Short Report

Imaging neuromuscular junctions by confocal fluorescence microscopy: individual endplates seen in whole muscles with vital intracellular staining of the nerve terminals

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ABSTRACT

The mammalian neuromuscular junction has been extensively studied by different methods to understand better the biological aspects of its normal development, ageing and pathological conditions, such as disorders of neuromuscular transmission. In the present report, a new technique is described that combines confocal microscopy with the use of a vital nerve terminal dye (4-Di-2-ASP) and rhodamine-alpha-bungarotoxin to stain postsynaptic acetylcholine receptors in the same endplate. Nerve terminals in the sternomastoid muscles of living adult mice were stained with 4-Di-2-ASP, which labels intracellular compartments of the nerve terminal containing mitochondria. Slides of these muscles were viewed by confocal microscopy and images were stored on magnetic optical discs. This procedure was compatible with subsequent acetylcholine receptor staining with rhodamine-α-bungarotoxin and observation under the confocal microscope. Classical features of the adult neuromuscular junction were displayed, such as the branched-pattern distribution of the nerve terminals and receptors and their complete colocalisation. In addition, nerve fibres from intramuscular nerve branches with their neighbouring cells, nuclei and muscle fibre striations could also be visualised. We conclude that the present technique can complement existing methods of investigation of nerve terminal anatomy and pathology, particularly where preservation of 3-dimensional relationships is required and intracellular disturbances involving mitochondrial organisation, such as ageing or other degenerative disorders, may be present.

Key words: Motor endplates; acetylcholine receptors; 4-Di-2-ASP; mitochondrial labelling.

INTRODUCTION

The morphology of the neuromuscular junction (NMJ) has been extensively investigated under different conditions, such as normal development (Balice-Gordon & Lichtman, 1993), degenerative disorders (Lyons & Slater, 1991) and ageing (Fahim et al. 1983). Generally, the morphological aspects of the neuromuscular junction are studied using techniques such as transmission electron microscopy (Kelly & Zacks, 1969; Matthews-Bellinger & Salpeter, 1978), scanning electron microscopy (Desaki & Uehara, 1987), in vivo observations (Balice-Gordon & Lichtman, 1990, 1993), and fluorescence video microscopy (Steinbach, 1981; Slater, 1982; Somasekhar et al. 1996).

Most methods currently used to study NMJs are limited in their ability to image the spatial relationship between nerve terminals and acetylcholine receptors, for example, in a given muscle fibre. In this respect, the use of confocal microscopy has opened up the possibility of imaging fine details and spatial relationships between cells in thick fixed specimens (Lichtman, 1994). For instance, 3-dimensional information about growth-related alterations in motor endplates has been obtained by confocal examination of fluorescently labelled endplates (Prakash et al. 1995).

Labelling of the pre and postsynaptic components...
is optimally performed in fixed tissues, where acetylcholine (ACh) receptors are labelled with rhodamine-
α-bungarotoxin and nerve terminals are labelled with
fluorescently tagged antibodies against ACh vesicles
(Deschenes et al. 1993) or axon membrane markers
such as calcium channels (Sugiura et al. 1995).

Alternative staining approaches to the nerve terminal
include the use of the vital mitochondrial marker 4-
Di-2-ASP (Lichtman et al. 1987; Magrassi et al.
1987). This marker has proved to be useful in the
interpretation of synaptic morphology during de-
velopment (Balice-Gordon & Lichtman, 1993), but its
use has been limited to in vivo experiments since it is
a vital-dependent marker.

In the present paper, we report a new method which
allowed us to combine in vivo 4-Di-2-ASP-dependent
staining with the higher resolution achieved with the
confocal microscope to obtain a detailed analysis of
nerve terminals in normal adult neuromuscular
junctions. 4-Di-2-ASP staining was compatible with
subsequent acetylcholine receptor (AChR) labelling
with rhodamine-bungarotoxin and confocal exam-
ination of the same endplate. The method will
complement existing techniques for the examination
of neuromuscular junctions, particularly where pres-
servation of 3-dimensional relationships and deter-
mination of the distribution of intracellular com-
ponents, such as mitochondria, are required.

MATERIALS AND METHODS

Adult female CF1/B strain mice (n = 5), 6–10 wk old,
were deeply anaesthetised with a single intraperitoneal
injection of chloral hydrate (0.5–0.6 mg/kg body
weight). The neck of the animal was opened by a
midline incision from the apex of the mandible to the
sternal notch. Both sternomastoid muscles were
exposed by lateral reflection of the salivary glands.
The muscles were bathed in situ with a 1 mm solution
of 4-Di-2-ASP (Molecular Probes, Eugene, OR) for
3–4 min. The muscles were then quickly removed and
the animals euthanised with 0.3 ml of a 10%
pentobarbital solution.

