Role of Lipid Microdomains in P/Q-type Calcium Channel (Ca\textsubscript{2.1}) Clustering and Function in Presynaptic Membranes*

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Lipid microdomains can selectively include or exclude proteins and may be important in a variety of functions such as protein sorting, cell signaling, and synaptic transmission. The present study demonstrates that two different voltage-gated calcium channels, which both interact with soluble N-ethyl-maleimide-sensitive fusion protein attachment protein receptor (SNARE) proteins but have distinct subcellular distributions and roles in synaptic transmission, are differently distributed in lipid microdomains: presynaptic P/Q (Ca\textsubscript{2.1}) but not Lc (Ca\textsubscript{1.2}) calcium channel subtypes are mainly accumulated in detergent-insoluble complexes. The immunoisolation of multiprotein complexes from detergent-insoluble or detergent-soluble fractions shows that the α1A subunits of Ca\textsubscript{2.1} colocalize and interact with SNARE complexes in lipid microdomains. The altered organization of these microdomains caused by saponin and methyl-β-cyclodextrin treatment largely impairs the buoyancy and distribution of Ca\textsubscript{2.1} channels and SNAREs in flotation gradients. On the other hand, cholesterol reloading partially reverses the drug effects. Methyl-β-cyclodextrin treatment alters the colocalization of Ca\textsubscript{2.1} with the proteins of the exocytic machinery and also impairs calcium influx in nerve terminals. These results show that lipid microdomains in presynaptic terminals are important in organizing membrane sites specialized for synaptic vesicle exocytosis. The cholesterol-enriched microdomains contribute to optimizing the compartmentalization of exocytic machinery and the calcium influx that triggers synaptic vesicle exocytosis.

When activated, the Ca\textsuperscript{2+} channels in many different cell types mediate a rapid Ca\textsuperscript{2+} influx that triggers important intracellular events (Ref. 1 and references therein). In neurons and neuroendocrine cells, the Ca\textsuperscript{2+} entering through voltage-gated Ca\textsuperscript{2+} channels acts as the second messenger of electrical signaling and initiates regulated exocytosis and synaptic transmission.

Five types of voltage-gated calcium channels have been identified in neurons on the basis of their physiological and pharmacological properties: L, N, P/Q, R, and T (recently referred to as Ca\textsubscript{1.1}, Ca\textsubscript{2.2}, Ca\textsubscript{2.1}, Ca\textsubscript{2.3}, and Ca\textsubscript{3}) (2). These distinct calcium channels show different compartmentalization on neuronal plasma membrane, e.g. Ca\textsubscript{2.2}, Ca\textsubscript{2.1}, and Ca\textsubscript{2.3} are mainly located in the nerve terminals, where they form clusters in specialized regions of the presynaptic membrane (the active zones), which also contain predockcd synaptic vesicles (Refs. 1, 3, and references therein). This clustered accumulation in specific membrane regions is presumably important for the roles of calcium channels in neurotransmitter release.

It has been proposed that the formation of channel clusters may involve cytoskeletal elements and/or interactions with modular adaptor proteins (4, 5), whereas another proposed mechanism is based on the formation of microdomains within the lipid bilayer that recruit specific proteins and form platforms for protein sorting and/or signal relay stations for intracellular signaling (see Refs. 6–9 for recent reviews). Recent data have demonstrated that various components of the synaptic vesicle fusion machinery, including the soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor (SNARE)1 proteins (10–12), syntaxin 1 (syn1), 25-kDa synaptosome-associated protein (SNAP-25), vesicle-associated membrane protein (VAMP-2, also called synaptobrevin), and synaptotagmin1, are localized in the cholesterol-enriched microdomains of neuroendocrine and epithelial cells (13–15). These findings suggest that lipid microdomains may be involved in defining sites for neurosecretory vesicle release and may help to concentrate other membrane proteins that are important for exocytosis.

On the basis of the above, we investigated the lipid microdomain distribution of the two voltage-gated calcium channel subtypes Ca\textsubscript{1.2} (Lc) and Ca\textsubscript{2.1} (P/Q). These channels are particularly interesting because they both interact with SNARE proteins (Refs. 1, 16–18, and references therein) but have different cell compartmentalization and play different roles in synaptic vesicle exocytosis. A large proportion of Ca\textsubscript{1.2} channels is concentrated in the soma and at the tips of dendritic spines, where they often colocalize with the β2-adrenergic receptor to form postsynaptic complexes regulating synaptic transmission (19, 20), whereas channels 2.1 are mainly clustered in the presynaptic terminals of many neurons, where they mediate the rapid calcium influx that triggers synaptic vesicle fusion (1, 21).

