ELSEVIER

MCN Molecular and Cellular Neuroscience

www.elsevier.com/locate/ymcne
Mol. Cell. Neurosci. 31 (2006) 515 – 524

Clustering transmembrane-agrin induces filopodia-like processes on axons and dendrites

Maik Annies,[a,1] Godela Bittcher,[b] Rene Ramseger,[a] Jürgen Löschinger,[c] Stefan Wöll,[a] Elmar Porten,[a] Christian Abraham,[a] Markus A. Rüegg,[b] and Stephan Kröger[a,*]

[a]*Department of Physiological Chemistry, University of Mainz, Duesbergweg 6, D-55099 Mainz, Germany*
[b]*Biozentrum, University of Basel, Klingelbergstrasse 70, CH-4056 Basel, Switzerland*
[c]*Max-Planck-Institute for Developmental Biology, Spemannstr. 35, D-72076 Tübingen, Germany*

Received 22 September 2005; revised 4 November 2005; accepted 7 November 2005
Available online 20 December 2005

The transmembrane form of agrin (TM-agrin) is primarily expressed in the CNS, particularly on neurites. To analyze its function, we clustered TM-agrin on neurons using anti-agrin antibodies. On axons from the chick CNS and PNS as well as on axons and dendrites from mouse hippocampal neurons anti-agrin antibodies induced the dose- and time-dependent formation of numerous filopodia-like processes. The processes appeared within minutes after antibody addition and contained a complex cytoskeleton. Formation of processes required calcium, could be inhibited by cytochalasine D, but was not influenced by staurosporine, heparin or pervanadate. Time-lapse video microscopy revealed that the processes were dynamic and extended laterally along the entire length of the neuron. The lateral processes had growth cones at their tips that initially adhered to the substrate, but subsequently collapsed and were retracted. These data provide the first evidence for a specific role of TM-agrin in shaping the cytoskeleton of neurites in the developing nervous system.

Keywords: Agrin; Synapse formation; Heparan sulfate proteoglycan; Axonal growth; Dendritic spine; Cytoskeleton

Introduction

Agrin is a heparan sulfate proteoglycan that is widely expressed in the central and peripheral nervous system as well as in non-neuronal tissues. Agrin's function is best understood in skeletal muscle where it plays a key role during formation, maintenance and regeneration of the neuromuscular junction (for review see Sanes and Lichtman, 2001; Bezakova and Ruegg, 2003). Little is known about agrin's role in other tissues, in particular in the CNS (for review, see Kröger and Schröder, 2002). Although CNS neurons from mice with a targeted deletion of the agrin gene form normal synaptic specializations in vitro and in vivo (Li et al., 1999; Serpinskaya et al., 1999), the acute suppression of agrin expression or function by antisense probes or antibodies influences the formation and function of synapses (Ferreira, 1999; Böse et al., 2000). Moreover, agrin isoforms are highly expressed by CNS neurons before synaptogenesis, suggesting additional functions for agrin for example during axonal elongation (Kröger, 1997; Kröger et al., 1996; Halfter et al., 1997; Mantych and Ferreira, 2001; Annies and Kröger, 2002). However, agrin's precise role during development of the CNS remains to be clarified.

Alternative splicing of the agrin cDNA generates agrin proteins that differ in their distribution and function (for review see Bezakova and Ruegg, 2003). In addition, alternative first exon usage results in the synthesis of either a secreted form (NtA-agrin) or a transmembrane form of agrin (TM-agrin; Burgess et al., 2000; Neumann et al., 2001). NtA-agrin specifically interacts with the laminin γ1-chain via its NtA domain and is stably incorporated into several basal laminae (Denzer et al., 1997; Kammerer et al., 1999). In contrast, in TM-agrin the NtA-domain is replaced by a non-cleaved signal peptide that anchors agrin as a type II transmembrane protein in the plasma membrane (Burgess et al., 2000; Neumann et al., 2001). NtA-agrin and TM-agrin are differentially expressed. While NtA-agrin is ubiquitously expressed in most basal lamina-containing tissues, TM-agrin is preferentially expressed in the CNS, particularly on neurites during the phase of active growth (Burgess et al., 2000; Neumann et al., 2001; Annies and Kröger, 2002).

The identification of a transmembrane form of agrin has led to the hypothesis that TM-agrin might serve as a receptor for transducing extracellular signals to the cytoplasm (Neumann et al., 2001). Here, we show that clustering of TM-agrin by polyclonal anti-agrin antibodies induced the formation of numerous filopodia-like processes extending from axons and dendrites of CNS and PNS neurons. The processes contained a complex cytoskeleton, were highly motile and resembled processes previously described during the initial formation of dendritic spines. Our results are consistent with a role for TM-agrin as a receptor or co-

* Corresponding author. Fax: +49 6131 3920136.
E-mail address: skroeger@uni-mainz.de (S. Kröger).
[1] Present address: Syngenta AG, CH-4002 Basel, Switzerland.
Available online on ScienceDirect (www.sciencedirect.com).

1044-7431/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.mcn.2005.11.005

receptor on neurites and suggest novel roles for TM-agrin during neurite growth and synaptogenesis.

