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ELECTRICALLY INDUCED RELEASE OF ACETYLCHOLINE FROM DENERVATED SCHWANN CELLS

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SUMMARY

1. Focal electrical stimulation of Schwann cells at the end-plates of denervated frog muscles elicited slow depolarizations of up to 30 mV in the muscle fibres. This response is referred to as a Schwann-cell end-plate potential (Schwann-e.p.p.).

2. Repeated stimulation sometimes evoked further Schwann-e.p.p.s, but they were never sustained for more than 30 pulses. Successive e.p.p.s varied in amplitude and time course independently of the stimulus.

3. The Schwann-e.p.p.s were reversibly blocked by curare, suggesting that they result from a release of acetylcholine (ACh) by the Schwann cells.

4. ACh release by electrical stimulation did not seem to occur in quantal form and was not dependent on the presence of calcium ions in the external medium; nor was it blocked by tetrodotoxin.

5. Stimulation which caused release of ACh also resulted in extensive morphological disruption of the Schwann cells, as seen with both light and electron microscopy.

6. It is concluded that electrical stimulation of denervated Schwann cells causes break-down of the cell membrane and releases ACh, presumably in molecular form.

INTRODUCTION

At neuromuscular junctions of vertebrates, small packets of acetylcholine are randomly released from motor nerve terminals, giving rise to spontaneous miniature end-plate potentials (min.e.p.p.s) in the muscle fibres. While studying the changes which occur during denervation of amphibian end-plates, Katz & Miledi (1959) noted min.e.p.p.s after the axon terminal had completely degenerated. It was suggested that these potentials result from the liberation of packets of ACh (or some close analogue) by the Schwann cells which, after disintegration of the axon

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terminal, come into direct contact with the junctional folds of the muscle fibre (Birks, Katz & Miledi, 1960). 'Schwann min.e.p.p.s' also occur upon denervation of amphibian slow muscle fibres (Miledi & Stefani, 1970) and mammalian skeletal muscle (Miledi & Slater, 1968), although in the latter they are much less common than in the frog and disappear approximately 3 weeks after nerve section, when the Schwann cells withdraw from the muscle.

This spontaneous release of acetylcholine quanta from Schwann cells is unlike that from nerve terminals in that the rate of release is not increased by raising the potassium concentration or the osmolarity of the bathing fluid (cf. Birks *et al.* 1960; Miledi & Slater, 1968; Bevan, Grampp & Miledi, 1973*b*). The present paper describes the results of direct electrical stimulation of the Schwann cells in denervated end-plates of the frog, using focal extracellular electrodes (see Dennis, 1972, for a preliminary communication).

METHODS

All experiments were performed on the cutaneous pectoris muscle of *Rana temporaria*. The left c. pectoris was denervated by excising under anaesthesia (Miledi, 1960) a 2–4 mm segment of the motor nerve just proximal to its entry into the muscle. The frogs were subsequently kept in tanks at room temperature (19–23° C) until use.

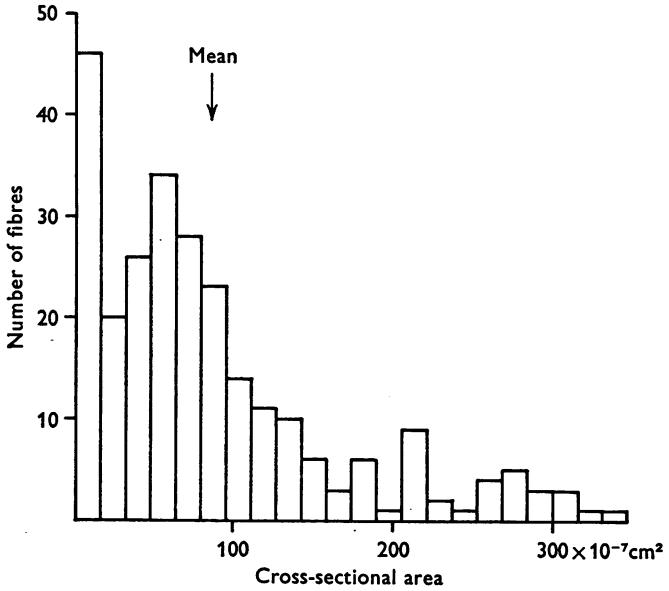
The muscle was mounted in a glass-bottomed chamber and viewed with Zeiss–Nomarski optics, using 10× oculars and a 40× water immersion objective which was electrically isolated from the microscope body by an ebonite neck. Glass micropipettes filled with 4 M potassium acetate were used for recording from the muscle fibres. Similar pipettes filled with 4 M sodium chloride and broken to a tip diameter of 2–4 μm were used for focal stimulation of the Schwann cells. The methods used for focal stimulation and recording have been previously described (Katz & Miledi, 1965). In experiments where attempts were made to record intracellularly from a Schwann cell and pass current through the same electrode, the signal was led into a W.P.I. type M4A electrometer which contains a current injection circuit.

The basic composition of the bathing medium was as follows (in millimoles): NaCl, 120; KCl, 2; $CaCl_2$, 108; glucose 10 and 5 mM sodium phosphate buffer, pH about 7·2. The anticholinesterase agent neostigmine methylsulphate was routinely included at a concentration of 10^{-6} g/ml. When lanthanum was added, the buffer was omitted from the Ringer solution to prevent precipitation of the lanthanum ions. In experiments with calcium-free solution, magnesium and in some cases also EGTA were added as indicated.

For electron-microscopic examination the muscles were stretched over a Perspex holder, and fixed in 1% osmium tetroxide buffered with veronal acetate. After 3–4 hr fixation small segments were cut from the end-plate regions, dehydrated in ethanol and embedded in Araldite (Ciba Ltd). Thin sections were doubly stained with uranyl acetate and lead citrate.

To identify in the electron-microscope selected muscle fibres and Schwann cells, a drawing was made of the region examined during the experiment using nerve bundles, blood vessels, melanophores, etc., as 'landmarks'. Adjacent muscle fibres were then impaled with the micro-electrode and damaged intentionally to produce

vacuolation of the muscle fibres. After some 10–60 min the muscle was fixed and the relevant area cut out and embedded in Araldite. Some of the landmarks could be seen in the block and the damaged fibres were easily identifiable in thin sections examined in the electron-microscope, or in the light microscope using thick sections stained with warm 1 % toluidine blue in 1 % sodium borate. Polaroid pictures were taken at various stages of the experiment to help in the identification.



Text-fig. 1. Distribution of cross-sectional areas in 257 muscle fibres from the cutaneous pectoris of *R. temporaria*. Muscle fixed in osmium tetroxide.

RESULTS

Visualization of Schwann cells

Nomarski interference microscopy was used throughout this study because it enhances the resolution of structural detail over that seen with ordinary brightfield optics (cf. McMahan & Kuffler, 1971; McMahan, Spitzer & Peper, 1972). One limiting factor is that the tissue must be relatively thin, otherwise this advantage is lost. The cutaneous pectoris muscle of the frog is composed of parallel fibres arranged in a planar sheet, 4–6 mm wide and one to four muscle fibres thick (cf. Pl. 1). It is therefore well suited for use with this type of optical system.

In frogs weighing about 20 g, the muscle fibres in the c. pectoris are 9–12 mm long, and have only one end-plate situated near the middle of the fibre. There are two or more muscle spindles each with two to seven muscle fibres (Pl. 1). Including these fibres, the total number of fibres

ranged from 194 to 257 in five muscles examined (average 220 fibres). The fibre size was quite variable, with some fibres being only about 1·5 μm in diameter while in others the major cross-sectional axis was about 100 μm. As illustrated in Text-fig. 1, the muscle fibre area was asymmetrically distributed around a mean value, $85{\cdot}3 \times 10^{-7}$ cm^2 in this case. The smallest fibre in this muscle had a cross-section of $1{\cdot}26 \times 10^{-7}$ cm^2, while the largest one was $335{\cdot}5 \times 10^{-7}$ cm^2 – a difference 266 fold! Similar results were obtained from the other muscle where areas were measured in the 194 muscle fibres; except that in this case the mean area was $61{\cdot}4 \times 10^{-7}$ cm^2, the largest fibre was $180{\cdot}0 \times 10^{-7}$ cm^2 and there was a 250-fold difference between this one and the smallest. In all muscles, there was a large number of small fibres (cf. Text-fig. 1), including the relatively small fibres in the muscle spindles. There are also many small fibres of presumed extrafusal origin. In some instances, one of these small fibres may lie closely apposed to a large one; or a group of small fibres is arranged in a cluster as though a large fibre had fragmented (Pl. 1). Fragmentation of muscle fibres has been observed after denervation of mammalian muscle fibres (Miledi & Slater, 1969). It might be that fragmentation of some muscle fibres, accompanied by growth of others, is part of a normal turnover of fibres in a muscle.

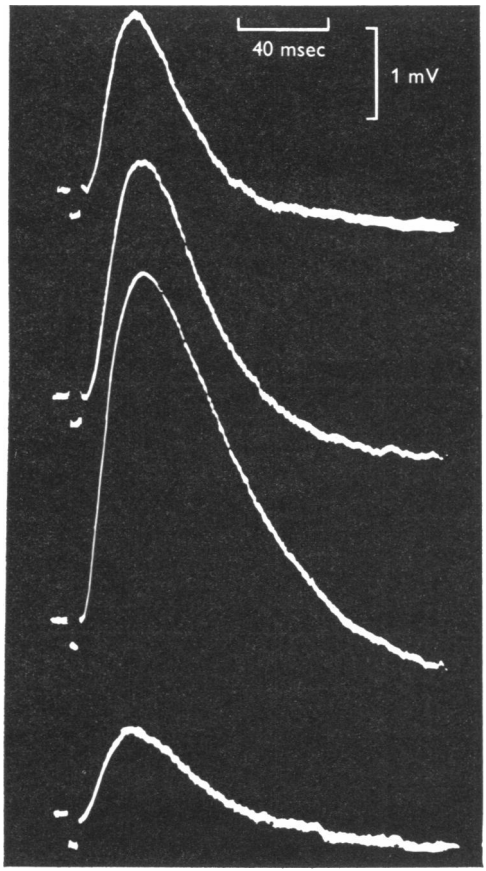
After disintegration of the nerve fibres the Schwann cells were located with Nomarski optics by tracing the fragments of degenerating myelin distally to their termination on a muscle fibre, thus indicating the general region of the old motor end-plates. In this area, a number of Schwann cell nuclei, usually between two and eight, could be seen (Pl. 2). Sometimes the projections of the synaptic gutter within which the unmyelinated nerve terminal used to lie, were also visible.

For the present experiments a muscle fibre was located which had a superficial end-plate with one or more accessible Schwann cells. The fibre was penetrated with the recording electrode, and checked for the presence of 'Schwann min.e.p.p.s'. A current-passing electrode was then placed on the surface of a Schwann cell, usually near the nucleus, and negative electric pulses of gradually increasing intensity were applied to the cell at about 1 sec intervals.

Muscle responses to electrical stimulation of Schwann cells

As electric pulses of increasing strength were being applied to Schwann cells in denervated muscles, at some current intensity a depolarization was evoked in the muscle fibre. Such responses could be obtained by stimulation of every Schwann cell 2 or more weeks after nerve section. The effective current intensities passed through the extracellular pipette were in the range of 10^{-7} to 10^{-6} A.

