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THE ROYAL SOCIETY

The frog neuromuscular junction revisited after quick-freezing–freeze-drying: ultrastructure, immunogold labelling and high resolution calcium mapping

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Until now, most ultrastructural studies on the neuromuscular junction have been carried out on samples first exposed to chemical treatments—with fixatives and/or dehydration agents—that are known to induce, or to be inadequate to prevent, artefactual changes of the native state. We report here on the potential of a physical approach to the preparation of samples that combines quick-freezing and freeze-drying (with or without exposure to OsO_4 vapours) followed by direct embedding of the samples in various resins. Thin sections from physically processed frog neuromuscular junctions, when compared to their chemically fixed counterparts, exhibit an overall excellent preservation, with the organelles retaining their native density and shape. These preparations were also investigated by electron spectroscopic imaging and electron energy loss spectroscopy, obtaining high resolution maps of native total calcium distribution within the nerve terminal. Finally, thin sections from analogously processed, however unfixed, preparations embedded in Lowicryl, were immunogold labelled before exposure to OsO_4. Nerve–muscle preparations treated this way exhibited adequate preservation of ultrastructure and revealed the distribution of synaptophysin with high sensitivity and resolution. In conclusion, we provide an overview of the potential of the quick-freezing–freeze-drying approach in the study of the neuromuscular junction function.

Keywords: election spectroscopic imaging; electron energy loss spectroscopy; endoplasmic reticulum; synaptic vesicles; synaptophysin

1. INTRODUCTION

During the last several decades, transmission electron microscopy has provided a great deal of information on the internal architecture of chemical synapses (Palay & Palade 1955; De Robertis 1967; Couteaux & Pécot-Dechavassine 1970). Particular interest was elicited by the presence of a large number of vesicles in nerve terminals, a distinctive feature soon to be considered as the morphological basis of chemical synaptic transmission (Ceccarelli & Hurlbut 1980). Starting from those preliminary observations, many studies have been published, mainly on frog neuromuscular junctions, in which the morphological modifications of nerve terminals have been investigated under all possible conditions of synaptic activity.

The current view of the ultrastructural organization of the neuromuscular junction is largely based on these classical electron microscopy studies, carried out on preparations that, as a first step of their processing, are chemically fixed and dehydrated by organic solvents (Hopwood 1969). In spite of their fundamental role in the progress of cell biology, these treatments have long been recognized to leave ground to artefactual changes of the native organization (Kellenberger *et al.* 1992). In particular, since chemical fixation is a slow process, cell activities do not stop at once but undergo progressive deterioration, with often unpredictable consequences. This is particularly critical at the neuromuscular junction, where chemical fixation unbalances vesicle turnover to the extent that, during the time required for complete blockade of membrane movements, vesicles are not retrieved after fusion but accumulate on to the presynaptic membrane (Hubbard & Laskowski 1972; Ceccarelli *et al.* 1979; Brewer & Lynch 1986). Moreover, chemical fixation, by perturbing the selective permeability of membranes and the energy control of cells, inevitably induces redistribution of water and small solutes among cytosol, nucleus, various membrane-bounded compartments and the extracellular space (Zierold 1991; Echlin 1992).

A treatment that blocks all activities almost instantaneously (typically in less than 1 ms) is quick-freezing, most often obtained by impacting living cells against a copper block cooled by liquid helium (Eränkö 1954; Van Harreveld & Crowell 1964). In these preparations freezing develops fast enough to prevent formation of ice crystals, and thus to preserve organization, in the few micrometres thick layer which directly faces the cooled

