Neuroscience 122 (2003) 659–668

CALCIUM-DEPENDENT MAINTENANCE OF AGRIN-INDUCED POSTSYNAPTIC SPECIALIZATIONS

L. J. MEGEATH,[a,b] M. T. KIRBER,[c] C. HOPF,[d] W. HOCH[d,e] AND J. R. FALLON[a]*

[a]*Department of Neuroscience, Brown University, Box 1953, 190 Thayer Street, Providence, RI 02912, USA*

[b]*Department of Cell Biology, Graduate School of Biomedical Sciences, University of Massachusetts Medical Center, Worcester, MA 01655, USA*

[c]*Whitaker Digital Imaging Microscopy Center, EBRC X-704, Boston University Medical Center, 650 Albany Street, Boston, MA 02118, USA*

[d]*Max-Planck-Institut für Entwicklungsbiologie, Abteilung Biochemie, Tübingen, Germany*

[e]*Department of Biology and Biochemistry, University of Houston, Houston, TX 77204, USA*

Abstract—Although much progress has been made in understanding synapse formation, little is known about the mechanisms underlying synaptic maintenance and loss. The formation of agrin-induced AChR clusters on cultured myotubes requires both activation of the receptor tyrosine kinase MuSK and intracellular calcium fluxes. Here, we provide evidence that such AChR clusters are maintained by agrin/MuSK-induced intracellular calcium fluxes. Clamping intracellular calcium fluxes after AChR clusters have formed leads to rapid MuSK and AChR tyrosine dephosphorylation and cluster dispersal, even in the continued presence of agrin. Both the dephosphorylation and the dispersal are inhibited by the tyrosine phosphatase inhibitor pervanadate. In contrast, clamping intracellular calcium at the time of initial agrin stimulation has no effect on agrin-induced MuSK or AChR phosphorylation, but blocks AChR cluster formation. These findings suggest an avenue by which postsynaptic stability can be regulated by modification of intracellular signaling pathways that are distinct from those used during synapse formation.

Key words: acetylcholine receptor, MuSK, neuromuscular junction.

Information processing in the nervous system depends upon appropriate patterning of synaptic connectivity. Synaptic organization is the result of competing, often experience-dependent factors that drive the formation, persistence and loss of synapses between neurons and their targets. These elements are critical for the precise development of the nervous system. For example, nerve–muscle connectivity is shaped by the activity-dependent elimination of synapses on multiply innervated myofibers (Lichtman and Colman, 2000). Dynamic patterning of synaptic connectivity persists throughout life in at least some areas of the nervous system and may be a basis for learning and memory (Muller et al., 2002).

Understanding how proper synaptic organization is achieved requires knowledge of the cellular and molecular mechanisms mediating synapse differentiation and elimination. The best understood aspect of synaptogenesis is the marshalling of neurotransmitter receptors to the postsynaptic membrane. High density arrays of receptors are characteristic of fast synapses throughout the nervous system (Hack et al., 2002; Heck et al., 2002). This clustering of neurotransmitter receptors is mediated by the coordinated action of distinct sets of cytoskeletal, transmembrane, and extracellular matrix molecules (Ango et al., 2002; Apel et al., 1997; Coleman et al., 2003; O'Brien et al., 1999; Wang et al., 1999).

Knowledge of the detailed mechanisms underlying postsynaptic differentiation is most advanced at the neuromuscular junction (NMJ). Formation of this synapse is directed by agrin, an extracellular matrix molecule secreted by the motor nerve terminal (McMahan and Wallace, 1989). Postsynaptic differentiation can be modeled in culture where the addition of agrin induces the formation of acetylcholine receptor (AChR) clusters on the myotube surface (see Sanes and Lichtman, 2001, for review). These agrin-induced AChR aggregates display many hallmarks of the postsynaptic apparatus including the selective concentration of MuSK, rapsyn, and a variety of other cytoskeletal, transmembrane and extracellular matrix components (Bowen et al., 1998; Gautam et al., 1996; Lieth and Fallon, 1993; Nitkin and Rothschild, 1990; Ruegg and Bixby, 1998; Wallace, 1988). An intracellular signaling cascade is triggered when agrin binds to a receptor complex consisting of the receptor tyrosine kinase MuSK and a putative co-receptor (Glass et al., 1996). Downstream of MuSK activation, the pathway leading to AChR cluster formation requires the cytosolic protein rapsyn (Gautam et al., 1995) and nitric oxide (Jones and Werle, 2000). Activated MuSK also leads to the tyrosine phosphorylation of AChRs, likely through the activation of a src-family kinase (Herbst et al. 2002; Luo et al., 2002; Meyer and Wallace, 1998; Mittaud et al., 2001; Mohamed et al., 2001; Smith et

*Corresponding author. Tel: +1-401-863-9308; fax: +1-401-863-1074.
E-mail address: justin_fallon@brown.edu (J. Fallon).

Abbreviations: AChR, acetylcholine receptor; BAPTA, tetraacetoxymethyl-1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetate; BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetrakis(acetoxy-methyl) ester; BSA, bovine serum albumin; α-BTx, α-bungarotoxin; DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol-bis(2-aminoethyl)-*N*,*N*,*N*′,*N*′-tetraacetic acid; MEM-H, minimal essential media with HEPES; NMJ, neuromuscular junction; PBS, phosphate-buffered saline; PTP, protein tyrosine phosphatase; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SFM, serum free medium.

0306-4522/03$30.00+0.00 © 2003 IBRO. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/S0306-4522(03)00602-X

al., 2001). Agrin is also necessary, probably through a less direct route, for presynaptic differentiation and synapse-selective transcription (Burgess et al., 1999; De Chiara et al., 1996; Gautam et al., 1996).

Calcium plays at least two roles in agrin-induced AChR clustering. Extracellular calcium is necessary for agrin to activate MuSK, while intracellular calcium plays an integral role in the agrin signaling pathway (Borges et al., 2002; Megeath and Fallon, 1998). Clamping intracellular calcium concentrations at the time of agrin addition inhibits AChR cluster formation. Notably, these calcium fluxes are likely to act downstream of the early events in the agrin signaling cascade, since they are not required for agrin-induced MuSK activation (Borges et al., 2002 and see below). Moreover, in previous work we have shown that in chick myotubes agrin-induced AChR phosphorylation is unaffected by clamping of calcium transients (Megeath and Fallon, 1998; but see Borges et al., 2002 and discussion). Together, these studies suggest that agrin-induced calcium transients may serve as a control point for postsynaptic differentiation.

Loss of synapses is essential for the normal development of the nervous system and is likely to be an integral player in the activity-mediated shaping of synaptic circuitry in learning and memory. However, the mechanisms underlying synaptic loss are poorly understood. About half of the nerve–muscle synapses initially formed in the embryo are eliminated in the first weeks after birth (Nguyen and Lichtman, 1996). Synaptic pruning is a tightly regulated process involving activity-dependent competition between neighboring synapses on the same muscle cell. Interestingly, the loss of synaptic constituents is asynchronous, with the postsynaptic apparatus dispersing before obvious signs of presynaptic withdrawal (Balice-Gordon and Lichtman, 1993; Culican et al., 1998). Proposed molecular mediators of this dispersal include proteases (Harding et al., 1996; Liu et al., 1994), calcium (Akaaboune et al., 1999), protein kinase C (Lanuza et al., 2001), Rho-GTPases (Weston et al., 2000), dystroglycan (Heathcote et al., 2000) and protein tyrosine phosphatases (PTPs; Dai and Peng, 1998). In addition, AChR cluster formation is inhibited by trkB activation, suggesting a role for neurotrophin signaling in regulating synaptic architecture (Gonzalez et al., 1999; Wells et al., 1999).

