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RAPSYN INTERACTS WITH THE MUSCLE ACETYLCHOLINE RECEPTOR VIA α-HELICAL DOMAINS IN THE α, β, AND ε SUBUNIT INTRACELLULAR LOOPS

Y. LEE, J. RUDELL AND M. FERNS*

Department of Anesthesiology and Physiology and Membrane Biology, University of California Davis, One Shields Avenue, Davis, CA 95616, USA

Abstract—At the developing vertebrate neuromuscular junction, the acetylcholine receptor becomes aggregated at high density in the postsynaptic muscle membrane. Receptor localization is regulated by the motoneuron-derived factor, agrin, and requires an intracellular, scaffolding protein called rapsyn. However, it remains unclear where rapsyn binds on the acetylcholine receptor and how their interaction is regulated. In this study, we identified rapsyn's binding site on the acetylcholine receptor using chimeric constructs where the intracellular domain of CD4 was substituted for the major intracellular loop of each mouse acetylcholine receptor subunit. When expressed in heterologous cells, we found that rapsyn clustered and cytoskeletally anchored CD4-α, β and ε subunit loops but not CD4-δ loop. Rapsyn-mediated clustering and anchoring was highest for β loop, followed by ε and α, suggesting that rapsyn interacts with the loops with different affinities. Moreover, by making deletions within the β subunit intracellular loop, we show that rapsyn interacts with the α-helical region, a secondary structural motif present in the carboxyl terminal portion of the subunit loops. When expressed in muscle cells, rapsyn co-immunoprecipitated together with a CD4-α helical region chimera, independent of agrin signaling. Together, these findings demonstrate that rapsyn interacts with the acetylcholine receptor via an α-helical structural motif conserved between the α, β and ε subunits. Binding at this site likely mediates the critical rapsyn interaction involved in localizing the acetylcholine receptor at the neuromuscular junction.

Key words: neuromuscular junction, synaptogenesis, agrin, postsynaptic membrane.

At the developing neuromuscular junction, presynaptic nerve terminals become precisely aligned with high-density aggregates of acetylcholine receptor (AChR) in the postsynaptic membrane, ensuring the high fidelity of transmission at this synapse. This process is regulated in part by agrin, a motoneuron-derived factor that plays an essential role in stably aggregating the AChR at nascent synapses. Agrin induces and/or stabilizes AChR clusters by signaling via a receptor complex consisting of the low-density lipoprotein receptor-related protein 4 (LRP4) and muscle-specific kinase (MuSK) (Glass et al., 1996; Kim et al., 2008; Zhang et al., 2008), and nerve-associated receptor clusters are absent in agrin, MuSK or LRP4 null mice at birth, resulting in perinatal lethality (DeChiara et al., 1996; Gautam et al., 1996; Weatherbee et al., 2006). MuSK signaling requires the adaptor proteins Dok7 and Tid1 (Beeson et al., 2006; Okada et al., 2006; Linnoila et al., 2008), and includes activation of the kinase PAK1 (Luo et al., 2002), geranylgeranyltransferase I (Luo et al., 2003), the small GTPases Rac, cdc42, and Rho (Weston et al., 2000, 2003), and the cytoplasmic tyrosine kinases src/fyn (Mittaud et al., 2001) and abl/arg (Finn et al., 2003), which may phosphorylate the AChR. However, the specific signaling events and protein interactions that mediate clustering of the AChR are still not clear (Strochlic et al., 2005).

One critical mediator of AChR localization is the intracellular, membrane-associated scaffolding protein, rapsyn. Rapsyn colocalizes precisely with AChR clusters *in vitro* and *in vivo* (Froehner et al., 1981; Burden, 1985; Noakes et al., 1993), and is estimated to be in approximately 1:1 stoichiometry with the AChR (Burden et al., 1983; LaRochelle and Froehner, 1986). Moreover, rapsyn clusters the AChR (and several other synaptic proteins) when they are co-expressed in heterologous cells (Froehner et al., 1990; Phillips et al., 1991b), and AChR clusters fail to form at neuromuscular contacts in rapsyn null mice (Gautam et al., 1995). In addition, rapsyn mutations in humans result in decreased AChR levels at the synapse, producing a severe myasthenic syndrome with impaired transmission and debilitating muscle weakness (Ohno et al., 2002; Maselli et al., 2003). In fact, up to 10% of congenital myasthenic syndromes may be due to rapsyn mutations (Engel et al., 2003; Maselli et al., 2003).

