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LIGHT AND ELECTRON MICROSCOPIC IDENTIFICATION OF NERVE TERMINAL SPROUTING AND RETRACTION IN NORMAL ADULT FROG MUSCLE

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SUMMARY

1. A combined light and electron microscopic study was performed on neuromuscular junctions of normal adult frogs.

2. In a previous investigation signs of new synapse formation, as well as abandoned former synaptic sites, have been observed in normal muscles (Wernig, Pécot-Dechavassine & Stöver, 1980*a*, *b*). Here we performed a detailed light and electron microscopic correlation to investigate those parts of junctions which, after staining for cholinesterase (ChE) and presynaptic axon terminals, were suspected either to be newly formed or sites abandoned by the presynaptic nerve and the Schwann cell.

3. Thin presynaptic nerve branches, enclosed by Schwann cell sheaths along most of their length, formed synaptic contacts with the muscle fibre only at small circumscribed areas. In these regions post-synaptic secondary folds (invariably present at mature synapses) were either missing or were less well developed. At these small contacts, binding sites for fluorescein-labelled α-bungarotoxin were usually present.

4. At other sites the ChE reaction product was present but an axon could not be detected in silver-stained preparations. Electron microscopic observation revealed post-synaptic secondary folds filled with ChE reaction product while the presynaptic axon and Schwann cell were missing. The sites with ChE remnants can thus be regarded as abandoned former synaptic contacts. No binding of fluorescein-labelled α-bungarotoxin could be detected at such sites.

5. These findings confirm earlier suggestions that synaptic contacts in frog muscle are normally undergoing continual remodelling.

6. The lack of binding sites for fluorescein-labelled α-bungarotoxin at abandoned synaptic sites suggests that a neural or Schwann cell factor is important for the maintainance of synaptic acetylcholine receptors.

INTRODUCTION

In a previous investigation on frog neuromuscular junction, signs of nerve terminal retraction and signs of nerve sprouting with formation of new synaptic contacts have

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been observed in normal muscles (Wernig, Pécot-Dechavassine & Stöver, 1980*a*, *b*). Such signs were described separately in the light microscope on preparations stained for cholinesterase (ChE) and in the electron microscope on randomly sampled ultrathin sections. The aim of the present investigation was to analyse the ultrastructure of those parts of a synapse which, from their light microscopic appearance, were candidates for sites of remodelling. Candidates for newly formed presynaptic contacts were spots which, after ChE staining, appeared as single rings or chains of rings (ChE rings, Wernig *et al.* 1980*a*, *b*). Candidates for vacated sites were ChE remnants that had no opposing presynaptic elements (cf. Letinsky, Fischbeck & McMahan, 1976; Letinsky & DeCino, 1980; Wernig *et al.* 1980*a*, *b*). In addition to describing the ultrastructure of these sites, the distribution of acetylcholine (ACh) receptors at both ChE rings and ChE remnants was determined using fluorescein-labelled α-bungarotoxin.

Short accounts of this investigation have been published (Wernig, Anzil & Bieser, 1981*a*; Wernig, Anzil, Bieser & Schwarz, 1981*b*).

METHODS

Cutaneous pectoris muscles of adult *Rana temporaria* were pinned out in plastic dishes, fixed with glutaraldehyde (0·9% in Ringer solution, pH 7·4, for 15 min at 20 °C) and stained for ChE (Karnovsky & Roots, 1964). After dehydration whole muscles were embedded in Epon. After curing, the Epon blocks were filed down to thin plates, covered with immersion oil and mounted on glass slides. With or without a glass cover-slip the embedded muscle could then be viewed with a 100 × oil-immersion objective in a standard microscope. After identifying sites of interest the block was trimmed and ultrathin sections were cut in a serial manner. Ultrathin sections were put on slot grids, stained with uranyl acetate (30 min) and lead citrate (9 min) and examined in a Zeiss EM 10.

For the binding studies freshly excised cutaneous pectoris muscles were incubated for 30–60 min at room temperature in about 10^{-6} g α-bungarotoxin/ml labelled with fluorescein isothiocyanate (FITC) (Suszkiw & Ichiki, 1976). For incubation, all fractions of labelled α-bungarotoxin, including the reversibly binding one (Suszkiw & Ichiki, 1976), were used. After incubation muscles were kept in normal Ringer solution for an additional 20–60 min at room temperature. After fixation (10 min each in 1 and 5% v/v formaldehyde in Ringer solution) the part of the muscle containing the synapses was cut out, mounted in Ringer solution on glass slides, covered with a glass cover-slip and examined in a fluorescence microscope with epi-illumination by blue light (450–490 nm). After all superficial synapses were photographed, the muscle was stained for axon and ChE (Wernig *et al.* 1980*a*), mounted and photographed again. In other experiments ChE stain was applied immediately after labelling with fluorescein–α-bungarotoxin.

