 min with 10 mM and after 25 min with 20 mM concentrations. For 10 and 20 mM concentrations, 2–3 min

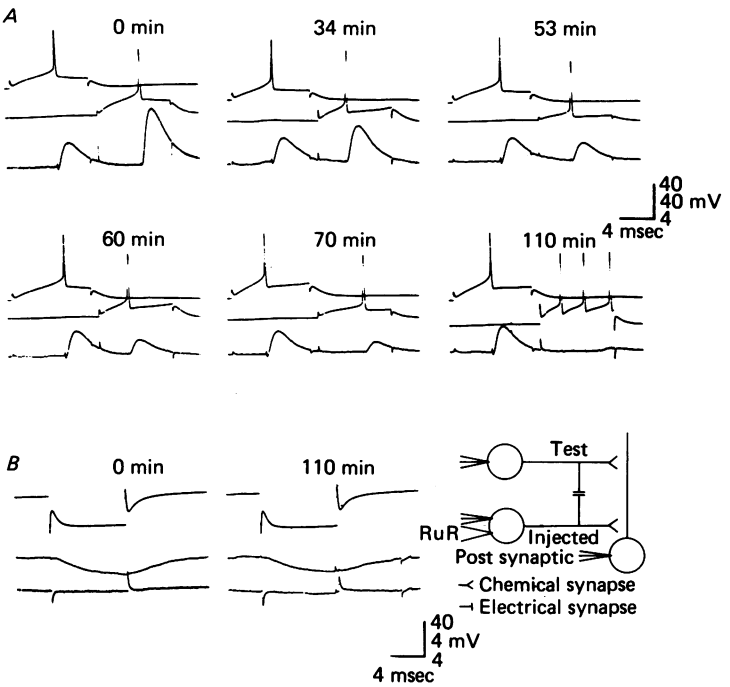

Fig. 1. Ruthenium red:intracellular ionophoretic injection. Recording from the two pre-synaptic interneurones (upper and middle traces) and a post-synaptic cell (lower traces) in *Aplysia* buccal ganglion. Middle traces correspond to injected interneurone recordings. *A*, the two pre-synaptic cells were stimulated with injected current to obtain an action potential and corresponding post-synaptic potentials. Post-synaptic cell was hyperpolarized to −80 mV to invert the p.s.p. for easier analysis. Ruthenium red was ionophoretically injected at time zero. Blockade of the p.s.p. was completed in 110 min. *B*, electrical coupling between the two interneurones. Current was injected into the test interneurone and records were obtained from it (upper traces) as well from the ruthenium red injection interneurone (middle traces). Lower traces represent post-synaptic cell. The electrical coupling ratio between the two interneurones was 0·05 before ruthenium red injection and 0·04 after complete blockade of synaptic transmission (t = 110 min). Insert: diagram of synaptic connexions between the two pre-synaptic interneurones and any one of several post-synaptic cells in *Aplysia* buccal ganglion. The two interneurones are coupled by electrical synapses. The chemical synapses are cholinergic and curare-sensitive, and the inhibitory post-synaptic response is caused by a chloride permeability increase.

after bath application, one could record a transient spontaneous firing of both pre- and post-synaptic cells, which indicated an activation of a large number of neurones. This activity led to p.s.p. facilitation, which was responsible for maintaining the amplitude of the p.s.p.s for a time (early part of the 10 and 20 mM curves of Fig. 2). When the test p.s.p. was blocked, high gain records showed that all the spontaneous

synaptic potentials disappeared, suggesting a general blockade of synapses. Slight recovery was observed after washing.