In the present study, we investigated the mechanisms mediating the maintenance of agrin-induced postsynaptic specializations. We find that agrin-dependent intracellular calcium fluxes are necessary for AChR cluster stability on cultured myotubes. Moreover, clamping intracellular calcium after AChR clusters have formed causes rapid tyrosine dephosphorylation of both MuSK and AChRs. The effects of clamping intracellular calcium fluxes are counteracted by treatment with a tyrosine phosphatase inhibitor. These findings indicate that the mechanisms mediating the formation and the maintenance of agrin-induced AChR clusters are distinct, and further suggest an avenue by which postsynaptic stability can be regulated by signaling pathways involving agrin, MuSK, and intracellular calcium transients.

EXPERIMENTAL PROCEDURES

Myotube culture

Embryonic chick myotube cultures were prepared as previously described (Nastuk et al., 1991). Briefly, myoblasts from embryonic day 11 chick embryos were dissociated and plated in medium containing minimal essential medium (α medium; Gibco, Gaithersburg, MD, USA) supplemented with 2% chick embryo extract, 10% horse serum, 100 U/ml penicillin G, and 2 mM L-glutamine. For ligand binding, immunoprecipitations, and AChR phosphorylation assays, cells were grown on plastic coated with 100 μg/ml gelatin (Sigma, St. Louis, MO, USA). For AChR clustering assays and calcium imaging, cells were grown on glass coverslips coated with 20 μg/ml poly-D-lysine (Sigma; MW>300,000) and gelatin. Myotubes were used 3–4 days after plating.

Drug treatment

Stock solutions of the acetoxymethylester of BAPTA (tetraacetoxymethyl-1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetate; BAPTA-AM; Molecular Probes, Eugene, OR, USA) were prepared in dimethyl sulfoxide (DMSO; vehicle; see Megeath and Fallon, 1998). BAPTA-AM or vehicle was diluted in serum free medium (SFM) consisting of minimal essential medium (α medium; Gibco), 2 mM L-glutamine (Gibco), 0.5% bovine serum albumin (BSA), 100 U/ml penicillin G, 5 μg/L insulin, 5 μg/L transferrin, and 5 μg/L selenium (all from Sigma). To load cells with BAPTA, cells were incubated in 50 μM BAPTA-AM for 1 h at 37 °C (final DMSO concentration 0.5%), then rinsed with SFM. Cells were loaded with 50 μM BAPTA-AM except where otherwise noted.

Pervanadate was prepared as previously described (Wallace, 1995). One part 500 mM hydrogen peroxide (Mallinckrodt, Hazelwood, MO, USA) was added to 50 parts 10 mM Na orthovanadate (Sigma) in modified Tyrodes solution (145 mM NaCl, 5 mM KCl, 5.5 mM glucose, 40 μM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM HEPES pH 7.4). The mixture was incubated for 10 min at room temperature and diluted in SFM for a final concentration of 100 μM immediately before use.

Calcium imaging

To load cells with Fura-2, myotubes were incubated in 0.5 μM Fura-2-AM (Molecular Probes) for 1 h at room temperature and then rinsed in SFM. Acetylcholine (20 μM; Sigma) was applied to myotubes for 6 s via a pressure ejection micropipette system (General Valve; Parker Instruments, Fairfield, NJ, USA). Fluorescence measurements were made with a high speed dual wavelength imaging system (IonOptix, Milton, MA, USA) with a custom built image intensifier (Intevac, Santa Clara, CA, USA) and modified OEM CCD camera module (Philips Photonics, Slatersville, RI, USA). Calibrations were performed in solutions using standard techniques and adjusted to reflect the intracellular environment. Using either cell-free or with permeabilized cell systems we have found that this Fura-2 imaging system can readily detect calcium concentrations below the normal resting level of 100 nM. Thus, any reduction in resting calcium levels that may have been induced by BAPTA treatment would have been observed in our experiments.

To calculate resting calcium levels, fluorescence measurements were made for 2 s prior to the addition of acetylcholine. Two to five regions were randomly chosen on cells in a field and measurements from each field were averaged. Means from four to six fields were averaged.

AChR clustering assays

Recombinant rat agrin containing inserts of 12, four, and eight amino acids at the *x*, *y* and *z* splice sites, respectively, was

produced in COS cells as previously described (O'Toole et al., 1996). Recombinant agrin was used at a concentration of 25 pM in SFM.

Cells grown on coverslips were incubated with or without agrin for 4 h at 37 °C. Immediately after this incubation, cultures were rinsed twice in SFM and BAPTA treatment was then begun. Cultures were rinsed twice in fresh media 1 h after the addition of BAPTA-AM. In some experiments BAPTA treatment was preceded by 10–20 min in EGTA rinse to quantitatively remove any bound exogenous agrin (Bowe et al., 1994). The rates of AChR cluster dispersal did not change following this treatment, indicating that residual agrin was not influencing cluster stability (data not shown).

To detect AChRs, 1 μg/ml rhodamine conjugated α-bungarotoxin (α-BTx) was added to the media 1 h before each culture was fixed. At different time points coverslips were fixed in methanol at −20 °C for 5 min, mounted in Citifluor (Pella, Redding, CA, USA), and viewed on a Nikon Eclipse microscope. For quantitation of AChR clustering, 20–30 myotube segments (200 μm in length) were randomly chosen from two to three coverslips. AChR clusters (defined as AChR aggregates ≥4 μm in diameter) were scored under rhodamine optics (Nastuk and Fallon, 1991) at 400×. The numbers of clusters in each condition were normalized relative to the amount of clusters observed after cells were stimulated with agrin for 4 h.

Ligand binding assays

Binding of α-BTx to myotubes was quantitated as previously described (Bowe et al., 1994). Myotubes grown on gelatin-coated removable 96 well strips (Immulon 4; Dynatech, Chantilly, VA, USA) were blocked for 1 h in minimal essential media with HEPES (MEM-H) with 1% BSA and 10% horse serum, and incubated for 30 min with 10 nM ^{125}I-α-BTx (10–20 μCi/μg; DuPont NEN, Boston, MA, USA). Wells were washed in MEM-H, immersed twice in Hanks' balanced salt solution with 1% BSA and 1 mM calcium, dried and counted. Nonspecific binding was determined by including 1 mM EGTA (in agrin binding experiments) or 100-fold excess competing unlabeled α-BTx. In each experiment, six individual wells were counted for each condition and the results of multiple experiments were pooled.

AChR extraction and isolation

AChRs from cultured myotubes were purified according to the method of Wallace et al. (1991) with minor modifications. Biotinylated α-BTx (Molecular Probes) was purified on an ImmunoPure Immobilized Monomeric Avidin column (Pierce). Myotube cultures were incubated for 4 h in agrin, loaded with BAPTA or vehicle in the presence of 1 μg/ml biotinylated α-BTx, washed twice in cold phosphate-buffered saline (PBS) and harvested in extraction buffer (5 mM EDTA, 5 mM EGTA, 20 mM Tris, pH 7.5, 20 mM glycine, 1% Triton X-100, 150 mM NaCl, 40 mM Na pyrophosphate, 50 mM NaF, 10 mM Na molybdate, 1 mM Na orthovanadate, 5 mM benzamidine, 1 mg/ml bacitracin, 10 mM *N*-ethylmalemide, 1 mM phenylmethylsulfonyl fluoride [PMSF], and 50 μg/ml each chymostatin, pepstatin, aprotinin, leupeptin, and antipain). Cell lysates were briefly centrifuged and the cell pellet was resuspended in extraction buffer by trituration through a 23 gauge needle. Samples were sonicated 20 s with a Branson 450 Sonifier at maximum power, incubated 15 min at 4 °C, then spun for 20 min at 3000×*g*. Solubilized AChR-biotinylated α-BTx complexes were incubated with streptavidin-sepharose beads (Sigma) for 1.5–2 h with constant mixing at 4 °C. Beads were washed four times in extraction buffer containing 1 M NaCl, twice in extraction buffer lacking NaCl and Triton X-100, and eluted in reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer at 60 °C for 10 min.