The results of the study show that Ca\textsubscript{2.1} but not Ca\textsubscript{1.2}
channels are widely distributed in lipid microdomains, in which they colocalize and interact with SNARE protein complexes as shown by immunoslotting. Altering the organization of synaptosomal membrane lipid microdomains by means of the cholesterol-complexing drug, methyl-β-cyclodextrin (MβCD), alters the distribution of Ca2+,1 and inhibits their interactions with SNAREs. Furthermore, cholesterol depletion reduces calcium influx in the synaptosomes in response to depolarization. Taken together, these data support the conclusion that lipid microdomains in presynaptic membranes are important for defining organized sites in which specific voltage-gated calcium channel subtypes and SNAREs are colocalized.

**EXPERIMENTAL PROCEDURES**

**Materials and Antibodies**

The Infinity cholesterol reagent, cholesterol, MβCD, saponin, n-octyl-β-D-glucopyranoside (octylglucopyranoside), and the antibodies against syn1, α1A and α1C (subunits of Ca2+,1 and Ca2+,2 channels, respectively), mouse IgG, rabbit IgG, horseradish peroxidase-coupled cholera toxin subunit B, and anti-rabbit and anti-mouse IgG conjugated with peroxidase were all from Sigma-Aldrich (Milan, Italy). The antibodies against the transferrin receptor was purchased from Zymed Laboratories (South San Francisco, CA). Ultrapure Triton X-100 solution (Surfactant subunit B, and anti-rabbit and anti-mouse IgG conjugated with peroxidase) were all from Sigma-Aldrich (Milan, Italy). The antibody to toxin subunit B, and anti-rabbit and anti-mouse IgG conjugated with peroxidase were all from Sigma-Aldrich (Milan, Italy). The Bio-Rad Protein Assay was from A. Malgaroli (DIBIT, Milan, Italy). The antibodies directed against synaptotagmin1 (22) were a gift from A. Malgaroli (DIBIT, Milan, Italy). The Bio-Rad Protein Assay was purchased from Bio-Rad.

**Rat Brain Subcellular Fractionation**

Rat brain fractionation was carried out by means of differential centrifugation, essentially as described previously (23, 24). Briefly, the dissected cerebral cortices were homogenized in 4 mEq HEPES-NaOH, pH 7.3, containing 0.32 mM sucrose and protease inhibitors (2 µg/ml pepstatin, 2 µg/ml aprotinin); the total homogenate was centrifuged for 10 min at 8,000 x g, and the postnuclear supernatant was collected and centrifuged as described previously (23) to yield a pellet corresponding to the synaptosomal fraction (P2) and a supernatant (S2). The S2 containing the remaining membrane-bound vesicles and organelles of the total homogenate was centrifuged at 165,000 x g for 2 h to yield a high-speed supernatant corresponding to the cytosol and a pellet (P3) enriched in cell body organelles (24).

**Cholesterol Extraction, Reloading, and Determination**

**Saponin Treatment**—The P2 samples (2000 or 700 µg of proteins) were resuspended in 250 µl of a buffer containing 150 mM NaCl, 2 mM EGTA, and 50 mM Tris-HCl, pH 7.5 (buffer A) containing 1% (w/v) saponin (proteindetergent ratios 1:1.25 or 1:3.6) and incubated for 30 min on ice.

**MβCD Treatment**—The P2 samples (660 µg of proteins) were resuspended in 250 µl of buffer A supplemented with protease inhibitors, incubated with or without 10 or 30 mM MβCD (final concentration) for 40 min at 37 °C and then centrifuged. The supernatants were completely removed, and the synaptosomal pellets were resuspended in the appropriate buffer for cholesterol reloading or Triton X-100 solubilization and gradient analysis.

**Cholesterol Reloading**—The cholesterol and MβCD inclusion complexes were prepared as described by Klein et al. (25). Briefly, 15 µg of cholesterol dissolved in methanol:chloroform (2:1) were added to 10 ml of a 5% MβCD solution and incubated for 90 min at 80 °C. After MβCD treatment, 2% PEG 6000 samples (500 µg) were centrifuged, and then in 320 µl of buffer A containing 10 mM cholesterol/MβCD complexes and incubated for 30 min at 37 °C. After reloading, the P2 samples were centrifuged, and the pellets were resuspended in buffer A for further analysis. Cholesterol concentrations were determined in aliquots of the untreated (control), MβCD-treated, and MβCD/cholesterol complex-treated P2 samples. The aliquots were centrifuged, the synaptosomal pellet was resuspended in a lysis buffer (0.5% Triton X-100, 0.5% deoxycholic acid-sodium salt, 150 mM NaCl, and 20 mM Tris-HCl, pH 7.2), and the amount of cholesterol was determined using the Infinity cholesterol reagent according to the Sigma protocol. The protein concentrations in the MβCD and cholesterol/MβCD-treated samples were determined using the Bio-Rad Protein Assay.