Action of ruthenium red on non-cholinergic synapses

(1) *Intracellular ionophoretic injection.* We tested the action of ruthenium red on non-cholinergic excitatory synapses of *Aplysia* cerebral ganglia (Fredman & Jahan-Parvar, 1975). The presynaptic and post-synaptic cells were the A and B groups

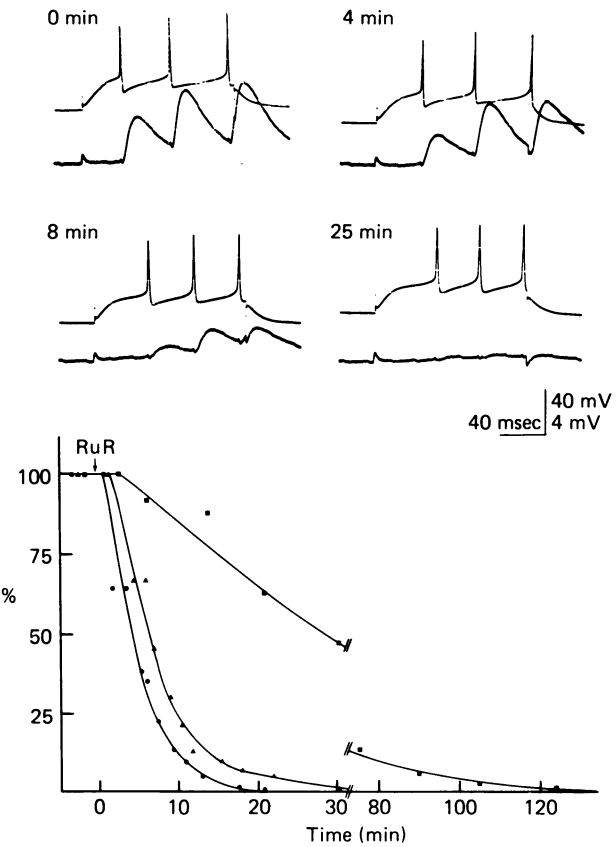

Fig. 2. *Upper half:* external application of ruthenium red by bath perfusion. Simultaneous intracellular recordings of a presynaptic interneurone (upper traces) and a post-synaptic cell (lower traces) in buccal ganglion. The dissected ganglion was perfused with 20 mM-ruthenium red in ASW. Control recordings in upper left corner. Blockade of p.s.p.s was almost complete in 20 min. *Lower half:* time course of transmission blockade in a buccal ganglion interneurone after bath perfusion of ruthenium red. Results are expressed as percent of maximum p.s.p. amplitude. Three different concentrations of ruthenium red were used, 5 mM (filled squares), 10 mM (filled triangles), 20 mM (filled circles).

described extensively by these authors. The monosynaptic nature of the connections was established with tetraethylamonium (TEA) injection by Hinzen & Davies (1978). The transmitter involved is not known but serotonin (5HT) appears to be a good candidate (Hinzen & Davies, 1978). After intracellular ionophoretic injection of ruthenium red into the presynaptic cell, full blockade of transmission developed in

2–2·5 hr, with a visible effect in 50 min (Fig. 3). The tested synaptic potential showed spontaneous changes in their amplitude which did not obscure the overall result but made it difficult to express the time course of the depression as a continuous curve. As with the buccal ganglion cells, the e.p.s.p. produced by a test interneurone in the same post-synaptic cells was not affected (Fig. 3).

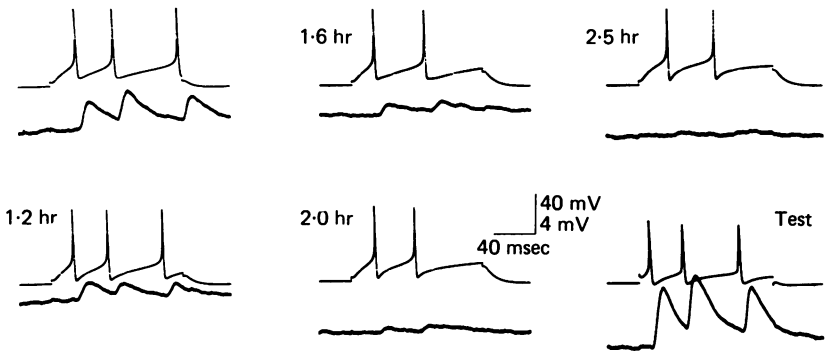

Fig. 3. Intracellular ionophoretic injection of ruthenium red in a non-cholinergic interneurone in the cerebral ganglion. Simultaneous intracellular recordings show e.p.s.p.s produced in a post-synaptic cell (lower traces) by presynaptic action potentials (upper traces). Post-synaptic cell was left at its resting potential (-50 mV in this experiment). Test record (lower right corner) was obtained in the same post-synaptic cell but by stimulation of a different, non-injected interneurone at the end of the experiment. Time in hours.

