

Inhibitory Action of Ruthenium Red on Neuromuscular Transmission

(presynaptic nerve terminal/ Ca^{++} transport)

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ABSTRACT The effect of Ruthenium Red on synaptic transmission was examined at isolated junctions of the frog, by conventional methods for stimulation and intracellular recording. Ruthenium Red (2.5–10.0 μM) reduces the synaptic potential to subthreshold levels. An analysis of this phenomenon shows that the main action of Ruthenium Red is on the presynaptic nerve terminal where it decreases the number of quanta of transmitter liberated by the nerve impulse. It has the following additional effects: a reduction in the amplitude of the spontaneous miniature end plate potentials; an increase in their frequency; and an increase in delayed release of transmitter after a nerve impulse. Some of these results are discussed in terms of the known inhibitory action of Ruthenium Red on calcium transport across mitochondrial membranes.

Calcium ions play an essential role in synaptic transmission at the neuromuscular junction (1). Their main action is on the presynaptic nerve terminal, where the influx of calcium ions apparently links the nerve impulse to the process of secretion of neurotransmitter (2). The inorganic dye Ruthenium Red (3) is a powerful inhibitor of calcium transport across the mitochondrial membrane (4, 5). Hence, it would be expected that if the calcium transport systems in the mitochondria and in the nerve terminal membrane share common properties, Ruthenium Red might act as an inhibitor of evoked release of transmitter. If, in addition, significant amounts of Ruthenium Red penetrate into nerve fibers (6), it is expected that the mitochondrial uptake of calcium would be inhibited, which in turn might increase the intracellular free $[\text{Ca}^{++}]$ and, consequently, the spontaneous release of transmitter. Initial experiments that support this view are reported.

MATERIALS AND METHODS

Experiments were done at room temperature (about 20–24°) on a cutaneous pectoris preparation of frog (*Rana pipiens*), which was isolated and bathed in standard Ringer's solution of the following composition: 115 mM NaCl–2.0 mM KCl–1.8 mM CaCl_2 –1 mM NaH_2PO_4 . The pH of the solution was 6.8–7.0. Occasionally the divalent cation composition of the medium was altered by isoosmotic substitution for sodium. In several experiments, phosphate was omitted.

The nerve was stimulated with a suction electrode at a rate of 0.2–0.5 pulses/sec. Conventional, KCl-filled micropipettes were used for intracellular recording (resistances 20–80 $\text{M}\Omega$). For recording of synaptic potentials, muscle fibers were im-

paled about 50–100 μm from the terminals, which were visually identified under Nomarski interference optics (7).

For estimation of the number of quanta liberated by the nerve impulse (quantal content = m), two parameters were measured: R , the amplitude of the endplate potential, and a , the amplitude of the spontaneously released miniature endplate potential. Then, $m = \bar{R}/\bar{a}$ (8). When R was more than 2 mV, a correction for nonlinear summation was applied (9). When \bar{a} was too small to be measured accurately, m was estimated by the coefficient of variation method (8).

Ruthenium Red was obtained from Sigma Chemical Co.

RESULTS

Neuromuscular Blockade by Ruthenium Red. In 14 experiments the muscle was made to contract by maximal stimulation of the motor nerve, every 5 sec. On addition of Ruthenium Red (2.5–10 μM), the contractions were abolished within 10 min. Intracellular electrodes near the endplate still recorded subthreshold endplate potentials (Fig. 1). The amplitude of these endplate potentials varied between 1 and 20 mV, de-

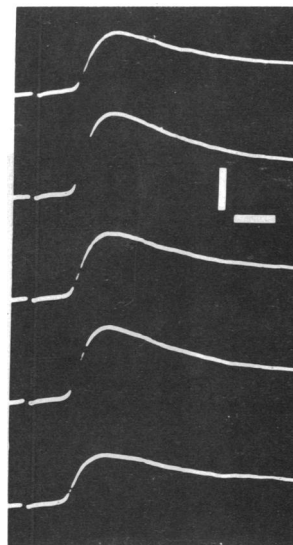


FIG. 1. Neuromuscular blockade by Ruthenium Red. The preparation was bathed in standard Ringer's solution with the addition of 5 μM Ruthenium Red. Note that all endplate potentials are subthreshold. Calibration: 2 mV, ordinate; 2 msec, abscissa. Frequency of stimulation, 0.2 pulses/sec.