**Detergent Solubilization and Sucrose Gradient Fractionation**

The proteins from the untreated P2 and P3 samples, or the P2 samples treated with saponin, MβCD, or cholesterol/MβCD, were degraded in 250 µl of buffer A (supplemented with protease inhibitors) containing a final concentration of 1% (w/v) Triton X-100 (protein: detergent ratios 1:2.5 or 1:3.6). The samples were then incubated for 30 min on ice. The detergent-insoluble complexes were separated by centrifugation on discontinuous gradients as described previously (15, 26). Briefly, 250 µl of buffer A containing 2.4 mM sucrose was added to 250 µl of each sample, which was then placed in a centrifuge tube and overlaid with 1 ml of 0.9 M, 0.5 ml of 0.8 M, 1 ml of 0.7 M, and 1 ml of 0.6 M of sucrose solutions (all prepared in buffer A). The discontinuous gradient were centrifuged at 335,000 g for 15 h at 4 °C. The gradient fractions were collected from the top and the total protein concentration in each fraction was determined using a Bio-Rad Protein Assay. After densitometric analysis of the protein bands, the fractions were analyzed by SDS-PAGE and Western blotting. The sucrose concentration in each fraction was determined by refractometry; the mean values of seven gradients from three different experiments are shown.

**Immunoslotting of Ca2+,1 and SNARE Complexes**

The immunocomplexes from the sucrose gradient fractions were isolated by means of immunoprecipitations or immunoaffinity columns. For the immunoslotting of the proteins colocalized in lipid microdomains, particular care was taken to preserve the properties of the lipid microdomains. All of the procedures were carried out on ice. Aliquots of the gradient fractions were adjusted to 0.3 mM sucrose with buffer A without detergent and incubated with anti-syn1, VAMP-2, or SNAP-25 monoclonal antibodies prebound to protein G beads. As a control, equal sample amounts were immunoprecipitated with non-immune mouse IgG prebound to protein G beads. After overnight incubation at 4 °C with gentle mixing, the beads were centrifuged at 2000 x g for 3 min, washed three times with buffer A containing 0.3% Tween 20, and resuspended in Laemmli’s sample buffer (27). The immunocomplexes and aliquots (1.5 µl) of the samples used for immunoslotting (inputs) were analyzed by SDS-PAGE.

For the immunoslotting of the protein complexes, immunoaﬃnity column purification or immunoprecipitations were carried out after solubilization of the lipid microdomains. Equal aliquots (1.5 ml) of fractions 3 and 7 were diluted (to 6 ml) in buffer A containing 0.3 M sucrose and 1% Triton X-100 and then incubated for 30 min on ice, followed by 15 min at 25 °C to solubilize the lipid microdomains. The samples were incubated overnight at 4 °C with anti-syn1 antibodies conjugated with protein G-Sepharose (Amersham Pharmacia Biotech). The beads were then washed three times with 0.2% octylglucopyranoside in PBS, and the protein complexes were eluted with 0.2 M glycine, pH 2.2, containing 0.2% octylglucopyranoside, neutralized with potassium phosphate, and separated on polyacrylamide gels.

The proteins in the gels were stained with SYPRO Orange (Bio-Rad) and analyzed using a Versa-Doc 3000 imaging system (Bio-Rad) or transferred to nitrocellulose filters for immunoblotting.

**Immunoblotting and Gαq/11 Detection**

The samples underwent SDS-PAGE on 6, 10, or 12% polyacrylamide gels and were then blotted onto nitrocellulose (NC) membranes (Schleicher & Schuell, Dassel, Germany) with 0.45-µm pores. The Western blots were analyzed as described previously (24). For all antibodies except anti-α1A and α1C, the blots were blocked overnight in 5% non-fat milk in Tris-buffered saline (TBS), washed in a buffer containing 5% non-fat milk and 0.3% Tween 20 in TBS (immunoblotting buffer), incubated for 2 h with the primary antibody diluted in immunoblotting buffer, and then washed and incubated with the appropriate peroxi-
dase-conjugated secondary antibodies. After another series of washes, the blots were incubated in a 1:5000 dilution of chemiluminescent substrates (Pierce). The antibodies directed against the calcium channel subunits were diluted from 1:400 to 1:2000 in 1% bovine serum albumin, 0.1% Tween 20 in TBS. To detect ganglioside Gαq, the samples were separated on 15% polyacrylamide gels, transferred to NC membranes, and incubated with peroxidase-conjugated choleratoxin subunit B diluted 1:100,000 in immunoblotting buffer. After extensive washing, peroxidase was detected using chemiluminescent substrates. Alternatively, 0.5–1 µl of each gradient fraction was spotted directly onto NC membranes, which were then probed with peroxidase-conjugated choleratoxin subunit B as described above.
Fig. 1. Different distribution of Ca_{2.1} and Ca_{1.2} in the lipid microdomains of synaptosomes and cell soma. The proteins in the fractions (Fr. No. 1–8) and the pellets (P) collected from discontinuous sucrose gradients of synaptosomes (P2) and cell soma membranes (P0) lysed in Triton X-100 were analyzed by Western blotting for the distribution of synaptotagmin1 (Syt), munc-18-1 (M-18), syn1 (Syn1), SNAP-25 (SN-25), VAMP-2 (VAMP2), and the pore-forming subunits of Ca_{2.1} (α1A) and Ca_{1.2} (α1C) and the transferrin receptor (TrR). The distribution of the G_{M1} marker was determined by labeling with peroxidase-conjugated cholera toxin. The sucrose concentrations in the fractions are indicated. Blots representative of four (P2 gradients) or two independent experiments (P3 gradients) are shown.