Surprisingly, the molecular mechanism by which rapsyn localizes the AChR remains unknown. Functional studies on rapsyn have revealed an amino terminal (N-terminal) myristylation site required for rapsyn's targeting to the plasma membrane (Phillips et al., 1991a), 7 tetratricopeptide repeats (amino acid [aa] 6–279) that mediate rapsyn self-association (Ramarao and Cohen, 1998; Ramarao et al., 2001), and a coiled-coil domain (aa 298–331) and cysteine-rich RING structure (aa 363–402) required for interaction with AChR and β-dystroglycan, respectively (Ramarao and Cohen, 1998; Bartoli et al., 2001; Ramarao et al., 2001). Rapsyn's site of interaction on the AChR is unclear, however, and it is unknown whether rapsyn binds one or more subunits of the pentameric AChR (2α, β, δ and γ (fetal) or ε (adult) subunits) and if binding is constitutive or regulated. Indeed, each subunit contains a large intra-

*Corresponding author. Tel: +1-530-754-4973; fax: +1-530-752-5423.
E-mail address: mjferns@ucdavis.edu (M. Ferns).
Abbreviations: aa, amino acid; AChR, acetylcholine receptor; C-terminal, carboxyl terminal; LRP4, low-density lipoprotein receptor-related protein 4; MuSK, muscle specific kinase.

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cellular loop between the third and fourth transmembrane domains that could potentially interact with rapsyn, and consistent with this, structural studies on the AChR have revealed an accessory protein, that is presumably rapsyn, lying immediately beneath the channel in association with the loops (Miyazawa et al., 1999). One candidate interaction site is the β subunit loop, as cross-linking studies have shown that rapsyn is in close proximity to this subunit (Burden et al., 1983), and rapsyn also interacted with the β subunit intracellular loop in a modified yeast two-hybrid assay (Bartoli et al., 2001). On the other hand, it has been reported that rapsyn can associate with all of the AChR subunits when individually expressed in heterologous cells, at least in intracellular aggregates (Maimone and Merlie, 1993; Huebsch and Maimone, 2003). In this study, we have used chimeric proteins in which CD4 is fused to the large intracellular loop of each of the AChR subunits to define the subunits and domains responsible for interaction with rapsyn.

EXPERIMENTAL PROCEDURES

CD4-subunit loop constructs

To generate the CD4-subunit loop chimeras, a BglII restriction site was introduced by site-directed mutagenesis at the end of the transmembrane domain of the mouse CD4 cDNA. The intracellular domain of CD4 was then excised and the large intracellular loops of the AChR subunits were ligated into this site. The loops were obtained by PCR of cDNAs for each of the mouse AChR subunits, and all the constructs were then sequenced. All the CD4-loop constructs were expressed in the mammalian expression vector pcDNA3.

To generate CD4-β loop α-helix constructs, a BglII site was introduced at the end of the CD4 intracellular domain and PCR fragments comprising segments of the α-helix were ligated into this site (denoted CD4^{ct}-β). This CD4 tail spacer was used to position the short α-helical segment further from plasma membrane. We also generated CD4-α and ε loop α-helix constructs, by replacing the β loop α-helix with the analogous region of the α and ε subunits.

Cell culture and transfection

COS cells were grown in Dulbecco's modified eagle medium with high glucose (DMEM-HI), supplemented with 10% fetal bovine serum and 100 U/ml penicillin–streptomycin. For biochemical experiments, cells growing in 10 cm dishes were transfected using the $CaPO_4$ method. For immunostaining experiments, cells growing in eight well chamber slides (Nalge Nunc Itl., Naperville, IL, USA) were transfected in parallel.