The muscles were mounted on a slide in phosphate-
buffered saline (PBS) and covered with a coverslip
(absolute thickness of the muscles 0.7–0.8 mm
and coverslip thickness 0.17 mm) and examined with
a Noran real time laser scanning imaging system
linked to a Nikon Optiphot microscope, using a ×10
objective for lower power assessment and a ×60 oil
objective for confocal examination. The excitation
source was an argon ion laser (excitation wavelength
490 nm for 4-Di-2-ASP). Full frame 512×512 pixel
images of nerve terminals were collected and stored on
magnetic optical discs.

The muscles were then placed on a Sylgard dish,
washed with PBS and fixed for 30 min with a cold
solution of 2% paraformaldehyde and 0.1% glutaral-
dehyde in PBS. The muscles were washed with PBS for
15 min and stained with rhodamine-α-bungarotoxin
(2 mg/ml, Molecular Probes) for 30 min at room
temperature. After washing with PBS, the muscles
were mounted under a coverslip in Vectashield
(Vector) and observed under the confocal microscope
(excitation wavelength 560 nm). By using the images
of nerve terminals collected before as a guide, the
same endplates were relocate and images of their
AChRs were obtained.

RESULTS

We observed nerve terminals and AChR distribution
at adult normal neuromuscular junctions using real
time laser scanning confocal microscopy. Nerve
terminals were visualised by intravitral staining with
the 4-Di-2-ASP dye which stains the mitochondria of
living nerve terminals. AChRs were visualised in fixed
muscles with α-bungarotoxin labelled with rhoda-
mine, after imaging nerve terminals in the same
muscle fibre. By using this technique, we were able to
observe the morphological characteristics of the adult
neuromuscular junction described below.

The preterminal axon was clearly seen in all cases,
containing mitochondria as indicated by its bright
staining (Figs 1a, c, 2d). Some terminals showed
‘dotted-type’ staining (Fig. 2d), while most of the
terminals showed a more uniform distribution of the
label, so that continuous branches were seen (Fig.
1a, c). In many cases, axons from intramuscular
nerve branches could be followed until they ended as
preterminal axons (Fig. 2b, c). At the endplate region,
the nerve terminal divided into about 5–8 branches
(Fig. 1a). Cells with long processes were seen closely
associated with or adjacent to nerve fibres. Depending
on the focal plane, the myelin sheath was seen and the
axon seemed to be covered with long cell processes
(Fig. 2b). brightly stained round or oval structures
were seen at the preterminal axon (Fig. 1a) and at the
endplate region (Fig. 1c).

At the muscle fibre level, the striations represented
by alternate bright and dark bands were clearly seen
under the fluorescent channel (Fig. 1c), as also were
round dark structures representing endplate nuclei or
muscle fibre nuclei (Fig. 1a).

After 3–5 min of observation, the 4-Di-2-ASP dye
seemed to spread out and a bright signal was mostly
detected at the endplate region around the terminals (Fig. 2d), rather than at the nerve terminals themselves. At this point, the muscle was stained for AChR viewing at the same endplates seen before with 4-Di-2-ASP. Receptors colocalised with nerve terminal branches (Fig. 1b, d) in a branched-pattern distribution typical of normal adult endplates and this pattern of staining was also seen when the muscle had not been previously stained with 4-Di-2-ASP (Fig 2a).

**DISCUSSION**

In the present investigation, we studied adult neuromuscular junction pre and postsynaptic components using a vital staining technique combined with confocal microscopy. Nerve terminals were visualised with 4-Di-2-ASP in muscles freshly removed from the animal and AChRs were stained with fluorescently tagged alpha-bungarotoxin in the same muscles, after fixation. The present technique contributes to the extensive literature on NMJ morphology by permitting for the first time the study of an intracellular compartment of nerve terminals containing mitochondria in a non-in vivo experiment, combined with the higher resolution achieved with the confocal microscope.

The 4-Di-2-ASP dye has been described as a vital nerve terminal dye since it depends on live mitochondria to work as a label (Magrassi et al. 1987; Rich & Lichtman, 1989; Balice-Gordon & Lichtman,
Fig. 2. (a) Motor endplate labelled with rhodamine-α-bungarotoxin alone, without previous 4-Di-2-ASP staining. AChRs show a typical branched-pattern distribution and the images obtained are similar to those seen when the mitochondrial marker is used before receptor staining. In b, c and d, the 4-Di-2-ASP staining permitted the observation of intramuscular nerve branches and, in many cases, isolated axons. One such axon is shown in b, covered by long and thin cell processes (arrows), assumed to belong to a Schwann cell. A cell nucleus (asterisk) along with the processes of the cell is seen nearby. These axons could be easily followed along their entire length and, not uncommonly, until their termination at the endplate region, which is seen in c. Some terminals had a dotted-type staining (d, arrows) and after 3–5 min of observation with 4-Di-2-ASP, a bright material started to deposit at the endplate region (d, arrowheads). A, motor axon. Bar: a, b, d, 10 μm; c, 20 μm.