RESULTS

We first describe the ultrastructure of the thin presynaptic nerve branches which are apparently forming new synaptic contacts. Next we describe ultrastructural results from former synaptic sites which have evidently been abandoned by the nerve. Finally, the distribution of ACh receptors at new and abandoned sites is analysed.

Newly formed synaptic contacts

Thin presynaptic nerve branches were found which only made a few circumscribed contacts with the muscle fibre. This can be seen in Fig. 1 which is a reconstruction of a thin nerve branch based on electron micrographs from serial ultrathin transverse sections. The axon is enveloped by its Schwann cell (stippling) along most of its length

and contacts the muscle fibre only through small openings in this sheath. At such sites the enlarged axon is filled with synaptic vesicles; there is ChE reaction product in the synaptic cleft and electron-dense material is present beneath the subsynaptic membrane. All these features are characteristic of synaptic contacts. Secondary folds, however, which are always present at mature synaptic contacts, were absent from the sections obtained from these parts of the nerve branch. This suggests that the synaptic contacts are newly formed. At other sites along the nerve branch the axon is enlarged (e.g. at asterisk in Fig. 1), but is still totally enclosed by its Schwann cell. ChE is missing in the cleft as are the typical specializations of the muscle fibre membrane (folds, electron-dense material).

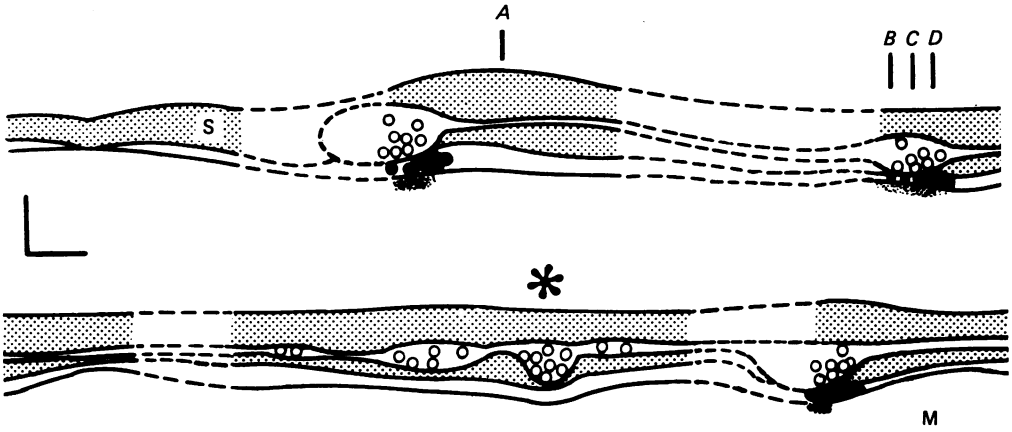

Fig. 1. A reconstruction of the distal part of a thin presynaptic nerve branch in cutaneous pectoris muscle of normal frog (*R. temporaria*, body length 6 cm). Ultrathin transverse sections were cut in intermittent series from a ChE-stained muscle. The parts of the drawing at which the Schwann cell (S) is lightly stippled are based on an analysis of sections; the open parts (dotted lines) are simply the postulated intervening portions. At several sites the axon is enlarged and is in contact with the muscle fibre (M) through openings in the Schwann cell sheaths; note ChE reaction product (black) in the cleft and thickenings at the subsynaptic membrane (heavy stipple). At other sites the enlarged axon is still completely enclosed by the Schwann cell (asterisk). The vertical lines marked *A–D* indicate the positions of the sections shown in Pl. 1 *A–D*. Bars: 1 μm.

Detailed information on this presynaptic nerve and its synaptic contacts are shown in the electron micrographs of Pl. 1 (*A–D*). An opening in the Schwann cell sheath, allowing direct axon–muscle cell contact, starts to form at *B*, has a diameter of about 0·7 μm at *C* and is closed again at *D*. The axon is reduced in size and is enclosed by the Schwann cell in *A* (arrow indicates axon). The position of the sections depicted are indicated by the lines marked *A–D* in Fig. 1.