(2) *Bath application.* When applied in the bath, ruthenium red blocked synaptic transmission with the same speed as in the buccal ganglion. A complete blockade of post-synaptic potentials could develop in 15 min with 20 mM-ruthenium red. In all these experiments, resting membrane potentials input resistances and pre-synaptic spike amplitudes measured at the somatic level remained constant.

Ruthenium red action site

(1) *Ca spike and ruthenium red.* Ca channels were suggested as the possible sites of action of ruthenium red by Alnaes & Rahamimoff (1974). The depression of transmission at the neuromuscular junction by ruthenium red applied in the bath was tentatively explained by these authors as resulting from the blocking action of this dye on the Ca channels of the nerve terminal membrane. To investigate this possibility in our preparation, we studied the effect of ruthenium red on neurones showing Ca spikes. In some *Aplysia* neurones, spikes show a large Ca^{2+} component. Ca^{2+}-dependent spikes can be recorded from these neurones in the presence of tetrodotoxin (TTX), a well known blocking agent of Na^+ channels, or in Na-free ASW (Hagiwara, 1974). In presynaptic neurones of the buccal ganglia and in the presence of TTX (10^{-4} M) the remaining spike was found to be small even when it appeared able to induce a post-synaptic response. In general, it is considered that the voltage-dependent Ca channels have similar properties in all membranes so far studied (Hagiwara, 1974). We used abdominal ganglion cells having large Ca^{2+} spikes to investigate effects of ruthenium red on voltage-sensitive Ca influx. We chose cells L_1 to L_6 using 10^{-4} M-TTX or Na^+-free ASW as a test for Ca^{2+} spiking. As shown by Geduldig &

Junge (1968), it was necessary to depolarize the cells to -30 to -20 mV to activate Ca channels, and repetitive stimulations enhanced the Ca spike amplitude. The Ca-dependent nature of these spikes was further confirmed by blocking them with 2×10^{-3} M-La added to the bath.

Ionophoretic intracellular injection or bath application of ruthenium red (Fig. 4) had no effect on the threshold or the size of these spikes. The concentrations were

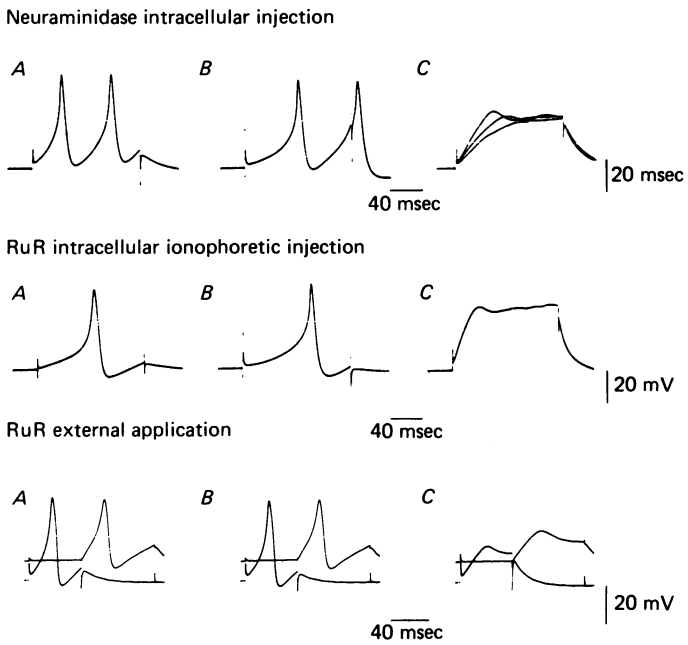

Fig. 4. Lack of effect of ruthenium red and neuraminidase on Ca action potentials. The first line corresponds to air pressure neuraminidase injection, the second line to ionophoretic injection of ruthenium red and the third line to bath application of 20 mM-ruthenium red. (In this last case simultaneous recordings were made from two different cells). Records in column *A*: spikes from L_1–L_6 cells in three different abdominal ganglia perfused with Na-free ASW (NaCl replaced with Tris-Tris HCl). Records in column *B*: same cells one hour after treatment. Records in column *C*: same cells after perfusion of 4 mM-$LaCl_3$. Note in line 1, superimposed traces for various intensities of stimulation.

similar to those used to block synaptic transmission. These results make it unlikely that an action on Ca^{2+} spiking was the mechanism by which ruthenium red depressed synaptic transmission.

