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Guanylate cyclase and cyclic GMP-dependent protein kinase regulate agrin signaling at the developing neuromuscular junction

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Abstract

During formation of the neuromuscular junction (NMJ), agrin secreted by motor axons signals the embryonic muscle cells to organize a postsynaptic apparatus including a dense aggregate of acetylcholine receptors (AChRs). Agrin signaling at the embryonic NMJ requires the activity of nitric oxide synthase (NOS). Common downstream effectors of NOS are guanylate cyclase (GC), which synthesizes cyclic GMP, and cyclic GMP-dependent protein kinase (PKG). Here we show that GC and PKG are important for agrin signaling at the embryonic NMJ of the frog, *Xenopus laevis*. Inhibitors of both GC and PKG reduced endogenous AChR aggregation in embryonic muscles by 50–85%, and blocked agrin-induced AChR aggregation in cultured embryonic muscle cells. A cyclic GMP analog, 8-bromo-cyclic GMP, increased endogenous AChR aggregation in embryonic muscles to 3- to 4-fold control levels. Overexpression of either GC or PKG in embryos increased AChR aggregate area by 60–170%, whereas expression of a dominant negative form of GC inhibited endogenous aggregation by 50%. These results indicate that agrin signaling in embryonic muscle cells requires the activity of GC and PKG as well as NOS.

Keywords: Neuromuscular junction; Synapse formation; Agrin; Muscle cells; Acetylcholine receptors; Nitric oxide; Cyclic GMP; Guanylate cyclase; Cyclic GMP-dependent protein kinase; *Xenopus* embryos

Introduction

Formation of the neuromuscular junction (NMJ), a well-studied synapse, is a complex process which occurs over a period of days to weeks in embryos of different species (Sanes and Lichtman, 1999b). Assembly of the postsynaptic apparatus, and, indirectly, the nerve terminal, is directed by agrin released by motor axons (Gautam et al., 1996; Cohen et al., 1997; Jones et al., 1997). Postsynaptic proteins, including acetylcholine receptors (AChRs), are directed by agrin to aggregate into stable structures anchored to the actin cytoskeleton (Dai et al., 2000; reviewed in Godfrey and Schwarte, 2003). Agrin acts in muscle cells through a membrane tyrosine kinase, MuSK, a component of the agrin receptor (DeChiara et al., 1996; Glass et al., 1996, 1997). However, signaling steps downstream of MuSK activation leading to aggregation of postsynaptic AChRs and other proteins are not well defined. Understanding the molecular mechanisms involved in assembly of the neuromuscular junction will provide a basis for comparison with signaling events in formation and plasticity of synapses in the central nervous system (CNS), and may also suggest therapeutic approaches for neuromuscular diseases.

Nitric oxide synthase (NOS), an enzyme that synthesizes the free radical gas nitric oxide (NO), is concentrated postsynaptically at the NMJ (Chao et al., 1997; Yang et al., 1997), and NO

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regulates acetylcholine release from motor nerve terminals (Wang et al., 1995). NOS is also localized postsynaptically at some CNS synapses, and NO has been implicated as a retrograde signaling molecule in long-term potentiation (Hawkins et al., 1998). NOS activity is required for agrin signaling of AChR aggregation in chicken skeletal muscle cells (Jones and Werle, 2000) and in *Xenopus* embryo muscles (Godfrey and Schwarte, 2003). Overexpression of NOS in *Xenopus* embryos greatly increases AChR aggregate area at embryonic NMJs, whereas NOS inhibitors block endogenous AChR aggregation 50–90% and completely block agrin-induced aggregation in cultured embryonic muscle cells (Schwarte and Godfrey, 2004).

Common downstream effectors of NO signaling in cells include guanylate cyclase (GC), the enzyme that synthesizes cyclic GMP (cGMP), and cyclic GMP-dependent protein kinase (PKG; Hofmann et al., 2000). Agrin-induced aggregation of AChRs into large, dense clusters in the membrane of cultured chicken muscle cells requires activity of both GC and PKG (Jones and Werle, 2004). Here we asked whether GC and PKG activities are also necessary for synaptic aggregation of AChRs during formation of the embryonic NMJ. Inhibitors of both GC and PKG reduced AChR aggregation at the NMJ, and blocked agrin-induced increases in AChR aggregation in cultured embryonic muscle cells. Conversely, overexpression of either GC or PKG increased AChR aggregate area at the NMJ, as did a cyclic GMP analog, but a dominant negative form of GC inhibited synaptic aggregation. These data strongly suggest that GC and PKG are involved in agrin signaling of postsynaptic differentiation at the embryonic NMJ and in cultured muscle cells.