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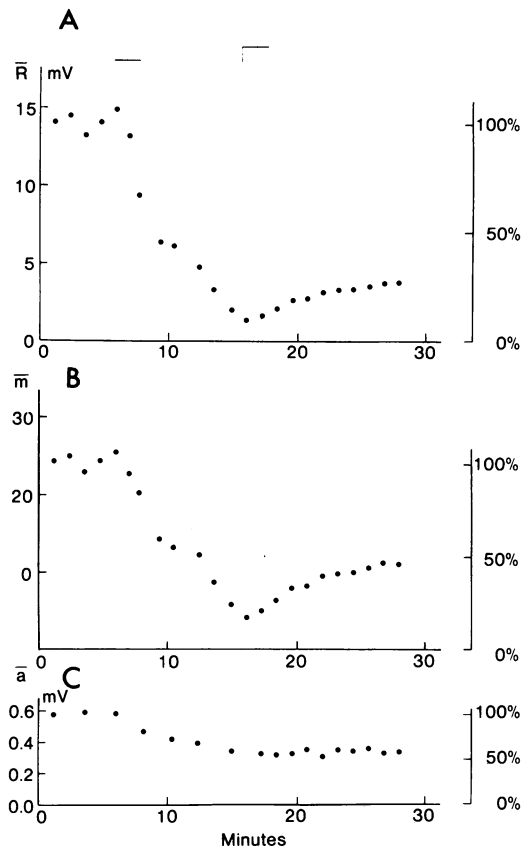


FIG. 2. Presynaptic and postsynaptic effects of Ruthenium Red. Neuromuscular transmission was blocked by a Ringer's solution containing 0.6 mM Ca and 4 mM Mg. (A) Endplate potential amplitude. Each point represents the average of 10 measurements. At the first mark 5.0 μ M Ruthenium Red was added. At the second mark the preparation was washed with the control solution. (B) Quantal content. (C) Miniature endplate potential amplitude. Experimental details in B and C correspond to those in A.

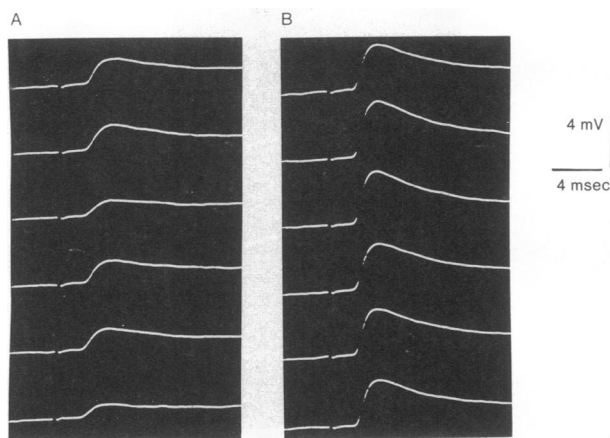


FIG. 3. The effect of Ca on a preparation blocked by Ruthenium Red. (A) Sample records in the presence of 3.6 mM Ca. Quantal content = 42.5 (estimated on a series of 210 responses by the coefficient of variation method). (B) Sample records in 7.2 mM Ca. Quantal content = 112. (Total length of series, 290 responses.)

pending on the concentration and the duration of the exposure to Ruthenium Red.