\[ ^{125}\text{I}-\text{Labeled α-Conotoxin MVIIC Binding} \]

\[ \text{P2 samples were resuspended in a buffer containing 145 mM choline chloride, 5 mM KCl, 1.2 mM CaCl}_2, 1.2 mM MgCl}_2, 2.4 mM NaH}_2\text{PO}_4, 10 mM glucose, and 10 mM HEPES-NaOH, pH 7.4 (buffer B). Each sample was incubated with 3 \mu M \text{of } ^{125}\text{I}-\text{CTxMVIIC for } 40 \text{ min at } 37^\circ\text{C} \text{ and then filtered through GF/B filters presoaked in } 1\% \text{ polyethyleneimine. After washing in buffer B, the radioactivity of the filters was counted in a gamma counter. Each point was evaluated in triplicate, with nonspecific } ^{125}\text{I}-\text{Labeled α-CTxMVIIC binding being evaluated for each group by means of the parallel incubation of samples in the presence of an excess of unlabeled toxin (100 nM).} \]

\[ \text{Ca}^{2+} \text{ Influx} \]

\[ \text{The synaptosomes (P2) were fixed with 2\% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, for 2 h at 4^\circ\text{C}, post-fixed with 1\% osmium tetroxide for } 1 \text{ h at room temperature, washed extensively, dehydrated in ethanol series, and then embedded in Epon. Sections of a pale-gold, silver interference color were obtained with a Reichert Ultracut E ultramicrotome, collected on copper grids, counterstained with a saturated aqueous solution of uranyl acetate and Reynold's lead citrate, and finally examined using a Philips CM10 transmission electron microscope.} \]

\[ \text{Electron Microscopy} \]

\[ \text{RESULTS} \]

Calcium Channels and SNAREs in Detergent-resistant Membranes Isolated from Synaptosomes—Over the last 10 years, various procedures have been developed for isolating the detergent-insoluble complexes obtained by treating cells or membranes with weak detergents at low temperature. These detergent-resistant complexes identify lipid microdomains that are thought to resemble the self-organized lipid-protein complexes present in the membrane before detergent treatment (Refs. 6–9 and references therein). We analyzed the lipid microdomains in rat brain fractions enriched in nerve terminal (synaptosome) or cell soma membranes following well-established procedures. The pellets enriched in synaptosomes (P2) were separated from the homogenates of rat brain cortices by means of differential centrifugation (23). The supernatants containing the remaining membranes and organelles of the cell soma (including the endoplasmic reticulum and Golgi apparatus) (24) were centrifuged at high speed to obtain P3 pellets. Low-density, Triton X-100-insoluble complexes prepared from P2 and P3 were then separated from the soluble proteins using flotation equilibrium sucrose density gradients as described previously (15, 26). It has been shown previously that this method separates the Triton X-100-insoluble complexes (which peak in fractions 3 and 4) from the fully soluble and cytoskeletal proteins (which peak in fractions 7–8). In line with previous data (26), the soluble fraction marker transferrin receptor was only detected in the very high-density fractions (fraction 8 in the P2 gradients; fractions 7 and 8 in the P3 gradients; Fig. 1). On the contrary, the low-density marker G_{M1} was detected mainly in the low-density fractions (peaking in fractions 3–4).

