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Lipid raft localization of $GABA_A$ receptor and Na^+, K^+-ATPase in discrete microdomain clusters in rat cerebellar granule cells

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Abstract

The microdomain localization of the $GABA_A$ receptor in rat cerebellar granule cells was studied by subcellular fractionation and fluorescence- and immunogold electron microscopy. The receptor resided in lipid rafts, prepared at 37 °C by extraction with the nonionic detergent Brij 98, but the raft fraction, defined by the marker ganglioside GM_1 in the floating fractions following density gradient centrifugation, was heterogeneous in density and protein composition. Thus, another major raft-associated membrane protein, the Na^+, K^+-ATPase, was found in discrete rafts of lower density, reflecting clustering of the two proteins in separate membrane microdomains. Both proteins were observed in patchy 'hot spots' at the cell surface as well as in isolated lipid rafts. Their insolubility in Brij 98 was only marginally affected by methyl-β-cyclodextrin. In contrast, both the $GABA_A$ receptor and Na^+, K^+-ATPase were largely soluble in ice cold Triton X-100. This indicates that Brij 98 extraction defines an unusual type of cholesterol-independent lipid rafts that harbour membrane proteins also associated with underlying scaffolding/cytoskeletal proteins such as gephyrin ($GABA_A$ receptor) and ankyrin G (Na^+, K^+-ATPase). By providing an ordered membrane microenvironment, lipid rafts may contribute to the clustering of the $GABA_A$ receptor and the Na^+, K^+-ATPase at distinct functional locations on the cell surface.
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1. Introduction

The $GABA_A$ receptors belong to the superfamily of ligand-gated ion channels (Macdonald and Olsen, 1994) and mediate the majority of the fast inhibitory synaptic transmissions in the CNS by gating Cl^- through an integral membrane channel (Fritschy and Brunig, 2003; Schousboe, 1999; Whiting, 2003). Structurally, $GABA_A$ receptors are heteropentameric channels assembled from a multitude of subunits belonging to six subunit families (α, β, γ, δ, ε, ρ) and the expression of receptor subtypes show region- and neuron specificity (Burt, 2003; Carlson et al., 1998; Fritschy and Brunig, 2003; Macdonald and Olsen, 1994; Sinkkonen et al., 2004).

As polarized cells, neurons crucially depend on the segregation of proteins to specific microdomains at the cell membrane. For $GABA_A$ receptors, a high packing density depends upon expression of gephyrin, a 93 kDa peripheral membrane protein (Schmitt et al., 1987), originally identified in copurification with glycine receptors (Pfeiffer et al., 1982). Yet, it has not been possible to establish biochemically a direct link between gephyrin and the $GABA_A$ receptor (Kannenberg et al., 1997). Furthermore, some clustering of $GABA_A$ receptors reportedly persists in the retina of gephyrin null mutant mice (Fischer et al., 2000). Finally, association of gephyrin with $GABA_A$ receptors varies during development (Danglot et al., 2003). Thus,

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additional forces might contribute to clustering of the receptor (Kneussel, 2002).

The Na^+, K^+-ATPase is a major membrane component of nervous tissue, accounting for up to 50% of the total energy consumption and largely responsible for creation and maintenance of electrochemical gradients across the cell membrane (Stahl and Harris, 1986). Like the $GABA_A$ receptor, Na^+, K^+-ATPase is microdomain localized at the cell surface where it is typically clustered in axon initial segments and at the nodes of Ranvier (Salzer, 1997, 2003). The multivalent cytoskeletal adaptor protein ankyrin G is considered a key component in the organization of these clusters, linking Na^+, K^+-ATPase and ion channels to the underlying spectrin cytoskeleton (Jenkins and Bennett, 2002; Nelson and Veshnock, 1987).

Microdomain organization of proteins at the surface of cells can also be influenced by functional protein–lipid interactions. Thus, cholesterol and glycosphingolipids form ordered (l_o phase) microdomains (lipid rafts) (Brown and London, 1998; Hooper, 1999; Simons and Ikonen, 1997). By acting as lateral sorting platforms of proteins in the membrane, lipid rafts have been implicated in a variety of cell membrane-related processes, including surface receptor signaling (Bagnat et al., 2000; Simons and Toomre, 2000), endocytosis (Parton and Richards, 2003) and cholesterol transport (Simons and Ikonen, 2000).

