The effect of calcium-ionophores on acetylcholine release from Schwann cells

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After denervation of frog muscle, the Schwann cells release multimolecular amounts of acetylcholine, which evoke miniature endplate potentials in the muscle.

The effect of calcium ionophores (X-537A, A23187 and avenaciolide) and rabbit serum, all of which presumably raise the intracellular level of ionized calcium, was examined at normal and denervated endplates. The agents produced a large increase in the release of acetylcholine quanta from nerve terminals. In contrast, the release of acetylcholine quanta from Schwann cells was greatly depressed.

It is suggested that two systems may be involved in the release of substances from cells. One is activated by a rise in intracellular Ca\(^{2+}\), as in axon terminals; the other is inhibited, as in the Schwann cells.

Introduction

It is known that miniature endplate potentials (min. e.p.ps), due to a multimolecular action of acetylcholine (ACh) on the muscle membrane, are seen in denervated frog muscle fibres well after the axon has degenerated (Katz & Miledi 1959). These min. e.p.ps are caused by ACh-quanta discharged from the Schwann cells which, after denervation, proceed to occupy the synaptic position previously held by the axon terminals (Birks, Katz & Miledi 1960; Miledi & Slater 1968). It is known also that when Ca\(^{2+}\) is microinjected into a nerve terminal, it greatly accelerates the rate of release of transmitter quanta (Miledi 1973). Therefore, the question arises whether the release of ACh-quanta from the Schwann cell is also determined by the intracellular level of ionized calcium. The most direct way of probing this question would be to see if intracellular injection of Ca\(^{2+}\) into the Schwann cell increases the frequency of 'Schwann-cell min. e.p.ps'. Unfortunately, this is rather difficult to do without damaging the Schwann cells. A possible alternative is to try to move calcium ions into the Schwann cell using cationic ionophores which, although not entirely specific, are capable of transferring calcium ions across membranes (Pressman 1973; Reed & Lardy 1972).

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Methods

The experiments were performed on the sartorius muscle of the frog (*R. temporaria*) using techniques described previously (Katz & Miledi 1965). For denervation, one sciatic nerve was cut in the pelvis (Miledi 1960). After the operation the frogs were kept in tanks with running water at room temperature, and fed with a mixture of homogenized liver and cod liver oil. Ten to fourteen days after the operation, the sartorius muscles from both sides were dissected, and the unoperated side served as control. Individual endplates were identified under the compound microscope and 5–20 endplates were examined in each experiment. All experiments were carried out at room temperature.

The basic composition of the bathing medium was (mm): NaCl 116, KCl 2, CaCl$_2$ 1.8; phosphate buffer 1–2 (pH 7.1). Neostigmine methylsulphate was routinely added at a final concentration of $10^{-6}$ g/ml to inhibit cholinesterase.

The ionophores X-537 A (Hoffmann La Roche), A23187 (Eli Lilly) and avenaciolide (see Harris & Wimhurst 1974), which are very poorly soluble in water, were dissolved in ethanol. Small aliquots from this alcoholic solution were added to the normal frog Ringer solution giving a final ethanol concentration of 0.1–1%. At these concentrations ethanol has only small effects on min. e.p.ps at both innervated and denervated frog endplates (Okada 1967; Bevan, Grampp & Miledi 1976).

Normal rabbit serum was prepared by collecting blood without anticoagulant and allowing it to clot at room temperature for 2 h, followed by 12–20 h at about 4 °C. The clot was then spun down and the serum separated. The sera were dialysed for 20–24 h at 4–6 °C against normal frog Ringer with or without PO$_4$ buffer, and were used unheated to preserve complement which induces the release of acetylcholine quanta from frog axon terminals (Ito, Miledi & Vincent 1974).

Results

At innervated endplates, min. e.p.ps occur at a rate of a few per second, while at denervated endplates the frequency of detectable Schwann-cell min. e.p.ps is only a few per minute. Another important difference between the two types of min. e.p.ps is that ‘neuronal min. e.p.ps’ have an approximately Gaussian amplitude distribution, while that of Schwann-cell min. e.p.ps is highly skewed, with many of the smallest min. e.p.ps presumably submerged in the noise of the recording system. Therefore, recording conditions were usually chosen so that min. e.p.ps of 50 μV amplitude or less could be detected. Also, since the ionophores, or sera, were applied for periods of several hours, a few experiments were made to determine the stability of Schwann-cell min. e.p.p. frequency in the absence of any experimental treatment. There was no significant difference between the Schwann-cell min. e.p.p. frequency soon after dissection and that observed at the same endplates a few hours later (see also Bevan et al. 1976).
The effect of ionophore X-537A