We then examined the gradient distribution of the molecular components of the exocytic machinery and the α subunits of Ca_{2.1} and Ca_{1.2} (Fig. 1). The proteins from the fractions collected from P2 or P3 gradients were separated on 12% gels, transferred onto NC filters, and probed with polyclonal antibodies directed against synaptotagmin1 (22) and munc-18-1 (15) or commercially available monoclonal antibodies against the SNARE proteins syn1, SNAP-25, and VAMP-2. As shown in
Fig. 1, significant amounts of SNAREs and synaptotagmin1 were found to float in the low-density fractions (mainly in fractions 3–4) collected from the Triton X-100-treated P2 or P3 samples, although larger amounts were detected in the high-density fractions, as reported previously for neuroendocrine PC12 cells (13). However, unlike in PC12 cells, a small but significant amount of the syn1-accessory protein munc-18-1 was also found in the low-density fractions of the P2 gradients (Fig. 1).

Not all of the proteins known to play a role in exocytic machinery function floated. In line with previous results (13), immunoblotting did not detect synaptophysin (which interacts with VAMP-2 in synaptic vesicle membrane) or complexes (cytosolic proteins that selectively bind to assembled SNARE complexes) (10) in the low-density fractions of the P2 gradients (data not shown).

We then investigated the flotation gradient distribution of Ca,2.1 and Ca,1.2 channels. The blots were probed with commercially available polyclonal antibodies directed against the α1A or α1C subunits. Before being used on the gradient fractions, the antibodies were immunoblotted on P2 membrane proteins, in which they recognized bands of the expected molecular size; in 6% polyacrylamide gels, the anti-α1A antibody recognized a major band of ~190 kDa and a very minor polypeptide of ~210 kDa, which appears after longer exposure (data not shown) and is consistent with the results of previous work on rat brain and chromaffin cell membranes (30, 31). The antibody against the α1C subunit detected three bands of ~190, 200, and 220/230 kDa (data not shown), as reported previously (19). When the blots were immunostained with irrelevant rabbit IgG as a control, no bands of the same molecular sizes as those revealed by the specific anti-α 49 subunit antibodies were immunodetected.

Analysis of the flotation gradient fractions using the anti-α1A or α1C antibodies revealed that the Ca,2.1 α1A subunit (immunodetected as the polypeptide of ~190 kDa) was almost exclusively present in the lower-density fractions (fractions 3–4) of the P2 gradients (Fig. 1), whereas the α1C subunit was mainly detected in the high-density fractions, although a minor portion was also immunodetected in the lighter fractions (Fig. 1). Finally, the amounts of α1A and α1C found in the Triton X-100-treated P3 samples were much less than those detected in the P2 membranes, and only very faint immunoreactive bands were detected in the high-density fractions.

To analyze further the presence of Ca,2.1 in the gradient fractions and confirm the immunoblotting results, we used a detection system based on 125I-labeled α-CTxMVIIC binding. The toxin-binding experiments were performed in gradient fractions collected from the Triton X-100-treated P2 samples, and the data of multiple experiments (n = 3) were averaged. As shown in Fig. 2, the majority of the toxin labeling was again found in fractions 3–4, whereas almost no toxin binding was revealed in fraction 7. These results are similar to those obtained by means of immunoblotting and further demonstrate that the antibodies recognized α1A subunits. The proportionally higher amount of 125I-labeled α-CTxMVIIC binding activity in fraction 4 (in comparison with the immunoblotting distribution shown in Fig. 1) may be attributable to slight variations in the different experiments and the limited linear range of the enhanced chemiluminescence technique.

Taken together, these data strongly suggest that Ca,2.1 and a portion of the proteins of the exocytic machinery are localized in lipid microdomains isolated from synapses.

**Effects of Cholesterol Perturbation on Ca,2.1 and SNARE Protein Distribution in Detergent-insoluble Fractions**—Because cholesterol is critical for the formation and stability of lipid microdomains, we analyzed the effects of cholesterol perturbation on the buoyancy of SNARE proteins and α1A subunits using saponin and MβCD. Saponin interacts with cholesterol and efficiently disrupts lipid microdomains, whereas MβCD is capable of including cholesterol in its hydrophobic pocket, thus perturbing the organization of lipid microdomains (25, 32). Furthermore, MβCD/cholesterol complexes can be used to load membranes with exogenous cholesterol (25).

When P2 samples were incubated with MβCD, the effect on cholesterol levels was dose dependent; 10 mM MβCD partially modified the amount of cholesterol (19.4 ± 8% versus control, n = 4), but 30 mM reduced the level of endogenous cholesterol to 60% (60.7 ± 4.8%, n = 6) without inducing any significant change in protein concentration (data not shown). We therefore used 30 mM MβCD in the subsequent experiments. As shown in Fig. 3, in comparison with the mock-treated (control) samples, the Gm1 marker in the P2 samples treated with MβCD shifted to the higher-density gradient fractions. Peak Gm1 reactivity was found in fractions 2–4 of the control sample but in fractions 4–6 of the MβCD-treated samples, thus indicating that the organization of the detergent-resistant membranes was altered by the cholesterol-chelator (Fig. 3). In line with this, α1A also shifted to the higher-density fractions: in the control P2 samples, the pore-forming subunit of the channel was immunodetected in fractions 3 and 4 but was mainly found in fraction 5 of the parallel samples treated with MβCD and analyzed by means of gradient centrifugation (Fig. 3, A and B). Similarly, MβCD treatment shifted the SNARE proteins from fractions 3–4 to the bottom of the gradients, with a substantial aliquot being detected in fraction 5 (Fig. 3B).