Results

Anti-agrin antibodies induce processes on growing CNS axons

Cell surface clustering of transmembrane proteins using polyclonal antibodies has been widely used to activate downstream intracellular events (for review, see Heldin, 1995; Weiss and Schlessinger, 1998) and has been applied to specifically cluster agrin on myotubes (Uhm et al., 2001). Since previous studies had shown that retinal ganglion cells (RGCs) exclusively express the transmembrane form of agrin and that this form is present on the surface of growing axons in vivo and in vitro (Kröger et al., 1996; Neumann et al., 2001; Annies and Kröger, 2002), we first analyzed the response of RGC axons to the addition of anti-agrin antibodies. While RGC axons in the presence of preimmune sera or untreated axons had a smooth surface and extended straight (Figs. 1A, C), axons in the presence of the antibodies had a curvy appearance and were profusely lined along their entire length with filopodia-like protrusions (Figs. 1B, D). In anti-agrin antibody-treated cultures, the agrin immunoreactivity became more punctate (compare Figs. 1C, D) and puncta were associated with individual processes (arrowheads in Figs. 1B, D), indicating that the antiserum had clustered agrin. The same punctate staining was detected in live cells, showing that agrin was clustered on the surface of the axons and that it was not internalized within the 3 h of incubation (data not shown).

Process formation was observed independently of the substrate used (EHS tumor-derived laminin, rat tail collagen, matrigel, and retinal basal lamina). Moreover, preincubation of the substrates with anti-agrin antibodies did not cause process formation (data not shown). Thus, the effect of the antiserum is caused by its binding to the axonal surface.

Processes contain a complex cytoskeleton

To determine if the filopodia-like processes contained cytoskeletal elements, axons with or without processes were labeled with fluorescent phalloidin to reveal actin filaments. In contrast to the situation in control cultures (Fig. 1E), the antibody-treated cultures showed numerous lateral actin filaments emanating from the main bundle along the entire length of the axon (Fig. 1F), demonstrating that the processes contained actin filaments. Disruption of the actin cytoskeleton by parallel incubation of the axons with anti-agrin antiserum and 20 μM cytochalasin D for 3 h completely abolished the axon's responsiveness to the anti-agrin antibodies, without obvious signs of cytotoxicity (0.5 ± 0.2 processes per 110 μm axon segment compared to 25.2 ± 0. processes in untreated axons; mean ± SEM with $N = 3$), suggesting that intact actin filaments are required for process formation.

To further characterize the antibody-induced filopodia-like processes, we double-labeled them with fluorescent phalloidin and antibodies against several other cytoskeletal components. This staining showed that the processes contained synaptotagmin (Fig. 1H) and GAP-43 (Fig. 1J), but no neurofilament-68 (Fig. 1G). Likewise, the processes could be labeled with antibodies against acetylated tubulin and the phosphorylated form of the microtubule-associated protein 1B (data not shown). Weak β-tubulin immunoreactivity was detectable at the base particularly of the more complex processes (Fig. 1I). These results show that the processes contain a complex cytoskeleton suggesting that they might be motile structures. Note the curvy appearance of antiserum-treated axons and the elaborate structure of some processes (Figs. 1I, J).

To analyze the specificity of process formation we investigated the effect of several different anti-agrin antibodies. The activity of the antisera was determined by counting the number of lateral processes per axon segment of 110 μm length. The antisera were generated and characterized independently in several laboratories and shown to specifically react with agrin as determined by immunocytochemistry and Western blotting of different CNS tissues, including the retina (see Experimental methods). All polyclonal antisera tested induced similar processes on the surface of RGC axons (Fig. 2). In contrast, the corresponding preimmune sera (-pre lanes in Fig. 2) were inactive and only the background of spontaneously formed processes (control lane in Fig. 2) was observed. Several other antisera directed against extracellular epitopes expressed on RGC axons (including antibodies against N-CAM, β1-integrin, Thy-1, and Ng-CAM) showed no process-inducing activity (data not shown). The activity of the anti-agrin antisera could be blocked by preincubating the antisera with agrin (lane as-46 plus agrin in Fig. 2). Polyclonal antisera generated against short peptides within the NtA-domain of agrin (Neumann et al., 2001), or within the 19 amino acids of splice site B of the agrin protein (as-B19; Kröger, 1997) were not active in inducing processes on RGC axons. Likewise, two monoclonal anti-agrin antibodies (mAbs C3 and 5B1, respectively) alone or in combination did not induce process formation. Finally, Fab fragments that were generated from the anti-agrin antiserum #46 were not able to induce processes on RGC axons even at a concentration of 1 mg/ml (lane Fab as-46 in Fig. 2), demonstrating that the formation of processes was not the result of blocking a particular epitope of TM-agrin, but more likely due to the clustering of TM-agrin by bivalent anti-agrin IgG. These data also suggest that dimerization of TM-agrin by monoclonal antibodies is not sufficient for process induction.

The formation of processes is dose- and time-dependent

We next analyzed the concentration dependence of the anti-agrin antibody-induced formation of processes. Retinal ganglion cells were cultured for 3 h in the presence of increasing concentrations of anti-agrin IgG (affinity-purified from the rabbit antiserum #46) and the number of processes per axon segment was determined. As shown in Fig. 3A process formation was dose-dependent and reached a maximum at approximately 30 processes per 110 μm. Half maximal response was observed at an IgG concentration of approximately 0.8 μg/ml. Thus, the formation of processes by anti-agrin antibodies was saturable and could be observed at low antibody concentrations. The fact that affinity-purified anti-agrin IgG can induce these processes rules out the possibility that other serum components are required.

To determine the time course of process formation, we incubated RGC cultures with saturating concentrations of anti-agrin antibodies (20 μg/ml) for different periods of time and counted the number of processes per axon segment. As shown in Fig. 3B, processes formed within a few minutes, and the maximum was observed approximately 3 h after addition of the antibodies. We did not observe significantly more processes after prolonged