In agreement with the findings of Kita & Van der Kloot (1974), X-537A increased min. e.p.p. frequency at innervated endplates. For example, within 10–20 min after the application of 25 μM X-537A, the frequency of axonal min. e.p.ps was raised more than 100-fold (figure 1a). No such increase was seen when the ionophore was tested on Schwann min. e.p.ps: on the contrary, their frequency appeared to be reduced. For instance, 40 min after the application of 50 μM X-537A, the frequency of min. e.p.ps at 10 denervated endplates was reduced from 11.7 min⁻¹ to 1.0 min⁻¹ (P < 0.01). At this concentration X-537A reduced the resting potential to less than 50 mV within 1 h (cf. Devere & Nastuk 1975), and many fibres developed retraction clots. Therefore further experiments were done using 15 μM X-537A and again, only a decrease in Schwann min. e.p.p. frequency was observed (figure 1b). However, even with this concentration, 30 min after introducing the ionophore, the resting potential of the denervated muscle fibres fell from 90.3 ± 3.0 mV (n = 19) to about 80 mV, and 1 h later the mean resting potential was 64.6 ± 6.5. The loss in muscle fibre resting potential would lead to diminished min. e.p.p. size and partly account for the decrease in min. e.p.p. frequency. Therefore, other ionophores were tested, in the hope of avoiding this complication.
Avenaciolide is a dilactone (Brookes, Tidd & Turner 1963) which has ionophoric actions and causes the release of Ca$^{2+}$ and Mg$^{2+}$ from mitochondria (Harris & Wimhurst 1974). Its action on the neuromuscular junction will be described elsewhere (Harris & Miledi, unpublished). It will suffice to say here that avenaciolide, in micromolar concentrations, greatly raises the frequency of mini. e.p.ps in innervated muscles and that a large increase in mini. e.p.p. frequency is seen even when the muscles are bathed in Ca-free Ringer (zero Ca + EGTA), presumably because Ca is released from internal stores. In contrast, when avenaciolide was applied to denervated muscle, the frequency of Schwann-cell mini. e.p.ps was reduced to 1/10–1/100 of its original value within 30–60 min (figure 2). With the avenaciolide concentration (50 μM) used in the experiment illustrated in figure 2, the mini. e.p.p. frequency in the control muscle was already > 100 s$^{-1}$ 10 min after applying the ionophore. The decreased rate of Schwann-cell mini. e.p.ps must be due to a direct action of the ionophores on ACh-release, because 10–50 μM avenaciolide produced only a slight depolarization of the muscle fibres and had no obvious effect on mini. e.p.p. amplitude.

**Figure 2.** Effect of avenaciolide on frequency of Schwann-cell mini. e.p.ps in an endplate 13 days after denervation. Ten minutes after applying avenaciolide, the mini. e.p.p. frequency in the innervated endplates of the control muscle was > 100/s. Ethanol concentration after adding avenaciolide: 0.5%. Temp. 17 °C.

### The effect of A23187

Approximately 5–10 min after applying A23187 (25–50 μM), the frequency of axonal-mini. e.p.ps began to rise and within 20–30 min went up to > 100 s$^{-1}$ (figure 3b); but the frequency of Schwann-cell mini. e.p.ps was again reduced by the ionophore. For instance, in one of the examples illustrated in figure 3a, the frequency of Schwann mini. e.p.ps soon after dissection was $22.2 \pm 3.50$ min$^{-1}$ (± s.e.), which was not significantly different from that observed 2 h later (23.1 ± 3.85 min$^{-1}$). After applying A23187 (20 μM) the frequency of Schwann mini. e.p.ps declined progressively and 1 h later was 7.5 min$^{-1}$. This reduction in frequency...
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was not caused by exhaustion of ACh, because the frequency rose to 20.3 min⁻¹ (range 2–44 min⁻¹ in different fibres) when the medium was made hypotonic (60 %); a procedure which normally increases the rate of discharge of Schwann min. e.p.ps.

A23187 (20 μM) did not have a significant effect on the size of Schwann-cell min. e.p.ps. At 9 identified endplates the mean min. e.p.p. amplitudes were 0.25 ± 0.05 mV (± s.d.) and 0.24 ± 0.04 before and after the application, respectively, of the ionophore. Nor did A23187 cause a significant change in the resting membrane potential of the muscle fibres. Mean values of resting potential in 16 fibres were 86.9 ± 5.80 before, and 90.1 ± 5.64 after the treatment.

![Graph](image)

**Figure 3.** Effect of A23187 on the frequency of Schwann min. e.p.ps (a) and axonal min. e.p.ps (b). In (a) different concentrations were used on two muscles. ○, 20 μM, 14 days after denervation. ●, 50 μM, 11 days after denervation. Mean ± s.e. of 5–15 endplates. (b) shows the effect of 50 μM A23187 on two endplates of the control muscle.