At contact sites muscle and Schwann cell basal laminae appear to have fused; the margins of such a site are indicated by the horizontal arrows in *D*.

The ChE reaction products usually appear as black crystals. At some sites, however, the reaction product was lost from the section during cutting or was dissolved during section staining with lead citrate. Consequently, corresponding white holes with sharp edges or fuzzy spots remained visible; some of these artifacts, as well as the normal reaction product are marked by asterisks in *A–D*. Since larger

amounts of ChE reaction product apparently develop immediately at the lateral margins of a synaptic contact, the light microscopic appearance of isolated, spot-like synaptic contacts is in whole-mount preparations characterized by rings of ChE reaction product, subsequently called ChE rings (Pl. 1 *E*, asterisk). At such locations the ChE reaction product is more vulnerable to loss during histological preparation (cf. Pl. 1 *F* and *G*; Pl. 2 *B*).

For other thin presynaptic nerves with ChE rings, well-developed secondary folds were found in the post-synaptic membrane. This was the case for the ChE rings at the neuromuscular junction shown in surface view in Pl. 1 *E* (light micrograph after staining for ChE) and in longitudinal section in Pl. 1 *F* and *G* (representative electron micrographs from two of the ChE rings visible in *E*, asterisks).

The distal portions of six thin nerve branches with ChE rings were investigated by serial ultrathin sectioning. At the synaptic contacts secondary folds were missing in two branches and were more or less well developed in the others. Thus, synaptic contacts in different states of maturity are present at ChE rings.

The presence of isolated synaptic contacts at ChE rings is consistent with the electrophysiological observation (Bieser, Wernig & Zucker, 1984) that spontaneous transmitter discharge is present at these sites, but not in the intervening regions. In future it would be interesting to consider the ability of these thin nerve terminal branches to carry action potentials and to contribute to evoked transmitter release.

Abandoned synaptic sites

Deposits of ChE reaction product were regularly found in connexion with otherwise normal junctions; they are usually located beyond the distal portions of the nerve terminal branches and range in length from a few micrometres to a few hundred micrometres (Wernig *et al.* 1980*a*, *b*). In silver-stained preparations the nerve is missing over such deposits. These configurations are called ChE remnants (Wernig *et al.* 1980*a*, *b*). Plate 2 *A* shows a light micrograph of a part of a junction after staining for ChE. On the left an existing junction is apparent and a ChE remnant is visible to the right. In Pl. 2 *B*, a representative electron micrograph obtained from an ultrathin section cut parallel to the muscle fibre long axis is shown. There are several secondary folds visible (arrows), some filled with ChE reaction product. Reaction product outside the folds was partly lost from the sections leaving artifactual light areas visible above the secondary folds. Nevertheless, it is clear that presynaptic elements (nerve or Schwann cell) are missing here. This and the presence of well-developed secondary folds suggest that this is an abandoned former synaptic site.

In electron micrographs from other ChE remnants, secondary folds were less pronounced and occasionally were only recognized by their regular spacing and by the presence of ChE reaction product.

Distribution of α-bungarotoxin binding sites

The distribution of fluorescein-labelled α-bungarotoxin binding sites was compared to the exact location of the axon and to ChE reaction product at individual junctions. Several representative examples are shown in Pl. 3. At all parts of a junction with more-or-less continuous double lines of ChE reaction product, and at spot-like nerve–muscle contacts (ChE rings), intensely labelled bars of toxin-binding sites are present. Between ChE rings no binding sites are detectable (Pl. 3, arrows). These bars probably

represent the necks of the folds of the muscle fibre membrane where ACh receptors are concentrated (Matthews-Bellinger & Salpeter, 1978). In the junctions depicted, individual ChE rings contain one to seven bars occupying a distance of 3–12 μm along the muscle. This distribution pattern matches the extent and the number of folds found on electron micrographs from corresponding ChE rings (Pl. 1 *F* and *G*).

Other sites were found (Pl. 3, asterisks), where ChE reaction product was present (in palisades or diffuse arrangements) but where the presynaptic axon was missing (ChE remnants). In thirty-five such sites in nine muscles, fluorescein-labelled α-bungarotoxin could not be resolved, although a faint labelling might not be detectable by the methods employed (see Discussion).

