Motor Nerve Terminal Staining Combined with Catecholamine Histofluorescence or Immunocytochemistry

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ABSTRACT. A number of excellent techniques are available to stain and characterize different types of neurons and nerve terminals. However, because these different techniques are frequently not compatible, their usefulness in determining the relationships between specific axons and neuromuscular junctions is often limited. The goal was to develop specific procedures for simultaneous visualization of different types of unmyelinated axons and motor nerve terminals in the same preparation. First we modified the formaldehyde/glutaraldehyde staining solutions of the aqueous aldehyde fluorescence technique (Faglu) to observe catecholamine containing axons in whole mount amphibian skeletal muscle. The compatibility of this modified staining solution with other histological procedures made it possible to stain both motor nerve terminals with tetrazolium salts and, in the same preparation, to observe unmyelinated axons with aldehyde-induced catecholamine histofluorescence. This same general formaldehyde/glutaraldehyde staining procedure was also used with immunocytochemical techniques to visualize fluorescent antibody stained nerves and motor nerve terminals in the same whole mount preparation.

Key words: unmyelinated axons, motor nerve terminals, catecholamines, immunocytochemistry, skeletal muscle.

A number of powerful techniques have been developed to stain neurons (e.g., nerve staining using silver or gold salts, zinc iodide and osmium, or tetrazolium salts), and to selectively stain or identify specific neuron types (e.g., catecholamine histofluorescence, neurons filled with markers like horseradish peroxidase, and more recently, using antibodies to specific neuronal markers). By applying a variety of techniques, we have begun to study the diversity of innervation of vertebrate skeletal muscle. We have identified unmyelinated, nonmotor axons with catecholamines and several different bioactive peptides in close proximity to motor nerve terminals in amphibian skeletal muscle (Linden and Letinsky 1988, Oren 1986, Oren et al. 1989). However, because of incompatibilities in our staining techniques, we were not able to determine the exact relationship between histofluorescent or immunofluorescent axons and stained neuromuscular junctions in the same preparation. The ability to perform such an anatomical analysis would help to determine whether these axons play a role in the development and/or function of motor nerve terminals (see Linden and Letinsky 1988). This paper describes modifications of various experimental procedures to make simultaneous visualization of catecholamine (CA) histofluorescent or immunofluorescent axons and stained motor nerve terminals possible.

Several different methods have been developed to reveal CA-containing axons in the nervous system (generally reviewed by Moore and Loy 1978, e.g., Falck-Hillarp techniques, reviewed by Corrodi and Jons-
son 1967, glyoxylic acid techniques, reviewed by Bjorklund et al. 1972, and the aqueous aldehyde methods, Furness et al. 1977). Of these techniques, the aqueous aldehyde method (Faglu) developed by Furness et al. (1977) is the best suited for use with motor nerve terminal staining. It is rapid, simple, and does not require special equipment. Because this method involves treatment of the tissues with a mixture of formaldehyde and glutaraldehyde, it produces good fixation suitable for both light and electron microscopy (Furness et al. 1978) and it induces fluorescence in CA-containing structures. We have taken advantage of these properties to develop a method for simultaneously visualizing both formaldehyde/glutaraldehyde-induced CA fluorescence and motor nerve terminals stained with tetrazolium salts (Letinsky and DeCino 1980, Letinsky 1983). This same general procedure was modified to demonstrate simultaneously fluorescent antibody stained neurons and motor nerve terminals in the same whole mount preparation.

METHODS AND MATERIALS

All experiments used the cutaneous pectoris (CP) muscle of adult frogs (Rana pipiens and Rana catesbeiana). Animals were sacrificed by an overdose of tricaine methane sulfonate (MS222, Sigma, St. Louis, MO) and individual CP muscles were dissected and pinned out at rest length on Sylgard (Dow Corning, Midland, MI) plated plastic Petri dishes for staining (Letinsky and DeCino 1980, Letinsky 1983). This same general procedure was modified to demonstrate simultaneously fluorescent antibody stained neurons and motor nerve terminals in the same whole mount preparation.

Demonstration of CA-containing neurons was performed by a modification of the Faglu method (Furness et al. 1977, 1978) using a combined solution of 0.8–8% formaldehyde (EM grade, methanol free; Polysciences, Warrington, PA) and 0.15–0.75% glutaraldehyde (EM grade; Polysciences) in 0.09 M phosphate buffer (pH 7.2). CA histofluorescence staining was performed on fresh, unfixed or TNBT stained CP muscles as follows:

(1) 2–3 hr incubation in the appropriate prechilled F/G solution at 4 C in the dark.
(2) 2–3 min counterstaining with 0.5%
trypan blue in the appropriate prechilled F/G solution.

