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ABNORMALITIES IN NEUROMUSCULAR JUNCTION STRUCTURE AND SKELETAL MUSCLE FUNCTION IN MICE LACKING THE $P2X_2$ NUCLEOTIDE RECEPTOR

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Abstract—ATP is co-released in significant quantities with acetylcholine from motor neurons at skeletal neuromuscular junctions (NMJ). However, the role of this neurotransmitter in muscle function remains unclear. The $P2X_2$ ion channel receptor subunit is expressed during development of the skeletal NMJ, but not in adult muscle fibers, although it is re-expressed during muscle fiber regeneration. Using mice deficient for the $P2X_2$ receptor subunit for ATP ($P2X_2^{-/-}$), we demonstrate a role for purinergic signaling in NMJ development. Whereas control NMJs were characterized by precise apposition of pre-synaptic motor nerve terminals and post-synaptic junctional folds rich in acetylcholine receptors (AChRs), NMJs in $P2X_2^{-/-}$ mice were disorganized: misapposition of nerve terminals and post-synaptic AChR expression localization was common; the density of post-synaptic junctional folds was reduced; and there was increased end-plate fragmentation. These changes in NMJ structure were associated with muscle fiber atrophy. In addition there was an increase in the proportion of fast type muscle fibers. These findings demonstrate a role for $P2X_2$ receptor-mediated signaling in NMJ formation and suggest that purinergic signaling may play an as yet largely unrecognized part in synapse formation.

Key words: ATP, acetylcholine, knockout, mouse, nerve terminal, synapse.

It is well established that ATP is co-released with acetylcholine (ACh) from motor nerve terminals (Redman and Silinsky, 1994; Redman and Silinsky, 1996). While ACh is the transmitter that mediates via nicotinic receptors muscle contraction in mature animals, during early development both ATP, acting via P2X ion channel receptors (Ralevic and Burnstock, 1998) and ACh mediate muscle responses (Kolb and Wakelam, 1983; Henning, 1997; Heilbronn and Eriksson, 1998). $P2X_2$ receptor subunits are expressed during early postnatal development, when neuromuscular junction (NMJ) maturation and patterning occur, but disappear in the adult (Ryten et al., 2001). Furthermore, this receptor is re-expressed in the later stages of muscle regeneration in the mdx mouse model of muscular dystrophy (Ryten et al., 2004; Jiang et al., 2005). Also direct postjunctional responses to ATP reappear after denervation of chick skeletal muscle (Wells et al., 1995).

Electrophysiology, immunohistochemistry and reverse transcriptase polymerase chain reaction have demonstrated the expression of a range of purinoceptors in developing skeletal muscle, including $P2X_2$, $P2X_5$, $P2X_6$, $P2Y_1$, $P2Y_2$ and $P2Y_4$ (Kolb and Wakelam, 1983; Hume and Thomas, 1988; Thomas et al., 1991; Henning et al., 1992; Meyer et al., 1999a,b; Bo et al., 2000; Ruppelt et al., 2001; Ryten et al., 2001; Cheung et al., 2003). Specific roles in skeletal muscle fiber formation and function have been identified for two of these receptors. While activation of the $P2Y_1$ receptor has been shown to regulate acetylcholine receptor (AChR) and acetylcholinesterase (AChE) expression at NMJs (Choi et al., 2001; Ling et al., 2004), the $P2X_5$ receptor has been implicated in the regulation of myoblast activity and muscle regeneration (Ryten et al., 2002, 2004). The function of the $P2X_2$ receptor is less clear. However, the singular ability of this receptor type to interact with nicotinic AChRs to produce cross-inhibition of channel opening (Nakazawa, 1994; Barajas-López et al., 1998; Searl et al., 1998; Zhou and Galligan, 1998; Khakh et al., 2000), and the timing of $P2X_2$ receptor expression in development suggests a role for purinergic signaling in the late stages of NMJ formation and patterning.

In the present study we use the $P2X_2$ receptor knockout mouse ($P2X_2^{-/-}$) (Cockayne et al., 2005) to investigate the role of the $P2X_2$ receptor in NMJ development and skeletal muscle function.