The blocking of Ca channels in synapses depressed by injected neuraminidase was thought unlikely by Tauc & Hinzen (1974) because the small Ca component of the presynaptic spike persisted. When injected into the L_1–L_6 cells, neuraminidase did not affect the Ca spike (Fig. 4).

(2) *Ruthenium red and the electrotonic depolarization of synaptic terminals.* The axonal ramifications of invertebrate neurones and the peculiar axo-axonal localization of synapses require one to consider whether subtle spike conduction blocks could explain the failure of the synapse in our studies. Such a possibility was suggested to explain the action of various metabolic inhibitors in the neuromuscular junction (Krnjević &

Miledi, 1959). To investigate this, we tried to induce transmitter release in the absence of conducted spikes, by imposing steady depolarization on the pre-synaptic membrane. Transmitter release in the absence of detectable axonal spikes has been obtained by strong depolarization of pre-synaptic terminals in several preparations: neuromuscular

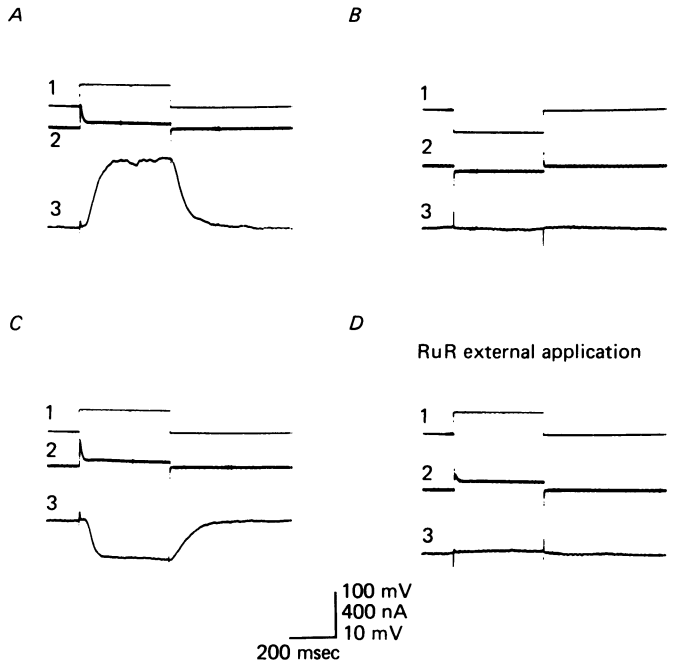

Fig. 5. Effect of imposed depolarization on transmitter release before (*A* to *C*) and after ruthenium red bath application (*D*). Upper traces represent imposed polarizations of the interneurone. Middle traces correspond to current recorded through the current voltage converter. Lower traces show the induced post-synaptic response. Throughout the experiment, the disected ganglia were bathed with 10^{-4} M-TTX in ASW. In *A*, the post-synaptic cell was hyperpolarized to -80 mV. In *B*, hyperpolarizing pulse of the same amplitude was imposed on the interneurone. The post-synaptic cell was hyperpolarized to -80 mV. Note the absence of coupling between the interneurone and the post-synaptic cell. In *C*, as in *A*, but the post-synaptic cell was depolarized to -30 mV. The inversion potential of post-synaptic response was -62 mV (not shown). In *D*, complete blockade of sustained release after ruthenium red bath application. The post-synaptic cell was maintained at -80 mV. Note the difference in outward current.

junction (Katz & Miledi, 1976*a*, *b*), squid giant synapse (Katz & Miledi, 1967*c*), Mauthner cell axon giant synapse (Martin & Ringham, 1974, 1975).