To investigate whether the effects on SNARE and α1A buoyancy were attributable to cholesterol removal, MβCD-treated P2 samples were incubated with MβCD/cholesterol complexes. After reloading, the cholesterol level was very similar to that observed in the control samples (98%). Analysis of Gm1 in the gradient fractions treated with MβCD/cholesterol complexes revealed a distribution pattern that was similar to that observed in the controls (compare Figs. 3 and 1), thus suggesting that the procedure at least partially restored lipid microdomain organization. Aliquots of SNARE proteins were also recovered in gradient fractions 3–4 (Fig. 3), once again with a distribution similar to that observed in the control P2 samples (see Fig. 1). On the contrary, cholesterol reloading had only a slight effect on the distribution of α1A, and only a very small portion of the Ca,2.1 subunits was recovered in the lighter fractions of the gradient (mainly in fraction 4); the majority of the protein was still detected in fraction 5 (Fig. 3). These results may indicate that cholesterol depletion modifies the physical prop-
Altered cholesterol levels in synaptosomal membranes modify the buoyant density of 
\( Ca_{2.1} \) and SNARE proteins. The untreated synaptosomes (Control, A) and those treated with MyCD (B) or MyCD/cholesterol (C) were lysed at 4 °C in 1% (w/v) Triton X-100 and loaded at the bottom of discontinuous sucrose gradients (see "Experimental Procedures"). Aliquots of the gradient fractions were analyzed by Western blotting for the distribution of the pore-forming subunits of \( Ca_{2.1} \) (\( \alpha 1A \)), synaptotagmin (Syn1), munc-18-1 (M-18), syn1 (Syn1), SNAP-25 (SN-25), and VAMP-2 (VAMP). The SNARE bands are representative of five (MyCD) or three (MyCD/cholesterol) separate experiments. A and B show the distribution of \( Ca_{2.1} \) in the control and the 30 mM MyCD P2 samples processed and analyzed in parallel. The distribution of the \( G_{M1} \) marker was determined by means of dot blots using peroxidase-conjugated cholera toxin. The asterisks indicate a nonspecifically labeled band of ~170 kDa, which was detected by enhanced chemiluminescence, even in the absence of the primary antibodies.

Properties of \( \alpha 1A \) subunits to such an extent that they cannot be fully restored by reloading, or that the association of \( \alpha 1A \) with lipid microdomains requires other lipids removed by MyCD that cannot be restored by means of cholesterol reloading.

Given the partial solubilization achieved with MyCD (possibly because of the incomplete removal of cholesterol from the P2 membranes), we incubated the samples with 1% saponin before Triton X-100 treatment. Two different protein:saponin ratios were used (see "Experimental Procedures"), and in both cases, \( G_{M1} \) was found in the very high-density fractions (Fig. 4), thus demonstrating that saponin affected the organization of the lipid microdomains more than the MyCD treatment (Fig. 3). In line with the distribution of \( G_{M1} \), the SNARE proteins and \( \alpha 1A \) were found mainly at the bottom of the gradients (Fig. 4). These results indicate that efficient disruption of lipid microdomains completely solubilizes \( Ca_{2.1} \) subunits and SNAREs.

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SNAP-25 (representative of five (MyCD) or three (MyCD/cholesterol) separate experiments. A and B show the distribution of \( Ca_{2.1} \) in the control and the 30 mM MyCD P2 samples processed and analyzed in parallel. The distribution of the \( G_{M1} \) marker was determined by means of dot blots using peroxidase-conjugated cholera toxin. The asterisks indicate a nonspecifically labeled band of ~170 kDa, which was detected by enhanced chemiluminescence, even in the absence of the primary antibodies.

... SNARE complexes were immunoprecipitated with monoclonal antibodies directed against syn1 or VAMP-2 from: 1) a pool of fractions 3 and 4 (3–4) containing detergent-resistant complexes; 2) a pool of fractions 5 and 6 (5–6) containing the majority of \( \alpha 1A \) subunits after MyCD treatment; and 3) fraction 7 containing the fully soluble proteins (Fig. 5A, input). In the absence of MyCD, substantial amounts of \( \alpha 1A \) were coimmunoprecipitated from the lipid microdomain-containing fractions (3–4) together with SNAP-25, VAMP-2, and synaptotagmin1 (Fig. 5, B and C). After MyCD treatment, although present with SNAREs in fractions 5–6 (see input), the \( \alpha 1A \) subunit was not coimmunoprecipitated with the use of anti-syn1 or VAMP-2 antibodies (Fig. 5, B and C). Similar results were obtained by means of immunoprecipitation with anti-SNAP-25 antibodies (data not shown).

