Co-segregation of AMPA receptors with G\textsubscript{M1} ganglioside in synaptosomal membrane subfractions

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Biochemical studies have suggested that certain synaptic proteins associate with lipid rafts to perform key functions within the synapse. However, variability in biochemical preparations raises questions as to which synaptic proteins actually associate with lipid rafts. In the present study, we use both electron microscopy and biochemistry to investigate AMPA (\(\alpha\)-amino-3-hydroxy-5-methylisoxazole-4-propionic acid) receptor localization in synaptic membrane subfractions prepared in two different ways, by Triton X-100 detergent treatment or without detergent by sonication at high pH. Immunogold electron microscopy shows that a detergent-resistant synaptosomal membrane subfraction consists of empty vesicles 0.1–1.0 \(\mu\)m in diameter.

A subpopulation of these vesicles labelled for glycosphingolipid G\textsubscript{M1} ganglioside, a marker of lipid rafts, and 46 % of the labelled vesicles also labelled for the AMPA receptor subunit GluR2. This co-segregation into specific vesicles does not depend on effects of detergent because a similar distribution of label was found in vesicles isolated without the use of detergent. Our results suggest that AMPA receptors localize within specific regions of synaptic membranes rich in G\textsubscript{M1} ganglioside.

Key words: detergent-free, detergent-resistant, electron microscopy, lipid raft, negative stain, postsynaptic density.

EXPERIMENTAL

Antibodies and immunoblotting
SDS/PAGE was carried out using 7.5 % minigels (Bio-Rad). Proteins were transferred on to nitrocellulose, and immunoblots were obtained using the following antibodies. Rabbit polyclonal anti-GluR2/3 (Millipore) and rabbit polyclonal anti-PSD-95 (postsynaptic density 95; custom made by New England Peptide) antibodies [14] were used at a 1:500 dilution in Western blot analysis. Mouse monoclonal extracellular anti-GluR2 antibody (Clone 6C4; Millipore) was used at a 1:100 dilution for immunocytochemistry and a 1:1000 dilution in Western blot analysis. Rabbit polyclonal anti-ganglioside G\textsubscript{M1} antibody (Abcam) was used at a 1:100 dilution for immunocytochemistry and a 1:1000 for dot blots. Anti-rabbit IgG alkaline phosphatase conjugate antibody (Millipore) was used in Western blot analysis at a dilution of 1:5000. Anti-mouse IgG alkaline phosphatase conjugate antibody (Millipore) was used in Western blot analysis at a dilution of 1:5000. Goat anti-mouse 10 nm gold antibody (British BioCell) was used at a 1:100 dilution to detect GluR2 and goat anti-rabbit 15 nm gold antibody (British BioCell) was used at a 1:100 dilution to detect G\textsubscript{M1} in all immunocytochemistry. For dot blots, fractions were dotted on to a nitrocellulose membrane and allowed to dry before being labelled with antibody.

Subcellular fractionation
Isolation of PSD and DRSM (detergent-resistant synaptosomal membrane) fractions using Triton X-100
The method of Carlin et al. [15] was used to obtain subcellular fractions from rat brains (Pel-Freez Biologicals) with modifications as detailed in Dosemeci et al. [16]. Briefly, a synaptosome fraction was obtained and treated with 0.5 %...
Triton X-100 at 4°C. Detergent-insoluble material was collected by centrifugation and further fractionated on sucrose gradients. The DRSM fraction was collected from the 0.32/1.0 M sucrose interface. Crude PSD fraction was collected from the 1.5/2.1 M sucrose interface and treated again with 0.5% Triton X-100/75 mM KCl and collected on a 2.1 M sucrose cushion to obtain the final PSD fraction used in the present study.

**Synaptosomal lipid raft fraction without detergent treatment**

The detergent-free preparation was adapted from previously published methods using high pH and sonication [3,17]. Synaptosomes were obtained as above and pelleted by centrifugation (20,000 rev./min for 30 min using a Sorwall SS34 rotor). The synaptosomal pellet was re-suspended in 0.5 M Na2CO3, (pH 11) and sonicated using a probe sonicator, five times for 20 s with 2 min intervals (Kontes Micro Ultrasonic Cell Disruptor; output control at 60). The resulting particulate material was fractionated using a sucrose gradient. Material from the 0.32/1.0 M and 1.0/1.5 M sucrose interfaces, as well as the 1.0 M sucrose layer, were collected. No visible material was present at the 1.5/2.1 M sucrose interface. Fractions were stored at −20°C in 33% glycerol [18].