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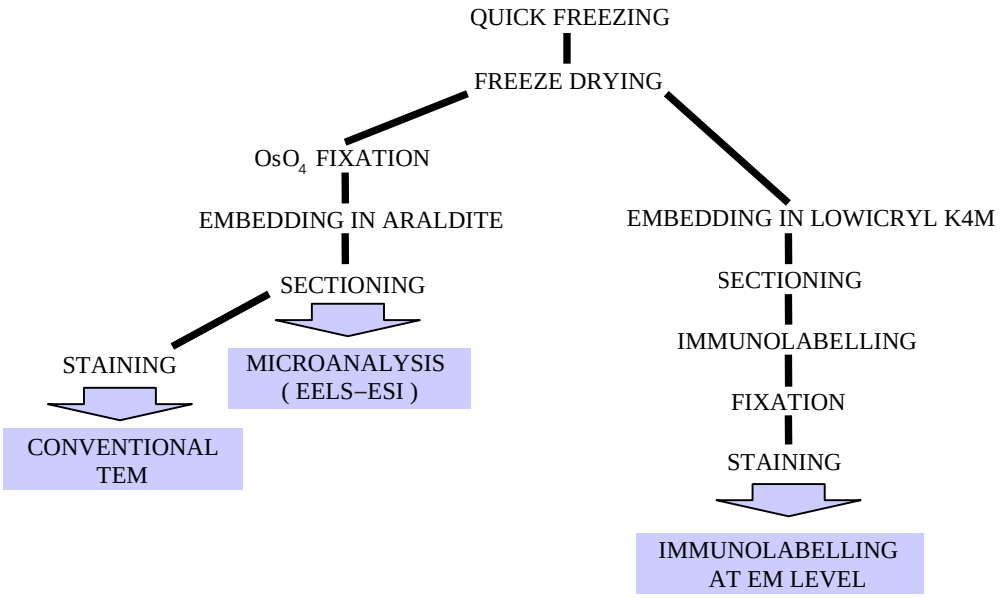

Figure 1. Unitary scheme of the different schedules employed in this work to alternatively: privilege the visualization of the ultrastructural organization (conventional TEM); avoid redistribution of diffusible elements (microanalysis by EELS–ESI); combine the preservation of recognizable structures with the maintenance of antigenicity (immunocytochemistry at EM level).

copper block, whereas the cell structure is more and more devastated in deeper regions by ice crystal growth. Therefore, thin sections of quick-frozen nerve–muscle preparations have been mostly employed to study cell surface events, including evoked exocytosis of synaptic vesicles (Heuser & Reese 1981; Torri Tarelli *et al.* 1985). In those studies, the extraction of frozen water from the samples was usually obtained by low-temperature exposure to organic solvents, i.e. freeze-substitution (Van Harreveld & Crowell 1964; Harvey 1982), a treatment which, however, can cause denaturation and washout of cell components. As an alternative to this procedure, water can be sublimated by long-term freeze-drying (Echlin 1992), a physical approach that grants molecular preservation and represents therefore a worthy complement to the high time resolution of quick-freezing. So far, the long recognized potential of the quick-freezing–freeze-drying (QF–FD) approach (Eränkö 1954) has been exploited only to a limited extent in investigating cell ultrastructure and composition by the use of ultrathin sections (Linner *et al.* 1986; Jorgensen & McGuffee 1987; Grohovaz *et al.* 1996; Pezzati *et al.* 1997).

We review here the potential of this approach in three different areas of investigation at the neuromuscular junction. At the ultrastructural level, it provides new insights into cell structure organization; moreover, its results are most appropriate for high-resolution immunocytochemistry carried out on unfixed nerve terminals and, especially, for mapping total calcium at the ultrastructural level by electron energy loss spectroscopy–electron spectroscopic imaging (EELS–ESI).

2. MATERIAL AND METHODS

(a) *Physical preparation (qf–fd) of the nerve–muscle preparation*

Cutaneous pectoris muscle preparations from frogs, *Rana pipiens*, were used. Pairs of muscles were dissected and pinned out in a chamber containing a Ringer solution (Grohovaz *et al.* 1996).