Syn1 and munc-18-1 were coimmunoprecipitated from the lipid microdomain fraction (3–4) but not from fraction 5–6 (Fig. 5B). On the other hand, SNAREs, synaptotagmin1, and munc-18-1 were largely coimmunoprecipitated from fractions 7, possibly because of the presence of preformed protein-protein complexes (Fig. 5, B and C).

When immunoprecipitations were carried out using nonimmune mouse IgG, neither the proteins of the exocytic complex nor the \( Ca_{2.1} \) \( \alpha 1A \) subunits were immunodetected by Western blotting using specific antibodies (data not shown).

Taken together, these data suggest that \( Ca_{2.1} \) and SNAREs are contained within the same detergent-insoluble complex, but this colocalization is not maintained after partial extraction of cholesterol with MyCD.

Ca 2.1 Interacts with SNARE Complexes in Lipid Microdomains—Because it has been widely demonstrated that the presence of preformed protein-protein complexes (Fig. 5, B and C).

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Taken together, these data suggest that \( Ca_{2.1} \) and SNAREs are contained within the same detergent-insoluble complex, but this colocalization is not maintained after partial extraction of cholesterol with MyCD.
to investigate the presence of other components of the exocytic complex and α1A subunits in the immunoisolated proteins. As shown in Fig. 6B, VAMP-2, synaptotagmin1, and α1A were immunoisolated with syn1 from fraction 3, but interestingly, no significant amount of munc-18-1 was immunoprecipitated. On the contrary, the complexes isolated from fraction 7 contained substantial amounts of munc-18-1, as well as SNAREs and synaptotagmin1. Because interactions between syn1 and munc-18-1 or the cognate SNAREs are mutually exclusive (33), these results suggest that, in lipid microdomains, syn1 is largely engaged in complexes with the cognate SNARE, synaptotagmin1, and Ca2.1 to form the molecular machinery for neurotransmitter release (Refs. 10, 16, 17, and references therein). The association of munc-18-1 with the membrane regardless of syn1 binding is in line with the data reported by Garcia et al. (34), who demonstrated that a pool of membrane-associated munc-18-1 does not interact with syn1.

Role of Lipid Microdomains in Nerve Terminal [42]Ca2+ Influx—We next examined whether modified cholesterol levels in synaptic membranes have effects on nerve terminal calcium influx. P2 samples were incubated with 10 or 30 mM MβCD or left untreated. Electron microscopy showed that the drug treatment did not lead to any major alterations in nerve terminal morphology (Fig. 7, A and B). When the efficiency of the calcium influx was monitored, we found that depolarization-evoked calcium uptake was affected in the MβCD-treated synaptosomes in a dose-dependent manner and was clearly visible after treatment with 30 mM. The Cd2+ channel blocker reduced the evoked uptake of calcium in both the control and MβCD-treated synaptosomes, and more sustained inhibition was obtained when EGTA was included in the depolarization buffer (data not shown), thus indicating that the calcium influxes occurred via voltage-gated calcium channels.

**DISCUSSION**

The aim of this study was to investigate the mechanisms involved in the localization and clustering of Ca2.1 subtypes in presynaptic membranes. Our experiments provide the first evidence that Ca2.1 calcium channels are largely accumulated in lipid microdomains isolated from synaptosomes, in which they colocalize with proteins of the exocytic complex. These conclusions are supported by a number of experimental findings: i) the distribution of the large majority of α1A subunits in the low-density, detergent-resistant membrane fraction; ii) the shift in the buoyant density of α1A after alterations in membrane cholesterol levels with saponin or MβCD (a criterion chosen to confirm the association of a protein with lipid microdomains enriched in cholesterol); and iii) the immunoisolation of detergent-insoluble complexes containing SNAREs, synaptotagmin1, and α1A.