Site of the Blocking Action of Ruthenium Red. To determine whether the site of action of Ruthenium Red is pre- or postsynaptic, it is necessary to measure the amplitudes of the evoked endplate potentials. This was done on preparations where the normal release of neurotransmitter was reduced by a medium containing 0.6 mM of Ca and 4 mM Mg (10, 11). Under these control conditions the amplitude of the synaptic response was subthreshold; in the experiment illustrated in Fig. 2 it was nearly 15 mV, with a mean quantal content of about 25. Addition of 5 μ M Ruthenium Red to the control perfusing medium (*first shaded block*), markedly reduced the amplitude of the synaptic response to less than 2 mV (Fig. 2A). This reduction was caused by a decrease in both the quantal content (Fig. 2B) and the size of the miniature endplate potential (Fig. 2C). However, a comparison of Fig. 2B with Fig. 2C shows that Ruthenium Red has a much more prominent effect on the quantal content (down to 20% of control), than on the size of the miniature endplate potential (50% of the control). Thus, it seems that the main action of Ruthenium Red, in these ranges of concentration, is on the presynaptic terminal, where it reduces the number of quanta liberated by the nerve impulse. Fig. 2 also shows that the action of Ruthenium Red reversed only slightly after a wash with the control solution (*second shaded block*).

When Ruthenium Red caused a decrease in the quantal content, successive endplate potentials fluctuated in amplitude, and occasionally a complete failure in release occurred. The number of these failures was accurately predicted by the Poisson theorem and, therefore, they were part of the normal stochastic behavior of the release process at low quantal con-

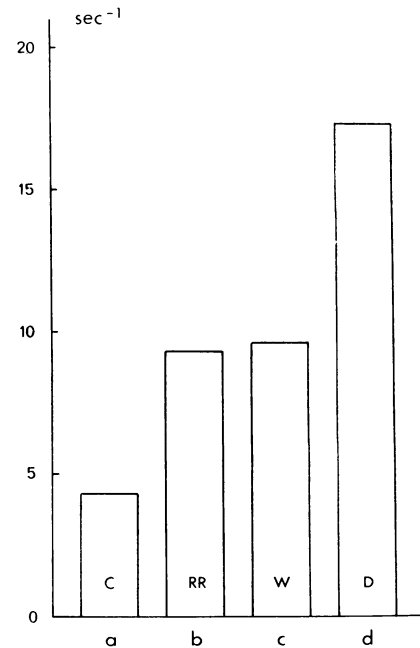


FIG. 4. Effect of Ruthenium Red on miniature endplate potential frequency. (a) Control ($n = 149$). (b) After addition of 5 μ M Ruthenium Red ($n = 146$). (c) After return to control ($n = 422$). (d) After addition of 0.5 mM Dicoumarol ($n = 531$). n is the number of events used for the frequency estimation.

tent, and were presumably not due to conduction block in the nerve terminals.

The reduction in amplitude of miniature endplate potentials was concentration dependent. While at 5 μ M their amplitude was about 50% of the control, at 7.5 μ M there was a further decrease to about 30%. (In one experiment, at 40 μ M Ruthenium Red, all synaptic potentials disappeared.) This apparent decrease in synaptic sensitivity did not arise from a decrease in muscle membrane resistance, since in two experiments there was an increase in input resistance after addition of Ruthenium Red.

The Opposing Action of Ca^{++} on Ruthenium Red Blockage.

Block of neuromuscular transmission by Ruthenium Red can be antagonized by an increase in the extracellular $[Ca^{++}]$. For example, in Fig. 3A, transmission had been blocked by 5 μ M Ruthenium Red in Ringer's solution containing 3.6 mM Ca. A doubling of the $[Ca^{++}]$ (to 7.2 mM) more than doubled the amplitude of the endplate potential (Fig. 3B). An analysis of variance revealed that the addition of calcium increased m to 235%, while the mean amplitude of the miniature endplate potential remained practically unchanged.

In several experiments, normal transmitter release was restored simply by a further increase in $[Ca^{++}]$. Thus, a shift from the standard 1.8 to 14.4 mM, brought the quantal content from 21.7 to 279.6, which is in the normal range of m (12).

No systematic experiments were made to determine the nature of the antagonism between Ruthenium Red and Ca^{++} , nor were the kinetics of the interaction studied in detail.

Ruthenium Red and Spontaneous Release of Transmitter.