Sol8 mouse muscle cells were maintained in DMEM-HI, supplemented with 20% fetal bovine serum, 100 U/ml of penicillin-streptomycin, and 2 mM L-glutamine. The myoblasts were transfected at 85% confluency using the $CaPO_4$ method, and then when confluent, the cells were incubated with fusion medium (DMEM-HI supplemented with 5% horse serum and 2 mM L-glutamine) to induce formation of myotubes.

Protein extraction, immunoprecipitation and Western blotting

To assay detergent extractability, transfected COS cells were rinsed, scraped off and pelleted in ice cold PBS. They were then re-suspended in extraction buffer (25 mM Tris, 25 mM glycine, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100 and the protease inhibitors PMSF, benzamidine and $Na_2S_4O_6$) and incubated for 10 min on ice, after which the insoluble proteins were pelleted by centrifugation (13,000 rpm for 5 min). The CD4 chimeric proteins were then immunoprecipitated from the soluble fraction with monoclonal antibody GK1.5 (BD Biosciences, San Jose, CA, USA) and protein G-agarose (Invitrogen, Carlsbad, CA, USA). In addition, the insoluble pellet was resuspended in 200 μl of loading buffer (SDS, glycerol, 5% β-mercaptoethanol and Bromophenol Blue). The immunoprecipitated proteins and 20% of the pellet fraction were separated on 10% polyacrylamide gels. They were then immunoblotted with monoclonal antibody H129.19 to CD4 (BD Biosciences), followed by an anti-rat HRP-conjugated secondary antibody and visualized using enhanced chemiluminescence. The intensity of the Western blot signals was quantified using Sci-Scan 5000 Bioanalysis (USB, Cleveland, OH, USA) or ImageGauge V4.22 software (FijiFilm, Valhalla, NY, USA).

To assay rapsyn association in transfected COS cells or Sol8 myotubes, the cells were extracted and CD4 immunoprecipitations performed as above. In the case of Sol8 myotubes, the AChR was also isolated using biotin-conjugated α-bungarotoxin and streptavidin agarose (Invitrogen–Molecular Probes, Carlsbad, CA, USA). Co-immunoprecipitated rapsyn was then detected by immunobotting with polyclonal antibody B5668, which we generated against a peptide encompassing rapsyn aas 133-153. The blots were reprobed for the AChR α-subunit using mAb210 (Babco, Berkley, CA, USA).

Immunostaining of transfected cells

Transfected COS cells were fixed with 2% paraformaldehyde/PBS, blocked with 10% horse serum/PBS and stained for surface CD4 with rat monoclonal antibodies GK1.5 or H129.19 (BD Biosciences). After washing with PBS, the cells were permeabilized in 0.5% Triton X-100/PBS for 10 min, and incubated with rapsyn monoclonal antibody 1234 (gift of Dr. Froehner, U. Washington) or rabbit polyclonal antibody B6766 (Lee et al., 2008) for 45 min. The cells were then incubated with Alexa 488– and 594–conjugated secondary antibodies (Invitrogen). In some experiments we confirmed that rapsyn clustered surface-expressed CD4 chimeras by incubating live cells with anti-CD4 antibodies at 4 °C for 15 min, and then fixing, permeabilizing and immunostaining for rapsyn.

To quantify rapsyn-induced clustering of CD4-subunit loops, we selected random fields and then scored all rapsyn-positive cells according to whether they had strong, weak or no clustering of CD4. Cells were defined as having strong clusters when CD4 staining overlapped precisely with rapsyn aggregates with little staining elsewhere, and weak clusters when significant CD4 staining was evident elsewhere on the cell surface.

RESULTS

Construction and expression of CD4-subunit loop chimeras

The muscle AChR is a pentamer composed of 2 α, β, δ and γ (fetal) or ε (adult) subunits. The subunits share $\sim$31% homology ($\sim$16% sequence identity) and have the same membrane topology, with a large intracellular loop between transmembrane domains 3 and 4 that is the most likely site of interaction with rapsyn. To define rapsyn's binding site on the AChR, therefore, we generated chimeric constructs where the intracellular domain of CD4 was substituted for the major intracellular loop of each AChR subunit (Fig. 1A, B). This allowed surface expression and circumvents the problem that individually expressed subunits are retained in the endoplasmic reticulum, with only fully assembled AChR being trafficked to the