Agrin-induced Acetylcholine Receptor Clustering Is Mediated by the Small Guanosine Triphosphatases Rac and Cdc42

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Abstract. During neuromuscular junction formation, agrin secreted from motor neurons causes muscle cell surface acetylcholine receptors (AChRs) to cluster at synaptic sites by mechanisms that are insufficiently understood. The Rho family of small guanosine triphosphatases (GTPases), including Rac and Cdc42, can mediate focal reorganization of the cell periphery in response to extracellular signals. Here, we investigated the role of Rac and Cdc42 in coupling agrin signaling to AChR clustering. We found that agrin causes marked muscle-specific activation of Rac and Cdc42 in differentiated myotubes, as detected by biochemical measurements. Moreover, this activation is crucial for AChR clustering, since the expression of dominant interfering mutants of either Rac or Cdc42 in myotubes blocks agrin-induced AChR clustering. In contrast, constitutively active Rac and Cdc42 mutants cause AChR to aggregate in the absence of agrin. By indicating that agrin-dependent activation of Rac and Cdc42 constitutes a critical step in the signaling pathway leading to AChR clustering, these findings suggest a novel role for these Rho-GTPases: the coupling of neuronal signaling to a key step in neuromuscular synaptogenesis.

Key words: nicotinic acetylcholine receptors • neuromuscular junction • guanosine triphosphate–binding proteins • Rac • Cdc42

Introduction

Acetylcholine receptors (AChRs) are diffusely distributed on the surface of cultured C2C12 muscle cells become aggregated into clusters upon treatment with neural agrin (Bowen et al., 1996; Ferns et al., 1996). This mimics the action of agrin secreted by motor neurons during the formation of neuromuscular junctions (Bowe and Fallon, 1995; Burden, 1998; Sanes and Lichtman, 1999). There is evidence that agrin initiates its effects by binding to a surface complex comprised of a recently characterized muscle-specific receptor tyrosine kinase (MuSK), and other as yet unidentified muscle surface components (DeChiara et al., 1996). The clustering of AChRs is accompanied by increased anchorage of these receptors to the cytoskeletal framework and loss of lateral mobility (Prives et al., 1982; Stya and Axlord, 1983). Furthermore, agrin induces the co-clustering with AChR of other synaptic proteins, including the actin-binding cytoskeletal proteins utrophin and α-actinin (Shadiack and Nitkin, 1991; A pel et al., 1997). These findings support the possibility that the clustering of AChR and other cell surface components can be mediated through agrin-induced cytoskeletal reorganization (Hoch et al., 1994).

Members of the Rho family of small GTPases, including Rac and Cdc42, have been shown to mediate focal reorganization of the actin cytoskeleton in response to extracellular cues in several cell types (Burr ridge and Chrzanowska-Wodnicka, 1996; Tapon and Hall, 1997; Hall, 1998). The activation of Rac by membrane signaling is known to produce actin reorganization at the cell periphery, giving rise to lamellipodia and membrane ruffles (Tapon and Hall, 1997; Hall, 1998). Cdc42 activation leads to the formation of actin-containing filopodia (Tapon and Hall, 1997) as well as to Rac activation (Keely et al., 1997). There is now considerable evidence that Rac and Cdc42 produce changes in cytoskeletal organization by controlling actin polymerization via multiple regulatory pathways (R aber et al., 1998; Yang et al., 1998; Sells et al., 1999; Van Leeuwen et al., 1999). Based on the demonstrated ability in other cell types of Rac/Cdc42 to link the signaling of surface receptors for growth factors to localized actin polymerization at the cell periphery (Hall, 1998), we have investigated the contribution of these small G proteins to...
agrin-induced AChR clustering in muscle cells. We show that agrin triggers the activation of Rac and Cdc42, and present evidence that this activation is crucial for agrin-induced AChR clustering. Moreover, like other actions of agrin, the stimulation of Rac and Cdc42 was not observed with nonmuscle cells.

Materials and Methods

Reagents and Cell Culture
Soluble recombinant neural agrin was prepared from COS cells with cDNA encoding the COOH-terminal half of agrin (Ferns et al., 1993), and concentrations were determined in comparison to purified agrin by Western blotting.