The effect of Ruthenium Red on spontaneous release of transmitter was to increase the frequency of the miniature endplate potentials. This was observed in three experiments, in which the initial mean amplitude of the spontaneous events was large enough so that even after the decrease in size by Ruthenium Red, the miniature endplate potentials were clearly above noise level. This increase in frequency is illustrated in Fig. 4. The first column (a) represents the frequency in standard Ringer's solution. Addition of 5 μ M Ruthenium Red, approximately doubled the frequency (column b). This effect of Ruthenium Red was not reversed by washing the sample in standard solution for 15 min (column c). Interestingly, another mitochondrial inhibitor, Dicoumarol (Na-Warfarin), which presumably operates on a different mechanism (13), could even further increase the frequency of the miniature endplate potentials (column d). Not only was the resting frequency of miniature endplate potentials increased by Ruthenium Red and Dicoumarol, but also the delayed release of transmitter (14). The miniature endplate potential frequency during an arbitrary period of 56 msec after the stimulus (f_A) was compared to the frequency before nerve stimulation (f_B) (Table 1). Under control conditions, this delayed release of transmitter ($f_A - f_B$) was only 0.18 miniature endplate potentials per sec, and increased to 0.98 miniature endplate potentials per sec and 3.66 miniature endplate potentials per sec after the addition of Ruthenium Red and Dicoumarol, respectively.

DISCUSSION

The results show that Ruthenium Red is a potent blocking agent of neuromuscular transmission. This substance acts at

TABLE 1. Release of transmitter and Ruthenium Red

	Quantal content m	Delayed release ($f_A - f_B$) (\pm SEM)	n_B	n_A
Control	25.09	0.18 \pm 0.06	48	94
Ruthenium Red (5.0 μ M)	4.02	0.98 \pm 0.11	83	173
After wash	11.23	1.14 \pm 0.10	135	286
Dicoumarol (0.5 mM)	20.35	3.66 \pm 0.12	265	608

f_A = frequency of miniature endplate potentials in 56 msec after the endplate potential (miniature endplate potentials/sec).

f_B = frequency of miniature endplate potentials in 35 msec before the endplate potential (miniature endplate potentials/sec).

n_A and n_B are the corresponding numbers of miniature endplate potentials.

comparable concentrations as the classic blocker of transmission, *d*-tubocurarine. The site of action is, however, different. While *d*-tubocurarine affects mainly the postsynaptic membrane (15), Ruthenium Red acts primarily on the transmitter release process. Since our main interest was the action of Ruthenium Red on the secretion by the presynaptic nerve terminal, the effects on the postsynaptic muscle membrane, namely the decrease in miniature endplate potential amplitude and the increase in membrane resistance, were not investigated in detail.

Three presynaptic effects of Ruthenium Red were observed: decrease in quantal content, increase in miniature endplate potential frequency, and increase in delayed release. The simplest explanation for the diminished quantal content would be that Ruthenium Red decreases the transport of Ca^{++} into the nerve terminal during activation, an effect analogous to that on the mitochondrial membrane. It should be noted that in the latter case Ruthenium Red stops both the active and the passive uptake of Ca^{++} (5). Alternative modes of action of Ruthenium Red, such as an effect on membrane polarization, have not been ruled out.

Singer *et al.* (6) have shown that Ruthenium Red can be detected inside the nerve (probably entering through the unmyelinated portions). If such entry also occurs at the frog motor nerve terminal, one would expect a partial inhibition of Ca^{++} uptake into the mitochondria. This uptake would increase intracellular $[Ca^{++}]$ and, thus, may account for the increase in miniature endplate potential frequency and delayed release.

A further increase in the frequency of the miniature endplate potentials and in delayed release can be achieved when the mitochondrial activity is more substantially inhibited by Dicoumarol (Table 1). Under those conditions not only was the spontaneous release enhanced, but also the release evoked by the nerve action potential. This result makes one wonder whether mitochondria may play an active physiological role in regulation of the intracellular calcium concentration similar to that in other cells (16) and in this way be partially responsible for the termination of transmitter release after a nerve impulse.

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