The accumulation in detergent-resistant membranes appeared to be more specific for Ca2.1; the behavior of the 1C subtype of Ca2.1.2 was different. The widespread distribution of α1A in lipid microdomains may play a role in the trafficking and subcellular compartmentalization (presynaptic versus post-synaptic membranes) of the channel. A recent study has shown that the interaction of α1A subunits with SNARE proteins is an important requirement for the efficient nerve terminal localization of Ca2.1 channels (35). Therefore, our results showing the codistribution of α1A and SNARE proteins suggest that lipid microdomains function as platforms for the recruitment and presynaptic localization of Ca2.1 channels and proteins of the exocytic machinery. This hypothesis is further supported by the observation that disrupting lipid microdomain organization inhibits the colocalization of Ca2.1 channels and SNAREs. However, specific localization in lipid microdomains is not sufficient to explain the subcellular dis-
Ca$_{2,1}$ interacts with SNARE complexes in lipid microdomains. In A and B, equal volumes of fractions 3 or 7 were incubated with anti-syn1 antibody covalently conjugated to protein G-Sepharose and then eluted from the beads and analyzed by SDS-PAGE. The proteins in the gels were stained with SYPRO Orange (A, the arrows indicate the position of SNAP-25 (SN-25) and syn1 or analyzed by Western blotting (B) using antibodies directed against α1A, synaptotagmin1 (Syt), munc-18-1 (M-18), and VAMP-2 (VAMP). The data are representative of three separate experiments.

thought to play an important role in the organization of presynaptic compartments (37, 38). Specific sequence domains and interactions with scaffolding proteins may therefore also be involved in the presynaptic targeting of Ca$_{2,1}$ channels.

Cholesterol depletion not only alters the distribution of α1A subunits but also affects calcium influx to nerve terminals. This effect may be explained by various mechanisms. The first implies the direct role of cholesterol in maintaining the correct molecular structure and function of Ca$_{2,1}$ and possibly of other calcium channel subtypes known to be localized in presynaptic membrane (1, 3). It has been reported that lipid composition affects the folding and activity (39–41) of voltage-gated K$^+$ channels. The K$_{1,5}$ and K$_{1,2}$ channel subtypes are specifically targeted to distinct lipid microdomains (40, 41), and if the cholesterol in the expressing cells is depleted with MβCD, the steady-state inactivation kinetics of the channels shifts in the hyperpolarization direction. The function of Ca$_{2,1}$ may therefore also be sensitive to specific chemical interactions with neighboring membrane components and the biophysical properties of the membrane environments. The disruption of lipid microdomains caused by cholesterol depletion may modify the membrane properties affecting channel activity and/or folding.

A second cause of impaired calcium influx to nerve terminals may be related to the altered distribution and inhibition of the interaction of Ca$_{2,1}$ with SNAPREs and synaptotagmin1 after cholesterol extraction with MβCD, which negatively influence channel activities (Refs. 16–18, 35 and references therein).

Finally, the possibility that cholesterol depletion alters the interaction of the α1A subunit with other important functional regulatory molecules such as neurexins cannot be excluded. Recent data have demonstrated that α-neurexins are essential for Ca$^{2+}$-triggered neurotransmitter release, which is impaired in neurexin-knockout mice, because Ca$^{2+}$ currents, Ca$_{2,2}$ and Ca$_{2,1}$ function, and synaptic neurotransmitter release are markedly reduced despite the apparently normal number of cell-surface calcium channels. This suggests that α-neurexins promote functional coupling between calcium channels and synaptic vesicle exocytosis and help the organization of the presynaptic domain (38). Interestingly, experimental evidence suggests that members of the neurexin superfamily are incorporated into lipid microdomains as a necessary step for cell surface sorting (42). In the light of these data, it can be speculated that disorganization of the lipid microdomains of presynaptic membranes impairs the interaction of neurexin with Ca$_{2,1}$ and contributes to the reduction in Ca$^{2+}$ influx in the nerve. Further experiments are required to define the mechanisms involved in the inhibition of Ca$^{2+}$ influx after alterations in the cholesterol content of presynaptic membranes.

In brief, our data demonstrate that Ca$_{2,1}$ channels are...
selectively accumulated in the lipid microdomains of nerve terminals, where they associate with the proteins of the exocytic complex. This compartmentalization may play an important role in organizing presynaptic membrane domains and may contribute to the coupling of calcium influxes and neurotransmitter release. Various lines of evidence indicate that neuronal lipid microdomains play a role in protein sorting and/or processing, neurotrophic factor signaling, axon guidance, and the localization and modulation of post-synaptic neurotransmitter receptors (Ref. 36 and references therein), and recent studies investigating the function of lipid microdomains in postsynaptic membrane organization have demonstrated the relevant role of these specialized microdomains in controlling the shape and number of spines and in stabilizing surface α-amino-3-hydroxy-5-methylisoxazole-4-propionyl acid receptors (43). However, although there is a considerable amount of information concerning postsynapses, little is known about the role of cholesterol-enriched microdomains in the organization of presynapses. Our results provide evidence that lipid microdomains also function in the molecular organization of specialized presynaptic membranes, where they play a role in the clustering of calcium channel subtypes with molecules of the exocytic machinery and their proper function.

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