(3) The solution was then removed as completely as possible.

(4) 4–24 hr drying of the muscle over P₂O₅ in a vacuum at 25 C in the dark.

The ideal F/G concentrations used for demonstration of only CA histofluorescence were 4–6/0.25–0.5. A slightly different F/G concentration ratio of 4/0.15 was used to optimize the combined TNBT-nerve terminal staining (this solution was also found to be compatible with our standard immunofluorescence procedures). Brief incubation in 0.5% trypan blue (Sigma) produced red muscle fiber fluorescence which masked the nonspecific F/G-induced muscle fiber fluorescence (Barker and Saito 1981). This greatly improved visualization of CA fluorescent axons. Once staining was completed, the CA fluorescence could be observed with water immersion optics, but the resolution was greatly improved by drying and mounting the tissue. This step was accomplished by removing as much of the final incubation solution as possible and drying the entire preparation (while still pinned out in the Sylgard lined Petri dish) for 4–24 hr in a vacuum desiccator at 25 C in the dark. Drying was accelerated by placing a 100 mm glass Petri dish containing fresh P₂O₅ (Polysciences) in the desiccator. The CP muscle was then infiltrated with xylene or Histosol (National Diagnostics, Somerville, N.J.) and mounted in toto between two coverslips (thickness 0) using Fluoromount (Gurr, England).

Combined α-melanocyte stimulating hormone (α-MSH) immunofluorescence and nerve terminal staining was performed as follows: (1) the nerve terminals were first TNBT stained as described above, (2) then the preparation was fixed for 16–24 hr in freshly made 4% formaldehyde in 0.09 M phosphate buffer (pH 7.2) at 4 C, and (3) the immunocytochemistry was performed using our standard immunofluorescence procedure (Oren et al. 1989).

The immunocytochemistry procedures are also briefly summarized here. First, the tissue was incubated in the primary antibody for 48 hr; then standard FITC-coupled secondary antibody fluorescence techniques were employed for visualization of the primary antibody (Oren et al. 1989). Finally, the intact CP muscle was mounted in toto in “No-Fade” mounting medium (Oren et al. 1989) between two coverslips (thickness 0).

Fluorescent axons were observed by means of epi-illumination using a Zeiss microscope equipped with an HBO 100 mercury lamp and the appropriate fluorescent filter sets (CA fluorescence: 405/6 excitation, LP435 barrier filter; FITC fluorescence: 485/20 excitation, BP 520–560 barrier filter). Zeiss 40/0.75, Leitz 50/1.0, and 100/1.2 water immersion or Zeiss 25/0.8 plan neofluar and 40/1.0 planachromat oil immersion objectives were used. Photographs were taken using Ilford XP1-400 film. A red suppression filter (KP 560) was sometimes used to enhance the green CA fluorescence.

RESULTS

Catecholamine Histofluorescence

The original Faglu method for staining CA-containing neurons (Furness et al. 1977) was modified for whole mount CP muscle preparations and to be compatible with other staining protocols. The level and quality of CA staining was affected by the aldehyde concentrations, temperature, incubation time, and the methods of drying and mounting the preparation. We tried other aldehydes in the staining procedure (e.g., acrolein and glyoxal), but these did not induce staining; only the combination of formaldehyde and glutaraldehyde produced CA fluorescence (Fig. 1).

Glutaraldehyde was essential for CA fluorescence because formaldehyde alone never produced fluorescence. However, glutaraldehyde fixation also caused intense muscle fiber fluorescence which, under some circumstances, was so bright
that it completely obscured the visualization of any CA axon fluorescence. This was seen when the formaldehyde concentration was held constant (in the optimal range of 4–6%). Then changes in glutaraldehyde concentration from 0.15–1.55% produced progressive increases in both CA fluorescence and background muscle fiber
fluorescence. While low glutaraldehyde concentrations (approximately 0.15%) yielded the least intense muscle fiber fluorescence, this concentration produced relatively weak CA fluorescence. Any glutaraldehyde concentration over 0.5% was not usable because the background muscle fiber fluorescence was always so intense that it obscured visualization of the CA fluorescence.