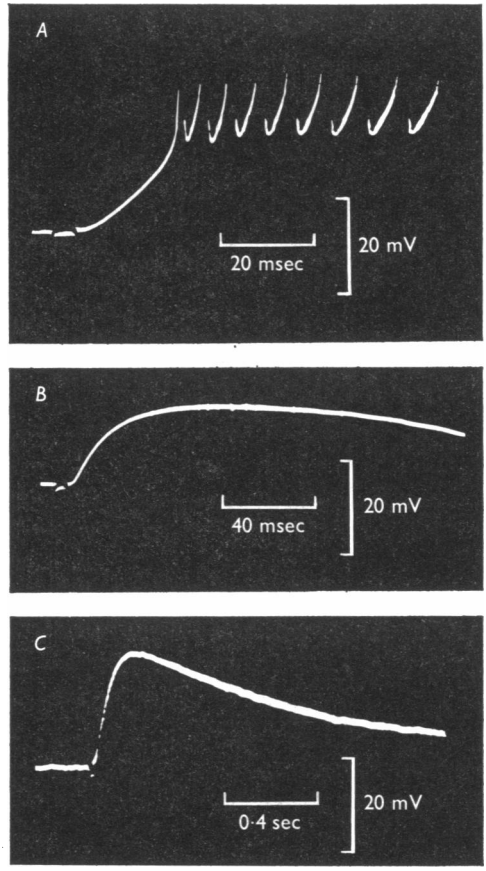
With negative polarity the muscle depolarization started after the end of the current pulse and rose slowly to a peak (e.g. Text-figs. 2 and 3). Once such a depolarization, or 'Schwann end-plate potential' (e.p.p.), was elicited, further responses could usually be triggered by additional pulses, but as illustrated in Text-fig. 2, successive Schwann-e.p.p.s varied in wave



Text-fig. 2. Part of a series of responses elicited by repeated stimulation of one Schwann cell with pulses of constant intensity. Note the apparently random fluctuation in amplitude of the Schwann-e.p.p. Some stimuli within the series (not illustrated) failed to evoke e.p.p.s. AC recording. Rising phases of the responses retouched.

form and amplitude even though the pulse intensity remained constant. That is, once threshold had been exceeded the responses were practically independent of the pulse size. In a few instances, increasing or decreasing the current pulse seemed to produce concomitant changes in e.p.p. amplitude, but in view of the limited number of e.p.p.s obtainable, and their

great variability, no clear relationship was established. After several Schwann-e.p.p.s had been obtained they invariably decreased in amplitude and soon ceased entirely; the maximum number which could be triggered from individual Schwann cells varied from one to thirty. Once a cell had ceased to respond, it was not possible to elicit any further e.p.p. by either

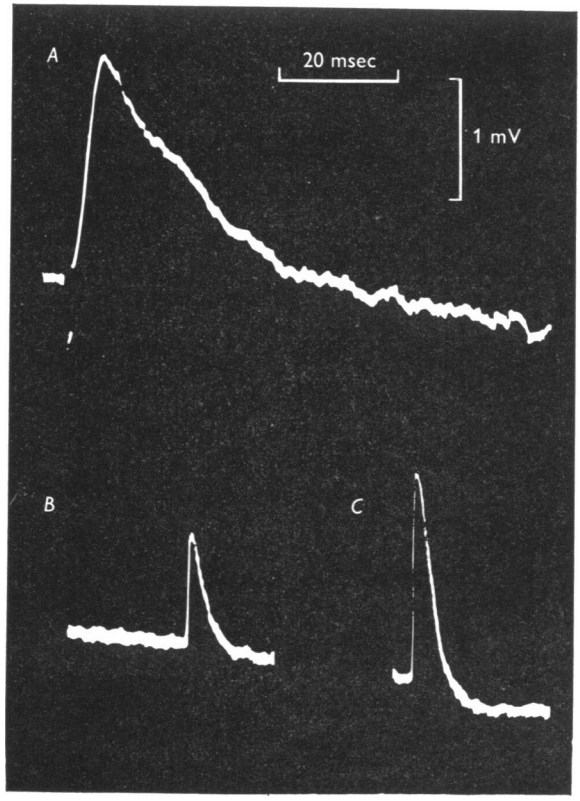


Text-fig. 3. Long-lasting Schwann-e.p.p.s recorded in three different muscle fibres. In *A* the amplitude was sufficient to trigger a train of action potentials in the muscle fibre; only the slowly rising base of each shows in this photograph.

increasing the current intensity or moving the stimulating electrode to a new position on the *same* Schwann cell. Other Schwann cells on the same muscle fibre would, however, still respond.

The maximum e.p.p. evoked by stimulating Schwann cells varied from a few mV to over 30 mV, often exceeding the threshold of the muscle

fibre, as shown in Text-fig. 3*A*. There was also considerable variability in the rates of rise of the Schwann-e.p.p.s. The fastest reached a peak in 5–10 msec (Text-figs. 2, 4*A* and 8), while others took more than 100 msec (Text-fig. 3*C*) to reach maximum amplitude. Significantly, the time to peak of the responses elicited by Schwann cell stimulation was always longer than that of most of the spontaneous Schwann min.e.p.p.s seen at the same end-plate, even in cases where the amplitude of the two events



Text-fig. 4. A relatively fast-rising Schwann-e.p.p. (*A*) compared with two spontaneous Schwann min.e.p.p.s (*B* and *C*), all recorded at the same denervated end-plate. The rates of rise of the evoked responses were always slower than those of the faster min.e.p.p.s, even when the amplitudes were similar. AC recording. Rising phases retouched.

was similar. Text-fig. 4*A* shows the fastest of forty-nine e.p.p.s elicited from five Schwann cells on one fibre, compared with two of the spontaneous min.e.p.p.s recorded at that same end-plate. As with the rise time, there was also a great variability in the rate of decay of the depolarization; sometimes the potential returned to its resting level within 50 msec or so,

as in Text-fig. 4A, whereas in other instances (cf. Text-fig. 3) the depolarization lasted up to 2 sec.

When the frequency of the Schwann min.e.p.p.s was noted before current application, and again after all of the accessible Schwann cells had been stimulated in the manner described above, there was invariably a decrease in the rate of occurrence of the spontaneous min.e.p.p.s. For example, in one muscle fibre, after four accessible Schwann cells were stimulated, the average min.e.p.p. frequency was reduced from 13 to 3 per minute.

Chemical nature of the response

To test whether these Schwann-e.p.p.s are mediated by acetylcholine (ACh), the Schwann cells in four muscles were stimulated after a potent antagonist, D-tubocurarine (D-TC), was added to the bathing fluid. In the presence of D-TC 10^{-5} g/ml. it was no longer possible to elicit the usual depolarizations; in thirty muscle fibres no responses were seen upon stimulation of seventy-five Schwann cells, and only small responses (less than 0·5 mV) resulted from stimulation of four others. Although no Schwann-e.p.p. was recorded in the presence of D-TC, the stimulated Schwann cells always underwent the characteristic change in appearance which is described below and which in normal Ringer solution is associated with the generation of a Schwann-e.p.p. The effect of curare was readily reversible. Even in muscles which had been exposed to D-TC for more than 2 hr, stimulation of the Schwann cells after washing out the curare elicited the usual Schwann-e.p.p.s. When the same concentration of curare was applied to a normally innervated muscle, at room temperature and in the presence of neostigmine, the end-plate potentials had an average amplitude of 4·3 mV (fifteen end-plates, range 0·9–9·2 mV).

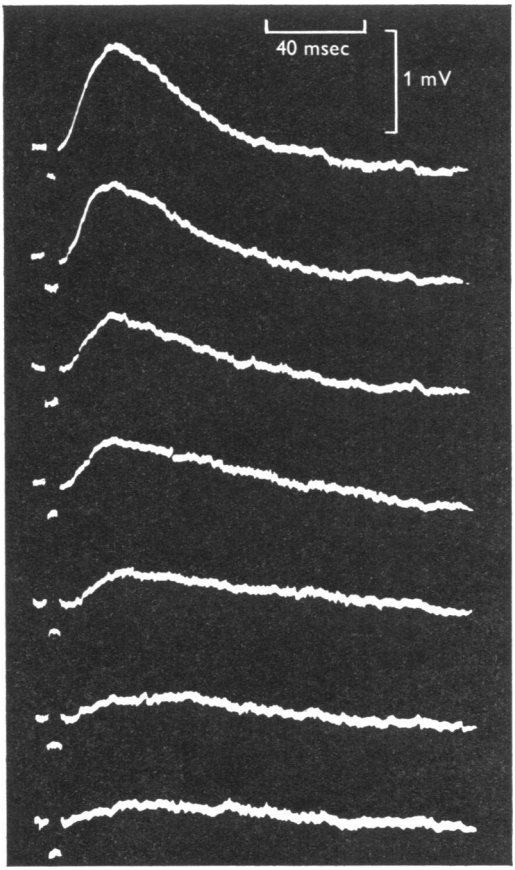
The pulses used to stimulate the Schwann cells were usually accompanied by electrokinetic movements near the electrode tip, and sometimes even local pulsation of the Schwann cell and muscle fibre was seen. Focal stimulation sometimes also elicited small local muscle contractions. However, these movements were still observed in the curarized muscle and therefore cannot be responsible for the depolarizing Schwann response. Furthermore, when the current electrode was placed directly on the muscle surface, away from the end-plate region, electric pulses like those usually applied to the Schwann cell did not produce significant depolarization in the muscle fibre, even when they evoked local contractions which could be seen by visual inspection. Additional evidence ruling out an artifact as a possible explanation of the Schwann-e.p.p.s comes from experiments carried out during the 'silent period' (Birks *et al.* 1960), when no Schwann min.e.p.p.s are detected in the denervated muscle (see below).

From all these experiments we conclude that the muscle fibre depolariza-

tion observed on stimulation of Schwann cells results from the release of ACh or a closely analogous substance from the Schwann cells, and we will henceforth assume that it is ACh.

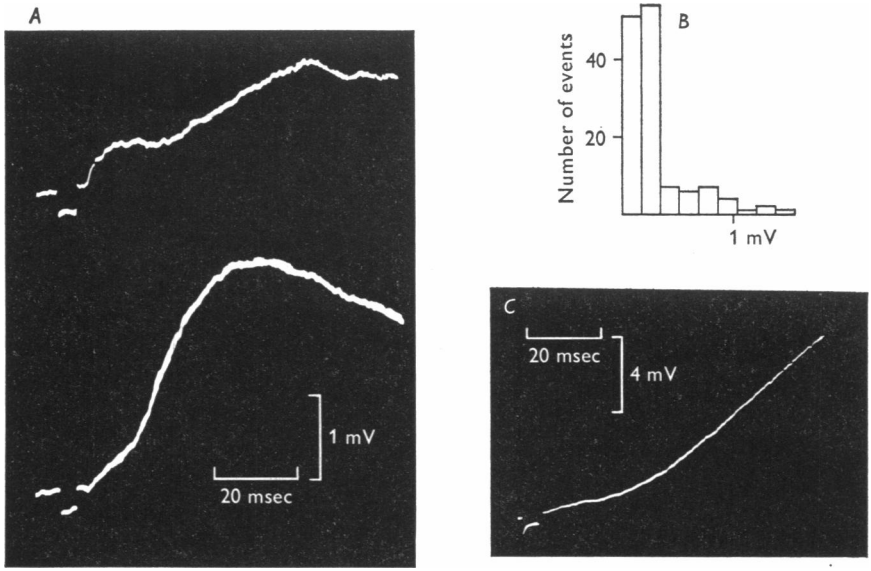
Mode of release of ACh upon stimulation

It is well known that both spontaneous, and depolarization-evoked, release of transmitter from nerve terminals takes place in quantal packets, and the same applies to the spontaneous release from Schwann cells at denervated end-plates (Birks *et al.* 1960). The question arises whether the response to electrical stimulation of the Schwann cells is also quantal.



