EXERCISE MODULATES SYNAPTIC ACETYLCHOLINESTERASE AT NEUROMUSCULAR JUNCTIONS

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Abstract—Acetylcholinesterase plays a major role in neuromuscular transmission and is regulated by neuromuscular activity. Since fast-twitch motor units are recruited with increased motor demand, we examined acetylcholinesterase regulation in rat leg muscles following treadmill training. Total acetylcholinesterase and specifically the membrane-bound tetramer increased in exercised fast-, but not slow-twitch muscles, while other isoforms remained unchanged. Synaptic acetylcholinesterase increased markedly in neuromuscular junctions of trained fibers, without concomitant changes in synaptic acetylcholine receptor, thus elevating synaptic acetylcholinesterase/receptor ratios. Electron microscopy showed that acetylcholinesterase increased in postjunctional folds and primary cleft, where it was added adjacent to the postsynaptic muscle membrane. Thus, although the primary acetylcholinesterase at the neuromuscular junction is the collagen-tailed asymmetric isoform associated with synaptic basal lamina, physiological demands such as strenuous exercise, or potentially pathological conditions, can selectively recruit the membrane-bound acetylcholinesterase tetramer to the synapse for optimal synaptic transmission. © 2016 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: neuromuscular junction, acetylcholinesterase molecular forms, acetylcholine receptor, fast-twitch, treadmill.

INTRODUCTION

Acetylcholinesterase (AChE) is an important component of central and peripheral cholinergic vertebrate

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Abbreviations: AChE, acetylcholinesterase; AChR, acetylcholine receptor; CNS, central nervous system; ColQ, non-catalytic collagenous subunit; DTAF, dichlorotriazinyl amino fluorescein; EDL, extensor digitorum longus; Fas, fasciculin 2; GrP, gracilis posterior; IOD, integrated optical density; Iso-OMPA, tetra-isopropyl pyrophosphoramide; NMJ, neuromuscular junction; PC, primary cleft; PJM, postjunctional membrane; PL, plantaris; PRiMA, proline-rich membrane anchor; Rd-BTX, rhodamine-α-bungarotoxin; Sol, soleus; TA, tibialis anterior; TRITC, tetramethylrhodamine.

synapses, where it hydrolyzes acetylcholine and is thus involved in termination of synaptic transmission (Katz and Miledi, 1973). As established already in the 1930s, AChE in muscle is highly concentrated at the neuromuscular junction (NMJ), where it modulates nerve-evoked muscle activation (for a review, see e.g. Couteaux, 1998). AChE is highly conserved in mammals, and appears in multiple molecular forms that are catalytic subunit oligomers, which arise as a consequence of alternate splicing of a single gene (Legay, 2000; Massoulié, 2002). The isoforms can be classified as globular and asymmetric. The globular forms consist of monomers, dimers and tetramers (G_1, G_2 and G_4) of the catalytic subunits. The G_1 form is soluble, whereas the G_2 and G_4 forms are membrane-bound, although soluble G_4 species have also been described (Massoulié, 2002). The asymmetric isoforms are composed of one, two or three catalytic subunit tetramers (A_4, A_8 and A_{12}) attached to a non-catalytic collagenous subunit (ColQ) (Krejci et al., 1999). The A_{12}-AChE is considered to be the principal form at NMJs (Hall, 1973; for reviews see Legay, 2000; Massoulié, 2002; Rotundo, 2003; Massoulié and Millard, 2009), although the presence of other forms cannot be excluded (Anglister et al., 1994a). The major form in the central nervous system (CNS) is the amphiphilic G_4 isoform, which is attached to the membrane by hydrophobic non-catalytic subunit called PRiMA (proline-rich membrane anchor) (Fernandez et al., 1996; Perrier et al., 2002). Conversely, AChE is itself influenced by neuromuscular (and/or motor) activity. Long-term denervation diminishes AChE activity in rat muscles, notably of the A_{12} form, with recovery to control levels upon reinnervation or muscle stimulation (Hall and Kelly, 1971; Vigny et al., 1976; Lømo et al., 1985; Sketelj et al., 1992; Fernandez et al., 1996). However, several studies provide evidence that the tetramer G_4 AChE in muscle is regulated by motor activity (Fernandez and Donoso, 1988; Jasmin and Gisiger, 1990; Fernandez et al., 1996; Boudreau-Larivière et al., 1997). Accordingly, short-term denervation results in an increase in G_4 levels in denervated fast-twitch muscles (Gregory et al., 1989; Hodges-Savola and Fernandez, 1991). Moreover, exercise induces marked and selective changes in levels of G_4-isoform in skeletal muscles, without concomitant modification of the levels of other isoforms (Fernandez and Donoso, 1988; Jasmin and Gisiger, 1990; Gisiger et al., 1991, 1994). While running or walking increase the level of G_4 AChE in flexor fast-twitch leg muscles, but respectively decrease or increase its level in extensor fast-twitch leg muscles (Fernandez

and Donoso, 1988; Jasmin and Gisiger, 1990), swimming results in elevation of G_4-AChE in all fast-twitch leg muscles (Gisiger et al., 1991). However, the location and distribution of the G_4 form in muscle, and more specifically, of the molecules added following exercise, have not been determined. The aim of this study was to investigate the influence of a short course of fast-walk training on AChE activity and isoforms in fast-twitch leg muscles of rats, with specific focus on the concentration and distribution of synaptic AChE. Whereas no change was observed in the current study in synaptic nicotinic acetylcholine receptor (AChR) levels, training produced a marked increase in synaptic AChE activity, thus elevating the AChE/AChR ratio at the NMJ. The increase in synaptic AChE could be ascribed to addition of amphiphilic (membrane-anchored) G_4 AChE levels, thus suggesting a role for this isoform in neuromuscular function.