We also found that CA fluorescence was dependent not only on the concentrations, but also on the ratio of the aldehydes (F:G) in the staining solution. Varying the F:G ratio greatly affected the CA fluorescence. This was studied by comparing CA fluorescence for different ratios while keeping the glutaraldehyde concentration in the optimal range to maximize CA nerve staining. An F:G ratio of 4:1 (e.g., F/G = 2/0.5) gave weak CA fluorescence, while ratios from 8:1 to 12:1 gave approximately the same levels of fluorescence, and 16:1 gave the most intense CA fluorescence observed. F:G ratios greater than 16:1, and up to 32:1, gave relatively weak CA fluorescence and were not used. Thus the range of ratios between 8:1 and 16:1 was best for optimizing CA fluorescence. Correspondingly, background fluorescence also varied with changes in the F:G ratio. Generally, an F:G ratio of 2.5:1 to 4:1 produced too intense background fluorescence; however, ratios between 8:1 and 16:1 yielded background fluorescence of acceptable intensity. While ratios greater than 20:1 gave less background muscle fiber fluorescence, the CA fluorescence was also attenuated. Figure 1A shows an example of fluorescent CA-containing axons stained using the optimal ratio (8:1) achieved with 4% formaldehyde and 0.5% glutaraldehyde (i.e., 4/0.5 F/G). In combined staining experiments described below a 6/0.15 F/G solution was found to be optimal.

In addition to the formaldehyde and glutaraldehyde concentrations, the quality of CA histofluorescence was also affected by the staining conditions. The initial experiments were done at room temperature (22–25°C) on an exposed laboratory bench. However, more consistent results occurred when the staining was performed at 4°C in the dark. This lower temperature helped to reduce the background muscle fiber fluorescence. The time in the F/G staining solution was also important. After 30 min in F/G solution (4/0.5) CA fluorescence was present, but weak. Two to three hours in this F/G solution was optimal and longer staining caused the background fluorescence to become too intense. CA fluorescence was present when the pH of the staining solutions was between pH 6.5–8, with the most intense CA fluorescence seen at pH 7.2. Acidic pH (pH 5–6) caused extremely bright background muscle fiber fluorescence with no CA staining. Staining at pH 8–9 produced less background muscle fiber fluorescence but no CA fluorescence.

Visualization of fluorescent CA-containing axons was possible immediately following the F/G procedure by using water immersion objectives. There was no obvious decrease in the axonal fluorescence when the preparations were kept in either normal frog Ringer’s or 0.09 M phosphate buffer for up to 1–2 hr. However, the best visualization of the CA fluorescence required dehydration. The fluorescence of wet preparations, even when mounted between coverslips and viewed with high numerical aperture oil immersion objectives, was still much less intense than when viewed after drying and mounting (Furness et al. 1977). Several methods of drying the preparation were tried. Both air drying using a hair dryer for 10 min (Furness et al. 1978) or drying in an oven worked, but the excessive heating compromised staining with other procedures. To overcome this, we dried the tissue over P₂O₅. P₂O₅ is hygroscopic and within 3–4 hr at room temperature individual CP muscles were completely dry. This process was further accelerated by drying the muscles in a vacuum desiccator. Longer drying times (up to 18–24 hr) were required for larger muscles, but we tried to keep this step to a minimum because excessive time in P₂O₅ made the muscles extremely brittle and increased the back-
ground muscle fiber fluorescence. Once the muscle had been dried, it could not be re-exposed to water. Even a brief exposure to any aqueous solution completely removed the CA fluorescence but this had no effect on the background muscle fiber fluorescence. Therefore, once dried, the tissue was immediately infiltrated with xylene or Histosol and embedded between coverslips in nonaqueous mounting medium.

The specificity of the CA fluorescence was assayed by using the neurotoxin 6-OHDA which is known to deplete catecholamines. A single injection of the frog with 6-OHDA completely abolished F/G-induced CA fluorescence for 8–10 days, but did not effect the nerve terminal staining.

**Neuromuscular Junction Staining Combined with CA Histofluorescence or Immunocytochemistry**

The ability to visualize simultaneously CA histofluorescent or immunofluorescent axons and stained neuromuscular junctions required using staining and fixation solutions which were compatible with each procedure. First we attempted to modify two standard neuromuscular stains to use in conjunction with F/G-induced CA fluorescence: the Karnovsky procedure (Karnovsky 1964, Letinsky and DeCino 1980) for staining postsynaptic acetylcholinesterase activity and nerve terminal staining with tetrazolium salts (Letinsky and DeCino 1980, Letinsky 1983). We then extended these procedures to combine TNBT nerve terminal staining with immunocytochemistry.