Text-fig. 5. Exhaustion of Schwann-e.p.p.s. Final responses from a longer series elicited from one Schwann cell with current pulses of fixed intensity and duration. The amplitude of successive responses decreased by less than 0·4 mV. After this decline, the amplitude of the response could not be restored by stronger stimulation. DC recording.

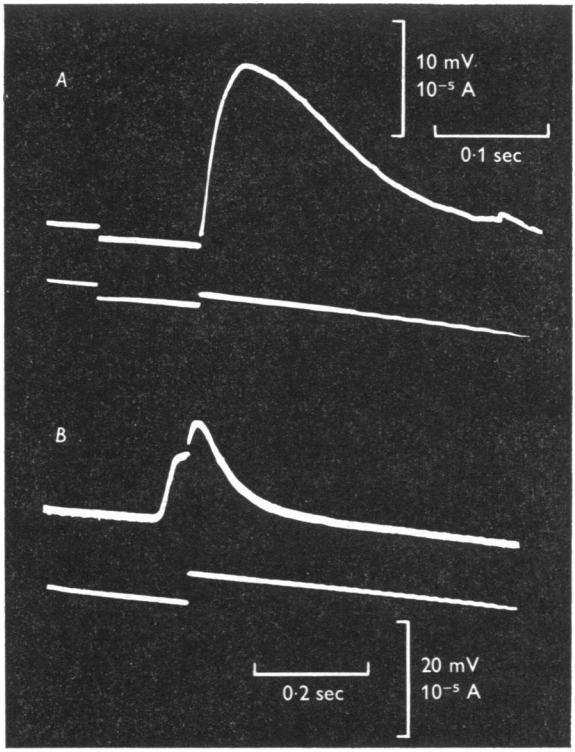
The amplitude of a series of e.p.p.s from a given Schwann cell did not seem to vary in discrete steps, as would be expected if release of normal-sized quanta were involved. The decaying final responses from a series evoked by constant stimuli repeated once every 2 sec are illustrated in Text-fig. 5. In this instance there is only a gradual decline from one e.p.p. to the next. The absence of obvious steps in size was most apparent with such small responses, which often seemed to be continuously graded down to the noise level.



Text-fig. 6. *A*, Schwann-e.p.p.s with slow inflexions on the rising phase. Two different muscle fibres. The rates of rise of these responses are much slower than those of the spontaneous min.e.p.p.s illustrated in Text-fig. 3. *B*, histogram of the amplitudes of spontaneous Schwann min.e.p.p.s, and (*C*) a slowly rising Schwann-e.p.p. elicited at the same denervated end-plate as *B*. Note that there are no obvious inflexions on the rising phase of the slow response. DC recordings.

Furthermore, if the Schwann-e.p.p. were composed of separate events, each resembling a spontaneous Schwann min.e.p.p., then one would expect to see inflexions due to these individual constituents. Such inflexions were not clearly observed. Occasionally we did see an inflexion on the rising phase of a response (as in Text-fig. 6*A*) but it had a lower rate of rise than that of spontaneous min.e.p.p.s. It may be that these relatively slow inflexions arose from a sudden increase in the area of the Schwann cell from which ACh was being released. On rare occasions a min.e.p.p. was seen on the falling phase of a Schwann e.p.p., as in Text-fig. 7*A*, but this was so infrequent that it may have been a random occurrence.

In considering whether the evoked release from Schwann cells is quantal, it must be remembered that amplitude histograms of the min.e.p.p.s recorded at denervated end-plates are always markedly skewed toward the origin, as illustrated in Text-fig. 6*B* (see also Birks *et al.* 1960; Miledi, 1960). The response shown in Text-fig. 6*C* was evoked by stimulating a Schwann cell at the same end-plate and, again, there are no obvious



Text-fig. 7. Schwann-e.p.p.s evoked by negative pulses of long duration. Each pair of traces consists of a voltage record from within the muscle fibre and a monitor of the current pulse used for Schwann stimulation (lower trace). Responses taken from two different muscle fibres. In *B* the pulse duration was 0·25 sec. DC recording.

inflexions on the slowly rising phase of the muscle depolarization. Even when we looked at the falling phase of long-lasting Schwann-e.p.p.s with higher gain we saw membrane potential fluctuations resembling 'ACh-noise' (cf. Katz & Miledi, 1972), but no discrete events comparable to the spontaneous Schwann min.e.p.p.s.

Schwann-e.p.p.s in low calcium medium

Some calcium is required in the bathing medium before depolarization of a normal axon will cause release of packages of transmitter, and it was therefore of interest to determine whether the evoked release of ACh from Schwann cells has a similar calcium dependence. In two experiments calcium was omitted and 10 mM magnesium added to the bathing fluid, while in two others the bath contained no calcium ions, and 4 mM magnesium + 3 mM-EGTA. After equilibrating the muscles for 2 hr in these solutions it was still possible to elicit Schwann-e.p.p.s which were as large and as long-lasting as those seen in normal solution, and the e.p.p.s showed the usual variability from one cell to the next. In a further experiment the calcium concentration was raised to 10 mM. Again, the Schwann-e.p.p.s appeared the same as with normal solution. It was concluded that the stimulus-evoked release of ACh from Schwann cells does not require the presence of calcium in the external fluid.

Influence of agents which interfere with transmitter release from nerve terminals

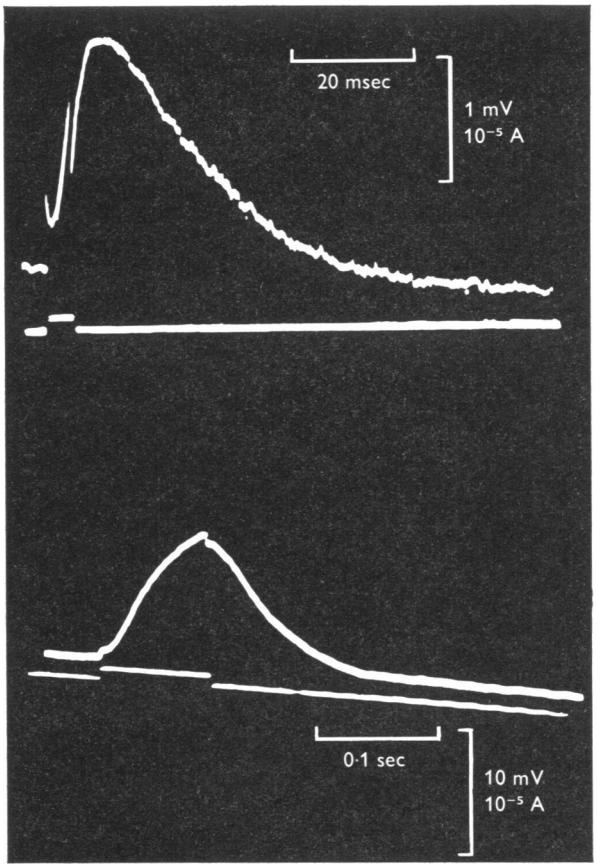
Action potential propagation in neurones is blocked by low concentrations of tetrodotoxin, and in consequence that poison interferes with the 'explosive' release of transmitter caused when an impulse arrives at a motor nerve terminal. In the presence of tetrodotoxin it is still possible, however, to evoke transmitter release by graded focal depolarization of the axon terminal (Katz & Miledi, 1967*a*, *b*). On the other hand, lanthanum ions abolish the transmitter release by nerve impulses (Heuser & Miledi, 1971), as well as the transmitter release induced by electrical pulses applied directly to nerve terminals (Miledi, 1971). If the electrically induced release of ACh from Schwann cells involved a mechanism similar to that which operates during normal neuromuscular transmission, then tetrodotoxin or lanthanum might be expected to abolish the Schwann-e.p.p. However, neither tetrodotoxin, 10^{-6} g/ml., nor 0·5 mM lanthanum had any influence on the Schwann cell's ability to produce e.p.p.s when electrically stimulated.

Effect of duration and polarity of stimulus

The Schwann-e.p.p.s illustrated so far were all elicited using brief negative current pulses. With this polarity the e.p.p. commenced after cessation of the stimulus, even when the pulse duration was increased to as much as 200 msec (see Text-fig. 7*A*). On a few occasions depolarization of the muscle fibre did begin before the end of the pulse (Text-fig. 7*B*), but this was seen rarely, even though a special search was made. Further-

more, there is still the possibility that when this occurred the electrode lost contact with the Schwann cell, which would be equivalent to turning the pulse off.

When the stimulus polarity was reversed it was still possible to elicit a Schwann-e.p.p., but in this situation the response began while the pulse was on. A disadvantage in using this polarity was that penetration of the muscle fibre with the current electrode, which occasionally occurred, gave a depolarizing artifact that was sometimes difficult to distinguish from the e.p.p.



Text-fig. 8. Schwann-e.p.p.s evoked by positive current pulses. With this polarity the responses began during the current pulse. Two responses from different muscle fibres. Lower trace in each pair monitors the current pulse. DC recording. Rising phase of upper response retouched.

An attempt was made to record and pass current from *within* the Schwann cells at denervated end-plates by using fine micro-electrodes of 50–200 MΩ resistance. Impalements were made by advancing the electrode until it just touched the

Schwann cell, and then gently tapping the table; or else brief pulses of current of either polarity were passed through the micropipette. Penetrations were rather poor, with the highest 'resting potential' being only about 15 mV, which is probably no more than a fraction of the true membrane potential.

After such a penetration, the relationship of the onset of the Schwann-e.p.p. to stimulus polarity was the same as observed when stimulating 'outside' with a blunt electrode. That is, with a negative (hyperpolarizing) pulse the response began at the 'off', and with positive polarity it began during the pulse. This suggests that even the blunt electrodes were effectively making electrical contact with the Schwann cell interior, perhaps due to a decrease in membrane resistance during the strong pulse (cf. Methods, Katz & Miledi, 1967*b*).

Development of the ability to release ACh

After section of the motor nerve there is a 'silent period', between the cessation of spontaneous transmitter release from the degenerating nerve terminal and the initiation of quantal release from the Schwann cells (Birks *et al.* 1960; Harris & Miledi, 1972; Bevan *et al.* 1973*a*). It was of interest to see if release of ACh could be evoked electrically from Schwann cells during the silent period, when there is no spontaneous release. We stimulated in the usual manner sixty-two Schwann cells at twenty different terminals which appeared to be in the silent period, as judged by the fact that no min.e.p.p.s were detected during at least 1 min of observation. Fifty-eight cells gave no response, three gave e.p.p.s of about 0·5 mV and from one there was a response of 1·0 mV. Since Schwann min.e.p.p.s may have a very low frequency when they first appear, it is possible that the 1 min period may not have been long enough to ascertain the absence of Schwann min.e.p.p.s. All of these cells had the typical vacuolated appearance after stimulation (see below) indicating that they had been adequately stimulated. Thus it seems that the Schwann cells release little or no ACh in response to electrical stimulation during the 'silent period' when they do not release quanta spontaneously.

In spite of the large variability, it seems that the magnitude of the e.p.p.s elicited by Schwann stimulation increased with time after denervation. 5–10 days after nerve section the largest responses evoked were roughly 5–8 mV and did not reach the threshold for discharge of the muscle fibres, whereas 20 days after denervation, responses of 10–30 mV were frequently encountered.