Isolated AChRs were electrophoresed on 5–15% gradient SDS-polyacrylamide gels and transferred to nitrocellulose. The blots were then blocked in PBS supplemented with 1% BSA. Tyrosine phosphorylated polypeptides were detected with anti-phosphotyrosine mAb 4G10 (diluted 1:1000; Upstate Biotechnology Inc., Lake Placid, NY, USA). After incubation with primary antibody, blots were washed in PBS, incubated for 2 h with horseradish peroxidase-linked sheep anti-mouse IgG (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA), and immunolabeled bands were visualized with enhanced chemiluminescence. Blots were prepared for successive Western blotting according to the method of Sugiyama et al. (1997). The blots were liberally rinsed in PBS, stripped with 0.2 M glycine, pH 2.5, and 0.1% Tween 20, then reprobed with mAb 61 (generously provided by J. Lindstrom, University of Pennsylvania) to detect AChR β-subunits. Bound antibodies were visualized using alkaline phosphatase conjugated goat anti-mouse IgG (Boehringer Mannheim, Indianapolis, IN, USA) and an alkaline phosphatase-based kit (Promega, Madison, WI, USA).

MuSK extraction and isolation

Myotube cultures were stimulated with agrin for 4 h, loaded with BAPTA, EGTA, or vehicle, washed twice in cold PBS with 1 mM Na orthovanadate and harvested in scrape buffer (50 mM Tris–HCl pH 7.4, 0.15 M NaCl, 0.1 M NaF, 10 mM Na pyrophosphate, 2 mM EDTA, 2 mM EGTA, 1 mM Na orthovanadate, 1 mM benzamidine, 0.1 mg/ml PMSF, 21 μg/ml aprotinin, and 1 μg/ml each leupeptin and pepstatin A). Cell lysates were spun briefly, resuspended by trituration through a 23 gauge needle in lysis buffer (scrape buffer supplemented with 10% glycerol and 1% Triton X-100), incubated for 20 min at 4 °C with gentle mixing and spun for 22 min at 16,000×*g*. Either rabbit antisera pAb Cyt-MuSK or pAb Ecto-MuSK (Hopf and Hoch, 1998a,b) was then added to the supernatants. Except where otherwise noted, pAb Cyt-MuSK was used throughout. Cell extracts were mixed with protein A beads (RepliGen Corporation, Cambridge, MA, USA) for 1 h, the beads washed with lysis buffer, and the immunoprecipitates eluted in reducing SDS-PAGE sample buffer at 60 °C for 10 min. Samples were probed with mAb 4G10 or anti-MuSK antibodies (kindly provided by David Glass, Regeneron Pharmaceuticals, Tarrytown, NY, USA) by Western blotting as described above with modifications. Secondary antibodies, goat anti-mouse IgG conjugated to alkaline phosphatase (Boehringer Mannheim), were preadsorbed by incubation with 10% normal rabbit serum (JR Scientific) overnight with agitation, followed by centrifugation (30 min, 16,000×*g*). Immunolabeled bands were visualized using alkaline phosphatase or chemiluminescence.

RESULTS

Clamping intracellular calcium fluxes accelerates AChR cluster dispersal

Both agrin binding to cells and agrin-induced AChR clustering require extracellular calcium (Nastuk et al., 1991; Wallace, 1988). Therefore, to selectively manipulate intracellular calcium fluxes we used BAPTA, a rapid, high affinity calcium buffer (Kd approximately 100–180 nM; Deisseroth et al., 1996; Megeath and Fallon, 1998; Roberts, 1993; Stern, 1992). Myotubes were loaded with BAPTA via its AM ester form (see Experimental Procedures). To directly assess the effects of BAPTA treatment we measured intracellular calcium levels using Fura-2 ratiometric imaging. As expected, resting intracellular calcium concentrations in BAPTA-loaded cells were indistinguishable from control (vehicle only) myotubes (105%±3.1 of control; un-

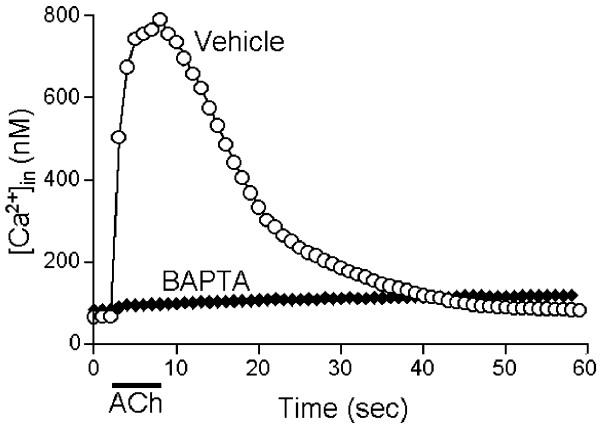

Fig. 1. Intracellular calcium fluxes are clamped in BAPTA-loaded myotubes. Cultured myotubes were loaded with either BAPTA or vehicle as described in Experimental Procedures. Intracellular calcium concentrations were monitored by Fura-2 imaging. A 6 s pulse of acetylcholine (ACh; bar) was applied using a pressure pipette. In vehicle-treated cells (○), ACh caused a rapid, transient increase in intracellular calcium. In contrast, ACh-induced intracellular calcium fluxes are greatly diminished in BAPTA-loaded cells (◆). Representative traces are presented. Similar results were obtained in seven other experiments.

paired *t*-test, $P>0.05$; $N=4$). To test the efficacy of BAPTA treatment, we used acetylcholine pulses to evoke intracellular calcium fluxes. Acetylcholine-induced intracellular calcium fluxes were abolished in BAPTA-treated cells (Fig. 1). Thus, BAPTA clamps intracellular calcium concentrations in cultured myotubes without disrupting resting levels of this ion. We also used Fura imaging to test the stability of BAPTA in myotubes. Complete inhibition was observed up to 2 h post-loading. Robust inhibition was still observed at least 4 h after loading, where nearly 80% of the calcium response was blocked (78%±6.7%).

To test the role of intracellular calcium fluxes in the maintenance of AChR clusters, myotubes were first stimulated with agrin for 4 h to induce cluster formation. Agrin was then washed out and the number of AChR clusters per myotube was counted at successive time points. Clusters dispersed gradually in control cultures (Fig. 2). Twenty percent fewer clusters were observed after 1 h and half the clusters ($t_{1/2}$) dissipated in approximately 3 h. In contrast, clamping intracellular calcium fluxes with BAPTA markedly accelerated AChR cluster dispersal. The $t_{1/2}$ was <1 h and virtually all the clusters dispersed within 2 h of agrin washout. Loading cells with increasing concentrations of BAPTA-AM resulted in increasing rates of AChR cluster dispersal (Fig. 2F). As in our previous studies (Megeath and Fallon, 1998), no effects of BAPTA loading on cell morphology or viability were observed at any of the tested concentrations.

The rapid loss of AChR clusters following clamping of intracellular calcium fluxes could be the result of either AChR internalization or diffusion of AChRs away from the cluster. To distinguish between these possibilities, we used ^{125}I-α-BTx binding to determine the levels of surface AChRs in cells stimulated with agrin and then treated with BAPTA or vehicle for 1 h. Surface AChR levels were not significantly altered in BAPTA- or vehicle-treated cells (93%±9 and 81%±12, respectively, where 100% is the level of AChRs measured immediately after 4 h of agrin treatment; $P>0.05$, two-way ANOVA; $N=3$). In addition, neither BAPTA treatment nor vehicle altered surface AChR levels in cells that had not been stimulated with agrin (91%±13 and 91%±16, respectively; $N=3$). Thus, the redistribution of surface AChR is the most likely explanation for the accelerated dispersal of AChR clusters induced by clamping intracellular calcium.

We next asked whether the accelerated AChR cluster destabilization occurred in the face of persistent agrin stimulation. In previous studies we showed that BAPTA treatment did not alter the level of agrin binding to the cell surface (Megeath and Fallon, 1998). Myotubes were stimulated with agrin for 4 h and then the cells were treated with BAPTA in the continued presence of agrin. AChR clusters rapidly disperse under these conditions, with a $t_{1/2}$ of approximately 1 h (Fig. 2G). These findings suggest that the accelerated destabilization results from the modulation of intracellular signaling pathways.