**Acetylcholinesterase Staining**

The general results of combining the Karnovsky procedure with F/G CA staining were poor. When acetylcholinesterase staining followed the F/G procedures (F/G = 2–4/0.2–0.5 for 0.5–2 hr), there was decreased CA fluorescence, too much background muscle fiber fluorescence, and the Karnovsky reaction product was too light. Even increasing the potassium ferricyanide concentration threefold, which speeds up the Karnovsky reaction (Letinsky, unpublished results), did not help, and produced even more intense muscle fiber fluorescence. Increasing the pH in steps from 6 (the pH optimum for the Karnovsky reaction) to 7.2 did decrease the background fluorescence but hindered the Karnovsky reaction. When the Karnovsky reaction was run prior to the F/G staining, it was sometimes possible to see the junctional acetylcholinesterase reaction product and CA fluorescence. However, the intense, Karnovsky-induced increase in muscle fiber fluorescence still resulted. Again, modifications of the potassium ferricyanide concentration, the pH, and the reaction time of the Karnovsky procedure and of the F/G procedure did not improve the combined staining. It has so far not been possible to obtain suitable acetylcholinesterase staining and good CA histofluorescence without intense background muscle fiber fluorescence.

**TNBT Staining of Motor Nerve Terminals**

Nerve terminal staining with tetrazolium salts worked in conjunction with the F/G procedure. However, TNBT staining could not be performed after the F/G procedure because the glutaraldehyde necessary for terminal staining (Letinsky and DeCino 1980) caused intense background muscle fiber fluorescence which completely obscured CA fluorescence. Combined CA histofluorescence and motor nerve terminal staining was possible when terminal staining was done first using F/G-type solutions in each step instead of the usual glutaraldehyde solution (Fig. 1B,C). Both NBT and TNBT worked in this combined procedure. Because NBT staining was darker and more grainy than TNBT staining (Letinsky and DeCino 1980, Letinsky 1983), it tended to obscure the CA fluorescence. Therefore, we preferred the lighter but more even terminal staining obtained with TNBT. To maximize the nerve terminal staining without compromising the CA fluorescence, we first tried different TNBT and 1-methoxy-
PMS concentrations in a standard F/G solution of 4/0.25. Varying the TNBT concentration between 0.5–3 mg/ml produced progressively darker nerve terminal staining, but concentrations greater than 1 mg/ml obscured the CA histofluorescence. When the 1-methoxy-PMS concentration was increased beyond the optimal 0.1 mg/ml (range 0.05–0.3 mg/ml), the CA fluorescence was decreased. After the optimal 1 mg/ml TNBT and 0.1 mg/ml 1-methoxy-PMS concentrations were determined, we varied the F/G solutions used for TNBT terminal staining. Glutaraldehyde concentrations in the F/G-TNBT staining solution were varied between 0.15–0.5%, with the best combination of nerve terminal and CA fluorescence occurring between 0.15–0.25%. When the formaldehyde concentration in the F/G-TNBT staining solution exceeded 4%, the nerve terminal staining was too grainy and faint. The initial TNBT nerve staining procedures did affect the F/G histochemistry slightly. The best combined TNBT nerve staining and CA fluorescence was achieved when different F/G solutions were used for the CA histofluorescence step (i.e., 6/0.15) and for the TNBT nerve terminal staining step (i.e., 4/0.15).

The use of F/G-type solutions in the TNBT procedure also made it possible to combine nerve terminal staining with immunocytochemical techniques (Fig. 2). After nerve terminals were stained with TNBT as described above, the preparations were fixed and our standard immunocytochemical procedures for demonstrating α-MSH-like immunoreactivity in motor nerve terminals were performed (Oren et al. 1989). These immunocytochemical procedures gave excellent results and did not require further modification. The TNBT staining parameters were varied as described above to maximize terminal staining without impairing the antibody fluorescence. Manipulation of each variable to produce darker nerve terminal staining always obscured visualization of the α-MSH immunofluorescence. The same TNBT staining protocol described above produced the best combination of antibody associated fluorescence and TNBT nerve terminal staining.