DISCUSSION

Indications of new synapse formation previously found in randomly sampled sections (Wernig *et al.* 1980*a*, *b*) can now be ascribed with some confidence to thin nerve branches with different numbers of small isolated synaptic contacts. In the light microscope these synaptic contacts appear as ChE rings and they are associated with thin nerve terminal branches of different lengths (a few micrometres to a few hundred micrometres) (Wernig *et al.* 1980*a*). ChE rings are also found at nerve terminal branches with portions of apparently continuous contacts (continuous double lines of ACh reaction product); at such branches ChE rings are either located at the most distal portions or in between longer segments with continuous contacts.

In the present investigation only distally located ChE rings have been investigated, but it is conceivable that new synapse formation also occurs at other sites with ChE rings.

New synapse formation

The thin nerve terminal branches described here in some detail are enclosed by Schwann cells over long distances (27 μm in Fig. 1). The axon is enlarged at sites at which it directly contacts the muscle fibre. The degree of maturity of these contacts differs, as judged from the presence and shape of the post-synaptic secondary folds. It is conceivable that new contacts develop with local swelling of the axon which eventually displaces adjacent parts of the Schwann cell. With further development the axon could enlarge the original bulbous expansions and develop additional ones, finally leaving small Schwann cell cytoplasmic bridges in between them. The more-or-less regularly spaced thin Schwann cell bridges interposed between nerve and muscle fibre in otherwise continuous contacts (Couteaux & Pécot-Dechavassine, 1973), could thus be remnants of the Schwann cell envelope present in young nerve branches. On a longer time scale, however, extensive nerve sprouting must also occur, since the total length of synaptic contacts and the total number of nerve terminal branches contributing to a single junction increase sharply with increasing body size of an animal (Wernig *et al.* 1980*b*; Wernig, Anzil, Marciniak & Bieser, 1981). The present findings suggest that thin axonal sprouts initially grow within their Schwann cell sheaths until they enlarge locally and form new contacts.

Abandoned sites in the synapse

Since secondary folds usually develop some time after synapse formation (Kullberg, Lentz & Cohen, 1977), their presence at ChE remnants defines the latter as former

synaptic sites from which the nerve has retracted. The variable ultrastructural appearance of these regions might be due to different periods of time after nerve retraction; similarly, after denervation the synaptic gutter disappears and folds become increasingly shallow (Verma, 1980). The lack of binding sites for α-bungarotoxin at ChE remnants indicates that an axonal or Schwann cell related factor is important for maintainance of synaptic receptors. After denervation, density of ACh receptors in the former subsynaptic membrane remains high for an unknown period of time (at least several weeks, Dreyer & Peper, 1974). In contrast to sites with ChE remnants, however, Schwann cells remain present in the denervated synaptic gutters for several months (Birks, Katz & Miledi, 1960; Letinsky *et al.* 1976; A. Wernig & M. Krause, unpublished observations).

Conclusions

In addition to the continual formation of new synaptic parts within neuromuscular junctions, the nerve apparently retracts at other sites and ChE remnants remain detectable for unknown periods of time. The time scales of nerve sprouting, synapse formation and nerve retraction are still unknown as are the mechanisms governing them (cf. Nudell & Grinell, 1982). The present findings, however, support the hypothesis that the frog neuromuscular junction is in the process of continual remodelling. Whether synapse remodelling is an exclusive feature in muscles of poikilotherms like the frog is uncertain. However, some recent observations on mammalian muscles are comparable to features described here (cf. Cardasis & Padykula, 1981; Wernig, Carmody, Anzil, Hansert, Marciniak & Zucker, 1984); this suggests that synapse remodelling may be a common phenomenon.