Immediately before the experiment, the preparation was gently draped over a foam cube stuck to the surface of a specially designed holder and pinned into place. The muscle was carefully blotted with filter paper to remove excess Ringer solution, the holder was plugged into the plunger of the freezing device (Cryoblock mounted on a Cryofract 190, Reichert Jung, Paris) and the preparation was finally quick-frozen against the liquid helium-cooled copper block. The frozen specimens were stored in liquid nitrogen until they were further processed. The freeze-drying was performed in an ultra high vacuum chamber (Cryofract 190) under conditions that prevent redistribution of both membranes and diffusible elements (Grohovaz *et al.* 1996). At the end of the drying cycle (3–4 days), when pressure and temperature had been restored to room values, the specimens were removed from the vacuum apparatus and treated according to one of the schedules summarized in figure 1 and described in detail below.

(b) *Conventional transmission electron microscopy*

QF–FD specimens were exposed overnight to OsO_4 vapours and then directly embedded in Araldite. Thin sections were cut from specimens with a diamond knife in an Ultracut microtome (Reichert Jung), collected on uncoated grids and examined in a Hitachi H7000 (Hitachi Ltd, Tokyo) after staining with uranyl acetate and lead citrate.

(c) *Immunocytochemistry*

Unfixed QF–FD specimens were directly infiltrated with Lowicryl K4M (Polysciences, Warrington, PA), transferred to transparent moulds and polymerized under UV light. Thin sections, immunostained with antisynaptophysin purified antibodies (provided by Dr F. Valtorta) and protein A-colloidal gold particles, were exposed to 1% OsO_4 in 0.12 M phosphate buffer, pH 7.3, stained with uranyl acetate and lead citrate, and finally examined in a Hitachi H7000 (Hitachi Ltd, Tokyo) by conventional EM microscopy (for further details, see Pezzati *et al.* 1997).

(d) *Microanalysis, EELS–ESI*

Ultrathin (15–25 nm) sections from QF–FD samples, processed as described above (except they were maintained

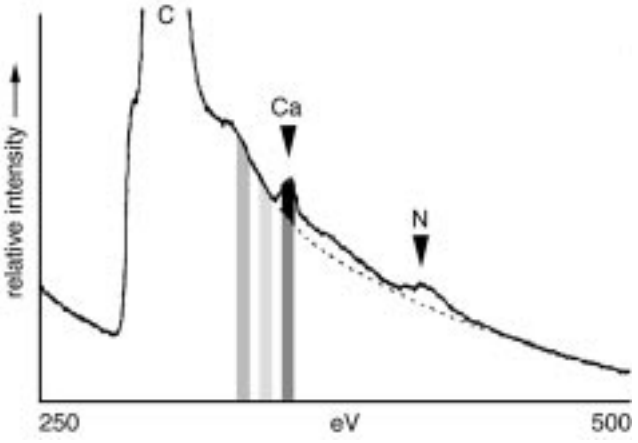

Figure 2. Electron energy loss spectrum recorded from a QF–FD specimen in the region 250–500 eV. The C, Ca and N core edges are clearly visible. The net calcium signal (black area) at 350 eV was obtained by subtracting the background (dark grey area) calculated by the power law on two pre-edge windows at 330 and 340 eV (light grey areas). The same approach was employed to obtain calcium maps from images.

Figure 3. Thin section electron microscopy of a frog neuromuscular junction processed according to the QF–FD procedure and examined by conventional transmission electron microscopy. The orderly arrangement of the myofilaments documents the good preservation of the ultrastructure in the most superficial layers of the specimen. The nerve terminal shows its characteristic complement of synaptic vesicles (v) that appear filled by dense material, in contrast with conventionally prepared specimens. Similarly, other structures look very dense, such as triads (t) or mitochondria (m) ($\times 56\,000$).