Structural changes upon Schwann stimulation

Light-microscopy. When a Schwann cell was stimulated with a pulse strong enough to evoke a response in the muscle fibre there was a rapid and dramatic change in the appearance of the stimulated cell: it became swollen, and large vacuoles formed in the vicinity of the nucleus (Pl. 2). This change was a sufficiently rapid and reliable index that simply by

watching the cell while increasing gradually the intensity of successive stimuli, the observer could judge when the pulse had reached threshold for evoking the e.p.p. The changes in Schwann cell appearance were observed even when the external medium had 3 mM-EGTA and no calcium ions. It was clear from these observations that 'effective' stimuli caused gross alterations in the Schwann cells, and to obtain further information on the nature of these changes, the Schwann cells were examined with the electron-microscope.

Electron-microscopy. To identify stimulated Schwann cells on selected muscle fibres, the adjacent fibres were damaged by coarse impalements with the micro-electrode. This led to vacuolation in the muscle fibres, which could then be easily identified. With this procedure, disruption of muscle fibre structure varied with the amount of damage, and with the time elapsed before fixing the muscle. However, it was possible to monitor the damage by observing the fibres with the water immersion lens. We usually fixed the fibres when they had attained a slightly granular appearance, were less transparent, and their striations were less well defined, at which time the electron-microscopic structure of the fibres was like that illustrated in Pl. 3. As damage progressed the fibres became opaque, the sarcoplasm broke up and retracted, but even when the fibre had been reduced to practically an empty tube, the Schwann cells – and axon terminals if the fibre was innervated – were not obviously altered.

Unstimulated Schwann cells. In denervated muscles the Schwann cells consist of a central nucleated portion, from which long processes arise and extend for many microns. Their structure varies greatly from cell to cell, as well as in different regions of the same cell. In some cases the Schwann cell appears simply as a round nucleus surrounded by a thin layer of cytoplasm (Pl. 4). In others the cell is composed of several compartments, with the nuclear region usually lying close to the synaptic muscle membrane (cf. Pl. 7*A*).

The Schwann cell cytoplasm contains many small mitochondria, well developed smooth and rough endoplasmic reticulum, Golgi complex, lysosomes, lipid granules, microtubules, gliofilaments, etc. (cf. Pls. 3–6, 7*A*, 8*A*). It also contains many clear vesicles which are of special interest because of their possible involvement in transmitter release. The vesicles are of different sizes (Pls. 5, 6 and 7*A*) and some are seen opening into the synaptic cleft, or elsewhere along the surface of the Schwann cell. These openings seem more common in the Schwann cell than in normal axon terminals, where the frequency of min.e.p.p.s is much greater, and may thus correspond mostly to the pinocytotic vesicles seen in many other cells. In addition, the Schwann cell contains coated vesicles (Pls. 8*A*, 5 and 6) and some coated tubes. It is possible that these, together with

some of the clear vesicles, represent stages in a secretory cycle which is seen more clearly after stimulating quantal transmitter release from axon terminals (Heuser & Miledi, 1971; Heuser & Reese, 1973).

Centrioles are occasionally seen in the juxtanuclear region of the Schwann cell (Pls. 5 and 6; see also Miledi & Slater, 1968). In some instances, like the lower one in Pl. 6, the centriole serves as a basal body of a cilium. These structures seem to be more common in denervated than in normal Schwann cells.

Stimulated cells. After ACh is released from a Schwann cell by electrical stimulation, its appearance is markedly changed. The nucleus rounds up and the chromatin aggregates in masses disposed around the periphery of the nucleus (Pls. 7*B*, 8*B* and 9); instead of being diffusely distributed as in control cells (see Pls. 3–6, 7*A*, 8*A*). The nucleolar material is dispersed and shows up as small dark aggregates (compare Pl. 3 with 9 and 7*B*). The nuclear envelope, with its outer layer studded with ribosomes, and the nuclear pores structure still remain, preventing the chromatin from dispersing in the cytoplasm.

After effective stimulation the endoplasmic reticulum is markedly swollen. Large 'vacuoles' appear, and some, formed from the rough endoplasmic reticulum, are lined with ribosomes (Pls. 10 and 8*B*). The mitochondria are also large and swollen, perhaps due to an increase in the intracellular concentration of calcium ions. Lysosomes do not appear to be greatly affected.

Another interesting feature of stimulated cells is that their cytoplasm appears to be relatively empty when compared with non-stimulated cells. Some clear vesicles and coated vesicles remain after stimulation, especially in compartments which are relatively less affected by the stimulation, but in general the picture looks as if the Schwann cell had discharged much of its contents, with vesicular membranes presumably being incorporated into the distended cisternae and surface membrane.

During the experiment, the stimulating micro-electrode was usually placed in the region of the Schwann cell nucleus, and we could not tell with certainty whether that region was in synaptic contact with the muscle. The electron-microscopic study showed that though this was usually the case, some stimulated nuclear regions were not in immediate contact with the muscle (cf. Pl. 9). It seems likely, therefore, that release of ACh from a distance contributes to the large spectrum of amplitudes and time courses of Schwann-e.p.p.s described above.

Even though the electric pulse was applied in the nuclear region, there is no doubt that a large part, perhaps all, of the Schwann cell is affected by the stimulus. For instance, Pl. 10 shows a Schwann cell process situated approximately 50 μm away from the nucleus.

It is interesting that after gross structural change of a Schwann cell by electrical stimulation, neighbouring Schwann cells on the same end-plate appeared normal under the electron-microscope. This suggests that the various Schwann cells of an end-plate are not electrically coupled, or that the coupling is weak and insufficient to allow enough current to spread to neighbouring cells and stimulate them.

DISCUSSION

It is clear that 1 or 2 weeks after denervation, all the Schwann cells elicit a Schwann end-plate potential when electrically stimulated. These e.p.p.s are caused by the release of ACh, or a similar substance, from the Schwann cell, as evidenced by the fact that the responses are greatly reduced by D-TC. Initially we wondered whether the lysosomal contents were being released and were acting on the muscle membrane, thus contributing to the Schwann-e.p.p., but this does not seem likely, because curare could completely abolish the response, and because lysosomes did not appear to be disrupted by the stimulation. Thus the present experiments lend further support to the proposal, put forward by Birks *et al.* (1960), that after denervation the Schwann cells release ACh, and also throw some light on the nature of the silent period (cf. p. 444).

Since the Schwann cells only grow into direct apposition with the junctional folds after degeneration of the axon terminal, it could be thought that the lack of min.e.p.p.s, or Schwann-e.p.p.s, during the silent period merely reflects insufficient contact between Schwann cells and muscle fibre. However, electron-microscopic observations indicate that during the silent period the Schwann cell has already established extensive contacts with the muscle fibre. Furthermore, from experiments in which actinomycin D was found to prevent the appearance of Schwann min.e.p.p.s, Bevan *et al.* (1973*a*) suggested that the Schwann cell is induced to synthesize, and release, ACh quanta only after denervation. On this scheme, the failure to elicit Schwann-e.p.p.s during the silent period indicates that at this time the Schwann cells do not contain much ACh.

It is known that, when calcium ions are present in the external medium, the amount of transmitter released from axon terminals can be finely controlled by applying graded depolarizing pulses to the terminal: depolarization of the axon membrane leads to an influx of calcium which causes the release of transmitter from the ending (Katz & Miledi, 1967*b*, 1969). In contrast, the evoked release of ACh from the Schwann cell did not require the presence of calcium in the external fluid, and was an explosive event over which we had relatively little control. All this suggests that the Schwann cell lacks the potential-dependent calcium 'gates' or

any of the subsequent steps which are normally involved in transmitter release from axon terminals, and raises the question of how the electric pulse is acting on the Schwann cell.

It has previously been shown that application of hyperpolarizing pulses to motor endings can cause high frequency bursts of min.e.p.p.s (del Castillo & Katz, 1954; Katz & Miledi, 1965, 1967*b*). Furthermore, in the giant synapse of the squid, a sudden discharge of transmitter may occur when the presynaptic membrane is hyperpolarized – sometimes by as little as 30–50 mV, with pulses of about 20–100 msec duration (R. Miledi, unpublished). This release of transmitter by hyperpolarization of the axon membrane resembles in many respects that from the Schwann cell. For instance, in both cases the responses occur suddenly and can be elicited only a limited number of times. Moreover, as already mentioned, the Schwann-e.p.p. can be obtained in media with very low calcium; similarly the axon terminal response to hyperpolarizing pulses can still be evoked in media with 0 calcium + EGTA (R. Miledi, unpublished): while in both cases calcium is not required, it could be that calcium is still involved in this type of transmitter release if the hyperpolarizing pulses were to mobilize calcium from sources such as membranes or mitochondria.

It has been suggested that the release of transmitter caused by hyperpolarizing the axon is a consequence of a dielectric break-down of the nerve terminal membrane (del Castillo & Katz, 1954; Katz & Miledi, 1965), and a similar membrane break-down may be responsible for the electrically induced release of ACh from Schwann cells. If one accepts this interpretation then there is an interesting difference in that the break-down in the Schwann cell can be readily obtained with current pulses of both polarities, whereas in the nerve terminals it is difficult to produce 'break-down release' with depolarizing pulses. An alternative, but less likely explanation of ACh release from Schwann cells is that the stimulating electrode actually produces in the Schwann cell membrane a hole through which ACh leaks. In any case, the 'effective' electric stimulation of a Schwann cell is a drastic procedure which disrupts its internal structure and has probably no functional significance.

Another interesting question raised by our experiments concerns the form in which the ACh is released from Schwann cells. The spontaneous release of ACh which evokes the Schwann min.e.p.p.s occurs in multimolecular amounts, as is also the ACh released by break-down of the axon membrane. On the other hand, the electrically induced release of ACh from Schwann cells does not seem to occur in quantal form. The limitations for detecting quantal events, in the present experiments, are quite significant, because most of the min.e.p.p.s caused by ACh released from denervated Schwann cells lie within the noise of the recording system.

If, as we believe, these small spontaneous potentials result from the release of packets of ACh, then the electrically evoked release could be made up of many of these very small quanta. The present experiments do not allow us to discriminate between this possibility and the alternative, that the ACh released by break-down of the Schwann membrane is in molecular form. We can say, however, that the larger packets of ACh, those which give rise to min.e.p.p.s of more than about 0·4 mV, do not seem to be released by electrical stimulation; so by analogy, perhaps most of the ACh is released in molecular form. Further insight into this question may come when Schwann-e.p.p.s are analysed with the same statistical treatment used to study the molecular events produced by ACh action on the muscle fibre membrane (cf. Katz & Miledi, 1972).

If the electric pulses cause the ACh to be released in molecular form, we then have a ready explanation for the somewhat puzzling finding that the ACh is released at the termination of a hyperpolarizing pulse; namely, that the positively charged ACh ions would be retained in the cell by the negative pulse. An alternative, but less attractive explanation would be that the negative pulse prevents vesicles from colliding, or reacting, with the Schwann membrane.