EXPERIMENTAL PROCEDURES

Materials

Materials were purchased from Sigma–Aldrich Inc. (St. Louis, MO, USA), unless specified otherwise.

Animals and training

Female Sprague–Dawley rats (175–199 g), 9–10 weeks old, were obtained from Harlan Laboratories (Jerusalem, Israel). Rats were housed, bred, cared for and treated under controlled conditions, using a protocol approved by the ethics committee (IACUC) of the Hebrew University of Jerusalem. The Hebrew University is an AAALAC International accredited institute.

Rats in "trained" groups were exercised by fast walking on a "motor-driven" horizontal treadmill at a speed of 13.2 m/min, with 2 min sprints of 18 m/min every 8 min, for 1 h each day (six repeated cycles), five days a week, for two weeks. The "control" group rats were randomly chosen from the same litter. Both "trained" and "control" rats were sacrificed 8–10 h after the last training session, by anesthesia with an overdose of isoflurane.

Muscle preparations

The following hind limb muscles were removed immediately after sacrifice: extensor digitorum longus (EDL), soleus (Sol), gracilis posterior (GrP), tibialis anterior (TA) and plantaris (PL).

For labeling of whole muscles and single fibers, freshly dissected muscles were pinned in a Sylgard®-coated dish in mammalian Krebs–Ringer solution (128 mM $NaCl$/4 mM KCl/2 mM $CaCl_2$/1 mM $MgSO_4$/1 mM NaH_2PO_4/25 mM $NaHCO_3$/30 mM glucose, pH 7.3), and immediately processed as required (see below).

For preparation of frozen sections, to be followed by labeling and microscopic examination, isolated muscles were embedded in OCT (Optimal Cutting Temperature compound; Tissue-Tek, Sakura Finetek USA, Inc., Torrance, CA), positioned so as to optimize sectioning through NMJs, and quick-frozen via isopentane in liquid

N_2. In all cases muscles were stored at $-70\,°C$ until further study.

For subsequent extraction and biochemical analysis of AChE activities and isoforms, muscles were weighed immediately after dissection (wet weight), quick frozen in liquid N_2, and stored at $-70\,°C$ until further processing.

Muscle extracts

Frozen muscles were thawed, cut into small pieces (1–2 mm), and homogenized with ice-cold extraction buffer (1:8 w/v) containing: 1 M $NaCl$/1% Triton-X-100/20 mM EDTA/10 mM phosphate, pH 7.3, and a cocktail of protease inhibitors (0.1 mM PMSF, 20 U/ml aprotonin, 20 µg/mg pepstatin, 1 mM benzamidine, 1 mM N-ethylmaleimide, 0.1 mg/ml bacitracin), to maintain the intrinsic pattern of the molecular forms of AChE (Silman et al., 1978). Homogenization was in a Heidolph type P7R1 motor-driven glass/glass homogenizer (Heidolph Instruments GmbH, Schwabach, Germany) kept on ice, at a medium speed of $\sim$90 s, until homogenization appeared complete, and no discernible particulate material remained. The homogenates were divided into appropriate aliquots, quick frozen in liquid N_2, and stored at $-70\,°C$ for further analysis (of protein content, AChE activity and isoforms).

Protein determination

For analysis of total protein content, homogenate samples were diluted to contain 3–5 µg protein/assay in detergent-free aqueous solution. Protein determination was according to Bradford (1976), in microtiter plates using an ELISA reader spectrophotometer at 595 nm (PowerWave$_x$340, Bio-Tek Instruments Inc., USA).

AChE assay

AChE activity in the tissue extracts (and in fractions from sucrose gradients, see below) was determined by a radiometric assay, with ^{3}H-acetylcholine (^{3}H-ACh; NEN, PerkinElmer Life Sciences Inc, Waltham, MA) as substrate, monitoring the ^{3}H-acetate generated during ACh hydrolysis (Johnson and Russell, 1975). AChE activity units (eu = enzyme unit) express nanomoles of ACh hydrolyzed per min at room temperature. Specific AChE activities in muscle samples were displayed per protein (eu/mg) or per muscle weight, as specified.

Velocity sedimentation for AChE isoforms

To determine the sedimentation coefficients values of the various AChE molecular forms, samples were analyzed by sucrose gradient centrifugation, as described earlier (Anglister et al., 1994a; Blotnick et al., 2012). A sample of homogenate (70–100 µl) was layered on top of a 10 ml 5–20% linear sucrose gradient formed over a 0.5 ml cushion of 50% sucrose, all made up in 10 mM phosphate, pH 7.3, containing 1 M $NaCl$ and 1% Triton-X-100. Gradients were sedimented at 38 k rpm for 16 h at $4\,°C$ in a SW41Ti swinging bucket rotor (Beckman Coulter Instruments Inc, Fullerton, CA, USA).