**The effect of rabbit serum**

It has been shown recently (Ito et al. 1974), that unheated rabbit serum contains a factor which causes massive release of transmitter from frog motor endings. This action of serum is due to an attack by the complement system (see Müller-Eberhard 1969) on the nerve terminals. In contrast to its effect at normal endplates, rabbit serum reduced the frequency of Schwann-cell min. e.p.ps (figure 4), without altering their amplitude or causing a significant reduction in the resting potential of the muscle fibres.
Figure 4. Effect of unheated rabbit serum on min. e.p.p. frequency. (a) min. e.p.ps at two endplates in the control muscle. (b) Schwann-cell min. e.p.ps in a muscle 12 days after denervation; mean ± s.e. of 2–4 endplates. The bathing fluid was replaced by rabbit serum (diluted 50%) at time 0.

Discussion

These results emphasize further the previous conclusion that the mechanism of release of ACh from Schwann cells differs from that which operates at axon endings (Birks et al. 1960; Miledi & Slater 1968; Dennis & Miledi 1974; Bevan et al. 1976). It is clear that when the level of ionized calcium inside the axon terminal is raised by an intracellular injection of Ca\(^{2+}\), independently of any membrane depolarization, the rate of release of transmitter quanta is greatly increased (Miledi 1973). The present experiments show that the ionophores X-537A, avenaciolide and A23187 raise the rate of release of ACh-quanta from motor endings by more than 100-fold. Although the mode of action of the ionophores is still not clear, it seems likely that they raise the level of ionized calcium inside the axon by allowing external Ca\(^{2+}\) to go in, or by releasing it from internal stores: a situation similar to that encountered at the adrenal medulla and neurohypophysis (Garcia, Kirpekar & Prat 1975; Nakazato & Douglas 1974; Nordmann & Currell 1975).

In contrast to the large stimulating effect on the release of ACh-quanta from axons, the ionophores and the rabbit serum not only failed to raise the frequency
of Schwann-cell min. e.p.ps, but markedly reduced it. Since ethanol depresses the rate of Schwann min. e.p.ps (Bevan et al. 1976), the decrease in min. e.p.p. frequency caused by the ionophores may to some extent be due to the use of ethanol as solvent (cf. Methods). However, this can only account for a small part of the decrease in frequency for which a different explanation must be sought. If, as seems likely, the ionophores raise the level of intracellular Ca\(^{2+}\) in the Schwann cell, we then have a system where an increase in ionized calcium inside the cell leads to a reduction in transmitter release: quite the opposite of what happens in nerve terminals. The most attractive explanation of our results is to postulate that membranes can have two types of sites involved in the release of transmitter or other substances. On this hypothesis, in the Schwann cell the sites would be inhibited by a rise in intracellular Ca\(^{2+}\), while in the axon the sites are activated by Ca\(^{2+}\), Sr\(^{2+}\) and possibly also La\(^{3+}\) (Miledi 1973; Heuser & Miledi 1971). Axon membranes may also contain release sites like those in the Schwann cell, but their existence would be difficult to demonstrate because their activity would be dominated by that of Ca\(^{2+}\)-activated sites.

In the axon terminals, depolarization of the membrane opens gates for Ca\(^{2+}\) and the consequent influx of Ca\(^{2+}\) evokes the release of transmitter quanta (Katz & Miledi 1967, 1969). On the other hand, there is no increase in the release of ACh-quanta when the Schwann-cell membrane is depolarized by K\(^{+}\) (Birks et al. 1960; Miledi & Slater 1968) or by electric pulses. In the latter case, ACh is indeed released, but not in recognizable ‘quantal packets’ and only if the pulses are made so strong that they cause membrane breakdown (Dennis & Miledi 1974). This could mean that the Schwann-cell membrane lacks the potential-dependent Ca\(^{2+}\) gates, though it is still possible that the gates are present but any Ca\(^{2+}\) which enters is very ineffective.

Another interesting question which remains unsolved concerns the mode in which multimolecular amounts of ACh are released to evoke Schwann min. e.p.ps. ACh could be released from preformed packages, such as the synaptic vesicles of axon terminals. Similar vesicles are seen in Schwann cells but they are not very numerous. Alternatively, the ACh could be released by a less specific process of exocytosis, from vesicles of the type encountered in all cells and which may serve to extrude material from the cell. Or the Schwann min. e.p.ps could be a consequence of a leakage, through transient membrane gates, of free cytoplasmic ACh; in which case, the size of the min. e.p.ps would be determined by the open-time of the gates. In any case, it seems that the release mechanism is inhibited by an increase in the level of ionized calcium inside the Schwann cell.
References