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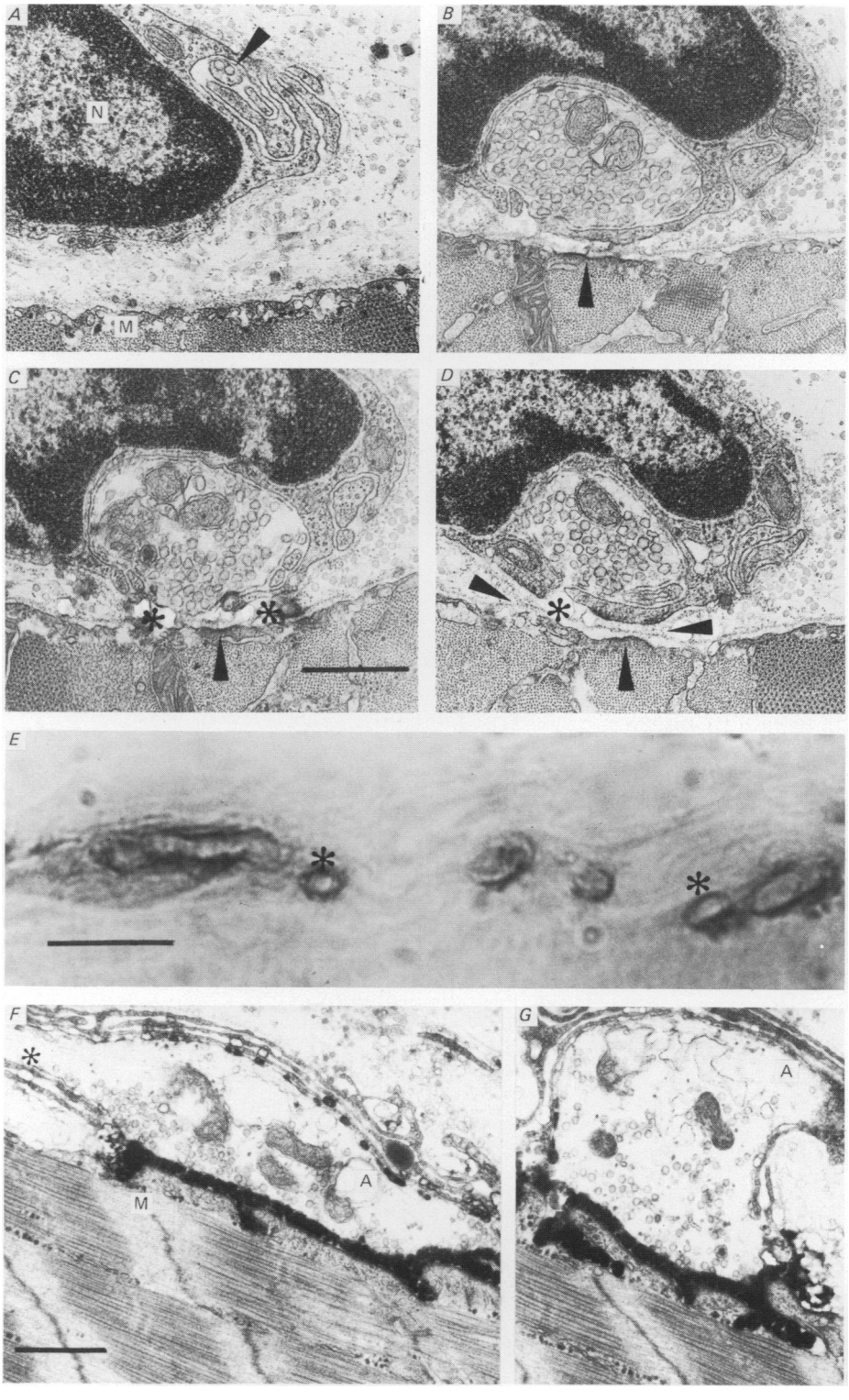
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