EXPERIMENTAL PROCEDURES

Animals

$P2X_2^{-/-}$ mice were generated by introducing a deletion encompassing exons 2–11 into the mouse $P2X_2$ gene (see Cockayne et al., 2005 for details).

Immunohistochemistry and histology

Four female wild-type and four $P2X_2^{-/-}$ mice were killed by CO_2 asphyxiation and death was confirmed by cervical dislocation according to Home Office (UK) regulations covering Schedule 1

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Abbreviations: ACh, acetylcholine; AChR, acetylcholine receptor; E, embryonic day; NMJ, neuromuscular junction; P, postnatal day; PBS, phosphate-buffered saline; $P2X_2^{-/-}$, $P2X_2$ receptor knockout mouse.

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procedures. All experiments conformed to the Royal Free and University College Medical School guidelines on the ethical use of animals; experiments were designed to minimize the use and suffering of animals. The soleus muscles were rapidly removed. Muscle samples used for the assessment of muscle fiber number, muscle fiber size or muscle fiber type were cut such that the mid-belly region was isolated, covered with OCT compound and frozen in liquid nitrogen–cooled isopentane. Cryostat sections (12 μm) were cut to produce transverse muscle sections. Sections showing the largest muscle bulk were collected on gelatinized slides, stained with Toluidine Blue and used for assessment of muscle fiber number and size. For assessment of muscle fiber type by demonstration of myofibrillar ATPase we followed the method described by Dubowitz (1985) with preincubation of fresh frozen muscle sections in alkali buffer (pH 9.4). In order to immunostain for fast skeletal muscle myosin, cryostat sections were fixed in 4% formaldehyde in 0.1 M phosphate buffer for 2 min, rinsed several times in phosphate-buffered saline (PBS) and incubated overnight with a monoclonal antibody (diluted 1:100 in 10% goat serum in PBS) that exclusively recognizes fast skeletal muscle myosin (Sigma Chemical Co., Poole, UK). Immunoreactivity was visualized by incubation with goat anti-mouse fluorescein-conjugated secondary antibody (Stratech Scientific, Newmarket, UK). Muscle samples used for visualization of NMJs were pinned at proximal and distal ends to sylgard and fixed in 4% paraformaldehyde in PBS for 45 min, rinsed briefly and sunk in 20% sucrose in PBS. Following fixation, muscle fibers were teased apart using fine forceps and incubated with Texas Red–labeled α-bungarotoxin (Invitrogen Ltd., Paisley, UK) diluted 1:1000 in PBS. To immunostain for synaptophysin, teased muscle fibers were incubated overnight in rabbit anti-synaptophysin antibodies (Synaptic Systems, Goettingen, Germany) diluted 1:100 in 10% goat serum in PBS. Immunoreactivity was visualized using goat anti-rabbit fluorescein-conjugated secondary antibody (Stratech Scientific). Images were captured using a Leica (Heerbrugg, Switzerland) confocal microscope and the sum pixel function Leica analysis software to collapse z-series stacks taken through entire NMJs.

Analysis of AChR expression at NMJs

Teased muscle fibers stained with Texas Red–labeled α-bungarotoxin and photographed as described above, were used for analysis of AChR expression at NMJs. Using Scion Image analysis software (NIH, USA) the number of AChR clusters per NMJ and total area of AChR expression per NMJ were measured. A minimum of 45 NMJs (taken from at least three mice) were sampled to make up each experimental group. End-plate morphology (cluster number) in wild-type and $P2X_2^{-/-}$ soleus muscles were compared using the Mann-Whitney test since the data were clearly not normally distributed. The total area of AChR expression per NMJ in wild-type and $P2X_2^{-/-}$ soleus muscles was compared using a two-tailed unpaired Student's *t*-test. A probability level of $P<0.05$ was taken as significant in all tests.