Materials and methods

cDNAs; RNA synthesis

The cDNA encoding green fluorescent protein (GFP; S65T mutant) was obtained from Dr. Richard Dorsky (University of Utah). The cDNAs coding for rat soluble GC subunits α and β (Chinkers et al., 1989; Yuen et al., 1994) were a gift of Dr. David Garbers (University of Texas Southwestern Medical School), and a dominant negative GC α1 construct (D529A mutant; Yuen et al., 1994) was obtained from Dr. Peter Yuen (National Institutes of Health). The cDNAs encoding bovine cGMP-dependent protein kinase Iα (PKG; Wernet et al., 1989) and a constitutively active form of human PKGIα (PKG-GFP fusion protein; Browning et al., 2001) were from Dr. Bonnie Firestein (Rutgers University) and Dr. Darren Browning (Medical College of Georgia), respectively. Protein coding sequences of all cDNAs were amplified by PCR. Sense primers contained the SP6 RNA polymerase promoter and the 5′ untranslated sequence from *Xenopus* beta globin found in the pCS2 vector (Godfrey et al., 2000; Rupp et al., 1994). RNA was synthesized using SP6 polymerase and the mMessage Machine kit (Ambion; Godfrey et al., 1999).

RNA injection

Embryos of *Xenopus laevis* were obtained by *in vitro* fertilization (Moon and Christian, 1989) and injected with synthetic RNAs at the one-cell stage using a Nanoject II injector (Drummond) fitted with glass micropipets (20 μm diameter tip). Embryos were injected (Moon and Christian, 1989) with 4.6–9.2 nl nuclease-free water containing GFP RNA (1–2 ng) alone or combined with RNAs encoding GC or PKG (3–12 ng).

Screening embryos and labeling acetylcholine receptors

Following injection of RNAs, embryos were transferred into 0.1 × modified Barth's solution (MBS; Gurdon and Wickens, 1983) and allowed to develop to stage 31 (Nieuwkoop and Faber, 1994), then screened for GFP fluorescence. Embryos were fixed and AChRs were labeled with 1.5 μg/ml Alexa 594-α-bungarotoxin (Invitrogen) as described (Schwarte and Godfrey, 2004).

Confocal microscopy and image analysis

AChR aggregates were imaged using a confocal microscope (Zeiss LSM 510). Six stacks of images were acquired from three to six embryos for each condition, two stacks of 4 optical sections (at 1 μm intervals) from each of myotomes 4, 5, and 6 (Godfrey et al., 1999). The images were taken through a 40 × objective with a 2.5-fold optical zoom (total magnification 100×). Optical sections were imaged to show innervated (medial) portions of the muscles in which AChR aggregates formed a continuous line, and were centered along the intermyotomal septa where NMJs form. Since muscle cells at this stage were about 10 μm in diameter, each 4 μm stack imaged primarily one set of cells. The area of AChR aggregates in a montage of all 4 images in each stack was measured with Metamorph image analysis software (Universal Imaging Corp.). Threshold was set to mark aggregates in each series of images, and aggregate area was converted to a percentage of the total area of the montaged images. Statistics were calculated as described (Godfrey et al., 1999).

Treatment of embryos with GC and PKG inhibitors and a cGMP analog

Embryos were exposed to the GC inhibitor 1*H*-[1,2,4]oxadiazolo[4,3-alpha] quinoxalin-1-one (ODQ), the PKG inhibitor Rp-8-pCPT-cGMPS (Rp-8), or 8-bromo-cyclic GMP (8-Br-cGMP) from 26 h of development (stage 24) to stage 31, a period of 18–20 h at 16–18 °C. Reagents (Biomol or EMD Biosciences) were dissolved in 1× MBS. In some experiments, penetration of inhibitors into muscles was facilitated by removing the skin overlying the trunk myotomes and dissolving inhibitors in cell culture medium. AChR aggregates were labeled and their area was quantified as described above. Skin was removed from myotomes of embryos treated with Rp-8 in some experiments with 8-Br-cGMP, and matching controls, but not for ODQ treatments. Removing skin did not delay development.

Cell culture studies

Myotomal muscles were removed from stage 21–23 embryos and were dissociated (Peng et al., 1991). Cells were cultured in 0.4 cm^2 wells (NUNC Lab-Tek #178599; cells from 0.7–1 embryo per well), which were mounted on glass coverslips with Sylgard (Dow Corning) and coated with a basement membrane extract (E.C.L., Upstate) as described (Schwarte and Godfrey, 2004). Cultures were used for experiments after 1–3 days, when the cells had attached and spread. Cultures were pretreated with inhibitors for 2 h prior to adding 6–12 ng/ml agrin (half-maximal dose = 3.5–5 ng/ml) with or without inhibitors for 14 h. After 16–18 h at 22 °C, cells were labeled 1 h with Alexa 594-α-bungarotoxin (1.5 μg/ml), rinsed twice with culture medium and fixed 10 min in 95% ethanol at −20 °C. Cultures were mounted in a glycerol-based mounting medium containing *n*-propyl gallate to retard fading of fluorescence (Valnes and Brandtzaeg, 1985). Aggregates of AChRs (≥ 1 μm) were counted in each of 20 cells per well in duplicate wells for each condition.

Results

Inhibitors of guanylate cyclase and cGMP-dependent protein kinase reduce endogenous AChR aggregation at the embryonic neuromuscular junction, but a cyclic GMP analog greatly increases AChR aggregate area in vivo

To determine whether the activities of guanylate cyclase (GC) and cGMP-dependent protein kinase (PKG) are required