**Cholesterol depletion**

The depletion of cholesterol on subfractions previously isolated from synaptosomes was accomplished by treating the sample with 0.5% saponin in 1 mM Hepes buffer for 15 min at 25°C, while shaking.

**EM**

Isolated fractions were transferred without fixation or resin embedding on to glow discharged, formvar-coated, 400 mesh, copper grids (SPI Supplies) by floating the grid on 10 μl drops of each fraction, diluted in 20 mM Hepes, for 10 min. Grids were then transferred on to 30 μl drops of 0.5% casein in PBS (BioFX) block for 1 h before being transferred on to 30 μl drops of primary antibody diluted in 0.5% casein in PBS block. Primary antibodies in 0.5% casein block were applied in separate drops for 1 h each; controls were merely placed in casein block for this step. All grids were washed in 0.05% Tween 20 in casein block for 30 min before being placed in 30 μl droplets of secondary antibodies diluted in Tween 20. It has been shown that Tween 20 does not significantly solubilize membrane [8]. The secondary antibody with the larger gold particle was applied first, and each secondary antibody was applied for 1 h. Grids were finally washed in three solutions consecutively: 0.05% Tween 20 in PBS for 30 min; PBS for 20 min; and 5 mM Hepes for 10 min. The samples were then passed through three drops of 1% uranyl acetate, 30 s each, dried and examined at 200 Kev with a JOEL 200CV electron microscope. All of the presented immunocytochemistry data were taken from micrographs of double-immunolabelled samples.

**Counts of gold label**

Individual vesicles were measured for cross-sectional area and the numbers of gold label, in micrographs of the DRSM fraction that featured multiple vesicles with well-defined membrane boundaries. Cross-sectional area was measured with ImageJ software (NIH) and gold labels were counted manually. Two particles of the same diameter, clustered within 15 nm, were counted as one label. In micrographs of detergent-free fractions, where vesicles were in aggregates, a grid with squares of 0.1 μm² (approximately the average area of a DRSM vesicle) was laid over the micrograph and counts per square were recorded in every square entirely filled by vesicles.

In control samples (primary antibody omitted), secondary antibodies, conjugated to 5 and 10 nm gold, labelled only 4.4 and 6.8% respectively of the vesicles in the DRSM fraction, with no vesicles labelled with more than one immunogold in any of the control micrographs.

**RESULTS AND DISCUSSION**

Biochemical analyses of synaptosomal membrane subfractions have shown that synaptic proteins associate with Triton X-100-insoluble lipids [1,7,9]. We have used biochemistry and EM to investigate the association of the glycosphingolipid Gm, ganglioside with AMPA receptors in structures isolated in synaptosomal membrane subfractions.

**Biochemical and morphological differences in detergent-isolated fractions**

Treatment of membrane fractions with Triton X-100 has been extensively used as a strategy to assess the association of neuronal proteins within specialized lipid domains, which are thought to include lipid rafts [1,7,9]. In particular, AMPA receptors have been observed in detergent-insoluble fractions from synaptosomal membranes [7,9] and from a heavy membrane (P2) fraction [1]. The starting material, as well as the detergent concentration, appears to influence the distribution of other proteins, specifically PSD-95, in the detergent-resistant fractions. Using 0.5% Triton X-100, Suzuki et al. [7] observed relatively little PSD-95 in the cholesterol-rich light DRSM fraction compared with the heavy detergent-resistant PSD fraction. On the other hand, Suzuki et al. [9] used a lower concentration of Triton X-100 in a later study and found a relatively higher amount of PSD-95 captured in the synaptosomal raft fraction. Hering et al. [1] found higher amounts of PSD-95 in the light DRSM fractions relative to the heavy PSD subfraction when using 0.5% Triton X-100.