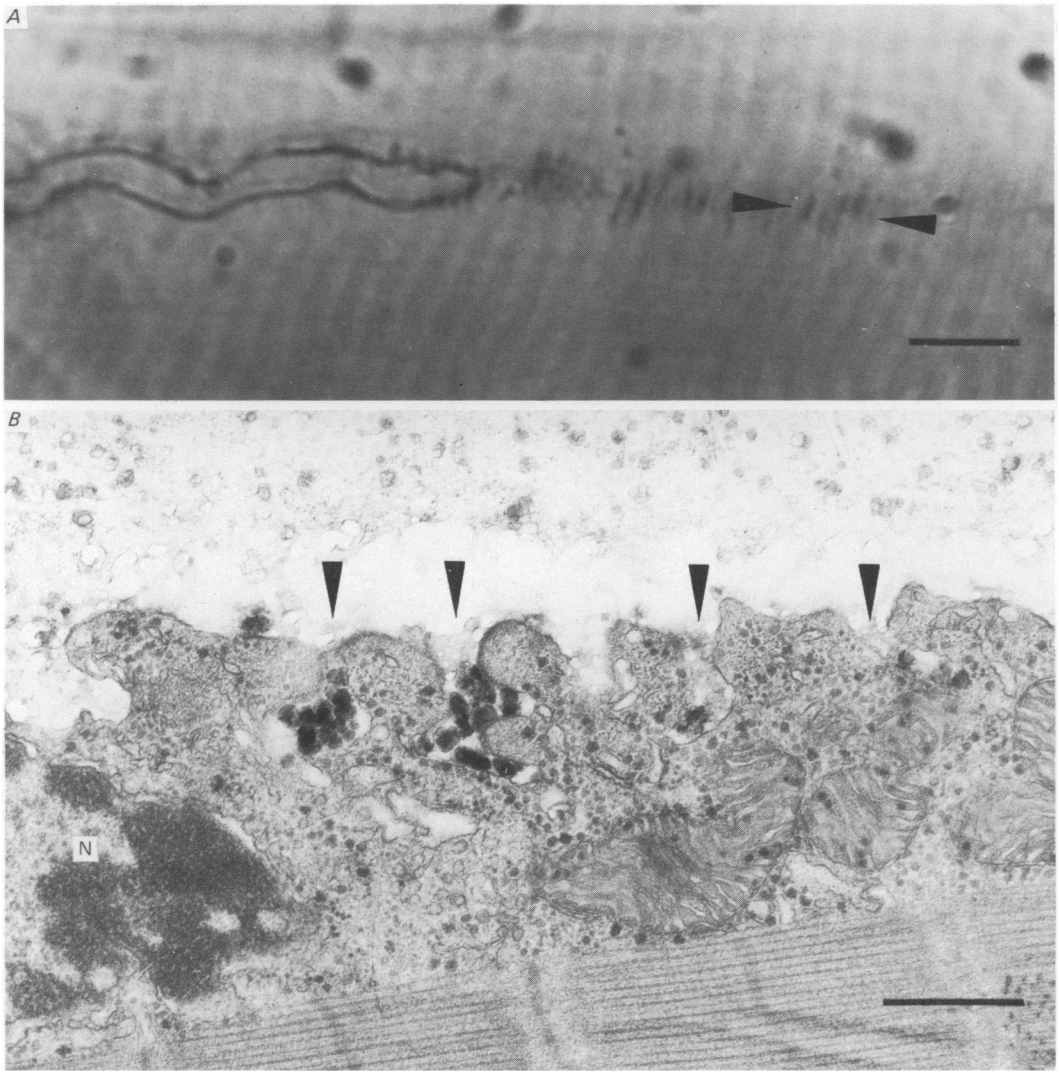
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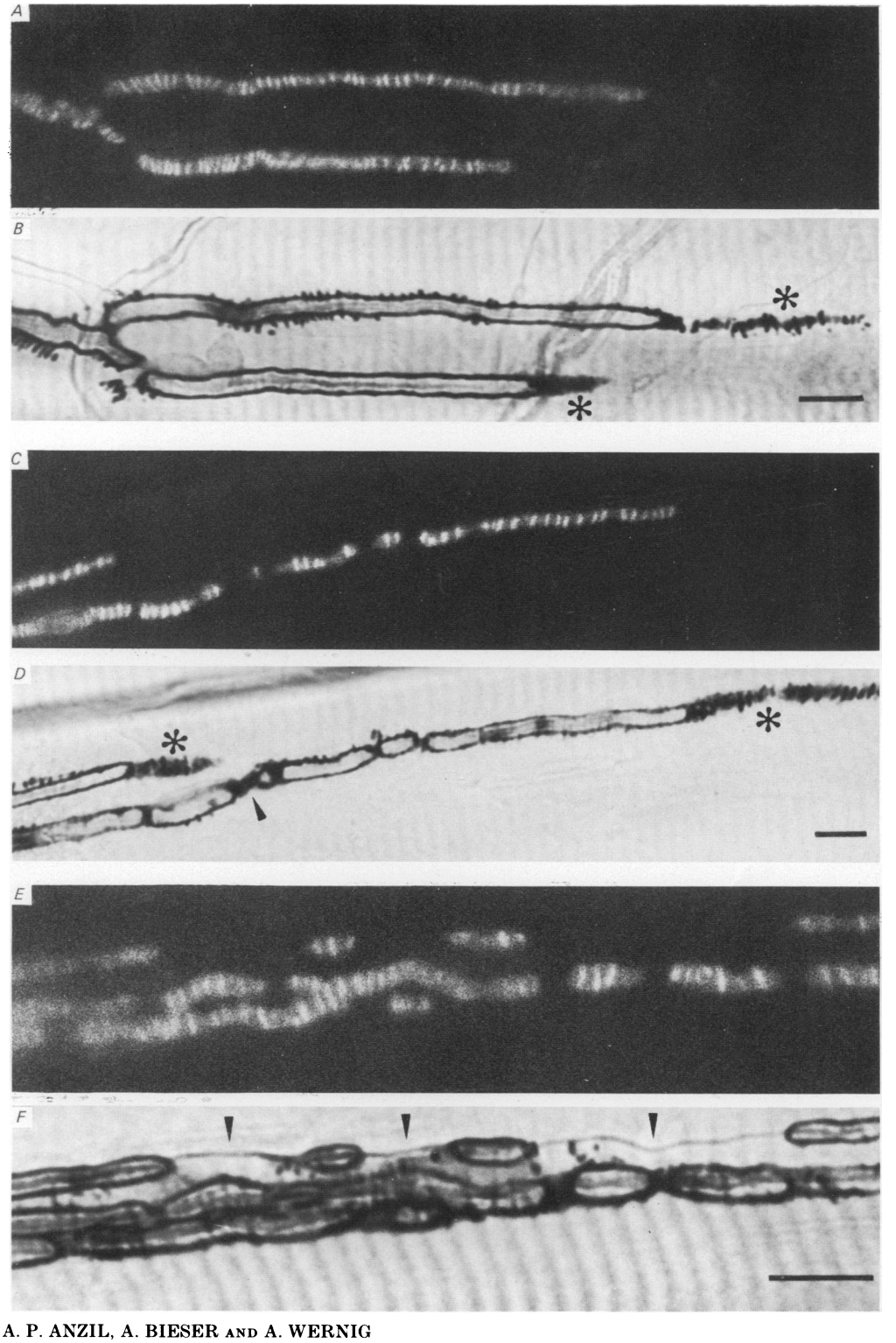