1993). Its use has therefore been limited to in vivo experiments, where skeletal muscles of living animals are bathed with the dye and the animal is kept alive to be studied over time (Rich & Lichtman, 1989; Balice-Gordon & Lichtman, 1993). We show here that 4-Di-2-ASP can also be used in muscles freshly removed from the animal and viewed under the confocal microscope. The images of nerve terminals obtained by this technique resemble those obtained by regular video microscopy in in vivo experiments, in that nerve terminals have a branched-pattern distribution, matching with AChR at the postsynaptic membrane. Our results suggest that the use of 4-Di-2-ASP in freshly removed muscles, combined with the fast scanning rate and better resolution of the confocal microscope, did not impair the ability of this stain to label nerve terminals, thus representing a suitable technique for the study of nerve terminal distribution also in non-in vivo experiments.

It has been reported that, during normal development, the 4-Di-2-ASP staining of nerve terminals changes over time in live animals from a continuous to
a spot-type staining. It was suggested that this altered staining pattern might be due to changes in the intracellular organisation of the nerve terminal, such as rearrangements of mitochondria as the terminal grows, related to the increase in number of release sites (Balice-Gordon & Lichtman, 1990). We also noticed that some terminals had a spot-type staining, mainly after longer periods of observation (3–5 min; Fig. 2d) and this may represent modifications of mitochondrial organization as well, since the muscle was not fixed at the time of 4-Di-2-ASP observation. Thus the use of 4-Di-2-ASP can be an alternative and complementary approach to the study of nerve terminal features, in addition to other dyes that stain their membranes (Balice-Gordon & Lichtman, 1993) and might be a potential tool for the study of the morphological alterations seen during ageing or in dystrophic animals, with the advantages of confocal microscopy.

Individual myelinated nerve fibres can easily be viewed in a peripheral nerve by combining aldehyde-induced fluorescence with confocal microscopy (Reynolds et al. 1994). In the present study using 4-Di-2-ASP staining, nerve fibres from intramuscular nerve branches could also be seen and followed over substantial lengths, until they end as nerve terminals at the endplate region, along with what we assume to be perineurial cells, Schwann cells or fibroblasts. Thus, the use of 4-Di-2-ASP as described here can be an additional tool for the investigation of polyinnervation, overcoming the limitations of classical methods such as silver staining (Pestronk & Drachman, 1978) by permitting analysis of whole nerve fibres without disruption of their neighbouring cells, with better resolution.

An interesting result observed with 4-Di-2-ASP staining was the presence of several nuclei at the endplate region. Confocal examination showed that these nuclei were located at 2 different depths. At a first and more superficial level, the nuclei were closely related to the nerve terminals. They appeared as slightly bright oval structures which we interpreted to be Schwann cell nuclei. It is known that terminal Schwann cells are distributed at the endplate region and their cytoplasmic prolongations and nuclei are typically seen in EM pictures of NMJs. Thus the present technique, if combined with a specific marker for Schwann cells, may be suitable to study the relationship between nerve terminals and Schwann cells.

On a second and deeper confocal plane, the nuclei seemed to belong to the muscle fibre itself, appearing as round and dark structures beneath the terminal or the endplate region. In a similar way, EM views of NMJs also show the presence of muscle fibre nuclei located in the subsynaptic sarcoplasm, called motor endplate nuclei. These nuclei, in contrast to the other muscle fibre nuclei, are related to the production of endplate molecules such as AChRs (Hall & Sanes, 1993). Thus, by permitting the observation of these nuclei in particular, the present technique may contribute to the study of the distribution of these nuclei in different situations, such as in regenerated muscle fibres.

One of the disadvantages of the present technique is the fact that the muscle, once removed and stained with 4-Di-2-ASP, must be viewed under the confocal microscope within a short period of time (5–10 min) since the label seems to lose its specificity thereafter, starting to spread out to the muscle fibre. However, the rapid scanning rate of the confocal microscope combined with its optical section capability and fast data acquisition overcomes this problem by permitting analysis of whole muscle and of several endplates (6–10 superficial endplates per muscle) within this short period of time. In addition, a permanent slide cannot be mounted for 4-Di-2-ASP staining, but only for rhodamine-alpha-bungarotoxin staining. However, the images taken from nerve terminals are stored on optical disks and can be further studied at a later time.

In conclusion, we have developed a method for imaging neuromuscular junction components by combining vital nerve terminal staining with 4-Di-2-ASP in a fresh muscle preparation and AChR staining with alpha-bungarotoxin in fixed muscle, with confocal microscopy. Application of the current method permitted clear visualisation of classical features of the nerve terminal and AChR distribution at adult endplates, in addition to other features such as Schwann cell and endplate nuclei and the image of whole nerve fibres with neighbouring cells. This technique will complement existing methods of investigation of nerve terminal anatomy and pathology, particularly where preservation of 3-dimensional relationships is required and intracellular disturbances involving mitochondrial organisation might be an issue, such as ageing or other degenerative disorders.

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REFERENCES