In buccal ganglion synapses, it was possible to change the amplitude of p.s.p.s by manipulating presynaptic membrane potential. Hyperpolarization of the presynaptic cell reduced p.s.p. amplitudes and depolarization increased them, as already shown at other *Aplysia* synapses by Shimahara & Tauc (1975) and Hinzen & Davies (1978). These results suggest a short distance between presynaptic soma and synaptic terminals. This distance has been estimated at few hundred microns (maximum 1 mm) from intracellular horse-radish peroxidase (unpublished results) or acetycholinesterase injections (Tauc *et al.* 1974).

Presumably because of a similarly small distance between soma and terminals in our system, a strong imposed depolarization of presynaptic terminals was able to liberate ACh when Na action potentials were blocked by Na-free ASW or TTX. Voltage clamping of pre-synaptic cell regions near the soma can be used to control transmitter release. As judged from the post-synaptic response, ACh was liberated

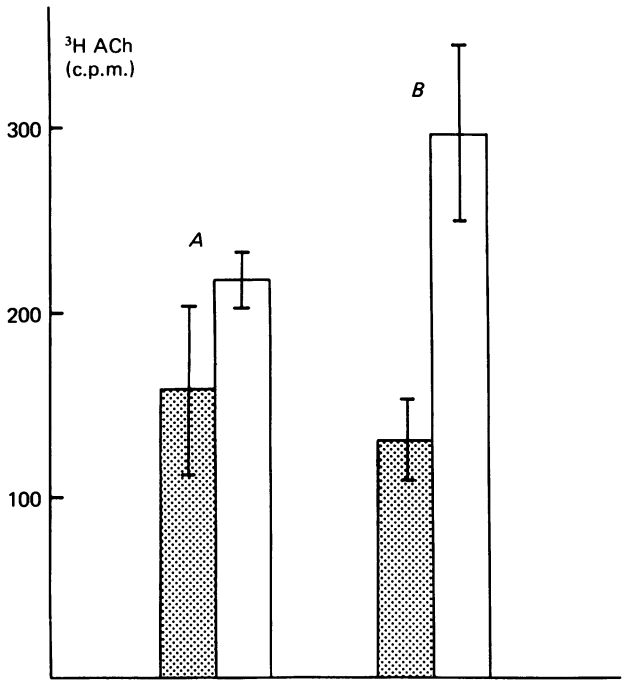

Fig. 6. Effect of ruthenium red on ACh pools (see Methods). Bound [³H]ACh and total [³H]ACh are represented respectively by punctate and white bars. *A*, results obtained from control neurones. *B*, data obtained after synaptic transmission was depressed with intracellular ruthenium red.

only when the soma membrane potential was brought to -10 mV; stronger depolarization produced increased responses. Using prolonged depolarization, the post-synaptic response can be maintained for several seconds. After injection of ruthenium red in the pre-synaptic cell or external application via the bath, sustained release of transmitter could no longer be obtained by imposed depolarization (Fig. 5). The transient outward current change can be tentatively attributed to blockade of self-inhibitory synapses described by Gardner (1977*b*) in these cells.

(3) *ACh receptors and ruthenium red.* ACh receptors or associated ionic channels situated on the post-synaptic cell are other possible sites of ruthenium red action. Interaction between ruthenium red and the ACh receptor was eliminated as an explanation for the present results by studying the effect of ionophoretic applicaton of ACh to post-synaptic neurones. As in most *Aplysia* neurones, extrasynaptic ACh receptors can be found on the soma and have the same properties as subsynaptic receptors (Tauc & Gerschenfeld, 1961). Ionophoretically applied ACh produced clear hyperpolarizating responses which reversed to depolarizing ones when the soma was

hyperpolarized, showing the same reversal potential as the i.p.s.p. The amplitude and the time course of the ACh responses were not changed when ruthenium red was added to the bath, in spite of the complete blocking of synaptic activities.