under dry N_2 atmosphere until embedding) were collected on to uncoated grids within 15 s of flotation in order to minimize contact with water, and left unstained. EELS–ESI analyses were performed in a CEM 902 (Zeiss, Oberkochen) electron microscope. An example of electron energy loss spectrum is illustrated in figure 2. Maps of net calcium distribution were obtained by computer-assisted processing of two images collected below the Ca edge (330 and 340 eV) and one image just below the Ca edge (350 eV). The final calcium map (coded in pseudocolours) was then superimposed on the image of the ultrastructural organization of the same field which appears in reverse contrast and was obtained at 240 eV, i.e. at an energy loss where most of the elements contribute to the image (Colliex 1986). The nature of calcium signals in the different regions of the maps was confirmed by EELS spectra (for further details, see Grohovaz *et al.* 1996).

3. RESULTS AND DISCUSSION

In the present work we report on the use of the physical, QF–FD approach to the frog neuromuscular junction, investigated not only by conventional thin-section electron microscopy but also by immunocytochemistry and EELS–ESI for the revelation of calcium. Although coordinate, the results obtained by these three experimental approaches exhibit independent aspects and are therefore treated in sequence.

(a) *Thin-section electron microscopy*

In spite of the apparently complete coverage of the ultrastructural detail of the neuromuscular junctions in the literature, at rest as well as under various experimental conditions, still new features can be appreciated by our QF–FD preparative procedure. The great richness in detail and the overall high density of cytoplasm and membrane-bounded organelles are the most prominent features of tissues treated this way. Enrichment in details was already recognized as a distinctive aspect of quick-frozen–freeze-substituted nerve terminals (Heuser & Reese 1981; Torri Tarelli *et al.* 1985). The use of a pure physical approach in the preparation of specimens further reduces the typical washout of structures that is caused by the exposure to the various solutions. As a result, inner cristae are barely recognized in mitochondria (Grohovaz *et al.* 1996), thus documenting the extraordinary density of the mitochondrial matrix, an expected counterpart of the very high protein concentration known to exist in this compartment. This dense appearance was already described in QF–FD PC12 cells (Pezzati *et al.* 1997) and seems therefore to be a general feature of eukaryotic cells, otherwise poorly preserved by chemical fixation treatments. Noticeably, also the so-called 'clear vesicles' appear very dense and compact, indicating that a great many components are extracted from their lumen during conventional sample processing (figure 3). Interestingly, the possibility that vesicles are filled with a dense matrix which may play a role in the kinetic control of neurotransmitter release has recently attracted attention (Rahamimoff & Fernandez 1997).

Although QF–FD has been available for several years (Eränkö 1954), its potential has been exploited to a very limited extent so far. This is somewhat surprising in view of the widely accepted belief that the cell structure is much better preserved by physical treatments (Echlin 1992), which should therefore set the standard for reference.

(b) *Immunocytochemistry*

In order to tune the physical approach to the requirements of immunocytochemistry, a more hydrophilic resin, Lowicryl, was employed for embedding instead of Araldite, and OsO_4 fixation was postponed to a step after immunolabelling. Nerve–muscle preparations processed according to this modified physical procedure still exhibit the native organization of their ultrastructure. Although the membranes are not resolved as clearly as in chemically fixed specimens, the organelles retain part of their

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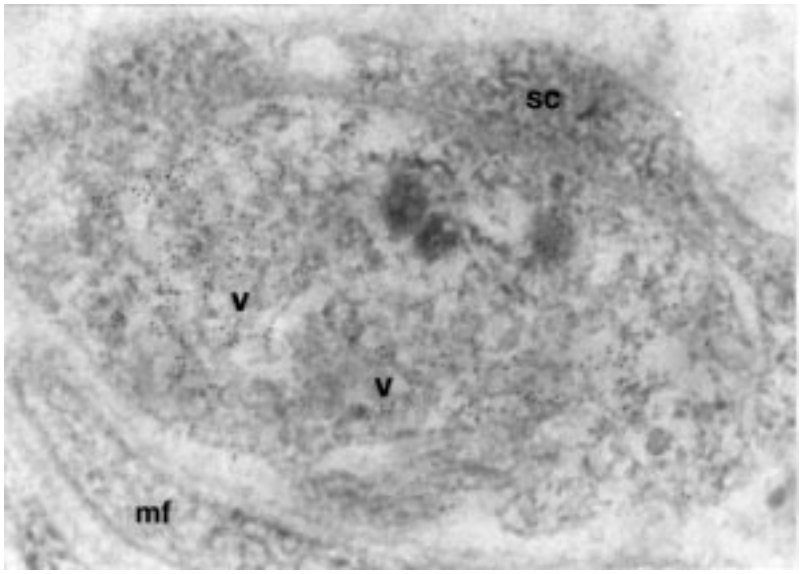