**DISCUSSION**

This series of experiments was undertaken to develop procedures for simultaneous visualization of unmyelinated axons and motor nerve terminals in the same whole mount nerve-muscle preparation. With these procedures it is now possible to observe the three-dimensional organization and spatial relationships between axons containing catecholamines or axons stained with specific antibodies and the distribution of motor nerve terminals in the same muscle. This was accomplished by using staining and fixation solutions of mixed aldehydes which were compatible with each staining technique and which allowed for adequate nerve terminal and fluorescent axon staining while not producing undesirable side effects. We found that a relatively simple modification of the formaldehyde/glutaraldehyde procedure (Furness et al. 1977) produced a general purpose staining solution, our F/G solution, which could be used successfully for both CA histofluorescence and motor nerve terminal staining with tetrazolium salts (Letinsky and DeCino 1980, Letinsky 1983). Most importantly, tetrazolium salt nerve terminal staining using the F/G solution also was completely compatible with our standard immunocytochemical procedures (Oren et al. 1989).

The modifications of the Faglu procedure worked well on whole mount amphibian muscle. CA fluorescence was seen routinely except in controls where catecholamines were depleted following 6-OHDA treatment. The F/G procedure worked well alone and in conjunction with nerve terminal staining with tetrazolium salts, but it could not be used in combination with acetylcholinesterase staining because of the intense background muscle fiber fluorescence that occurred. The same Karnovsky (1964) reaction does work in combination with similar CA
Fig. 2. Two examples of combined immunofluorescence and nerve terminal staining. (A) Motor nerve terminal processes stained with TNBT (white arrows) and nerve terminals and preterminal axons with α-MSH-like immunoreactivity (i.e., fluorescein secondary antibody fluorescence, small white arrowheads). The fluorescent preterminal axons pass in and out of the plane of focus in (B). Notice in (A) and (B) that both the α-MSH-like immunoreactivity and TNBT nerve terminal staining can be seen together (*) in parts of the terminal arborization. As shown earlier (Oren et al. 1989) the α-MSH-like immunoreactivity differs in intensity in different parts of the same nerve terminal (see (A) to the right of the two white arrows); (B) part of the terminal arborization (denoted by the two white arrows) does not have α-MSH-like immunoreactivity. Scale bar equals 30 μm.

histofluorescence performed on sectioned material (El-badawi and Schenk 1967; Nakamura and Torigoe 1979) and on whole mounts of isolated arteries (Kobayashi et al. 1981). However, these are much thinner preparations in which background fluorescence would not have been a problem. Another difficulty inherent in using F/G solutions for staining in whole mount skeletal muscle preparations was the background muscle fiber fluorescence produced by even the low levels of glutaraldehyde used. This background, glutaraldehyde-induced, muscle fiber fluorescence was often so intense that it was difficult or even impossible to see CA fluorescence or antibody labeled fluorescent axons. This limitation was overcome by exposing the muscle to trypan blue after the F/G staining. The trypan blue caused the muscle fibers to fluoresce in the red, thus masking the green, glutaraldehyde-induced muscle fiber fluorescence. This change in the muscle fiber fluorescence to red also produced enhanced color contrast so that green CA histofluorescence or fluorescein antibody fluorescence was more easily seen. Baker and Saito (1981) used this same approach to eliminate background autofluorescence in elastic fibers. In preliminary experiments, similar masking of glutaraldehyde-induced muscle fiber fluorescence by trypan blue works well to enhance the visibility of other fluorescent probes (Letinsky, unpublished results).

There are several unique and powerful experimental advantages to the staining procedures that we have developed. The F/G procedure for detecting catechol-
amines is rapid, reliable, inexpensive and easy. It permits the visualization of CA histofluorescent axons in whole mount skeletal muscle preparations in which the three-dimensional organization of the innervation and the muscle fibers is maintained. Also, these preparations are sufficiently well preserved so that they could be embedded and examined in the electron microscope (Furness et al. 1978; Letinsky and Wolowska, unpublished results). Most importantly, the F/G procedure is compatible with TNBT staining of motor nerve terminals. This permits concurrent evaluation of the spatial relationship between autonomic and motor innervation in the same muscle preparation. Another important result is that the use of F/G-type solutions now makes the tetrazolium salt nerve stain compatible with standard immunocytochemical methods. Thus, it is now possible to simultaneously visualize motor nerve terminals and virtually any structures stained by specific antibodies in the same whole mount preparation. Moreover, by varying the darkness of the tetrazolium salt nerve staining, it is potentially possible to visualize fluorescent markers or histochemically identified cellular organelles within stained axons or nerve terminals.

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