The role of intracellular calcium fluxes in MuSK and AChR phosphorylation and dephosphorylation

Agrin-induced MuSK phosphorylation is an early and essential step in the AChR clustering pathway. Since previous studies employed rodent myotubes, we developed an assay for MuSK tyrosine phosphorylation in chick muscle cells. We tested two antisera directed against the intracellular and the extracellular domains of rat MuSK (pAb Ecto-MuSK and pAb Cyt-MuSK, respectively; Hopf and Hoch, 1998a,b). Both antisera specifically immunoprecipitated a tightly spaced, tyrosine phosphorylated doublet centered at approximately 110 kD (Fig. 3). This mobility is similar to that observed for mammalian MuSK (Fuhrer et al., 1997; Glass et al., 1996; Jacobson et al., 1998). Although others have reported a relative mobility of approximately 140 kD for avian MuSK expressed in C2C12 cells (Glass et al., 1996), we did not detect any candidate MuSK polypeptides with this mobility. The differences between the reported mobility of avian MuSK and our results could be due to the gel systems used or to posttranslational modifications unique to C2C12 cells. Nonetheless, two different anti-MuSK antisera specifically immunoprecipitate an approximately 110 kD doublet that is tyrosine phosphorylated in response to agrin stimulation. We therefore conclude that this polypeptide doublet is avian MuSK.

In previous work we showed that blocking intracellular calcium fluxes inhibits agrin-induced AChR cluster formation but not agrin-stimulated AChR tyrosine phosphorylation (see Megeath and Fallon, 1998; and Fig. 3B). Since phosphorylation of MuSK precedes that of AChRs, this observation suggested that agrin-induced MuSK phosphorylation might also be insensitive to blockade of calcium transients. To test this prediction, cells were first treated with either BAPTA or vehicle and then incubated with agrin. As shown in Fig. 3B, agrin induced robust phosphorylation of MuSK and AChR β-subunits in both control and BAPTA-treated cells. In agreement with these

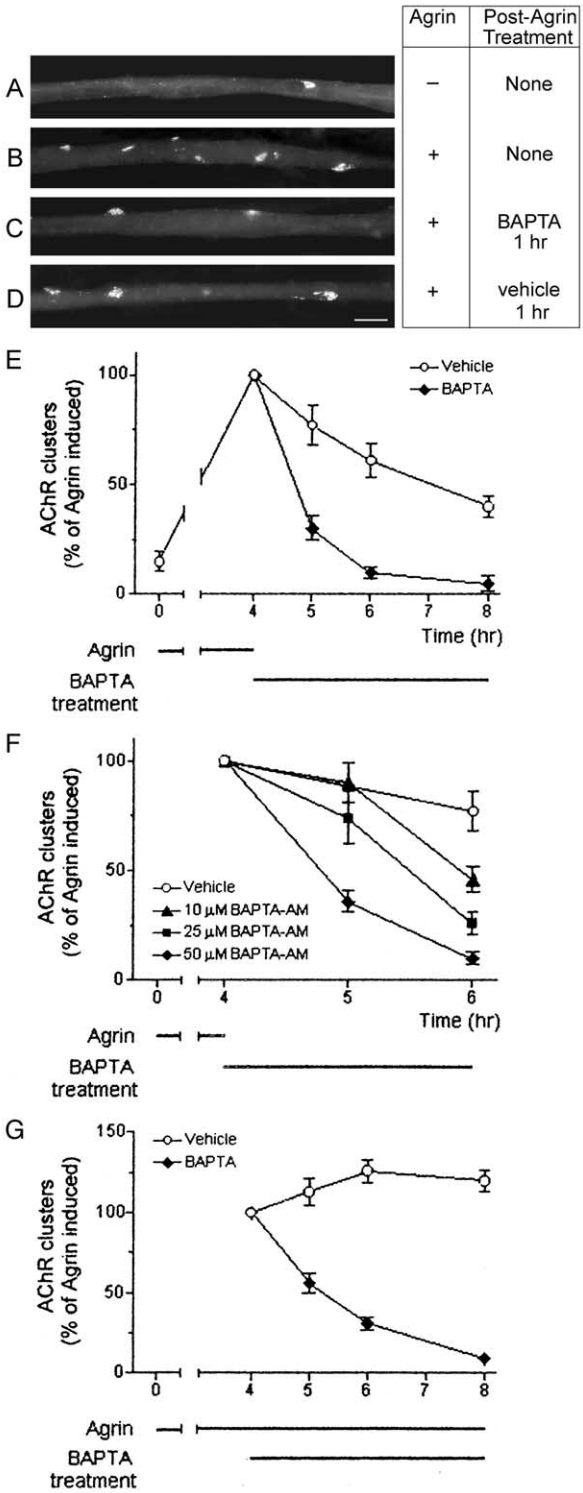

Fig. 2. Blocking intracellular calcium fluxes accelerates AChR cluster dispersal. (A–D) Cultured myotubes were stimulated with agrin as indicated. In (A) and (B) the cells were fixed immediately after the 4 h incubation. In (C) and (D) the agrin was washed out after 4 h and the cells incubated for an additional 1 h in the presence of BAPTA or vehicle alone. AChRs were labeled with rhodamine α-BTx and their distribution determined by fluorescence microscopy as described in Experimental Procedures. Note the reduction in the number of AChR clusters in the BAPTA-treated cells. Scale bar=20 μm. (E) Time course of dispersal. Myotubes were incubated with agrin for 4 h to induce AChR clustering. The agrin was then washed out and the cultures treated as indicated. AChR clusters were then counted as described in Experimental Procedures. In control cells AChR clusters gradually disperse following agrin withdrawal ($t_{1/2}$ approximately 3 h). Clamping intracellular calcium concentrations with BAPTA accelerates the rate of cluster dispersal ($t_{1/2}$<1 h). Values are mean±S.E.M. averaged from three separate experiments. (F) Dispersal of AChR clusters in cells loaded with varying concentrations of BAPTA-AM. After a 4 h agrin incubation, the agrin was removed and cells were treated with the indicated concentrations of BAPTA-AM. The most rapid dispersal is observed in cells loaded with 50 μM BAPTA-AM, the concentration used for all other experiments. Mean±S.E.M. (G) Agrin treatment does not prevent the destabilization of AChR clusters induced by BAPTA treatment. After 4 h of agrin incubation, BAPTA-AM is added to the agrin-containing media, causing rapid dispersal of AChR clusters. Mean±S.E.M.

findings, Borges et al. (2002) also observed normal levels of agrin induced MuSK phosphorylation in BAPTA-treated C2C12 cells. These data indicate that clamping intracellular calcium fluxes does not in itself inhibit agrin-induced MuSK tyrosine kinase activity. Moreover, these results place MuSK activation either upstream or parallel to the intracellular calcium-dependent steps in the agrin signaling pathway.

We next examined the relationship between AChR cluster dispersal and MuSK tyrosine phosphorylation. Myotubes were stimulated with agrin for 4 h. In one set of experiments the agrin was washed out and the level of MuSK phosphorylation was measured either immediately or after 1 h additional incubation in BAPTA or vehicle. MuSK phosphorylation declined 44%±9 in control cells but decreased 73%±10 in BAPTA-loaded myotubes relative to the level of agrin-induced phosphorylation at 4 h (average of four experiments; Fig. 3C). In other experiments BAPTA was added at 4 h but the agrin was not washed out. Remarkably, MuSK was also dephosphorylated (average decrease of 44%±8 in two experiments) in the BAPTA-loaded cells, despite the continued presence of agrin (Fig. 3D). Notably, the degree of MuSK dephosphorylation under both conditions correlated closely with the extent of AChR cluster dispersal (compare Fig. 2E to 3C and Fig. 2G to 3D). Thus, clamping intracellular calcium fluxes following the formation of AChR clusters results in rapid MuSK dephosphorylation.