(4) *Ruthenium red and ACh pools.* Methods have been developed for measuring ACh synthesis in a single neurone previously injected with a radioactive precursor (Koike *et al.* 1974; Eisenstadt & Schwartz, 1975). This technique permitted us to analyse ACh content of interneurones before and after ruthenium red treatment. We found that newly synthesized ACh was distributed into two compartments, as has been described in other preparations (Israel *et al.* 1968, 1970; Dunant *et al.* 1972; Israel & Dunant, 1975; Marchbanks, 1977). A so-called 'bound' pool was detected after homogenization of the ganglion in ASW. It probably represented intravesicular ACh, which was protected against endogenous acetylcholinesterase (AChE) liberated by homogenizations (Whittaker, Michaelson & Kirkland, 1963; Whittaker, Essman & Dowe, 1972). When the homogenization was done in presence of 5 % TCA to inactivate the endogenous AChE, higher quantities of ACh ('total ACh') were detected which suggested the presence of a 'free' pool of ACh which can be destroyed by AChE (Fig. 6). This 'free' ACh might have come from the cytoplasm. A cytoplasmic compartment of unbound ACh has been suggested as the source of ACh continuously leaving the nerve ending at rest (Katz & Miledi, 1977) and also of tonically released ACh during synaptic transmission (Tauc *et al.* 1974).

We compared 'free' and 'bound' [^{3}H]ACh in test and experimental situations. Due to experimental difficulties, only a limited number of preparations could be used (mostly three for each group). However, the results show clearly that after blockade by intracellular injection of ruthenium red the amount of bound [^{3}H]ACh did not change significantly, whereas total [^{3}H]ACh increased significantly after ruthenium red treatment ($P > 0{\cdot}05$ by Student's t test). Thus a large amount of 'free' [^{3}H]ACh was present after ruthenium red blockade (Fig. 6).

DISCUSSION

Intracellular action of ruthenium red

The observed results are in agreement with the hypothesis that ruthenium red exerted its action on these *Aplysia* synapses by binding to the SACS situated inside the terminal membrane and thus interfering with liberation of transmitter. The following points support this conception: ruthenium red did not alter spike mechanisms observable in the soma associated with transmitter release. Neither ionophoretic injection nor extracellular application of ruthenium red induced any change in the measurable electrical properties of the membrane. Spike size and duration did not change during the experiments. Moreover, transmitter release was obtainable in the absence of spikes by strong depolarization, and this release was also blocked by ruthenium red. Responses of post-synaptic neurone somata to ionophoretically applied ACh did not change after ruthenium red treatment. Since the close pharmacological resemblance of synaptic and somatic receptors is well documented (Ascher & Kehoe, 1975), blockade of synaptic receptors is a very improbable mechanism of action. When ionophoretically injected into post-synaptic cells, ruthenium red had no effect on synaptic transmission. All these data favour a presynaptic site of action.

A blockade of presynaptic Ca channels was suggested by Rahamimoff & Alnaes; 1973; Alnaes & Rahamimoff, 1975 to explain the depression of evoked end-plate potentials caused by ruthenium red at the neuromuscular junction. They compared its action on the nerve terminal to its action on mitochondrial membrane, where