Figure 4. Electron micrograph illustrating the distribution of synaptophysin at a QF–FD frog motor nerve terminal. The immunogold labelling with antisynaptophysin antibodies is restricted to the areas of the presynaptic terminal where synaptic vesicles (v) are highly concentrated. The other regions, such as the Schwann cell (sc), the synaptic cleft and the muscle fibre (mf) are virtually devoid of gold particles. (×81 000)

dense appearance and are still recognized. As a paradigm for the immunocytochemical approach, experiments were carried out with a polyclonal antibody addressed to the synaptic vesicle protein, synaptophysin (Valtorta *et al.* 1988). The gold particle labelling revealed the expected specific distribution pattern, strong and strictly confined to the areas of the presynaptic terminal rich in vesicles (figure 4). The extracellular space as well as the surrounding Schwann cell and the rest of the cytoplasm, including the muscle fibre, were consistently negative.

The interest of these immunocytochemical results is manifold. First, these results are consistent with the data present in the literature (Torri Tarelli *et al.* 1990). Second, our results document the potential of an experimental approach that has only been employed in a few studies (Jorgensen & McGuffee 1987; Pezzati *et al.* 1997) and which, in contrast, deserves wider use. In this respect, it should be emphasized that thin sections are chemically fixed by this approach only after the immunolabelling has been carried out. Therefore the technique is expected to be particularly valuable when antibody binding is severely affected by fixation. Moreover, the high sensitivity of the immunolabelling is not obtained at the expense of the cell structure, which appears also adequately preserved. Finally, this approach opens up the possibility of studying the time-dependent distribution of specific components that are known to undergo specific redistribution during synaptic activity.

(c) *Mapping of total calcium*

Because of its paramount physiological importance, the distribution of calcium has attracted attention in almost all types of cells. However, the majority of studies have been carried out on the fraction of calcium that is in the ionized form, while the information on the distribution of total calcium within intracellular membrane-bounded compartments remains still very limited. The micro-analytical studies of biological preparations, in fact, face two major problems: the rapid dissipation of calcium gradients following cell fixation; and the limitations of the revelation techniques, which are often inadequate in terms of sensitivity and/or resolution (Hall 1988; Le Furgey *et al.* 1988; Zierold 1991; Pozzan *et al.* 1994). Our recent studies (Grohovaz *et al.* 1996; Pezzati *et al.* 1998) have demonstrated, on the one hand, that the calcium distribution in living cells is adequately preserved in the QF–FD preparation and, on the other hand, that calcium can be revealed with high resolution by the EELS–ESI approach (Colliex 1986; Le Furgey 1988), provided that its content within the investigated organelles is in the 10^{-2} moles l^{-1} range.

Figure 5 illustrates a pair of ESI images recorded at a frog neuromuscular junction. Figure 5*a* (collected at 240 eV, i.e. below the carbon edge) provides a view of the cell ultrastructure. The subcellular organization, although shown in reverse contrast, is similar to that observed in conventional images (compare figure 5*a* to figure 3). The same image is shown in figure 5*b* with the calcium map, coded from dark red to yellow (low to high levels), superimposed on it.

