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Role of lipid rafts in agrin-elicited acetylcholine receptor clustering

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ABSTRACT

Emerging concepts of membrane organization point to the compartmentalization of the plasma membrane into distinct lipid microdomains. This lateral segregation within cellular membranes is based on cholesterol–sphingolipid-enriched microdomains or lipid rafts which can move laterally and assemble into large-scale domains to create plasma membrane specialized cellular structures at specific cell locations. Such domains are likely involved in the genesis of the postsynaptic specialization at the neuromuscular junction, which requires the accumulation of acetylcholine receptors (AChRs), through activation of the muscle specific kinase MuSK by the neurotropic factor agrin and the reorganization of the actin cytoskeleton. We used C2C12 myotubes as a model system to investigate whether agrin-elicited AChR clustering correlated with lipid rafts. In a previous study, using two-photon Laurdan confocal imaging, we showed that agrin-induced AChR clusters corresponded to condensed membrane domains: the biophysical hallmark of lipid rafts [F. Stetzkowski-Marden, K. Gaus, M. Recouvreur, A. Cartaud, J. Cartaud, Agrin elicits membrane condensation at sites of acetylcholine receptor clusters in C2C12 myotubes, J. Lipid Res. 47 (2006) 2121–2133]. We further demonstrated that formation and stability of AChR clusters depend on cholesterol. We also reported that three different extraction procedures (Triton X-100, pH 11 or isotonic Ca++, Mg++ buffer) generated detergent resistant membrane s (DRMs) with similar cholesterol/GM1 ganglioside content, which are enriched in several signalling postsynaptic components, notably AChR, the agrin receptor MuSK, rapsyn and syntrophin. Upon agrin engagement, actin and actin-nucleation factors such as Arp2/3 and N-WASP were transiently recovered within raft fractions suggesting that the activation by agrin can trigger actin polymerization. Taken together, the present data suggest that AChR clustering at the neuromuscular junction relies upon a mechanism of raft coalescence driven by agrin-elicited actin polymerization.

1. Principal findings

1.1. Ganglioside GM1 and cholesterol are concentrated at the rat neuromuscular junction

In a first attempt to identify *in situ* characteristic lipidic components of rafts, we looked for the presence of glycosphingolipids and cholesterol at the neuromuscular junction in cryostat sections of rat sternomastoïd muscles. Fluorescence microscopy experiments using RITC-

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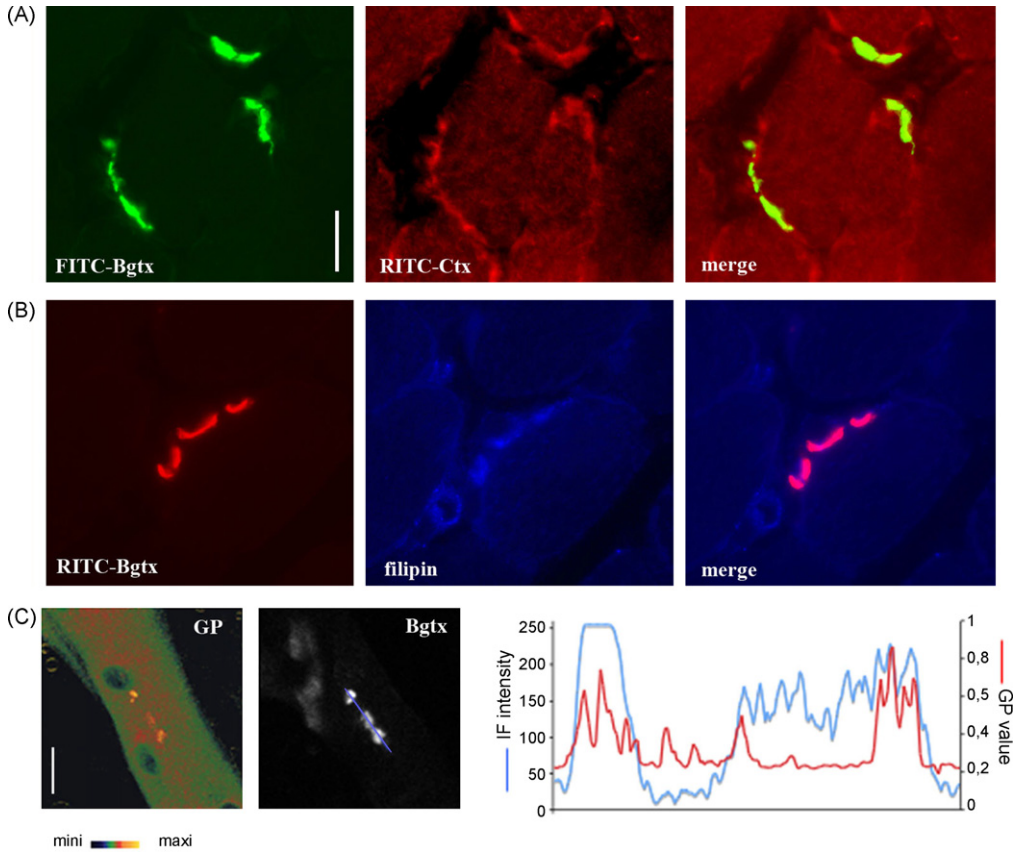