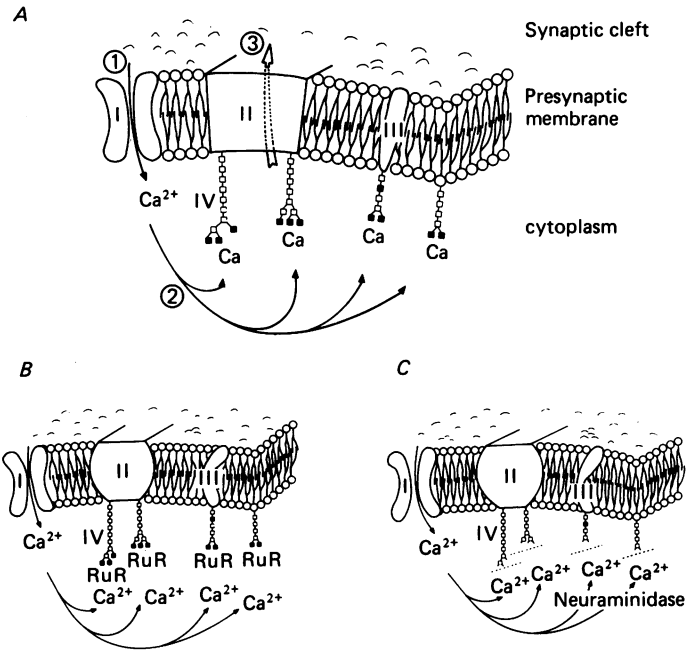

Fig. 7. Schematic illustration of proposed events in presynaptic membrane linking SACS to liberation of transmitter. Structures represented in the lipid bilayer are (1) voltage dependent Ca channels, (II) unknown membrane elements critical for transmitter release, (III) sialo-glyco-protein with a sialic acid moiety protruding into the cytoplasm, and (IV) glycosidic chain linked in a trigger-like relationship to other components of the release apparatus. Other glycosidic chains are attached to membrane lipids; possible external glycosidic chains are not represented. Sugars are represented by open squares, sialic acids by filled squares. Normal operation (*A*) is supposed to involve (1) Ca^{2+} entry through voltage-sensitive channels following terminal depolarization; (2) binding of Ca^{2+} to sialic acid moieties leading to (3), membrane conformational changes directly responsible for release of transmitter into the synaptic cleft. When ruthenium red binds to the negative charges of sialic acids (*B*) or when the sialic acids are cleaved by neuraminidase (*C*) this sequence of events cannot be completed.

Ca uptake mechanism is clearly inhibited by this drug. However, in the neuromuscular preparation no direct evidence of the blockade of voltage-dependent Ca^{2+} channels by ruthenium red was obtained, as presynaptic terminals are too small to be penetrated with micro-electrodes. Similarly, inhibition of [^{3}H]GABA release from synaptosomal preparations (Tapia & Meza-Ruiz, 1976) by ruthenium red has been interpreted as resulting from Ca^{2+} channel blockade and uptake of ^{45}Ca ions by synaptosomes was shown to decrease after treatment with ruthenium red (Swanson, Anderson & Stahl, 1974). These latter results are related to 'passive' Ca^{2+} influx and,

to our knowledge, no data are available which show voltage-dependent Ca^{2+} influx of the kind involved in regenerative spikes to be decreased by ruthenium red.

In *Aplysia*, we succeeded in obtaining direct evidence that ruthenium red has little effect on Ca spikes. Large Ca spikes recorded in the absence of Na^+ or with TTX persisted after extracellular or intracellular application of ruthenium red. As it seems reasonable to suppose that voltage-sensitive Ca^{2+} permeability in the somatic membrane is identical to that in the synaptic terminal membrane (Stinnakre & Tauc, 1973), we conclude that ruthenium red probably did not depress synaptic transmission by blocking voltage-sensitive calcium channels.

The strong depression of the amplitude of miniature end-plate potentials (m.e.p.p.s) (Fig. 7 in Alnaes & Rahaminoff, 1975) by ruthenium red has been suggested to have a post-synaptic origin. In view of our observations showing no clear effect on ACh post-synaptic receptors, it is possible to propose that m.e.p.p. depression was a result of impeded transmitter release.

We believe that ruthenium red has to be inside the terminal to be effective. This conception is supported by the fact that at least two orders of magnitude less ruthenium red is necessary to block transmission by intracellular injection than by application via the bath. Ruthenium red can probably penetrate the nerve membrane of neuromuscular junctions (Alnaes & Rahamimoff, 1975), since when added to the bath, the frequency of m.e.p.p.s increased, probably because of the increase of intracellular free Ca^{2+} liberated by ruthenium red from the mitochondria (Lehninger, 1970; Rose & Loewenstein, 1975). There is also some histological evidence of the penetration of ruthenium red (Singer, Krishnan & Fyfe, 1972).

We have not been able to visualize the penetration of extracellular ruthenium red (20 mM) by electron microscopy. Since only a very small amount of intracellular ruthenium red was necessary to block synaptic transmission, we also found that no staining of the terminals was detectable after injection of blocking quantities of the dye into the interneurone.

However, in the mammalian nervous system, micrographs of synapses (Fig. 1 in Tani & Ametani, 1970) clearly showed intracellular densities attributable to ruthenium red on the inner side of the presynaptic membrane.