The high-resolution calcium map of the nerve terminal reveals high levels of total calcium within a limited number of cell structures. High signals are appreciated only within synaptic vesicles and ER cisternae while the levels remain below detection in the cytosol as well as mitochondria (with the exception of tiny areas, often in clear apposition to cisternae; figure 5). The calcium nature of ESI signals was confirmed by EELS analysis: in the spectra, together with a prominent carbon signal (C-K edge), a calcium peak (Ca-$L_{2,3}$ edge) was present in the above-mentioned structures (see figure 2 as an example).

The possibility that synaptic vesicles can sequester calcium has long been suggested (Israel *et al.* 1980; Michaelson & Ophir 1980). This has also led to the proposal that these organelles may play an active role not only in the release of neurotransmitter but also in the control of calcium homeostasis, by their ability to store the element and later release it either in the cytosol on stimulation (Petersen 1996), or in the synaptic cleft during the fusion process (Parducz *et al.* 1994). At this stage, our data cannot address these controversial and still largely hypothetical issues, nevertheless they provide direct evidence for the high calcium content in these secretory organelles at rest.

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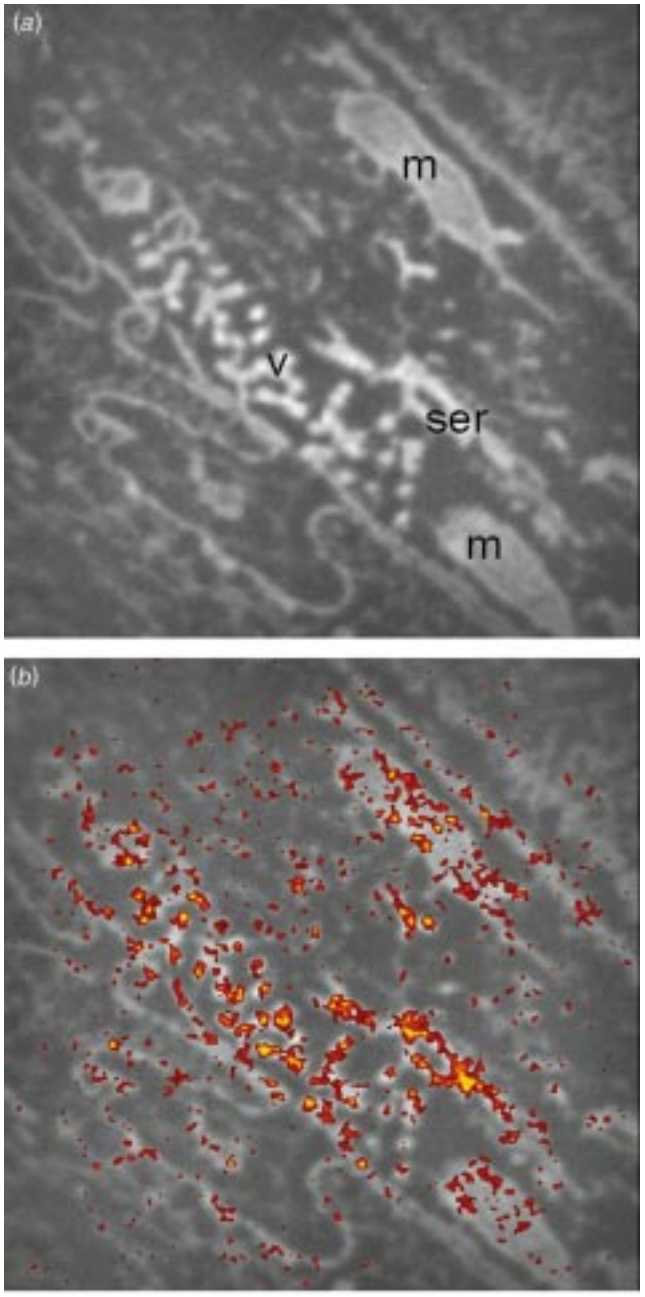