A later step in the agrin signaling pathway is the tyrosine phosphorylation of AChR β-subunits. In previous work we showed that the initial agrin-induced tyrosine phosphorylation of AChR was unaffected by BAPTA treatment (Megeath and Fallon, 1998). To test whether intracellular calcium transients play a role in maintaining AChR tyrosine phosphorylation, myotubes were treated with agrin for 4 h and then loaded with BAPTA or vehicle as described above. In vehicle-treated cells the level of AChR β-subunit tyrosine phosphorylation was only slightly diminished 1 h after agrin withdrawal (Fig. 4; compare lanes 1 and 4). In contrast, after 1 h of BAPTA treatment phosphotyrosine was undetectable on the AChR β-subunit (Fig. 4, lane 5). Similar results were observed when agrin was

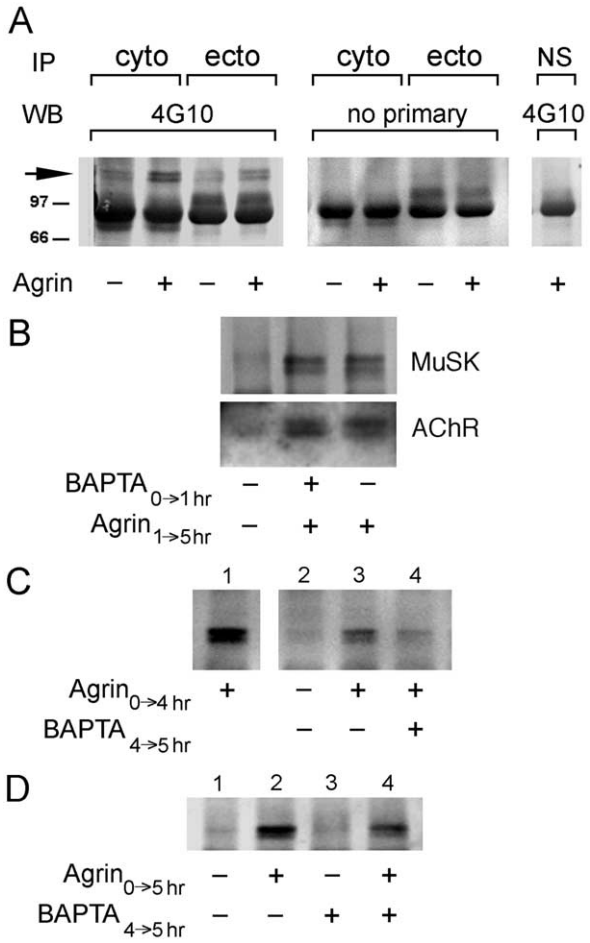

Fig. 3. MuSK is rapidly dephosphorylated when calcium fluxes are clamped after agrin stimulation. Primary chick myotubes were stimulated with agrin and then treated with BAPTA and vehicle as indicated. The cell extracts were then immunoprecipitated and probed with anti-phosphotyrosine antibody mAb 4G10. (A) Identification of avian MuSK. Two different antisera to rodent MuSK (pAb Ecto-MuSK and pAb Cyt-MuSK, directed against the extracellular (ecto) and intracellular (cyto) domains of MuSK, respectively) specifically immunoprecipitate a tyrosine phosphorylated polypeptide doublet of approximately 110 kD (arrow) from cultured chick myotubes. This doublet was not immunoprecipitated by normal rabbit serum (NS). Agrin (25 pM agrin for 15 min) increased the tyrosine phosphorylation of the doublet. The band at approximately 90 kD is non-specific. No specific signal was detected in the absence of mAb 4G10 (no primary). (B) Initial agrin-induced MuSK and AChR phosphorylation are insensitive to BAPTA treatment. Myotubes were loaded with either BAPTA or vehicle for 1 h, then stimulated with agrin for 4 h. Agrin induced comparable levels of MuSK phosphorylation in both BAPTA- and vehicle-treated cells. Similar results were observed with phosphorylation of the AChR β-subunit (see also Megeath and Fallon, 1998). (C) MuSK dephosphorylation is accelerated when intracellular fluxes are clamped *after* agrin-induced AChR clusters have formed. Myotubes were incubated in the presence or absence of agrin for 4 h. Cells were either assayed immediately (lane 1), or washed and treated for 1 h with vehicle (lanes 2, 3) or BAPTA (lane 4). MuSK phosphorylation declined 44%±9 in control cells but 73%±10 in BAPTA-loaded myotubes. Similar results were observed in three other experiments. (D) MuSK dephosphorylation following BAPTA treatment in the continued presence of agrin. Myotubes were incubated in the presence or absence of agrin for 5 h, with BAPTA treatment during the last hour as indicated. MuSK phosphorylation decreased 44%±8 (relative to agrin-only controls at 5 h; average of two experiments) in BAPTA-treated cells.

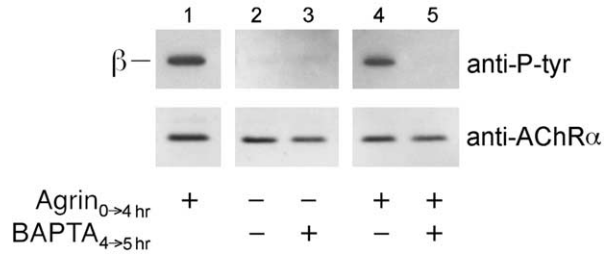

Fig. 4. AChRs are rapidly dephosphorylated when calcium fluxes are clamped after agrin-induced AChR clusters have formed. Myotubes were treated with or without agrin for 4 h. The cultures were then washed and the levels of AChR β-subunit tyrosine phosphorylation was then assessed immediately (lane 1) or following a 1 h treatment with BAPTA or vehicle as indicated (lanes 2–5). In vehicle-treated cells, agrin-induced AChR β-subunit tyrosine phosphorylation remained elevated 1 h hour after agrin withdrawal (compare lanes 1 and 4). In contrast, tyrosine phosphorylation of AChR β-subunit was undetectable following 1 h of BAPTA treatment (lane 5). The basal level of AChR tyrosine phosphorylation was unchanged by BAPTA treatment alone (compare lanes 2 and 3). AChR β-subunit tyrosine phosphorylation was assessed as described in Experimental Procedures. Equivalent loading of samples was verified by stripping the blots and reprobing with antibodies specific for the AChR α-subunit (bottom panel).

present throughout the experiment (data not shown). Thus, clamping intracellular calcium fluxes after agrin stimulation results in the dephosphorylation of AChR β-subunits.

Tyrosine phosphatase inhibitor treatment retains MuSK phosphorylation and AChR clusters

The results reported above show that the consequences of clamping intracellular calcium fluxes correlate with the state of AChR clustering. If AChRs (and presumably MuSK) are not clustered, clamping calcium fluxes does not perturb agrin-induced tyrosine phosphorylation of MuSK or AChR. On the other hand, both MuSK and AChR are rapidly dephosphorylated if calcium transients are clamped after AChR clusters have formed. These observations suggest that there may be a link between agrin-induced intracellular calcium transients and a tyrosine kinase or phosphatase.

To probe further for such a link we used the protein tyrosine phosphatase inhibitor pervanadate. Treatment with pervanadate alone did not alter the rate of AChR cluster dispersal (Fig. 5a; see also Wallace, 1988). However, pervanadate treatment blunted the BAPTA-accelerated dispersal of AChR clusters. Next, we assayed MuSK phosphorylation in BAPTA or vehicle-loaded cells. We observed that pervanadate inhibited MuSK dephosphorylation in BAPTA-treated cells (Fig. 5B). This effect was robust and reproducible. Quantification of five separate experiments showed that over 70% of agrin-induced MuSK phosphorylation was sustained in cells treated with both pervanadate and BAPTA. These results indicate that MuSK phosphorylation is regulated by the activity of a tyrosine phosphatase as well as by intracellular calcium fluxes.