Measurement of muscle fiber number

A minimum of five samples, each taken from different mice ($n=4$), was used to assess muscle fiber number in wild-type or $P2X_2^{-/-}$ soleus muscles. Soleus muscle sections prepared as described above were used for analysis and photographed under $\times$2.5 magnification using a Nikon digital camera. Muscle fiber numbers were counted by enlarging the screen image and using a grid (to prevent accidental re-counting of fibers). Wild-type and $P2X_2^{-/-}$ soleus muscle fiber numbers were compared using an unpaired Student's *t*-test. The numbers of fast (type II) muscle fibers, as demonstrated by myofibrillar ATPase were measured in a similar manner and expressed as a % of the total number of muscle fibers counted. The percentage of fast type muscle fibers in wild-type and $P2X_2^{-/-}$ soleus muscle was compared using an unpaired Student's *t*-test.

Assessment of the incidence of centrally nucleated fibers

A minimum of four samples from different mice ($n=4$) was used to assess the incidence of centrally nucleated fibers in wild-type or $P2X_2^{-/-}$ soleus muscles. Using the method described above, the total number and the number of centrally nucleated muscle fibers were counted. The incidence of centrally nucleated muscle fibers (expressed as a percentage of the total number of muscle fibers) in wild-type and $P2X_2^{-/-}$ muscle was compared using an unpaired Student's *t*-test.

Measurement of muscle fiber cross-sectional area

A minimum of five different samples, each taken from different mice, was used to assess muscle fiber cross-sectional area in wild-type or $P2X_2^{-/-}$ soleus muscles. Soleus muscle sections prepared as described above were used for analysis, and photographed under $\times$20 magnification using a Nikon digital camera. Two sections per muscle were analyzed. Images were viewed on a computer screen and a 200 μm$\times$200 μm grid was applied to each image. Random selection of muscle fibers was achieved by making fiber area measurements only on fibers overlying points of grid intersections. Cross-sectional fiber area was measured using Scion Image analysis software and expressed in μm^2. In this way at least 70 muscle fibers (approximately 8% of muscle fibers) were sampled from each image and a total of 702 wild-type and 719 $P2X_2^{-/-}$ soleus muscle fibers were measured. Average cross-sectional fiber areas (calculated for each muscle sample) from wild-type ($n=5$) and $P2X_2^{-/-}$ ($n=5$) soleus muscles were compared using a two-tailed unpaired Student's *t*-test.

Electron microscopy

Soleus muscles from three wild-type and three $P2X_2^{-/-}$ animals were fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer, washed, post-fixed in 1% osmium tetroxide (OsO_4), dehydrated in graded alcohol and embedded in resin. Thin longitudinal sections were cut at 80 nm thick, stained with uranyl acetate and lead citrate and examined under a JEM-1010 electron microscope. To quantify fold density, the total length of terminal contact was measured on photomicrographs and the number of junctional folds that could be seen was recorded. Measurements from wild-type and $P2X_2^{-/-}$ muscles were compared using a two-tailed unpaired Student's *t*-test.

Physiology

The soleus muscle from five wild-type and five $P2X_2^{-/-}$ mice was dissected free and placed in continually gassed (95% O_2/5% CO_2) modified Krebs solution (mM): NaCl, 133; KCl, 4.7; $NaHCO_3$, 16.4; $MgSO_4$, 0.6; NaH_2PO_4, 1.4; glucose, 7.7; and $CaCl_2$, 2.5; pH 7.3 at 37$\pm$1 °C. One end of the muscle was attached to a rigid support and the other to a Grass FT03C force displacement transducer via silk ligatures and mounted in 10 ml organ baths. Mechanical activity was recorded using PowerLab Chart for Windows (Version 4; ADInstruments New South Wales, Australia). An initial load of 1 g was applied to each soleus muscle, which was allowed to equilibrate for 60 min. Contractions were elicited by electrical stimulation of the muscle via two platinum wire rings 2.5 mm in diameter and 1 cm apart through which the soleus muscles were threaded.

Nerve-mediated and muscle-mediated twitch responses

Twitch contractile responses due to the stimulation of nerves were achieved by using: 100 V, 0.1 ms duration and one twitch/min. By extending the duration of stimulation to 5 ms direct stimulation of