Figure 5. Mapping of total calcium by ESI. (*a*) A view of the nerve terminal ultrastructure (image collected at 240 eV, i.e. below the carbon edge). In (*b*) the same image exhibits in addition the superimposed calcium map expressed in false colours coded from dark red (low) to yellow (high). The synaptic vesicles (v) and some smooth endoplasmic reticulum cisterna (ser) are positive for calcium, whereas other structures and organelles show distinctly lower signals (e.g. mitochondria, m) or remain below threshold. Reproduced with permission from Grohovaz *et al*. (1996).

4. CONCLUSIONS

Our work demonstrates that the use of physical fixation can provide extensive advantages over the conventional preparative techniques in the study of chemical synapses. Even the simple observation of the ultrastructural organization reveals aspects that add information to what is generally accepted, based on the results obtained with fixed preparations. However, the QF–FD approach is especially valuable for specific cytological studies: immunocytochemistry, for which the high sensitivity and high resolution potential of the method are exploited; and calcium distribution, of which we report here a high-resolution map in the nerve terminal. A major advantage of this approach, which was not exploited in this study, is its high temporal resolution; this feature is expected to yield valuable information on the physiological events which occur on the millisecond time-scale.

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REFERENCES

Brewer, P. A. & Lynch, K. 1986 Stimulation-associated changes in frog neuromuscular junctions. A quantitative ultrastructural comparison of rapid-frozen and chemically fixed nerve terminals. *Neuroscience* **17**, 881–895.

Ceccarelli, B. & Hurlbut, W. P. 1980 Vesicle hypothesis of the release of quanta of acetylcholine. *Physiol. Rev.* **60**, 396–441.

Ceccarelli, B., Grohovaz, F. & Hurlbut, W. P. 1979 Freeze-fracture studies of frog neuromuscular junctions during intense release of neurotransmitter. II. Effects of electrical stimulation and high potassium. *J. Cell Biol.* **81**, 178–192.

Colliex, C. 1986 Electron energy-loss spectroscopy analysis and imaging of biological specimens. *Ann. NY Acad. Sci.* **483**, 311–325.

Couteaux, R. & Pécot-Dechavassine, M. 1970 Vésicules synaptiques et poches au niveau des zones actives de la jonction neuromusculaire. *C. R. Acad. Sci.* **271**, 2346–2349.

De Robertis, E. 1967 Ultrastructure and cytochemistry of the synaptic region. *Science* **156**, 907–914.

Echlin, P. 1992 *Low temperature microscopy and analysis*. New York & London: Plenum Press.

Eränkö, O. 1954 Quenching of tissues for freeze-drying. *Acta Anat.* **22**, 331–335.

Grohovaz, F., Bossi, M., Pezzati, R., Meldolesi, J. & Torri Tarelli, F. 1996 High resolution ultrastructural mapping of total calcium, EELS–ESI analysis of a physically/chemically processed nerve–muscle preparation. *Proc. Natl Acad. Sci. USA* **93**, 4799–4803.

Hall, T. A. 1988 Capabilities and limitations of probe methods for the microanalysis of chemical elements in biology, a brief introduction. *Ultramicroscopy* **24**, 181–184.

Harvey, D. M. R. 1982 Freeze-substitution. *J. Microsc.* **127**, 209–221.

Heuser, J. E. & Reese, T. S. 1981 Structural changes after transmitter release at the frog neuromuscular junction. *J. Cell Biol.* **88**, 564–580.

Hopwood, D. 1969 Fixatives and fixation: a review. *Histochem. J.* **1**, 323–360.

Hubbard, J. I. & Laskowski, M. B. 1972 Spontaneous transmitter release and ACh sensitivity during glutaraldehyde fixation of rat diaphragm. *Life Sci.* **11**, 781–785.