Molecular target of ruthenium red

Ruthenium red blocked synaptic transmission in both cholinergic and non-cholinergic synapses. Its action is thus not dependent on the chemical nature of the transmitter. If our above conclusion regarding the intracellular action of ruthenium red is correct, the most obvious target for it appears to be sialic acids of the inner presynaptic membrane, since it is known to have a high affinity for such molecules. The synaptic blocking action of ruthenium red is similar to that of neuraminidase, an enzyme cleaving sialic acids of gangliosides and sialoglycoproteins (Tauc & Hinzen, 1974). There is of course a fundamental difference in the mechanisms of action: whereas the neuraminidase cleaves sialic acid bonds, ruthenium red binds to the negative charges of the terminal sialic acid moieties of these macromolecules. If one accepts the evidence that neither post-synaptic effects nor changes in Na or Ca spikes are necessary for synaptic blockade by these compounds, impeded transmitter release

secondary to modification of gangliosides or sialoglycoproteins becomes still more plausible mechanism for their action.

Experimental means exist for further investigation of this possibility. Any sialic acid molecules which participate in release must be on or very close to the sites where it occurs. The time between Ca entry and the measurable post-synaptic response was measured to be 200 μsec in the squid giant synapse (Llinas, 1977); thus Ca^{2+} movements inside the terminals before its binding to intracellular receptors leading to transmitter release must be very limited. This requires that such receptors be close to Ca channels, possibly on the membrane itself. Anatomical localization of sialic acid on synaptosomal membrane, though difficult, is within the range of available techniques. A demonstration that it is relatively concentrated near the region of synaptic specialization would favour, and the opposite finding would weigh heavily against, the theory presented here. Martin & Miledi (1978) recently examined the morphology of the squid giant synapse after injection of Ca^{2+} directly into the presynaptic terminal. They demonstrated dense deposits, presumably Ca binding sites, which were more prominant on the inner surface of the presynaptic membrane than elsewhere (Fig. 4 in Martin & Miledi). It would be of great interest to compare the topography of such deposits with sialic acid distribution.

The widely held view that transmitters are released from vesicles by exocytosis could lead one to explain the blocking of neuraminidase and of ruthenium red as intereference with fusion of synaptic vesicles to the presynaptic membrane. In fact, this has been suggested on the basis of an *in vitro* model (Dahl, Gratzl & Ekerdt, 1978). Otherwise, if a cytoplasmic ACh pool in the vicinity of the terminal membrane can be the source of immediately releasable transmitter (Tauc *et al.* 1974), sialic acid-containing molecules would be well situated to act as intracellular Ca receptors involved in the release process. Were such Ca^{2+} binding blocked by neuraminidase or ruthenium red, release could not occur and the cytoplasmic ACh pool would increase. Our biochemical results are consistent with this mechanism; the constancy of the bound pool indicates that the excess ACh did not come from vesicles.

An alternate explanation of the action of neuraminidase and ruthenium red is possible. These substances might affect mitochondria or other organelles in such a way as to increase cytoplasmic free Ca, which, in sufficient quantity, could block transmission (Kusano, 1970). Against this possibility are the facts that blockade by ruthenium red or neuraminidase is irreversible, the quantity of intracellular Ca thus liberated is limited, and the excess Ca should be extruded from the cell, unless that transport mechanism was also affected. Furthermore, membranes from synaptosomal mitochondria contain at most only traces of sialoglycoproteins (Zanetta, Reeber, Ghandour, Vincendon & Gombos, 1975), unlike those from liver preparations (Lehninger, 1973; Gomez-Puyou, Tuena de Gomez-Puyou, Becker & Lehninger, 1972). If Ca^{2+} binding proteins in synaptosomal mitochondria are not sialoglycoproteins, an action of neuraminidase on these mitochondria appears improbable, and so far as we know, has not been described.

We propose a model, necessarily oversimplified, to account for the present data. We suppose that (1) Ca^{2+} enters during presynaptic depolarization and binds to negative sites of sialic acids, inducing in the presynaptic membrane conformational changes which are necessary for transmitter release, (2) such conformational changes

are no longer possible after the sialic moieties have been cleaved by neuraminidase or their Ca-binding sites occupied by ruthenium red (Fig. 7). This model represents a working hypothesis intended to attract attention to the possible importance of SACS in synaptic transmission.

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