A regular feature of our experiments was that only a limited number of Schwann-e.p.p.s could be obtained from a given Schwann cell, perhaps due to exhaustion of a store of ACh. Whatever the reason for this limitation turns out to be, it is interesting to speculate on the amount of releasable ACh. Let us say that from a Schwann cell one can evoke twenty Schwann-e.p.p.s and that, on average, each e.p.p. is equivalent to 40 min.e.p.p.s like those seen in normally innervated muscles. The cell would thus release an amount of ACh equivalent to 800 min.e.p.p.s. Say each min.e.p.p. is built up of 10^3 elementary events (Katz & Miledi, 1972), this would make a total of 8×10^5 elementary events. If we assume further, that to evoke this number of events, 100 times as many ACh molecules must be released from the Schwann cell (cf. Katz & Miledi, 1973), we then have about 10^8 molecules ($1{\cdot}7 \times 10^{-16}$ moles) of ACh *releasable* by electric pulses from a Schwann cell. If the volume of a Schwann cell were equivalent to a cylinder 2 μm in diameter and 200 μm long $= 6 \times 10^{-10}$ ml., then the concentration of ACh, if in solution, would be in the order of 3×10^{-4} M. Admittedly this is a very rough estimate, based on too many assumptions, but it does suggest that release of ACh from cytoplasmic solution is not an implausible explanation of our results.

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REFERENCES

BEVAN, S., GRAMPP, W. & MILEDI, R. (1973*a*). Induced transmitter release from Schwann cells and its suppression by actinomycin D. *Nature, New Biol.* **241**, 85–86.

BEVAN, S., GRAMPP, W. & MILEDI, R. (1973*b*). Further observations on Schwann-cell min.e.p.p.s. *J. Physiol.* **232**, 88–89*P*.

BIRKS, R., KATZ, B. & MILEDI, R. (1960). Physiological and structural changes at the amphibian myoneural junction, in the course of nerve degeneration. *J. Physiol.* **150**, 145–168.

DEL CASTILLO, J. & KATZ, B. (1954). Changes in end-plate activity produced by pre-synaptic polarization. *J. Physiol.* **124**, 586–604.

DENNIS, M. (1972). Electrically evoked release of acetylcholine from Schwann cells at denervated motor end-plates. *J. Physiol.* **226**, 79–81*P*.

HARRIS, A. J. & MILEDI, R. (1972). A study of frog muscle maintained in organ culture. *J. Physiol.* **221**, 207–226.

HEUSER, J. & MILEDI, R. (1971). The effect of lanthanum ions on function and structure of frog neuromuscular junctions. *Proc. R. Soc.* B **179**, 247–260.

HEUSER, J. E. & REESE, T. S. (1973). Evidence for recycling of synaptic vesicle membrane during transmitter release at the frog neuromuscular junction. *J. cell Biol.* **57**, 315–344.

KATZ, B. & MILEDI, R. (1959). Spontaneous subthreshold activity at denervated amphibian end-plates. *J. Physiol.* **146**, 44–45*P*.

KATZ, B. & MILEDI, R. (1965). Propagation of electric activity in motor nerve terminals. *Proc. R. Soc.* B **161**, 453–482.

KATZ, B. & MILEDI, R. (1967*a*). Tetrodotoxin and neuromuscular transmission. *Proc. R. Soc.* B **167**, 8–22.

KATZ, B. & MILEDI, R. (1967*b*). The release of acetylcholine from nerve endings by graded electric pulses. *Proc. R. Soc.* B **167**, 23–38.

KATZ, B. & MILEDI, R. (1969). Spontaneous and evoked activity of motor nerve endings in calcium Ringer. *J. Physiol.* **203**, 689–706.

KATZ, B. & MILEDI, R. (1972). The statistical nature of the acetylcholine potential and its molecular components. *J. Physiol.* **224**, 665–699.

KATZ, B. & MILEDI, R. (1973). The binding of acetylcholine to receptors and its removal from the synaptic cleft. *J. Physiol.* **231**, 549–574.

MCMAHAN, U. J. & KUFFLER, S. W. (1971). Visual identification of synaptic boutons on living ganglion cells and of varicosities in postganglionic axons in the heart of the frog. *Proc. R. Soc.* B **177**, 485–508.

MCMAHAN, U. J., SPITZER, Y. & PEPER, Y. (1972). Visual identification of nerve terminals in living isolated skeletal muscle. *Proc. R. Soc.* B **181**, 421–430.

MILEDI, R. (1960). Properties of regenerating neuromuscular synapses in the frog. *J. Physiol.* **154**, 190–205.

MILEDI, R. (1971). Lanthanum ions abolish the 'calcium response' of nerve terminals. *Nature, Lond.* **229**, 410–411.

MILEDI, R. & SLATER, C. R. (1968). Electrophysiology and electron-microscopy of rat neuromuscular junctions after nerve degeneration. *Proc. R. Soc.* B **169**, 289–306.

MILEDI, R. & SLATER, C. R. (1969). Electron-microscopic structure of denervated skeletal muscle. *Proc. R. Soc.* B **174**, 253–269.

MILEDI, R. & STEFANI, E. (1970). Miniature potentials in denervated slow muscle fibres of the frog. *J. Physiol.* **209**, 179–186.

EXPLANATION OF PLATES

The following abbreviations are used: cen, centriole; cv, coated vesicles; er, endoplasmic reticulum; g.c., golgi complex; lys, lysosome; mit, mitochondrion; N, nucleus; Ne, nuclear envelope; Np, nuclear pore; rib, ribosomes; Sch, Schwann cell; sf, synaptic folds.

PLATE 1

Light microscope picture of a cutaneous pectoris muscle in cross section. Muscle fixed in osmium tetroxide + veronal acetate buffer.

PLATE 2

Nomarski interference-contrast micrographs of the end-plate region of a denervated muscle fibre with three Schwann cell nuclei visible, taken before (*A*) and after (*B*) focal electrical stimulation of the Schwann cells. Note the characteristic swollen appearance of the nuclei after stimulation. One of the responses caused contraction of the muscle fibre and resulted in damage of the muscle fibre by the recording electrode, with a sustained local contraction which led to a reduction of sarcomere length and of the distance between the Schwann nuclei in *B*. The sarcomere spacing of the adjacent muscle fibre, showing at the bottom of each micrograph, is approximately 3 μm in both cases.

PLATE 3

Electron-micrograph of a marked muscle fibre used to identify an adjacent fibre containing stimulated Schwann cells. Note the vacuoles produced by intentional damage for marking the fibre.

All Figures from cutaneous pectoris muscles 14 days after cutting the nerve, except Pl. 4, which was obtained from a muscle 11 days after crushing the nerve. Muscles fixed in osmium tetroxide in veronal acetate buffer. Sections stained with uranyl acetate and lead nitrate. Calibration bars 1 μm.

PLATE 4

Nuclear region of a Schwann cell in synaptic contact with a denervated end-plate. Note the thin layer of cytoplasm around the Schwann nucleus.

PLATE 5

Juxtanuclear region of a Schwann cell on a denervated end-plate. Note a centriole (possibly the base of a cilium) and the endoplasmic reticulum.

PLATE 6

Centrioles in a Schwann cell from another denervated muscle. In near serial sections the lower cilium was seen emerging from the Schwann cell. Note the assorted vesicles and endoplasmic reticulum.

PLATE 7

Comparison between unstimulated (*A*) and stimulated (*B*) Schwann cells, in the same denervated muscle. Note the swollen mitochondria and rough endoplasmic reticulum as well as the dispersed chromatin in the stimulated cell.

PLATE 8

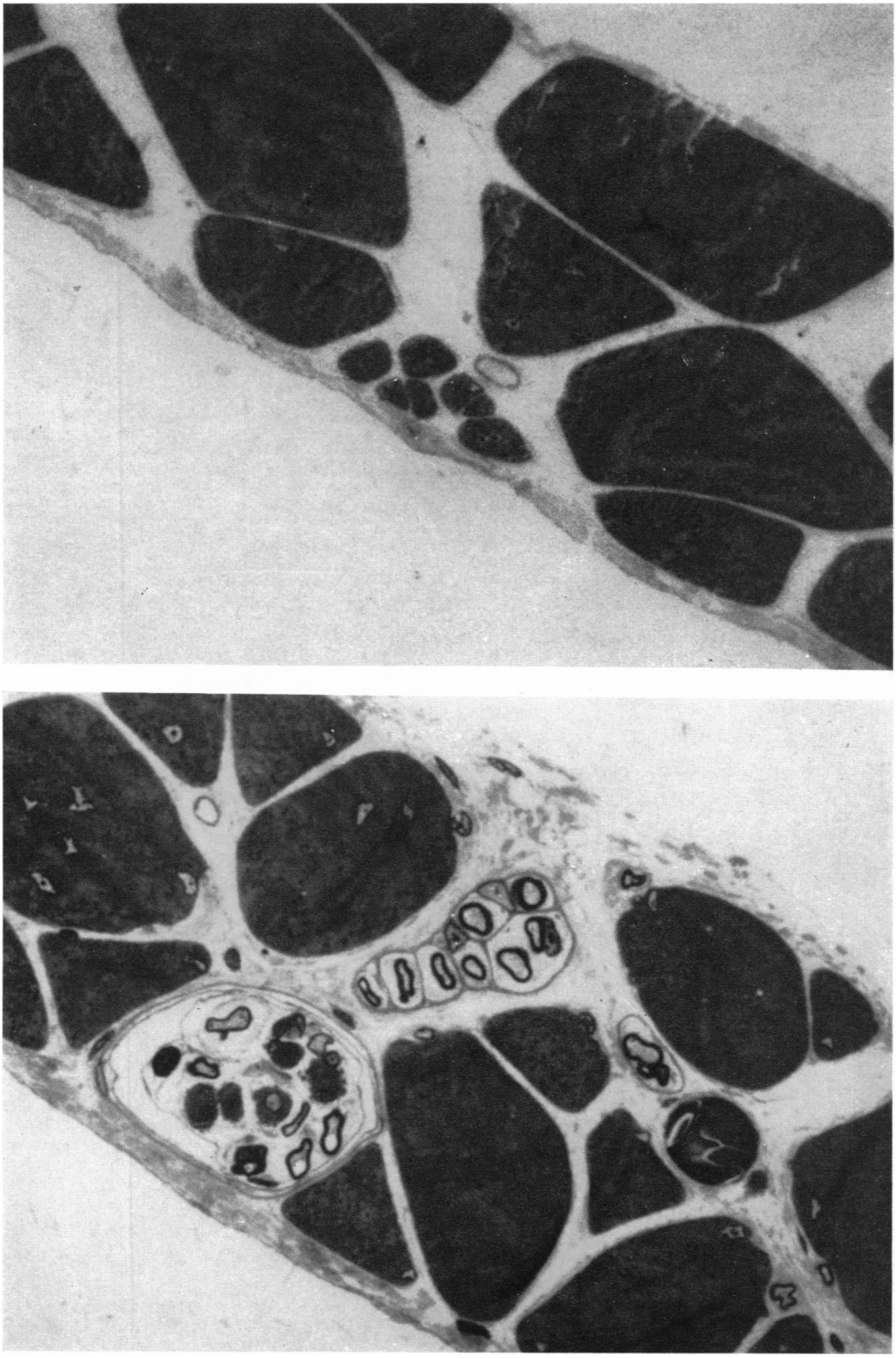
Another example of unstimulated (*A*) and stimulated (*B*) Schwann cells.