Israel, M., Manaranche, R., Marsal, J., Meunier, F. M., Morel, N., Frachon, P. & Lesbats, B. 1980 ATP-dependent calcium uptake by cholinergic synaptic vesicles isolated from Torpedo electric organ. *J. Membr. Biol.* **54**, 115–126.

Jorgensen, A. O. & McGuffee, L. J. 1987 Immunoelectron microscopic localization of sarcoplasmic reticulum proteins in cryofixed, freeze-dried, and low temperature-embedded tissue. *J. Histochem. Cytochem.* **35**, 723–732.

Kellenberger, E., Johansen, R., Maeder, M., Bohrmann, B., Stauffer, E. & Villiger, W. 1992 Artefacts and morphological changes during chemical fixation. *J. Microsc.* **168**, 181–201.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

LeFurgey, A., Bond, M. & Ingram, P. 1988 Frontiers in electron probe microanalysis: application to cell physiology. *Ultramicrosc.* **24**, 185–219.

Linner, J. G., Livesey, S. A., Harrison, D. S. & Steiner, A. L. 1986 A new technique for removal of amorphous phase tissue water without ice crystal damage: a preparative method for ultrastructural analysis and immunoelectron microscopy. *J. Histochem. Cytochem.* **34**, 1123–1135.

Michaelson, D. M. & Ophir, I. 1980 Sidedness of (calcium, magnesium) adenosine triphosphatase of purified Torpedo synaptic vesicles. *J. Neurochem.* **34**, 1483–1490.

Palay, S. L. & Palade, G. E. 1955 Fine structure of neurons. *J. Biophys. Biochem. Cytol.* **1**, 69–88.

Parducz, A., Loctin, F., Babel-Guerin, E. & Dunant, Y. 1994 Exo-endocytotic activity during recovery from a brief tetanic stimulation: a role in calcium extrusion? *Neuroscience* **62**, 93–103.

Petersen, O. H. 1996 Can Ca2+ be released from secretory granules or synaptic vesicles? *Trends Neurosci.* **19**, 411–413.

Pezzati, R., Bossi, M., Podini, P., Meldolesi, J. & Grohovaz, F. 1997 High-resolution calcium mapping of the endoplasmic reticulum-Golgi-exocytic membrane system. Electron energy loss imaging analysis of quick frozen-freeze dried PC12 cells. *Molec. Cell Biol.* **8**, 1501–1512.

Pozzan, T., Rizzuto, R., Volpe, P. & Meldolesi, J. 1994 Molecular and cellular physiology of intracellular calcium stores. *Physiol. Rev.* **74**, 595–636.

Rahamimoff, R. & Fernandez, J. M. 1997 Pre- and postfusion regulation of transmitter release. *Neuron* **18**, 17–27.

Torri Tarelli, F., Grohovaz, F., Fesce, R. & Ceccarelli, B. 1985 Temporal coincidence between synaptic vesicle fusion and quantal secretion of acetylcholine. *J. Cell Biol.* **101**, 1386–1399.

Torri Tarelli, F., Villa, A., Valtorta, F., De Camilli, P., Greengard, P. & Ceccarelli, B. 1990 Redistribution of synaptophysin and synapsin I during alpha-latrotoxin-induced release of neurotransmitter at the neuromuscular junction. *J. Cell Biol.* **10**, 449–459.

Valtorta, F., Jahn, R., Fesce, R., Greengard, P. & Ceccarelli, B. 1988 Synaptophysin (p38) at the frog neuromuscular junction: its incorporation into the axolemma and recycling after intense quantal secretion. *J. Cell Biol.* **107**, 2717–2727.

Van Harreveld, A. & Crowell, J. 1964 Electron microscopy after rapid freezing on a metal surface and substitution fixation. *Anat. Rec.* **149**, 381–385.

Zierold, K. 1991 Cryofixation methods for ion localization in cells by electron probe microanalysis: a review. *J. Microsc.* **161**, 357–366.

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