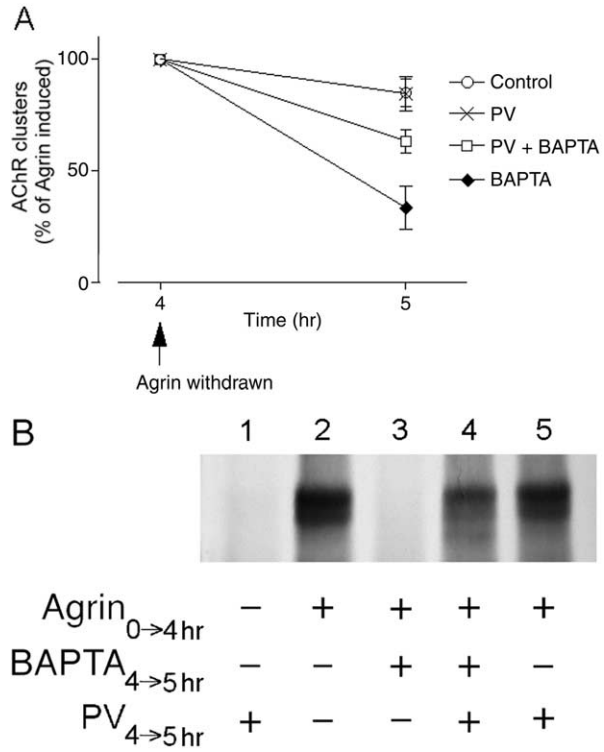

Fig. 5. MuSK is a substrate of a calcium-regulated PTP that regulates AChR dispersal. (A) A PTP inhibitor occludes BAPTA-induced AChR dispersal. Myotubes were stimulated with agrin for 4 h. The agrin was then washed out and the cells were treated with vehicle (control), pervanadate (100 μM), BAPTA, or pervanadate and BAPTA. Pervanadate alone did not alter the number of AChR clusters remaining 1 h post-agrin. However, pervanadate partially inhibited the dispersal of AChR clusters induced by BAPTA treatment. Values are mean±S.E.M. averaged from three separate experiments. (B) MuSK is a substrate of a calcium-regulated PTP. Pervanadate treatment rescues BAPTA-induced MuSK dephosphorylation. Myotubes were incubated with agrin or control media for 4 h and then treated for 1 h with BAPTA, pervanadate, and/or vehicle as indicated. Cell extracts were then immunoprecipitated with anti-MuSK and Western blotting was performed with anti-phosphotyrosine mAb 4G10. Pervanadate treatment had no effect on MuSK phosphorylation (lane 1 and compare lanes 2 and 5) while agrin induced robust MuSK phosphorylation (lane 2). The agrin-induced MuSK phosphorylation was reduced to baseline levels when cells were treated with BAPTA alone (lane 3). In contrast, MuSK phosphorylation remained high when cells were treated with a combination of pervanadate and BAPTA (compare lanes 2 and 4). (C) Quantification of MuSK phosphotyrosine levels from five separate experiments. Values were normalized to the agrin only condition, then averaged. Mean±S.E.M.

DISCUSSION

The central finding of this work is that AChR cluster maintenance is an active process mediated by agrin-induced intracellular calcium fluxes. Our results also indicate that distinct molecular mechanisms underlie the formation and the dispersal of agrin-induced AChR clusters. Moreover, these observations demonstrate that the modulation of intracellular signaling pathways can regulate the maintenance and dispersal of AChR clusters independently of MuSK activation by agrin. These findings suggest mechanisms that could mediate synapse formation and elimination *in vivo*.

A key technique in these experiments was the use of BAPTA to clamp intracellular calcium concentrations. This reagent was designed to be a specific and rapid calcium chelator (Tsien, 1980) and experiments in a wide array of cell systems have confirmed these characteristics (Deisseroth et al., 1996; Roberts, 1993; Whitlock and Lamb, 1999). Several lines of evidence indicate that this compound specifically blocks rapid intracellular calcium fluxes in myotubes and is not toxic to these cells. 1) Fura-2 imaging confirmed BAPTA's calcium buffering activity in chick myotubes (Fig. 1). 2) BAPTA treatment did not change the resting concentration of intracellular calcium. 3) The effects of BAPTA treatment are reversible (Megeath and Fallon, 1998). 4) The action of BAPTA is selective: while this compound suppressed agrin-induced AChR cluster formation, it did not inhibit the agrin-induced tyrosine phosphorylation of MuSK or the AChR β-subunit (Fig. 3 and Megeath et al., 1998). 5) None of the effects reported here were observed when EGTA, a calcium buffering compound with slower binding kinetics, was loaded into the myotubes (unpublished observations).

The dephosphorylation of clustered MuSK and AChR following calcium buffering could be due to either inhibition of kinase activity or to increased PTP activity. Several lines of evidence suggest a role for a PTP. First, BAPTA treatment of naive myotubes has no effect on agrin-induced MuSK or AChR tyrosine phosphorylation. Thus clamping intracellular calcium does not affect directly the tyrosine kinase activity of either MuSK or the enzyme that phosphorylates AChRs (which is likely to be distinct from MuSK; Fuhrer et al., 1997; Mittaud et al., 2001; Mohamed et al., 2001; Smith et al., 2001). Second, the rapid dephosphorylation of clustered MuSK and AChR in BAPTA-treated cells is observed even in the continued presence of agrin (Figs. 3 and 4). Moreover, previous work has shown that agrin binding to the cell surface is not altered by BAPTA treatment (Megeath and Fallon, 1998). Finally, the PTP inhibitor pervanadate inhibits both the MuSK dephosphorylation and the AChR dispersal observed when calcium transients are clamped.

An alternative model is that tyrosine kinase(s) may be stimulated by agrin to phosphorylate MuSK via a mechanism that is dependent upon intracellular calcium transients. Although the tyrosine phosphatase inhibitor pervanadate counteracts the effects of BAPTA treatment, these results by themselves do not conclusively establish PTP involvement in this pathway. In some cases pervanadate has been shown to inhibit, by indirect means, tyrosine kinase activity (Park et al., 2001). However, this alternative model must account for our observations that: 1) the level of MuSK phosphorylation decreases only when cells are treated with BAPTA *after* AChR clusters have formed; and 2) this dephosphorylation occurs even in the continued presence of agrin. Thus, this second model would require not only the presence of a calcium-dependent tyrosine kinase at AChR clusters, but also the selective inhibition of agrin-induced MuSK tyrosine kinase activity after cluster formation. Since there is no evidence for such inhibition, we favor the simpler model involving increases in PTP activity.

Dai and Peng (1998) also reported evidence for PTP activity in the dispersal pathway. In those experiments pervanadate was shown to inhibit AChR cluster dispersal, while injection of a constitutively active PTP drove their dispersal. They proposed a model that includes a ubiquitous PTP that serves as a sink for signals derived from localized tyrosine kinases. We observe that pervanadate alone did not alter the rate of AChR cluster dispersal (see also Wallace, 1995). At this point we do not know the basis for these differences. One explanation could be the different methods of AChR cluster induction in the two studies (HB-GAM-coated beads vs. agrin). In our view the data presented here best fit a model where both tyrosine kinases and phosphatases are localized to agrin-induced AChR clusters. Resolution of these questions will require the identification and localization of the specific PTP(s) involved in this process.

Pervanadate treatment only partially reversed BAPTA-induced AChR cluster dispersal and MuSK phosphorylation (Fig. 5). Although we suspect that this partial effect reflects incomplete phosphatase inhibition, it remains possible that additional (non-PTP) dispersal mechanisms could also be at work. Activity of the tyrosine phosphatases might not be totally blocked by pervanadate concentrations used (100 μM); however, we were not able to increase this dose since toxic effects were observed at higher concentrations. Moreover, we note that none of the available anti-MuSK antisera recognize chick MuSK on Western blots. Thus direct quantification of MuSK levels in the immunoprecipitates was not possible. Nonetheless it should be emphasized that the ability of pervanadate to inhibit MuSK dephosphorylation in BAPTA-treated cells (post agrin) was observed in five separate experiments and was statistically significant (Fig. 5C).

