Immunocytochemical electron microscopic study and Western blot analysis of myosin, paramyosin and miniparamyosin in the striated muscle of the fruit fly *Drosophila melanogaster* and in obliquely striated and smooth muscles of the earthworm *Eisenia foetida*

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Summary

Miniparamyosin is a paramyosin isoform (55–60 kDa) that has been isolated in insects (*Drosophila*) and immunolocalized in several species of arthropods, molluscs, annelids and nematodes. In this study, the presence and distribution of this protein, in comparison with that of paramyosin and myosin, has been examined in the striated muscle (tergal depressor of trochanter) of *Drosophila melanogaster*, and the obliquely striated muscle (body wall) and the smooth muscle (outer layer of the pseudoheart) of the earthworm *Eisenia foetida* by means of immunocytochemical electron microscopic study and Western blot analysis miniparamyosin, paramyosin and myosin antibodies from *Drosophila*. In the striated muscle of *D. melanogaster*, the three proteins were immunolocalized along the length of the thick filaments (A-bands). The distribution of immunogold particles along these filaments was uniform. The relative proportions miniparamyosin/paramyosin/myosin (calculated by counting the number of immunogold particles) were: 1/10/68. In the obliquely striated muscle of *E. foetida*, immunoreactions to the three proteins were also found in the thick filaments, and the relative proportions miniparamyosin/paramyosin/myosin were 1/2.4/6.9. However, whereas the distribution of both myosin and miniparamyosin along the thick filament length was uniform, paramyosin immunolabelling was more abundant in the extremes of thick filaments (the outer zones of A-bands in the obliquely striated muscle), where the thick filaments become thinner than in the centre (the central zone of A-bands), where these filaments are thicker. The relative proportions of paramyosin in the outer and of paramyosin in the central zones of A-bands were 4/1. This irregular distribution of paramyosin along the thick filament length might be actual but it may also be explained by the fusiform shape of thick filaments in the earthworm: assuming that paramyosin is covered by myosin, paramyosin antigens would be more exposed in the tips than in the centre of thick filaments. If miniparamyosin is, in turn, covered by paramyosin, the exposure of miniparamyosin antigens would be low even in the tips of thick filaments, and this might explain the scanty immunoreaction observed for this protein and the absence of a higher number of immunogold particles in the extremes of thick filaments. The distribution of the three proteins in the earthworm smooth muscle was as in the obliquely striated muscle, although the proportions miniparamyosin/paramyosin/myosin were 1/1.5/5.2; this is, immunoreactions to paramyosin and miniparamyosin were lower than in the obliquely striated muscle.

Introduction

It is well established that thick myofilaments of invertebrate muscle cells consisted of two major proteins: myosin and paramyosin. Recently a new protein, the miniparamyosin, has been isolated from the indirect flight muscle of *Drosophila melanogaster* (Bernstein et al., 1993; Maroto et al., 1995).

The myosin of invertebrate muscle is very similar to that of vertebrates and contain two heavy chains of about 200 kDa and four light chains of 20 kDa (Kendrick-Jones et al., 1976; Szent-Györgyi, 1973).
Paramyosin is only present in invertebrate thick myofilaments and has no known vertebrate homologue. The paramyosin molecule has high \( \alpha \)-helical content in which two monomers (~100 kDa) interact to form a coiled-coil (Cohen & Holmes, 1963; Lowey et al., 1963). Paramyosin filaments interact strongly with myosin, specifically with the \( \alpha \)-helical rod of light meromyosin. This protein has been localized in the thick myofilament core and it is probably surrounded by myosin (Epstein et al., 1985). Biochemical and genetics characterization of paramyosin as well as immunofluorescence light microscopy studies of this protein have been carried out in the nematode Caenorhabditis elegans (Epstein et al., 1993) and several arthropods (Beinbrech et al., 1988; Vinós et al., 1991, 1992; Becker et al., 1992; Schmitz et al., 1994). In addition, immunoelectron microscopic studies have been performed in C. elegans (Deitiker & Epstein, 1993), the lamellibranch mollusc Placopecten magellanicus (Castellani & Vibert, 1992), the gastropod mollusc Helix aspersa and the oligochaete annelids Eisenia fetida (Royuela et al., 1996).

Miniparamyosin is a distinct paramyosin isoform of 55–60 kDa, that, in D. melanogaster, is encoded by the same gene that paramyosin (Bernstein et al., 1993; Maroto et al., 1995). Using immunoblot analysis, Maroto and colleagues (1995) localized miniparamyosin in insects (D. melanogaster, D. viridis, Calliphora sp. and Locusta sp.), arachnids (Araneus sp.), crustaceous (Astacus sp.), molluscs (Mytilus sp. and Helix sp.) and annelids (Lumbricus sp.). These authors failed to detect this protein in nematodes (Caenorhabditis sp.).