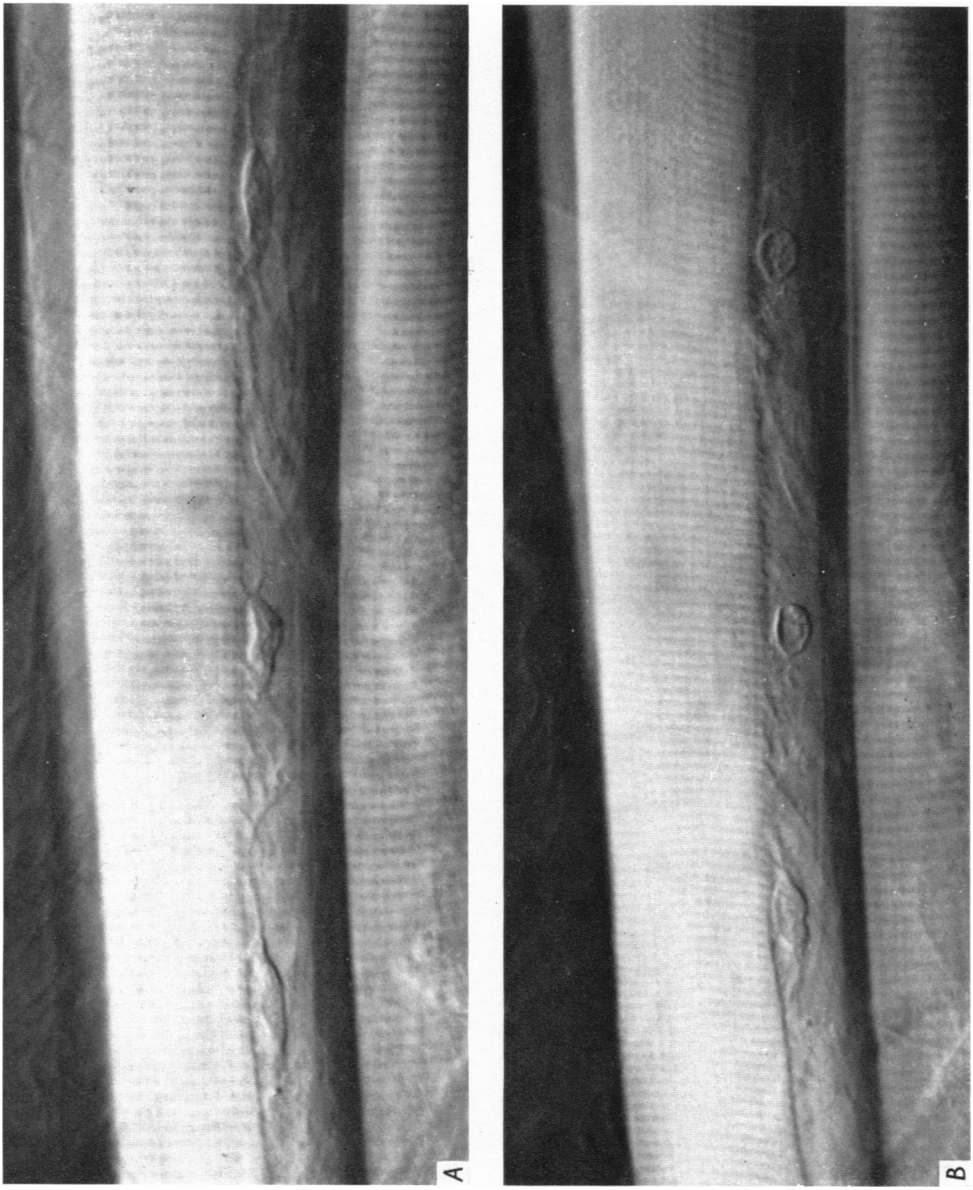
PLATE 9

Nuclear region of a stimulated Schwann cell. Notice that this nuclear region is not in synaptic contact with the muscle fibre. Same fibre as Pls. 7 and 8, but different Schwann cell. The dark aggregates in the nucleus are dispersed material from the nucleolus.

PLATE 10

Schwann cell process from a stimulated cell. Note the swollen mitochondria as well as the swollen endoplasmic reticulum studded with ribosomes.

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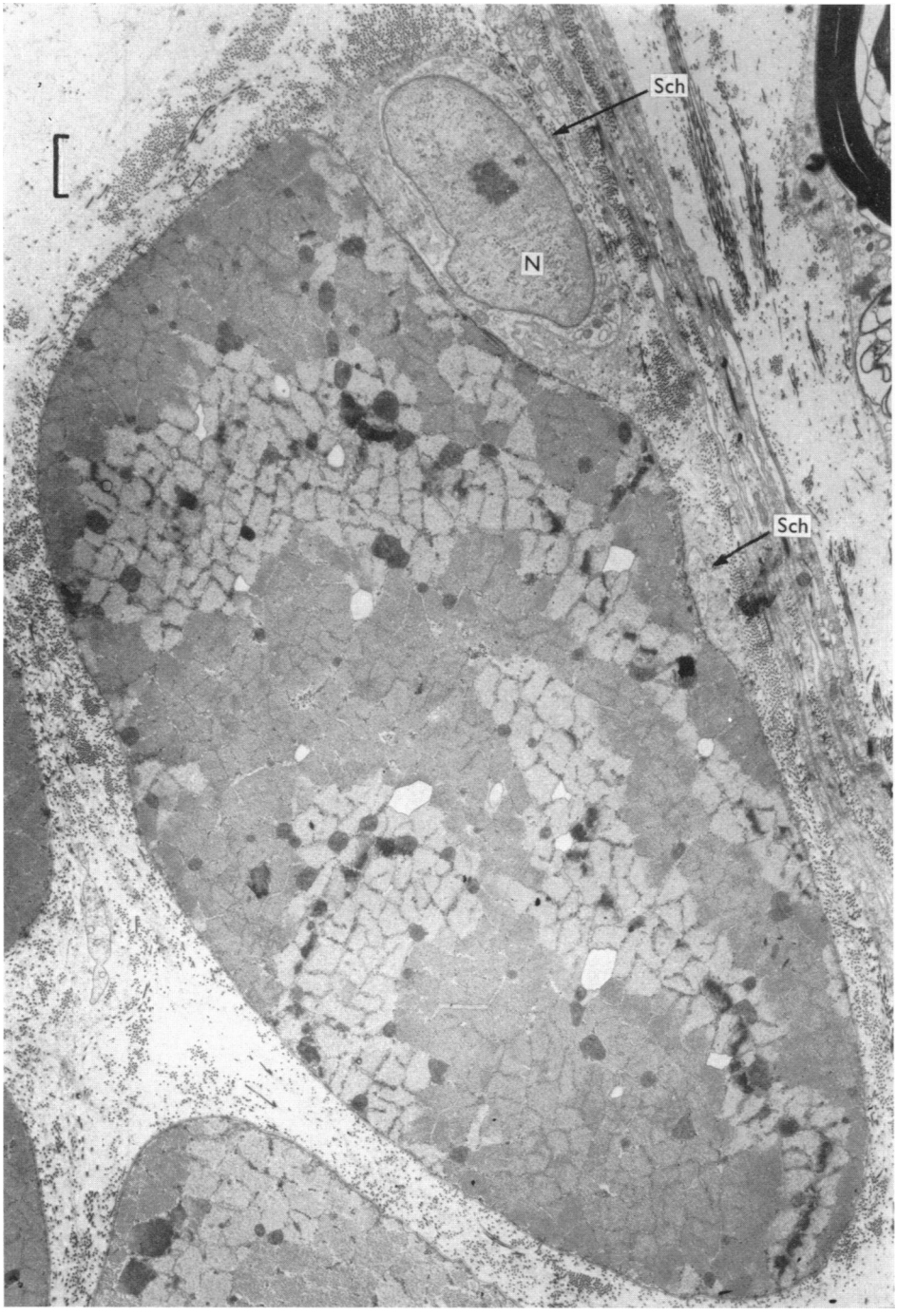
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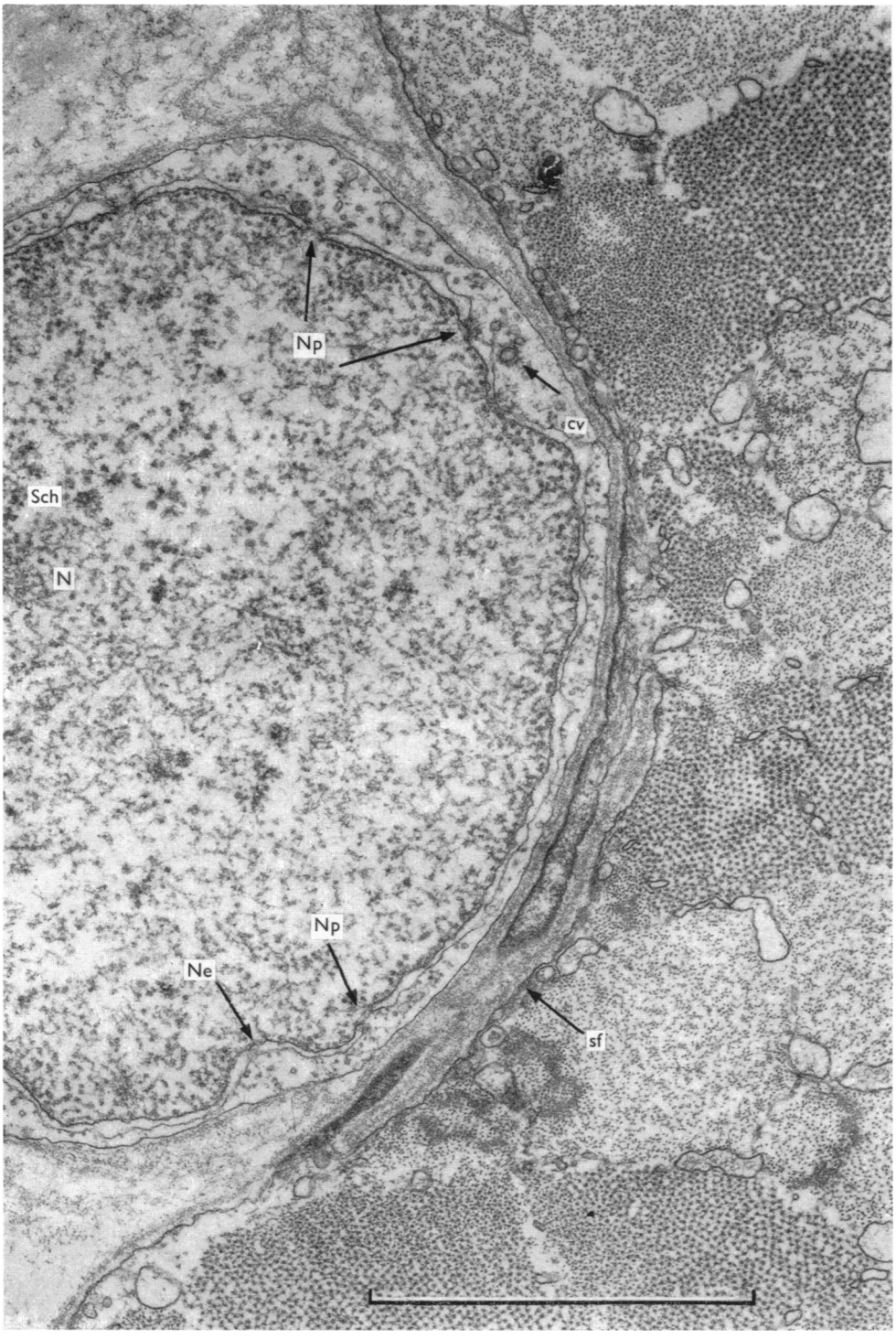