One implication of the phosphatase model is that elimination of AChR clusters may not occur via reversing the formation pathway. Rather, the events regulating AChR cluster formation appear to be distinct from the mechanism of maintenance and dispersal. Cluster formation relies upon increased kinase activity whereas cluster dissipation may require PTP activation. It is interesting to consider how synaptic architecture might be sculpted by balanced regulation of these kinases and PTPs. In addition, muscle activity results in influx of calcium through both the AChR and voltage-gated channels. Indeed, blockade of AChR activation results in rapid dispersal of AChRs in adult muscle (Akaaboune et al., 1999).

Important directions for future work are the identification of PTPs and kinases regulated by both calcium and agrin, and characterization of the intracellular calcium fluxes. Our attempts to visualize agrin-induced intracellular calcium transients with Fura-2 imaging have been unsuccessful, possibly because such calcium fluxes are highly localized or beyond the resolution of our methods (Megeath and Fallon, 1998). Candidate PTPs include the synapse-enriched non-receptor PTP from *Torpedo* electric organ that shows high specificity for AChR β-subunits (Mei and Huganir, 1991) and PTPs activated by binding phosphotyrosine residues on receptor tyrosine kinases, such as SHP-2 (Stein-Gerlach et al., 1998). Furthermore, it will be of interest to examine which tyrosine residues of MuSK will be important for cluster dissolution (Herbst and Burden, 2000).

Finally, there could be a mechanistic connection between the dispersal of AChR clusters observed *in vitro* and the selective postnatal synaptic elimination observed during development of mammalian muscle (Nguyen and Lichtman, 1996; Sanes and Lichtman, 1999). Studies of synapse elimination *in vivo* have revealed that the dispersal of AChR clusters occurs prior to withdrawal of presynaptic boutons (Balice-Gordon and Lichtman, 1993; Rich and Lichtman, 1989) and that synaptic maintenance is activity-dependent (Nguyen and Lichtman, 1996). We suggest that synapse disassembly may require tyrosine kinases and phosphatases situated at every NMJ. Such localized effectors could be regulated by synaptic activity, extracellular factors like agrin and by intracellular signals. In our studies the protective effect of agrin could be overridden by BAPTA treatment, and similarly an activity-driven signal from one synapse may destabilize a neighboring synapse *in vivo*.

Acknowledgements—J.R.F. was supported by grants from the National Institutes of Health (HD23924). M.T.K. was supported by NIH DK 47223. We thank J. Lindstrom and David Glass for generous gifts of antibodies. We also thank Beth McKechnie and Pamela Jo Zabel for expert technical assistance.

REFERENCES

Ango F, Robbe D, Tu JC, Xiao B, Worley PF, Pin JP, Bockaert J, Fagni L (2002) Homer-dependent cell surface expression of metabotropic glutamate receptor type 5 in neurons. Mol Cell Neurosci 20:323–239.

Akaaboune M, Culican SM, Turney SG, Lichtman JW (1999) Rapid and reversible effects of activity on acetylcholine receptor density at the neuromuscular junction in vivo. Science 286:503–507.

Apel ED, Glass DJ, Moscoso LM, Yancopolous GD, Sanes JR (1997) Rapsyn is required for MuSK signaling and recruits synaptic components to a MuSK-containing scaffold. Neuron 18:623–635.

Balice-Gordon RJ, Lichtman JW (1993) In vivo observations of pre- and postsynaptic changes during the transition from multiple to single innervation at developing neuromuscular junctions. J Neurosci 13:834–855.

Borges LS, Lee Y, Ferns M (2002) Dual role for calcium in agrin signaling and acetylcholine receptor clustering. J Neurobiol 50:69–79.

Bowe MA, Deyst KA, Leszyk JD, Fallon JR (1994) Identification and purification of an agrin receptor from *Torpedo* postsynaptic membranes: a heteromeric complex related to the dystroglycans. Neuron 12:1173–1180.

Bowen DC, Park JS, Bodine S, Stark JL, Valenzuela DM, Stitt TN, Yancopoulos GD, Lindsay RM, Glass DJ, DiStefano PS (1998) Localization and regulation of MUSK at the neuromuscular junction. Dev Biol 199:309–319.

Burgess RW, Nguyen QT, Son YJ, Lichtman JW, Sanes JR (1999) Alternatively spliced isoforms of nerve- and muscle-derived agrin: their roles at the neuromuscular junction. Neuron 23:33–44.

Coleman SK, Cai C, Mottershead DG, Haapalahti JP, Keinanen K (2003) Surface expression of GluR-D AMPA receptor is dependent on an interaction between its C-terminal domain and a 4.1 protein. J Neurosci 23:798–806.

Culican SM, Nelson CC, Lichtman JW (1998) Axon withdrawal during synapse elimination at the neuromuscular junction is accompanied by disassembly of the postsynaptic specialization and withdrawal of Schwann cell processes. J Neurosci 18:4953–4965.

Dai Z, Peng HB (1998) A role of tyrosine phosphatase in acetylcholine receptor cluster dispersal and formation. J Cell Biol 141:1613–1624.

De Chiara TM, Bowen DC, Valenzuela DM, Simmons MV, Poueymirou WT, Thomas S, Kinetz E, Compton DL, Rojas E, Park JS, Smith C, DiStefano PS, Glass DJ, Burden SJ, Yancopoulos GD (1996) The receptor tyrosine kinase MuSK is required for neuromuscular junction formation in vivo. Cell 85:501–512.

Deisseroth K, Bito H, Tsien RW (1996) Signaling from synapse to nucleus: postsynaptic CREB phosphorylation during multiple forms of hippocampal synaptic plasticity. Neuron 16:89–101.

Fuhrer C, Sugiyama JE, Taylor RG, Hall ZW (1997) Association of muscle-specific kinase MuSK with the acetylcholine receptor in mammalian muscle. EMBO J 16:4951–4960.

Gautam M, Noakes PG, Moscoso L, Rupp F, Scheller RH, Merlie JP, Sanes JR (1996) Defective neuromuscular synaptogenesis in agrin-deficient mutant mice. Cell 85:525–535.

Gautam M, Noakes PG, Mudd J, Nichol M, Chu GC, Sanes JR, Merlie JP (1995) Failure of postsynaptic specialization to develop at neuromuscular junctions of rapsyn-deficient mice. Nature 377:232–236.

Glass DJ, Bowen DC, Stitt TN, Radziejewski C, Bruno J, Ryan TE, Gies DR, Shah S, Mattsson K, Burden SJ, Distefano PS, Valenzuela DM, Dechiara TM, Yancopoulos GD (1996) Agrin acts via a MuSK receptor complex. Cell 85:513–523.

Gonzalez M, Ruggiero FP, Chang Q, Shi YJ, Rich MM, Kraner S, Balice-Gordon RJ (1999) Disruption of Trkb-mediated signaling induces disassembly of postsynaptic receptor clusters at neuromuscular junctions. Neuron 24:567–583.

Hack I, Koulen P, Peichl L, Brandstatter JH (2002) Development of glutamatergic synapses in the rat retina: the postnatal expression of ionotropic glutamate receptor subunits. Vis Neurosci 19:1–13.

Harding DI, Greensmith L, Connold AL, Vrbova G (1996) Stabilizing neuromuscular contacts increases motoneuron survival after neonatal nerve injury in rats. Neuroscience 70:799–805.

Heathcote RD, Ekman JM, Campbell KP, Godfrey EW (2000) Dystroglycan overexpression in vivo alters acetylcholine receptor aggregation at the neuromuscular junction. Dev Biol 227:595–605.

Heck WL, Slusarczyk A, Basaraba AM, Schweitzer L (2002) Subcellular localization of GABA receptors in the central nervous system using post-embedding immunohistochemistry. Brain Res Brain Res Protoc 9:173–180.

Herbst R, Avetisova E, Burden SJ (2002) Restoration of synapse formation in MuSK mutant mice expressing a MuSK/Trk chimeric receptor. Development 129:5449–5460.

Herbst R, Burden SJ (2000) The juxtamembrane region of MuSK has a critical role in agrin-mediated signaling. EMBO J 19:67–77.