The aim of the present study was to investigate the presence and distribution of miniparamyosin, in relation with that of myosin and paramyosin, in the striated muscle (tergal depressor of trochanter) of D. melanogaster, and in the obliquely striated muscle (body wall) and smooth muscle (outer muscular layer of the pseudoheart) of the oligochaetan annelid E. fetida (earthworm) by means of Western blot analysis and electron microscopic immunocytochemistry.

Materials and methods

The tissue studies were: tergal depressor muscle (transversely striated muscle) from D. melanogaster, and both muscular body wall (obliquely striated muscle) and outer layer of the pseudoheart (smooth muscle) from the earthworm (E. fetida, Oligochaeta). The animals were anaesthetized with ether, killed, and the above-mentioned muscles were removed for the following studies: conventional electron microscopy (two animals of each species); immunoelectron microscopy (five animals of each species); and Western blotting analysis (the number of animals that were necessary in each case to obtain 100 mg of muscular tissue). The primaries antibodies used (myosin, paramyosin and miniparamyosin antibodies) were obtained from rabbit. The source of antigens for the three antibodies was D. melanogaster. The procedure has been described previously (Maroto et al., 1995).

The specificity of the primary antibodies was tested by Western blotting analysis as described by Towbin and colleagues (1979). Tissues were homogenized in 0.5 M Tris-HCl buffer (pH 7.4) containing 1 mM EDTA, 12 mM 2-mercapto-ethanol, and 1 mM phenylmethylsulphonyl fluoride (PMSF). The homogenates were centrifuged at 10,000 g for 30 min. After boiling for 2 min at 98°C, aliquots of 20 µl homogenate were separated in SDS-polyacrylamide slab minigels (9% W/v), according to the procedure of Laemmli (1970). Separated proteins were transferred for 4 h to 0.25 A nitrocellulose paper and, thereafter, the nitrocellulose sheets were stained with Ponceau Red, soaked in blocking solution (1 M glucose, 1% BSA, 0.5% Tween-20, 10% glycerol in PBS, pH 7.3) overnight at 37°C and then incubated with the primary antibody at 1:1000 dilution in blocking solution for 3 h. After extensive washing with PBS-Tween-20, the sheets were incubated with a peroxidase-labelled second antibody (goat anti rabbit biotinylated immunoglobulin) (Biocell, Cardiff, UK), at 1/1000 dilution in blocking solution. The filters were developed with an enhanced chemiluminescence (ECL) Western blotting analysis, following the procedure described by the manufacturer (Amersham, UK). To compare differences in band intensities of myosin, paramyosin, and miniparamyosin, in each muscle type studied, the immunostained bands were directly quantified by densitometry using a scanner Omnimedia 6C3 and an image analyser Bio-image (Millipore Ibérica, Madrid, Spain).

For electron microscopy immunocytochemistry, tissues were fixed for 90 min in 0.1 st phosphate-buffered mixture of 2.5% paraformaldehyde and 0.5% glutaraldehyde (in equal proportions) (pH 7.4). Afterwards, the material was washed, dehydrated and embedded in Lowicryl K4M. Ultrathin sections were placed on drops of 0.2 M. Tris buffer containing 0.1% glycine and 1% BSA. Then they were incubated for 2 h at room temperature with the primary antibody at 1:20 dilution to paramyosin and miniparamyosin, and 1:150 dilution to myosin. After washing with Tris buffer, the sections were incubated with 15 nm gold-labelled IgG goat anti rabbit immunoglobulin (Biocell, Cardiff, UK), at 1/100 dilution in 20% goat serum-PBS buffer (pH 7.6) for 2 h at room temperature. After incubation with the second antibody, the sections were washed with Tris buffer and distilled water and counterstained with uranyl acetate for 20 min at room temperature. Double immunogold labellings for myosin/paramyosin, myosin/miniparamyosin, or paramyosin/miniparamyosin were carried out in some ultrathin sections of each muscle type. The procedure only differs for that of single immunolabelling in that, after incubation with the second antibody, the binding sites of unreacted immunoglobulins are denatured using hot paraformaldehyde vapour and a second single labelling procedure is carried out with larger gold particles (30 nm) (Wang & Larsson, 1985).

The specificity of the immunohistochemical procedure was checked by incubation of sections with: (1) non-