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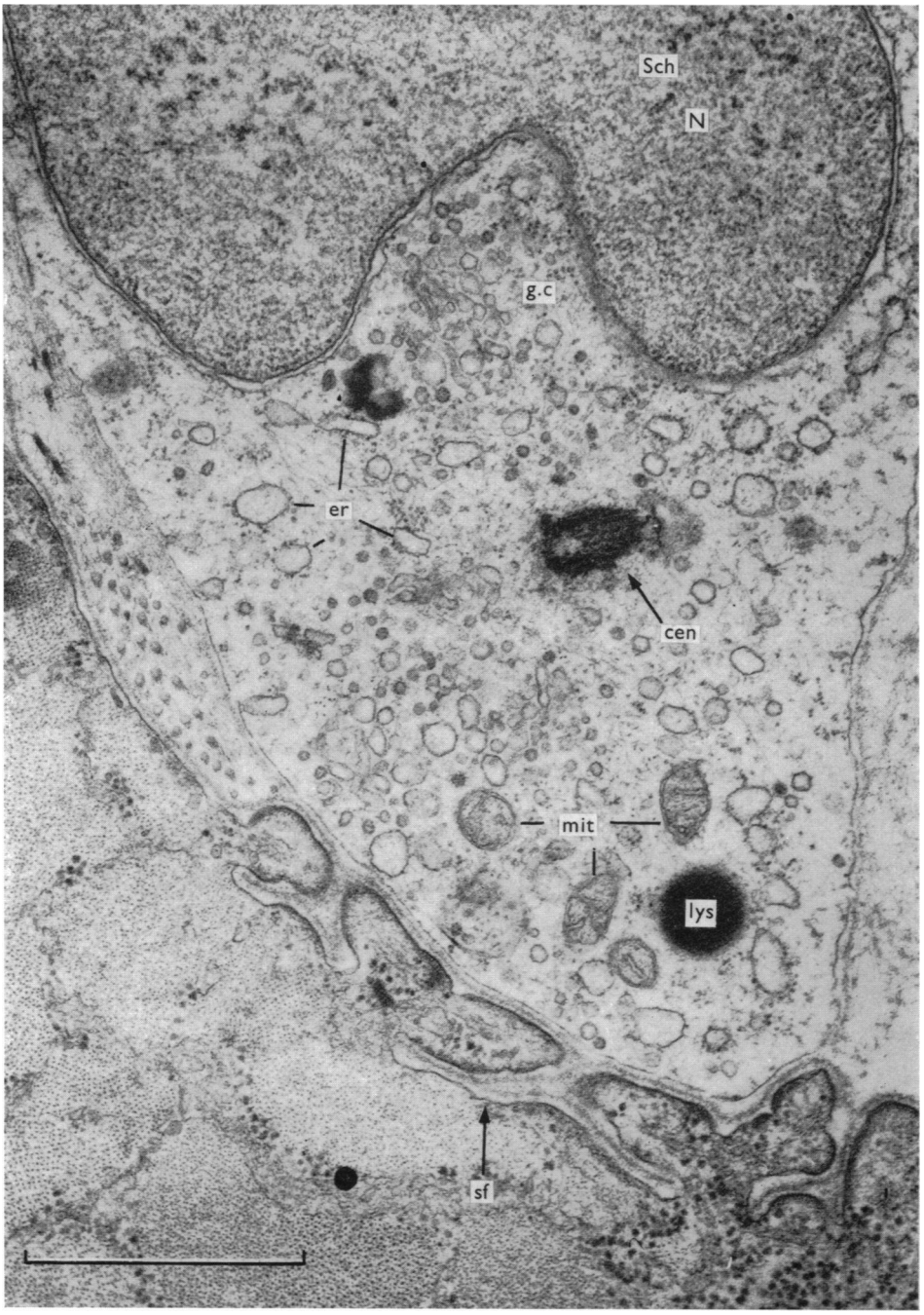
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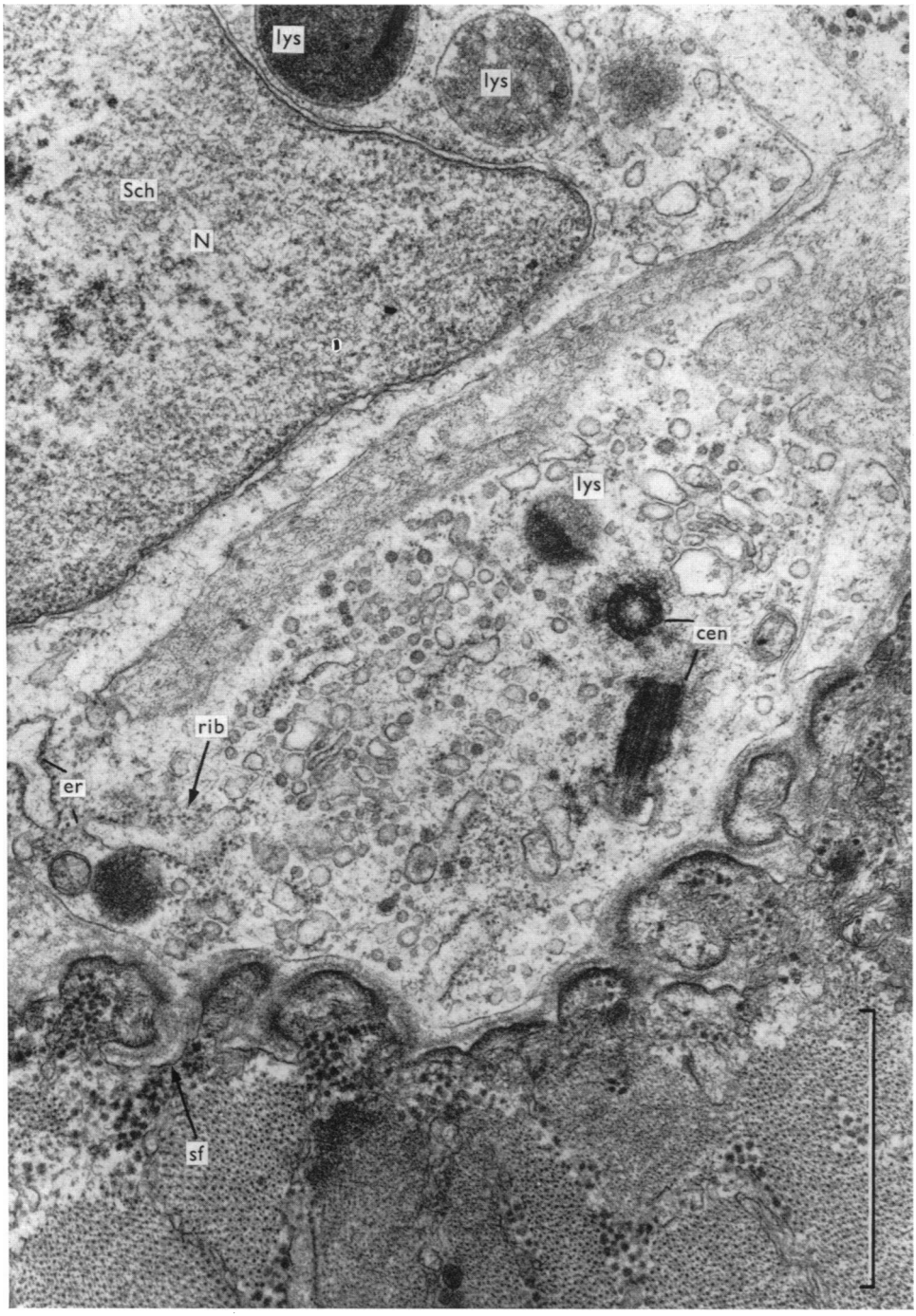