Expression plasmids encoding T7 epitope-tagged constitutively active Rac (RacV12), dominant interfering Rac (RacN17), myc epitope-tagged constitutively active Cdc42 (Cdc42V12), and dominant interfering Cdc42 (Cdc42N17) were provided by D. Bar-Sagi (State University of New York at Stony Brook). A plasmid-encoding GST fused to the Cdc42/Rac (p-21)—binding domain (GST-PBD) was a gift from S. Moorees and J. Brugge (Harvard University, Cambridge, MA.).

C2C12 mouse muscle cells were plated on 12-mm-diameter glass coverslips in 35-mm culture dishes for microscopy or in 100-mm culture dishes for kinase assays. The cells were cultured at 37°C with 5% CO2 in growth medium consisting of DME supplemented with 10% (vol/vol) fetal bovine serum and 5% (vol/vol) calf serum (Life Technologies). 2 d after plating, when the cells reached 50–70% confluence, the growth medium was replaced with differentiation medium consisting of DME containing 2% (vol/vol) horse serum (Life Technologies). During the subsequent 1.5–2 d, the myotube cultures were treated with 5 nM agrin for 15 min and then rinsed with ice-cold PBS supplemented with 1 mM Na3VO4, 1 μg/ml leupeptin, and 1 mM PM SF and incubated with 2 μg of GST-c-jun fusion protein beads (New England Biolabs, Inc.) at 4°C overnight. The beads were washed twice each with lysis buffer and kinase buffer (25 mM Tris, pH 7.5; 5 mM β-glycerophosphate; 2 mM DTT; 0.1 mM Na3VO4; and 1 μg/ml leupeptin). The JNK activity present in the immunoprecipitate was determined by resuspension in 50 μl of kinase buffer supplemented with 100 μM ATP. After 30 min at 30°C, the reactions were terminated by adding 25 μl of 3× sample buffer. The proteins were resolved by SDS-PAGE on 10% acrylamide gels and then transferred to nitrocellulose. The membranes were immunoblotted with antiphospho c-jun (Se63) antibody (New England Biolabs, Inc.). Immunocomplexes were visualized by ECL, and signals were quantitated using a Bio-Rad Laboratories imaging densitometer with Molecular Analyst software.

For the JNK assays using Rac and Cdc42 transfected cells, C2C12 cells were cotransfected using LipofectAmine reagent (Life Technologies) with FL A G epitope-tagged JNK1 and RacV12, RacN17, Cdc42V12, or Cdc42N17. After 2 d in differentiation medium, the C2C12 cells were treated with 5 nM agrin in DME at 37°C for varying amounts of time. The cells were then harvested and the transfected JNK was immunoprecipitated as previously described (Nimmual et al., 1998). The immunocomplexes were incubated in 50 μl of kinase buffer containing 10 μl of GST-c-jun phosphorylation was measured by autoradiography and quantitated using a PhosphorImager (Molecular Dynamics). Background levels of Rac or Cdc42 and JNK expression were determined by Western blotting and incubation with anti-JNK1, anti-J7, or anti-myec (Santa Cruz Biotechnology, Inc.) primary antibodies. Immunocomplexes were visualized by ECL.

Results

Dominant Interfering Mutants of Rac and Cdc42 Prevent Agrin-induced AChR Clustering

Under the culture conditions used here, AChR clusters, as visualized by fluorescence microscopy of myotubes surface-labeled with the rhodamine-tagged AChR ligand TMR-Bgt, were rarely seen in agrin-untreated cultures but were prominent in agrin-treated cells (Fig. 1 A, panels a and b). A grin-induced AChR clustering was clearly visible by 4 h of exposure and was maximal by 6 h. The crucial role of actin-based cytoskeleton in the formation of these AChR clusters is shown by the effect of the actin-disrupting toxin latrunculin B (Spector et al., 1989) on agrin-dependent AChR surface redistribution. AChR clustering induced by a 6-h exposure of myotubes to agrin (Fig. 1 A, panel b) was blocked in the presence of 0.2 μM latrunculin B (Fig. 1 A, panel c).