Fig. 1. (A and B) Raft-specific lipids GM1 and cholesterol were respectively detected by cholera toxin B and filipin labelings of cryostat sections of rat sternomastoïd muscles. Both raft markers colocalize with AChRs labeled by means of α-bungarotoxin in the postsynaptic membrane of the neuromuscular junctions. Bar equals 10 μm. (C) Membrane condensation at agrin-induced AChR clusters in C2C12 myotubes. Laurdan-labeled C2C12 myotubes were simultaneously imaged for the Laurdan intensity in two channels (400–450 and 450–530 nm) using two-photon laser scanning microscopy and for Alexa 555-conjugated α-Bgtx. Intensity images were converted to global polarization (GP) images as described in Ref. [2]. GP images were pseudo-coloured for low and high GP from blue to yellow, respectively. The confocal GP image (left panel) and the corresponding Alexa 555-α-Bgtx staining of AChRs (right panel) of the same field and focal depth of an agrin-treated myotube are shown. Plot profiles of the fluorescence intensity of the toxin (blue, left *y*-axis) and the GP values (red, right GP *y*-axis) along the line passing through the three AChR clusters disclosed high GP values corresponding to AChR clusters. Bar equals 10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

conjugated cholera toxin (CTX) as a marker of the ganglioside GM1 and filipin as a specific autofluorescent ligand of cholesterol, showed that both raft-associated lipids accumulated at the NMJ identified by means FITC or RITC-conjugated α-bungarotoxin (α-Bgtx), a specific ligand for AChR in the postsynaptic membrane (Fig. 1A and B).

1.2. Cholesterol is required for AChR cluster formation in C2C12 myotubes

To determine whether lipid rafts are involved in the genesis of AChR clusters, we used methyl-β-cyclodextrin (MβCD) as a drug to selectively deplete cholesterol from the cell membrane in C2C12. In our experimental conditions (10 mM MβCD, 30 min at 37 °C), about 60% of cholesterol was extracted from the membranes. In control myotubes incubated overnight with 5 ng/ml recombinant agrin (C-$Ag_{3,4,8}$, R&D Systems), numerous AChR clusters measuring up to 10–20 μm were detected at the cell surface by fluorescence microscopy using FITC-conjugated α-Bgtx as a marker for AChRs. Following cholesterol depletion, small dispersed AChR aggregates were observed instead of the large AChR clusters. In another series of experiments, we showed that cholesterol depletion before agrin treatment prevented agrin-induced AChR clustering (see Ref. [1]). To ascertain whether the AChR distributions observed in cholesterol-depleted cells reflect modifications in cluster formation rather than perturbations in AChR trafficking or targeting to the cell surface, we also quantified cell surface *vs.* intracellular AChR with ^{125}I α-bungarotoxin. We observed that control and MβCD-treated myotubes exhibit equivalent surface *vs.* intracellular AChR pools. Collectively, these data indicate that cholesterol is required for proper agrin-dependent AChR clustering at the surface of myotubes.

1.3. AChR clusters correspond to condensed membrane domains of the myotube surface

Lipid rafts, as entity, constitute a highly ordered membrane domain which is distinct from the more fluid surroundings. To demonstrate that AChR-rich microdomains