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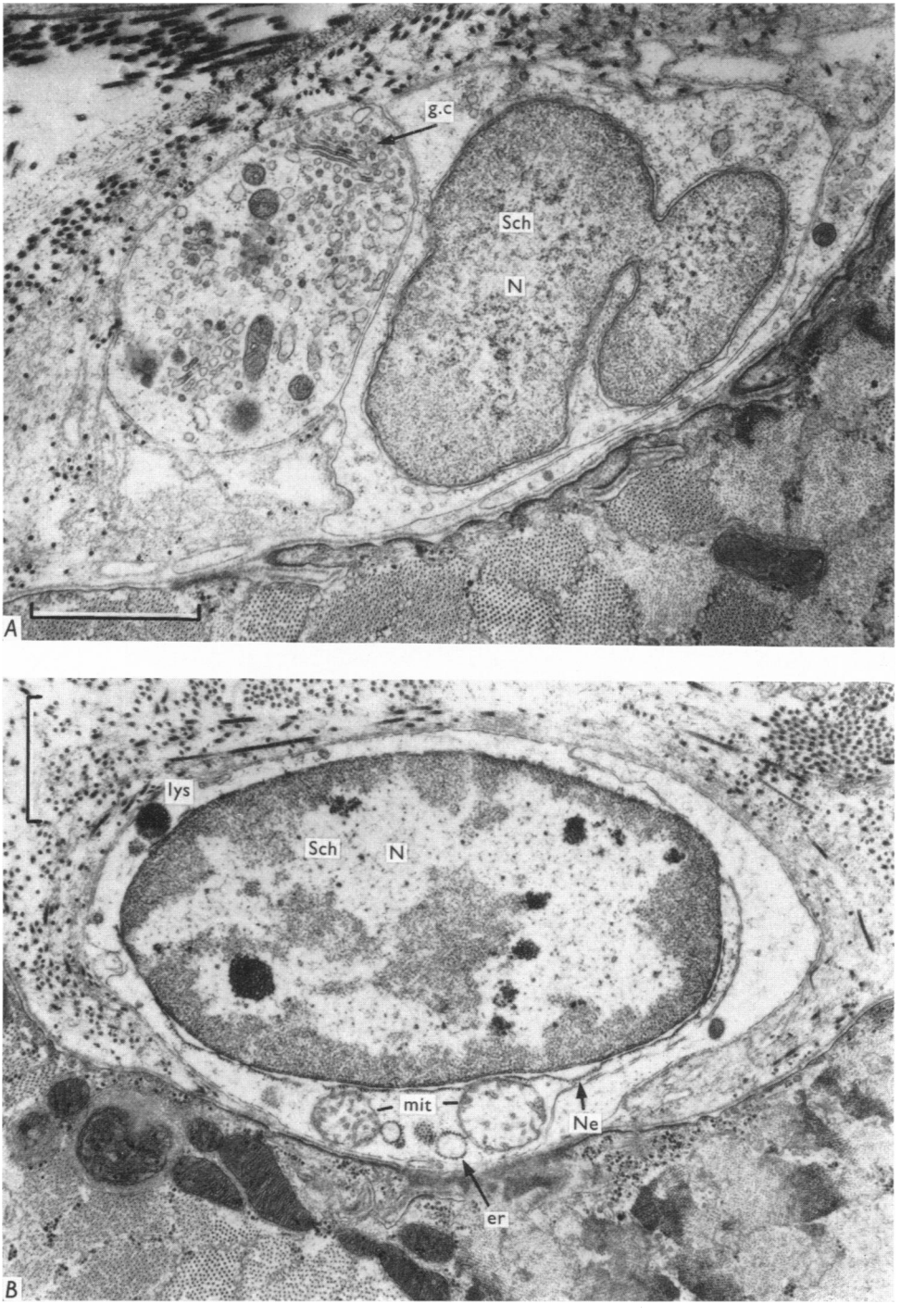
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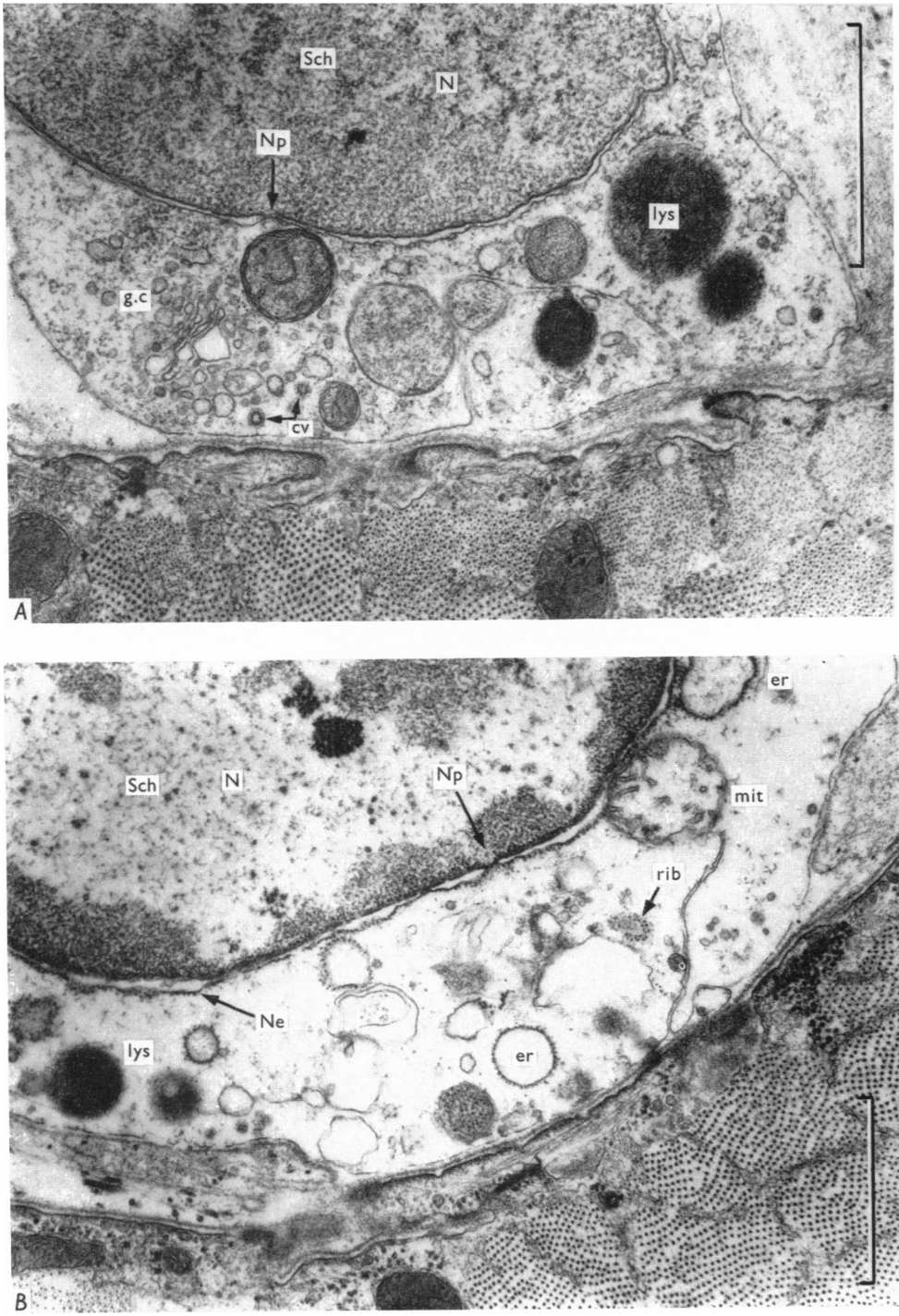
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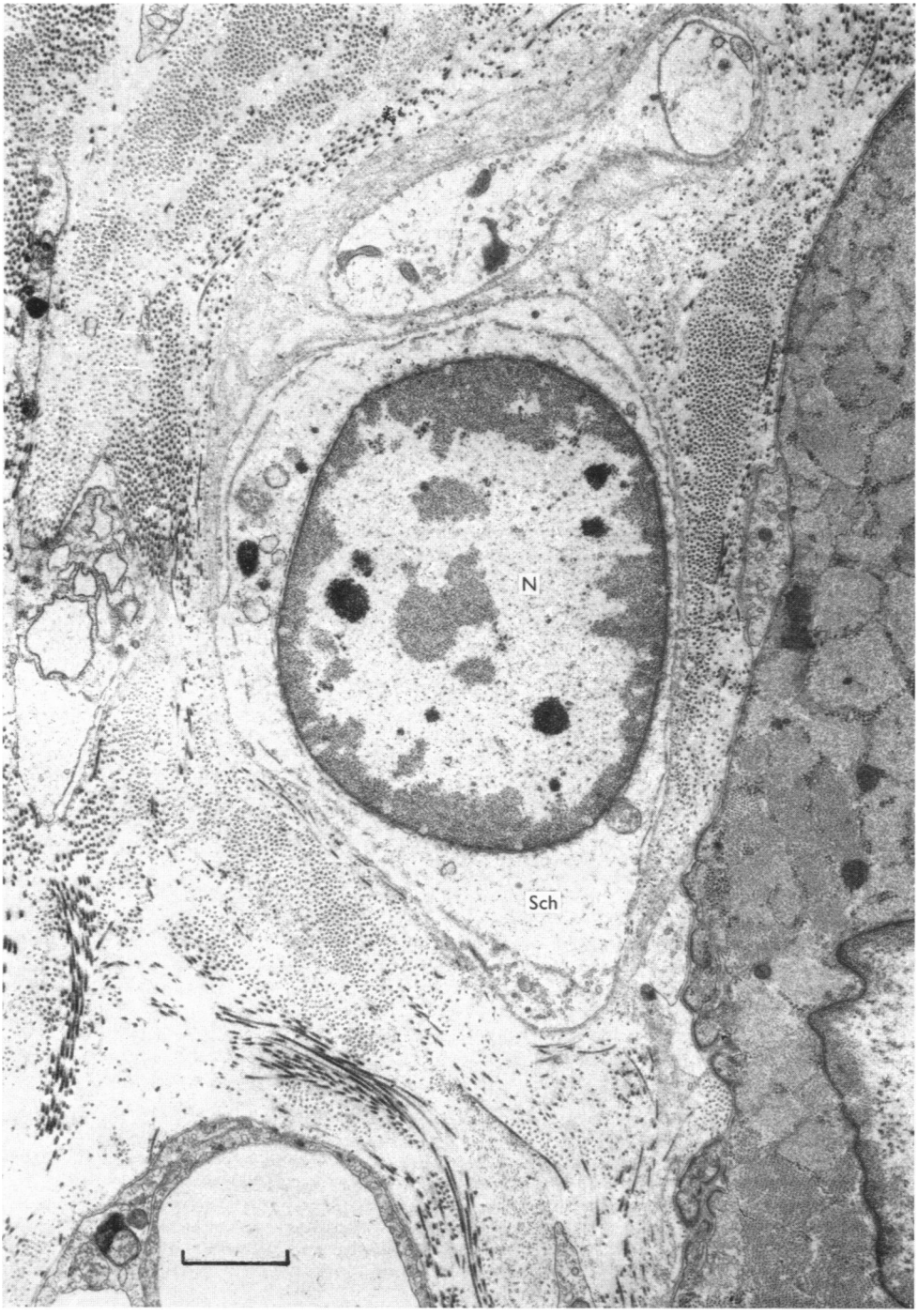

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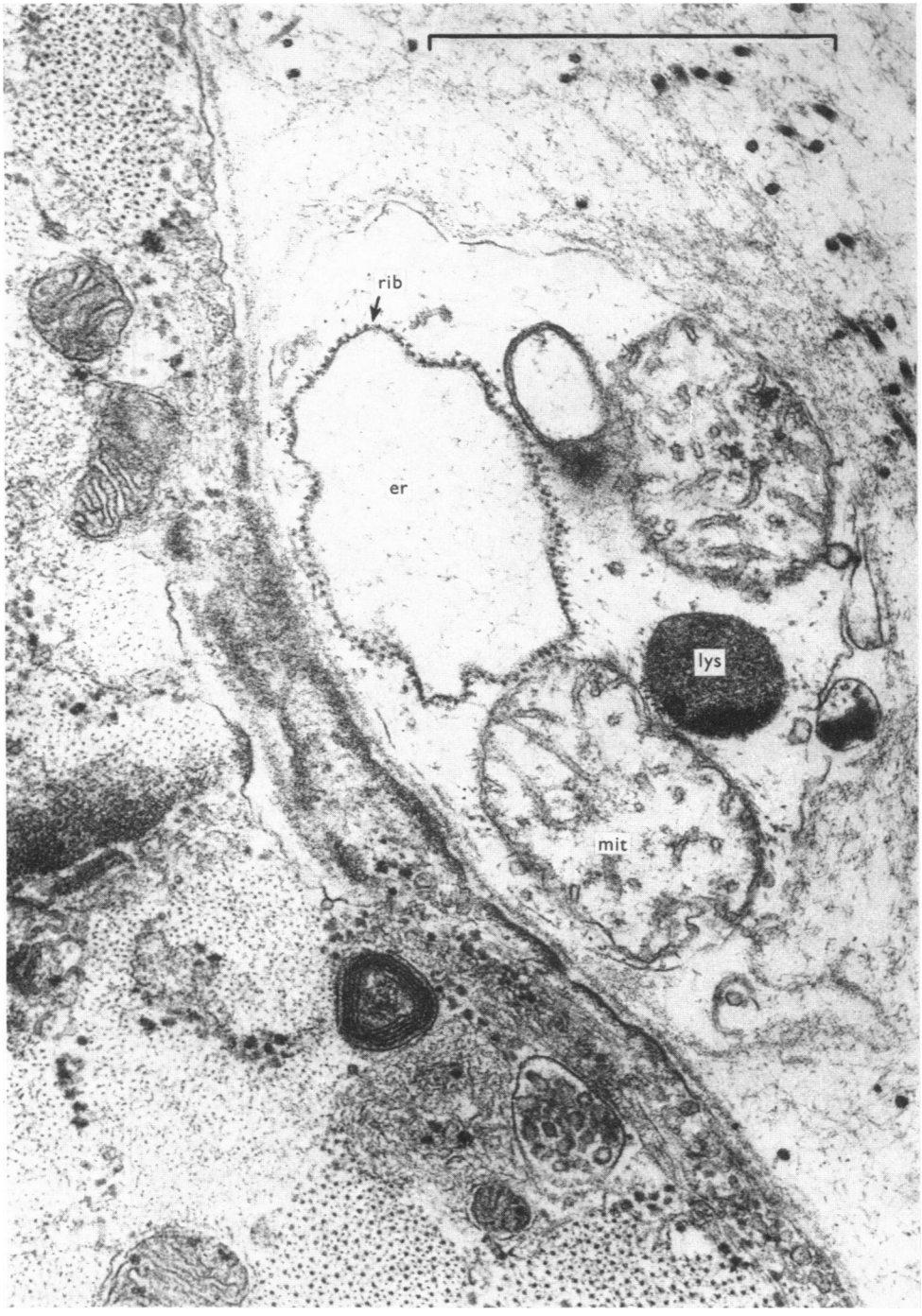

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