To determine the roles of Rac and Cdc42 in agrin-induced AChR clustering, C2C12 myotubes were transfected with dominant interfering (N17, GDP-bound) mu-
tants of Rac and Cdc42, and muscle differentiation was subsequently induced by switching the cultures from growth medium to differentiation medium, as described in Materials and Methods. 3 d after transfection, the effects of these mutants on AChR surface distribution in agrin-treated and -untreated myotubes were examined by fluorescence microscopy of TMR-Bgt–labeled cells. In contrast to the agrin-induced AChR clustering seen in cells transfected with vector only (Fig. 1 B, a and b), myotubes expressing RacN17 (Fig. 2 A, c and d) did not display AChR clustering after identical agrin treatment, or even at agrin concentrations 30-fold higher than required for maximal clustering in control myotubes (data not shown).

The inhibition of agrin-induced AChR clustering is not a general characteristic of dominant interfering mutants of small G proteins, as indicated by the observation that RasN17, a dominant interfering mutant Ras, did not block agrin-induced AChR aggregation (Fig. 2 A, e and f). Moreover, the differentiation of muscle cells expressing RacN17 and Cdc42N17 was not impaired, as was evidenced by their ability to undergo fusion (Fig. 2 A, a-d) and to display surface AChR at levels comparable to nontransfected myotubes (Fig. 2 A, b and d). Furthermore, these transfected myotubes were indistinguishable from mock-transfected and nontransfected myotubes in expression of muscle contractile proteins, including sarcomeric actin (Fig. 1 B, c and d) and myosin (data not shown), as detected by immunostaining.

Quantitative comparison of the number of AChR clusters on the surface of myotubes expressing either RacN17 or Cdc42N17 mutants versus control myotubes showed a
90% inhibition of agrin-induced AChR clustering by the dominant interfering mutants of both Rac and Cdc42 (Fig. 2 B). These findings indicate that Rac and Cdc42 activation is necessary for agrin-induced AChR clustering.

Constitutively Active Rac and Cdc42 Mutants Induce AChR Aggregation

To determine whether activation of Rac or Cdc42 is in itself sufficient to produce AChR aggregation, cells were transfected with the constitutively active mutants RacV12 or Cdc42V12 and the effects on AChR surface distribution were examined in agrin-untreated myotubes. Under these conditions, we found that the expression of either RacV12 or Cdc42V12 produced AChR aggregation (Fig. 3), partially mimicking the effect of agrin. However, although agrin treatment resulted in the formation of large AChR clusters (Fig. 1 A, b), RacV12 and Cdc42V12 induced the appearance of small, punctate AChR aggregates (1–5-µm diameter), referred to here as microclusters (Fig. 3 A, b and d). The induction of microclusters by both mutants is also evident from a quantitative comparison of the number of AChR microclusters on the surface of myotubes expressing either RacV12 or Cdc42V12 versus control myotubes (Fig. 3 B). Thus, it appears that activation of Rac and Cdc42 is sufficient for microcluster formation in agrin-untreated myotubes, but is insufficient for the aggregation of microclusters into full-sized clusters.

Agrin Treatment Causes Activation of Rac and Cdc42 in Myotubes

To determine whether Rac and Cdc42 are directly involved in agrin-induced AChR clustering, we examined the effects of agrin on Rac and Cdc42 activity. As shown in Fig. 4 A, exposure of intact myotubes to agrin caused strong activation of Rac as well as Cdc42, as measured in cell lysates by the selective binding of the activated forms of Rac (Fig. 4 A, a) and Cdc42 (Fig. 4 A, b) to GST-PBD, a GST fusion protein containing the Rac/Cdc42-binding domain of PAK (Sander et al., 1998). In addition, we used the JNK signaling cascade as a second, independent readout for activation of Rac and Cdc42 (Coso et al., 1995; Minden et al., 1995; Tan et al., 1996), and once again observed strong stimulation by agrin (Fig. 4 B, a). As shown by these assays, Rac/Cdc42 activation...
by agrin was not appreciable in undifferentiated C2C12 myoblasts (Fig. 4, A and B) or in HEK293 cells (Fig. 4 B), consistent with the selective expression in differentiated muscle of MuSK, the putative receptor for agrin (Valenzuela et al., 1996). Unlike agrin, anisomycin, an activator of the JNK pathway (Hazzalin et al., 1996), caused equivalent JNK activation in myoblasts and myotubes (Fig. 4 B), showing that JNK itself is unaffected by myotube differentiation. The possibility that agrin-induced activation of JNK is mediated by MuSK is further supported by the observation that JNK activation by agrin was transient (Fig. 4 B), with a similar time course as reported for other agrin-induced signals, such as the stimulatory effect of agrin on AChR phosphorylation (Ferns et al., 1996).