Hopf C, Hoch W (1998a) Dimerization of the muscle-specific kinase induces tyrosine phosphorylation of acetylcholine peceptors and their aggregation on the surface of myotubes. J Biol Chem 273: 6467–6473.

Hopf C, Hoch W (1998b) Tyrosine phosphorylation of the muscle-specific kinase is exclusively induced by acetylcholine receptor-aggregating agrin fragments. Eur J Biochem 253:382–389.

Jacobson C, Montanaro F, Lindenbaum M, Carbonetto S, Ferns M (1998) alpha-Dystroglycan functions in acetylcholine receptor aggregation but is not a coreceptor for agrin-MuSK signaling. J Neurosci 18:6340–6348.

Jones MA, Werle MJ (2000) Nitric oxide is a downstream mediator of agrin-induced acetylcholine receptor aggregation. Mol Cell Neurosci 16:649–660.

Lanuza MA, Garcia N, Santafe M, Nelson PG, Fenoll-Brunet MR, Tomas J (2001) Pertussis toxin-sensitive G-protein and protein kinase C activity are involved in normal synapse elimination in the neonatal rat muscle. J Neurosci Res 63:330–340.

Lichtman JW, Colman H (2000) Synapse elimination and indelible memory. Neuron 25:269–278.

Lieth E, Fallon JR (1993) Muscle agrin: neural regulation and localization at nerve-induced acetylcholine receptor clusters. J Neurosci 13:2509–2514.

Liu Y, Fields RD, Fitzgerald S, Festoff BW, Nelson PG (1994) Proteolytic activity, synapse elimination, and the Hebb synapse. J Neurobiol 25:325–335.

Luo ZG, Wang Q, Zhou JZ, Wang J, Luo Z, Liu M, He X, Wynshaw-Boris A, Xiong WC, Lu B, Mei L (2002) Regulation of AChR clustering by Dishevelled interacting with MuSK and PAK1. Neuron 35:489–505.

McMahan UJ, Wallace BG (1989) Molecules in basal lamina that direct the formation of synaptic specializations at neuromuscular junctions. Dev Neurosci 11:227–247.

Megeath LJ, Fallon JR (1998) Intracellular calcium regulates agrin-induced acetylcholine receptor clustering. J Neurosci 18:672–678.

Mei L, Huganir RL (1991) Purification and characterization of a protein tyrosine phosphatase which dephosphorylates the nicotinic acetylcholine receptor. J Biol Chem 266:16063–16072.

Meyer G, Wallace BG (1998) Recruitment of a nicotinic acetylcholine receptor mutant lacking cytoplasmic tyrosine residues in its beta subunit into agrin-induced aggregates. Mol Cell Neurosci 11:324–333.

Mittaud P, Marangi PA, Erb-Vogtli S, Fuhrer C (2001) Agrin-induced activation of acetylcholine receptor-bound Src family kinases requires Rapsyn and correlates with acetylcholine receptor clustering. J Biol Chem 276:14505–14513.

Mohamed AS, Rivas-Plata KA, Kraas JR, Saleh SM, Swope SL (2001) Src-class kinases act within the agrin/MuSK pathway to regulate acetylcholine receptor phosphorylation, cytoskeletal anchoring, and clustering. J Neurosci 21:3806–3818.

Muller D, Nikonenko I, Jourdain P, Alberi S (2002) LTP, memory and structural plasticity. Curr Mol Med 2:605–611.

Nastuk MA, Fallon JR (1991) Agrin binding to neurons of the embryonic chick spinal cord. Soc Neurosci Abstr 17:219.

Nastuk MA, Lieth E, Ma J, Cardasis CA, Moynihan EB, McKechnie BA, Fallon JR (1991) The putative agrin receptor binds ligand in a calcium-dependent manner and aggregates during agrin-induced acetylcholine receptor clustering. Neuron 7:807–818.

Nguyen QT, Lichtman JW (1996) Mechanism of synapse disassembly at the developing neuromuscular junction. Curr Opin Neurobiol 6:104–112.

Nitkin RM, Rothschild TC (1990) Agrin-induced reorganization of extracellular matrix components on cultured myotubes: relationship to AChR-aggregation. J Cell Biol 111:1161–1170.

O'Brien RJ, Xu D, Petralia RS, Steward O, Huganir RL, Worley P (1999) Synaptic clustering of AMPA receptors by the extracellular immediate-early gene product Narp. Neuron 23:309–323.

O'Toole JJ, Deyst KA, Bowe MA, Nastuk MA, McKechnie BA, Fallon JR (1996) Alternative splicing of agrin regulates its binding to heparin alpha-dystroglycan, and the cell surface. Proc Natl Acad Sci USA 93:7369–7374.

Park J, Hill MM, Hess D, Brazil DP, Hofsteenge J, Hemmings BA (2001) Identification of tyrosine phosphorylation sites on 3-phosphoinositide-dependent protein kinase-1 and their role in regulating kinase activity. J Biol Chem 276:37459–37471.

Rich MM, Lichtman JW (1989) In vivo visualization of pre- and postsynaptic changes during synapse elimination in reinnervated mouse muscle. J Neurosci 9:1781–1805.

Roberts WM (1993) Spatial calcium buffering in saccular hair cells. Nature 363:74–76.

Ruegg MA, Bixby JL (1998) Agrin orchestrates synaptic differentiation at the vertebrate neuromuscular junction. Trends Neurosci 21:22–27.

Sanes JR, Lichtman JW (1999) Development of the vertebrate neuromuscular junction. Annu Rev Neurosci 22:389–442.

Sanes JR, Lichtman JW (2001) Induction, assembly, maturation and maintenance of a postsynaptic apparatus. Nat Rev Neurosci 2:791–805.

Smith CL, Mittaud P, Prescott ED, Fuhrer C, Burden SJ (2001) Src, Fyn, and Yes are not required for neuromuscular synapse formation but are necessary for stabilization of agrin-induced clusters of acetylcholine receptors. J Neurosci 21:3151–3160.

Stein-Gerlach M, Wallasch C, Ullrich A (1998) SHP-2, SH2-containing protein tyrosine phosphatase-2. Int J Biochem Cell Biol 30:559–566.

Stern MD (1992) Buffering of calcium in the vicinity of a channel pore. Cell Calcium 13:183–192.

Sugiyama JE, Glass DJ, Yancopoulos GD, Hall ZW (1997) Laminin-induced acetylcholine receptor clustering: an alternative pathway. J Cell Biol 139:181–191.

Tsien RY (1980) New calcium indicators and buffers with high selectivity against magnesium and protons: design, synthesis, and properties of prototype structures. Biochemistry 19:2396–2404.

Wallace BG (1988) Regulation of agrin-induced acetylcholine receptor aggregation by Ca^{++} and phorbol ester. J Cell Biol 107:267–278.

Wallace BG (1995) Regulation of the interaction of nicotinic acetylcholine receptors with the cytoskeleton by agrin-activated protein tyrosine kinase. J Cell Biol 128:1121–1129.

Wallace BG, Qu Z, Huganir RL (1991) Agrin induces phosphorylation of the nicotinic acetylcholine receptor. Neuron 6:869–878.

Wang H, Bedford FK, Brandon NJ, Moss SJ, Olsen RW (1999) GABA(A)-receptor-associated protein links GABA(A) receptors and the cytoskeleton. Nature 397:69–72.

Wells DG, McKechnie BA, Kelkar S, Fallon JR (1999) Neurotrophins regulate agrin-induced postsynaptic differentiation. Proc Natl Acad Sci USA 96:1112–1117.

Weston C, Yee B, Hod E, Prives J (2000) Agrin-induced acetylcholine receptor clustering is mediated by the small guanosine triphosphatases Rac and Cdc42. J Cell Biol 150:205–212.

Whitlock GG, Lamb TD (1999) Variability in the time course of single photon responses from toad rods: termination of rhodopsin's activity. Neuron 23:337–351.

(Accepted 21 July 2003)