Lipid rafts in neuronal cells have been studied previously (Ledesma et al., 1998; Maekawa et al., 2003; Parkin et al., 1999) and could well be of importance for nerve cell functioning (Martens et al., 2004). Of neurotransmitter receptors, only the α7-subunit nicotinic acetylcholine receptor (Bruses et al., 2001), the AMPA-type glutamate receptor (Hering et al., 2003; Suzuki et al., 2001) and the $GABA_B$ receptor (Becher et al., 2001) has so far been localized to lipid rafts. To gain more insight into lipid raft organization and functioning in neuronal cells the membrane microdomain localization of the $GABA_A$ receptor and Na^+, K^+-ATPase in rat cerebellar granule cells was studied in the present work.

2. Materials and methods

2.1. Reagents

A rabbit antibody to rat $GABA_A$ receptor α1 subunit was kindly provided by Prof. Jean-Marc Fritschy (Institute of Pharmacology and Toxicology, University of Zürich, Switzerland). Mouse monoclonal antibodies to sheep kidney Na^+, K^+-ATPase α-subunit and rat gephyrin were from Affinity Bioreagents (Golden, CO, USA) and Transduction Laboratories (Lexington, KY, USA), respectively. Goat antibodies to rat gephyrin and rabbit antibodies to human protein kinase C βII (PKC βII) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and rabbit antibodies to asialo-G_{M1} were from Biotrend (Cologne, Germany). Gold-labeled goat anti-rabbit and goat anti-mouse antibodies were from Amersham Pharmacia Biotech. (Little Chalfont, UK). Alexa Fluor 488-Cholera toxin B subunit conjugate, Alexa Fluor 594-goat anti-mouse IgG conjugate and Alexa Fluor 488-goat anti-rabbit IgG conjugate were from Molecular Probes (Copenhagen, Denmark). The GABA agonist THIP (4,5,6,7-tetrahydroisoxazolo[5,4-*c*]pyridin-3-ol) was synthesized and kindly provided by Prof. P. Krogsgaard-Larsen (Dept. of Medicinal Chemistry, Danish University of Pharmaceutical Sciences, Copenhagen, Denmark). Cholera toxin B subunit (CTB), rabbit antibodies to cholera toxin, methyl-β-cyclodextrin and other reagents were obtained from Sigma (St. Louis, MO, USA).

2.2. Rat cerebellar granule cell culture

Primary cultures of dissociated cerebellum from 7-day-old rats, consisting of granule neurons with moderate contamination with astrocytes or other types of neurons, were prepared as previously described (Drejer and Schousboe, 1989). Briefly, cerebella were removed, chopped and dissociated by mild trypsinization and subsequent trituration in the presence of soybean trypsin inhibitor and DNAse. Dissociated cells were pelleted by centrifugation and resuspended in a sligthly modified Dulbecco's medium containing 10% (v/v) heat inactivated fetal calf serum, 24.4 mM KCl, 50 μM kainic acid, 0.2 mM glutamine, penicillin G (10^5 units/ml), insulin (10^{-4} IU/l) and *p*-aminobenzoic acid (7.3 μm). Cells were plated onto poly-L-lysine coated culture dishes (Sensenbrenner et al., 1978) and cultured at 37 °C in a humidified atmosphere of 5% CO_2 + 95% air. After 36–48 h of culture, cytosine arabinoside (20 μm final concentration) and the $GABA_A$ receptor antagonist THIP (150 μm final concentration) were added.

2.3. Isolation of lipid raft membranes

Cultured cerebellar granule cells were harvested, resuspended in 1 ml 25 mM HEPES-HCl, 0.15 M NaCl, pH 7.2, containing 10 μg/ml of aprotinin and leupeptin, and extracted for 15 min with either 1% (v/v) Brij 98 at 37 °C or 1% (v/v) Triton X-100 on ice. In some experiments, the cells were incubated for 1 h in the presence of 1% methyl-β-cyclodextrin at room temperature or 10 μg/ml CTB on ice prior to detergent extraction. The extracts were then mixed with an equal volume of 80% (w/v) sucrose in the same buffer. Detergent resistent membranes (DRM's or lipid rafts) were prepared by layering a 5–30% sucrose gradient in the same buffer on top of the extracts and centrifugation in a SW 40Ti rotor (Beckman Instruments, Palo Alto, CA, USA) for 18–20 h at 35.000 rpm (g_{max} = 217,000), as described previously (Brown and Rose, 1992; Danielsen, 1995). After centrifugation, the sucrose gradients were fractionated and proteins isolated by mixing gradient samples with an equal volume of acetone on ice, followed by centrifugation at

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