To ascertain that the stimulation of JNK by agrin did indeed reflect the activation of Rac or Cdc42 rather than other upstream activators of JNK, a kinase assay using transfected myotubes was used. Shown in Fig. 5 A are the findings obtained with C2C12 cells that were transfected with FLAG epitope–tagged JNK and, where indicated, RacV12 or RacN17. Subsequently, JNK activation was measured in the differentiated cultures with or without pretreatment with agrin. In these experiments the exogenously expressed JNK was immunoprecipitated with anti-FLAG antibody and its activation was assayed by measuring $^{32}$P-labelling of c-Jun in vitro (Nimnual et al., 1998). This approach allows measurement of JNK activation selectively in transfected cells. As can be seen in Fig. 5 A, when C2C12 cells transfected with JNK were treated with agrin there was a sizeable increase in c-Jun phosphorylation. In contrast, agrin did not activate JNK in cells cotransfected with the dominant negative RacN17. Moreover, in agrin-untreated myotubes expressing constitutively active RacV12, JNK activation was higher than the activation produced by agrin in cells expressing endogenous Rac. These findings confirmed that the level of phosphorylated c-Jun monitored by these assays was a measure of Rac activity, strengthening the conclusion that agrin induces Rac activation in differentiated C2C12 muscle cells.

To determine the influence of Cdc42 on agrin-induced JNK activation, measurements of JNK activation were...
carried out exactly as above using FLAG-tagged JNK-transfected C2C12 muscle cultures, but in this case we monitored the effects of coexpressing dominant negative Cdc42N17 or constitutively activated Cdc42V12. As shown in Fig. 5 B, the Cdc42 mutants had parallel effects to the analogous Rac mutants (Fig. 5 A). Thus, dominant negative Cdc42 blocked agrin activation of JNK, as measured by the phosphorylation of c-Jun, whereas constitutively activated Cdc42 produced markedly elevated c-Jun phosphorylation. Together, these findings provide strong evidence that agrin treatment of intact myotubes induces activation of Rac and Cdc42, as measured by three independent readouts.

Activation by Agrin of Rac and Cdc42

The novel finding in this study that agrin activates Rac/Cdc42 supports the notion that these Rho GTPases can mediate the effects of agrin signaling on AChR clustering. This activation was detected using three separate measurements and was demonstrated both with endogenous Rac or Cdc42 in nontransfected muscle cultures and with ectopically expressed Rac and Cdc42 in transfected myotubes. The most direct demonstration involved agrin-induced increases in the selective binding of activated (GTP-bound) Rac and Cdc42 to PAK, a downstream effector molecule.
that is thought to link Rac activation to actin polymerization (Manser et al., 1994; Arber et al., 1998; Sells et al., 1999). A different evidence for agrin stimulation of Rac/Cdc42 was obtained by recording the activation of a second downstream component, JNK, and the conclusion that JNK activation by agrin is indeed mediated by Rac and Cdc42 was confirmed by showing that this activation was blocked by overexpression of dominant negative mutants of either G protein. It is noteworthy that the activation of JNK and the polymerization of actin by Rac proteins are thought to occur through distinct effector pathways (Joneson et al., 1996).