In the present study, it was confirmed that Gm was more abundant in the light fraction isolated from the 0.32/1.0 M sucrose interface when compared with the 1.5/2.1 M sucrose interface. Western blot analysis showed the presence of GluR2 in both fractions with slightly higher levels in the 1.5/2.1 M interface fraction compared with the 0.32/1.0 M interface fraction. Western blots also indicated that the 1.5/2.1 M interface fraction had more PSD-95 compared with that found in the DRSM fraction (Figure 1, lower right inset). The present study, which uses conditions similar to those of Suzuki et al. [7], confirms their findings.

Thin-section EM indicated structural differences between the two synaptosomal raft fractions defined by Suzuki et al. [7]. While the fraction obtained using 0.5% Triton X-100 consisted mainly of smooth vesicles (Figure 1) [7], the fraction derived with 0.15% detergent had an abundance of electron-dense material [9]. Our micrographs of thin-sectioned 1.5/2.1 M interface, or...
Co-segregation of synaptic \( G_{M1} \) ganglioside with AMPA receptor subunits in detergent-derived membrane subfractions

The resolution afforded by EM is essential for determining the distribution of individual components in isolated lipid fractions. In the present study, we obtained electron micrographs of double-immunolabelled DRSMs which show that \( G_{M1} \) ganglioside and AMPA receptors aggregate within a subset of vesicles (Figure 2). In total, 60% of vesicles in the DRSM fraction were not labelled for \( G_{M1} \) or AMPA receptors (Table 1), showing that the majority of vesicles were labelled at a frequency distinguishably less than background (0.061 ± 0.029 and 0.149 ± 0.028 label/0.1 \( \mu m^2 \) respectively). The results indicated a biased distribution of lipids and proteins after detergent separation, with \( G_{M1} \)-tagged vesicles accounting for only 27% of the detergent-derived lipid vesicles. A subpopulation of vesicles labelled for the AMPA receptor subunit GluR2 accounted for 24% of vesicles in the detergent-derived fraction, and 46% of GluR2-labelled vesicles also contained label for \( G_{M1} \) (Table 1). Since \( G_{M1} \) is regarded as a marker for lipid rafts [1,2,9–13], these data agree with previous biochemical and light microscopy evidence suggesting that AMPA receptors associate with lipid rafts [1,2,7].

Saponin treatment destroys nearly all vesicles in the detergent-derived fraction

To further characterize the detergent-derived subfraction from synaptosomes, the fractions were treated with saponin, a cholesterol-depletion reagent. This strategy defines the role of cholesterol in maintaining the vesicles and helps assess whether the \( G_{M1} \)- and GluR2-positive vesicles are different with respect to cholesterol content. Saponin, although considered a relatively strong detergent, acts specifically on cholesterol [19,20]. For...
A single vesicle in the DRSM fraction is co-labelled for GM1 (10 nm gold, in circles) and GluR2 (15 nm gold, in squares). Other single vesicles (B–D) show the density of co-labelling varying from zero (B, label indicated as in A) to heavy (D, label indicated as in A). These graphs indicate the density of GM1 and GluR2 immunogold label for 276 vesicles in the DRSM fraction. The average background on the substrate between vesicles for GM1 and GluR2 labelling was 0.061 ± 0.029 and 0.149 ± 0.028 label/0.1 μm² respectively. Scale bars = 100 nm.

example, while a mild non-ionic detergent, such as Triton X-100, can practically destroy the cell membrane, saponin treatment merely produces holes and leaves the morphology relatively intact [20].

After saponin treatment of the DRSM fraction, thin-section EM of saponin-insoluble material revealed an almost complete disappearance of lipid vesicles (Figure 1B, inset). This result indicates that cholesterol is a major constituent of all lipid vesicles in the detergent-derived subfraction, and that segregation of GluR2/GM1 in a subset of vesicles is not related to cholesterol.