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EXPLANATION OF PLATES

PLATE 1

A–D, electron micrographs from transverse sections through the nerve terminal branch reconstructed in Fig. 1. The vertical lines in Fig. 1 indicate the positions of the sections illustrated. M, muscle fibre; N, nucleus of the Schwann cell. Thickenings at the subsynaptic membrane present in *B–D* are indicated by vertical arrows. The roughly horizontal arrows delimit a region of fusion of the basal laminae. Arrow in *A* points to the axon which is much reduced in diameter. Asterisks: crystals of ChE reaction product, corresponding white holes and fuzzy areas. Bar: 1 μm. *E*, light micrograph of a nerve–muscle whole mount showing the distal part of a nerve terminal branch after staining for ChE (Karnovsky & Roots, 1964). Several ChE rings are visible; the ones marked by asterisks are shown in *F* and *G*. Bar: 10 μm. *F* and *G*, electron micrographs of approximately longitudinal sections through the ChE rings marked by the two asterisks in *E*. A, axon; M, muscle fibre. Asterisk marks part of the axon not in contact with the muscle fibre and wrapped by Schwann cell sheaths. Note the black ChE reaction product in the synaptic cleft and in the well-developed secondary folds. Bar: 1 μm.

PLATE 2

A, light micrograph from a whole-mount preparation of the distal part of a nerve terminal branch with ChE remnant (to the right) after staining for ChE. The arrows indicate the part shown in the electron micrograph below (*B*) and the vertical plane of section. Bar: 10 μm. *B*, electron micrograph of part of the ChE remnant as indicated in *A*. The arrows point at the secondary folds, two of which are filled with ChE reaction product. Presynaptic elements are missing here. N, muscle cell nucleus. Bar: 1 μm.

PLATE 3

A–F, photomicrographs of parts of three different whole mounts, each preparation being labelled with fluorescein–α-bungarotoxin (*A*, *C*, *E*) and subsequently stained for ChE (*B*, *D*, *F*: *B* paired with *A*, *D* with *C*, *F* with *E*). Note the presence of one or several labelled bars at small ChE rings and the absence of binding sites in between ChE rings (arrows). At ChE remnants (asterisks) α-bungarotoxin label is absent. Bars: 10 μm.