**Rac and Cdc42 Mediate Agrin-induced Formation of AChR Clusters**

Our findings that the dominant negative mutants RacN17 and Cdc42N17 block the induction by agrin of AChR clustering suggest that Rac and Cdc42 activities are crucial for this pathway. This interpretation is supported by the results of the converse experiments—that AChR on the surface of myotubes transfected with the constitutively active mutants RacV12 and Cdc42V12 underwent aggregation without agrin treatment. However, under these conditions AChR aggregated into microclusters significantly smaller than the clusters induced by agrin treatment. This difference may reflect dissimilarity between the localized nature of the signal when triggered by agrin at the cell surface versus the more topologically diffuse signaling that arises from overexpression of constitutively activated mediators in the cytoplasm of transfected myotubes. Alternatively, activation of Rac and Cdc42 may be sufficient for microcluster formation but insufficient for the aggregation of microclusters to form full-sized clusters. Thus, even if localized in nature, the elevation of Rac/Cdc42 activity may be a necessary but not sufficient condition for the induction of fully developed clusters by agrin.

A decade after the discovery of agrin, the mechanism of agrin-mediated AChR clustering is still unresolved, and key players in both the agrin-signaling pathway and in the structural interactions underlying AChR cluster formation have yet to be identified. Until these findings, participation of only two components of this pathway, the receptor tyrosine kinase MuSK and rapsyn, a linker protein associated with AChR, had been shown to be crucial on the basis of mouse knockout studies. It was observed that AChR aggregation is absent at neuromuscular junctions in mice deficient in either MuSK or rapsyn, and agrin does not induce AChR clusters in cultured myotubes from mice lacking MuSK or rapsyn (DiChiara et al., 1996; Apel et al., 1997). In demonstrating that agrin-induced aggregation of surface AChR is dependent on the activation of Rac and Cdc42, our findings indicate that, like MuSK and rapsyn, these monomeric G proteins are crucial components in agrin-induced AChR clustering.

How might Rac and Cdc42 mediate the agrin-initiated clustering of AChR and other constituents of the neuromuscular junction? The ability of activated Rac/Cdc42 to induce reorganization of cortical actin by modulating the dynamics of actin polymerization is now well documented (for review see Hall, 1998). Moreover, surface AChR is thought to be attached to the actin cytoskeleton via a complex in which rapsyn links AChR to the actin-binding protein utrophin, and recent findings indicate that agrin-induced AChR aggregation involves the clustering of these diffusely distributed complexes at sites of MuSK activation (Apel et al., 1995; Fuhrer et al., 1999). According to our proposed scheme shown in Fig. 6, agrin-induced activation of Rac/Cdc42 produces highly localized reorganization of cortical actin cytoskeleton, resulting in redistribu-
tion of actin-anchored AChR-containing complexes into clusters.

In conclusion, our findings implicate Rac and Cdc42 in the coupling of signaling events initiated by the binding of agrin to the aggregation of surface AChR at neuromuscular synapses. It is plausible that these Rho GTPases can function to integrate multiple incoming signals to modulate AChR surface distribution. In addition to the agrin-MUSK pathway, there is substantial evidence that AChR clustering is regulated by other receptor tyrosine kinase-mediated pathways, such as BDNF acting through Trk receptors (Gonzalez et al., 1999), as well as by tyrosine phosphatases (Dai and Peng, 1998). Thus, by means of localized changes in actin-based cytoskeleton, Rho GTPases may serve to couple incoming signals from several phosphorylation pathways to AChR clustering at the neuromuscular junction.

We thank D. Bar-Sagi for valuable discussions as well as for plasmids encoding Rac, Cdc42, and Ras. We are grateful to M. Ferns (McGill University, Montreal, Canada) for the agrin constructs, R. Cerione for anti-Cdc42 antibody, and S. Moores and J. Brugge for GST-PBD cDNA. We are also grateful to G. Galan (Yale University, New Haven, CT) and M.-H. Ayman (State University of New York at Stony Brook) for critical comments on the manuscript.

This work was supported by grants from the National Science Foundation and M. uscular Dystrophy Association.

Submitted: 29 February 2000
R evised: 25 May 2000
A ccepted: 31 May 2000

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