Co-segregation of GM1 and GluR2 in subfractions isolated without detergent

Whether co-segregation of GM1 ganglioside and AMPA receptors in fractions isolated with detergent reflects their original state in the cell is open to question. Therefore we isolated an equivalent lipid fraction [5] without detergent to confirm that the segregation observed in the detergent-derived lipid fraction did not depend on detergent (Figure 3). The detergent-free disruption of synaptic membrane using high pH combined with sonication did not result in a fraction identical with the detergent isolation (Figure 3A, inset). Isolated fractions from the 0.32/1.0 M and 1.0/1.5 M sucrose interfaces, as well as the 1.0 M sucrose layer, were examined by Western blot and dot blots, as well as EM with immunocytochemistry. Our results indicated that the light vesicles collected at the 0.32/1.0 M sucrose interface have similar properties to the detergent-derived fraction collected at the same density interface. Dot blots showed that GM1 ganglioside had a heavier presence in the 0.32/1.0 M sucrose interface than in either the 1.0 M or 1.0/1.5 M fractions (Figure 3A, inset). The detergent-free fraction from the 0.32/1.0 M interface had a composition and distribution of PSD-95, GluR2 and GM1 similar to that of the detergent-derived fraction, as shown by immunobLOTS and EM (Figure 3). Electron micrographs of the immunolabelled vesicle fraction showed that vesicles were more aggregated than those in the DRSM fraction (Figure 3A). GM1 and GluR2 were clustered within these aggregates at concentrations higher than one gold label/0.1 μm² (Figures 3B and 3C). In total, 49% of regions with a high GluR2 concentration included clusters of GM1 (Table 2). Different stereological methods were required to determine label densities due to the aggregation, but the pattern of co-segregation in both samples appeared to be the same.

Although we have determined that co-segregation of GM1 ganglioside with AMPA receptors is not an effect of detergent, the temperature at which both isolations were performed could influence properties of the resulting fractions. One clear artefact is a vesicle size of 0.1–1.0 μm in the detergent-derived fraction; lipid membrane domains of this size are not likely to exist at the synapse under physiological conditions [10].

Biochemical and light microscopy studies have pointed to a connection between specialized lipid domains and AMPA receptors [1,2,7]. Examining lipid fractions from synaptic membranes by immunoelectron microscopy supports this idea. Our results indicated that the distributions of GM1 and AMPA
Co-segregation of GM1 with AMPA receptors

Figure 3  Detergent-free fraction shows DRSM-like segregation

Negative-stain immunogold labelling of vesicles in the detergent-free fraction (A). Vesicles in this fraction clump, obscuring their borders. Labels for GluR2 (10 nm gold, in circles) and GM1 (15 nm gold, in squares) were concentrated over one or a few vesicles. The density of labelling was measured by overlaying images with a 0.1 \( \mu \text{m}^2 \) grid, close to the average vesicle size measured in the detergent fraction (sample size indicated in upper left-hand region). (A, inset) Dot and Western immunoblots of synaptosomal subfractions were obtained for the detergent-free procedure showing relative levels of GM1, PSD-95 and GluR2/3. Scale bar = 100 nm. (B and C) Graphs indicating the label density frequencies for GM1 and GluR2 in 255 sample areas.

Table 2  Distribution of GluR2 and GM1 within sample areas of lipid vesicles obtained from synaptosomes by the detergent-free procedure

<table>
<thead>
<tr>
<th>GluR2 label</th>
<th>GM1 label</th>
<th>Number of sample areas</th>
<th>Percentage of sample areas (%)</th>
</tr>
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<tbody>
<tr>
<td>−</td>
<td>−</td>
<td>89</td>
<td>35</td>
</tr>
<tr>
<td>−</td>
<td>+</td>
<td>71</td>
<td>28</td>
</tr>
<tr>
<td>+</td>
<td>−</td>
<td>48</td>
<td>19</td>
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<tr>
<td>+</td>
<td>+</td>
<td>47</td>
<td>18</td>
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receptors are coupled, independent of the detergent interaction with the membranes. The association of AMPA, a major PSD receptor, with specialized lipid domains implies that neuronal communication could be influenced by the lipid composition of the synapse. GM1 ganglioside and AMPA receptor subunits co-segregate into the same vesicles, but do not strongly co-localize. The lack of co-localization indicated that there may be a property of co-segregated vesicles that attracts or traps specific lipids and proteins. For instance, Eggeling et al. [10] showed that areas of sphingomyelin and cholesterol hinder certain protein diffusion rates. It is possible that AMPA receptor subunits linger in lipid rafts without being specifically bound to any particular component.

AUTHOR CONTRIBUTION

Andy Cole designed and carried out experiments, analysed the data and wrote the manuscript. Ayse Dosemeci assisted in the design and performance of biochemical aspects of the research, analysis of the data and in the preparation of the manuscript. Thomas Reese assisted with the EM and morphological aspects of the research, as well as the analysis of data and preparation of the manuscript.

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