EM 3D contour maps provide protein assembly at the nanoscale within the neuronal porosome complex

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Summary

The neuronal porosome complex, the secretory machinery at the plasma membrane of nerve terminals, is a 12–17-nm cup-shaped lipoprotein structure possessing a central plug. Since the porosome is a membrane associated, multi-protein complex measuring >650 kD, it has precluded generation of 3D crystals for x-ray diffraction studies, nor structural analysis at the atomic level using solution magnetic resonance spectroscopy. These limitations were partially overcome in the current studies, furthering our understanding of the porosome structure. Using atomic force microscopy, electron microscopy and electron density and 3D contour mapping, finally provides at the nanoscale, the structure and assembly of proteins within the neuronal porosome complex. Results from this study demonstrate a set of eight protein units lining the porosome cup, each connected via spoke-like elements to a central plug region within the structure. The isolation of intact porosomes for near-atomic resolution using cryo-electron diffraction measurements, is finally possible.

Introduction

In the past decade, permanent supramolecular structures called porosomes or fusion pores have been identified at the cell plasma membrane in neurons, exocrine, endocrine and neuroendocrine cells, where membrane-bound secretory vesicles transiently dock and fuse to expel their contents to the outside during cell secretion (Schneider *et al.*, 1997; Cho *et al.*, 2002a, b, 2004, 2007; Jena *et al.*, 2003; Jeremic *et al.*, 2003). These findings demonstrate the porosome to be the universal secretory machinery in cells (Jena, 2005, 2007). The overall morphology, composition and reconstitution of

this secretory machinery in exocrine pancreas and in neurons are well documented (Schneider *et al.*, 1997; Cho *et al.*, 2002a, 2004, 2007; Jena *et al.*, 2003; Jeremic *et al.*, 2003); however, much remains to be understood regarding the fine structure and molecular details of the assembly of proteins within the porosome complex, especially in neurons. Porosomes are supramolecular lipoprotein structures, composed of several proteins (Jena *et al.*, 2003; Jeremic *et al.*, 2003; Cho *et al.*, 2004, 2007) such as SNAP-23/25, syntaxin, synaptotagmin, the ATPase N-ethylmaleimide-sensitive factor (NSF), cytoskeletal proteins (actin, α-fodrin and vimentin), calcium channels β3 and α1c, chloride ion channels ClC2 and ClC3, and in some cases their isoforms. Recent studies further demonstrate cholesterol to be an integral component of the porosome complex, required for retaining its integrity and intramolecular interactions (Cho *et al.*, 2007). Besides the fact that t-SNAREs and calcium channels are present at the base of porosomes (Jena *et al.*, 2003), and the likely association of actin at the opening of the structure to the cell exterior (Schneider *et al.*, 1997; Jena *et al.*, 2003), localization of various proteins within the complex remains to be determined. Ideally, atomic coordinates of the complex using either x-ray crystallography or solution nuclear magnetic resonance spectroscopy (NMR) would provide structural details at the atomic level. However, the size and complexity of the membrane-associated porosome, has precluded determination of its atomic structure, which ultimately would provide a molecular understanding of its function. Solution NMR has not been possible primarily due to the large molecular size of the porosome complex, which is beyond the operating limits of current NMR's. Similarly, x-ray crystallography has not been possible, due in part to the solubility problems of this membrane-associated complex. In the present study, however, these limitations have been partially overcome in furthering our understanding of the fine structure and nano-arrangement of proteins within the native porosome complex, using high-resolution atomic force microscopy (AFM) and electron microscopy (EM).

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Materials and methods

Synaptosome preparation

Synaptosomes were prepared from rat brains according to published method (Cho *et al.*, 2004, 2007). Whole brain from Sprague-Dawley rats (Jackson Laboratory, Bar Harbor, ME, USA) weighing 100–150 g, were isolated and placed in ice-cold buffered sucrose solution (5 mM Hepes, pH 7.4, 0.32 M sucrose), supplemented with protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). The brain tissue was homogenized using 8 to 10 strokes in a Teflon-glass homogenizer. The total homogenate was centrifuged for 3 min at 2500× **g**, and the supernatant fraction was further centrifuged for 15 min at 14500× **g**, to obtain a pellet. The resultant pellet was resuspended in buffered sucrose solution, and loaded onto a 3–10–23% Percoll gradient. After centrifugation at 28 000× **g** for 6 min, the enriched synaptosomal fraction was collected at the 10–23% Percoll gradient interface.

Immunoisolation of neuronal porosome

To isolate the neuronal porosome complex, SNAP-25 specific antibody conjugated to protein A-sepharose (Santa Cruz Biotechnology, Santa Cruz, CA) was used. Solubilized synaptosomal membrane preparations in ice-cold Triton-Lubrol buffer (0.5% Lubrol; 1 mM benzamidine; 5 mM Mg-ATP; 5 mM EDTA; 0.5% Triton X-100, in phosphate buffered saline (PBS), in the absence or presence of saponin (0.5% w/v) and supplemented with protease inhibitors (Sigma, St. Louis, MO), was used. SNAP-25 antibody conjugated to protein A-sepharose, was incubated with the solubilized homogenate overnight in the cold room, followed by washing (3×) with 40 vol of wash buffer (500 mM NaCl, 10 mM Tris, 2 mM EDTA, pH 7.5). The immunoisolated sample attached to the immunosepharose beads was eluted using low pH buffer to obtain the porosome complex, which was immediately brought to neutral pH.

Preparation of PC: PS-associated neuronal porosome complexes

All lipids were obtained from Avanti Polar Lipids (Alabaster, AL). Preparation of lipid vesicles and their reconstitution with proteins were performed using previously published procedures (Cho *et al.,* 2007). Briefly, a 10 mM lipid stock solution was prepared by mixing lipid solution in chloroform-DOPC (1,2-dioleoyl phosphatidylcholine): DOPS (1,2-dioleoyl phosphatidylserine) in 70:30 mol/mol ratios, or DOPC:DOPS:cholesterol in 56:24:20 mol/mol/mol ratios in glass test tubes. The lipid mixture was dried under gentle stream of nitrogen and resuspended in decane. The lipids were then suspended in buffer containing 10 mM Hepes-NaOH [pH = 7.5] and 140 mM NaCl by vortexing for 5 min at room temperature. Vesicles were prepared following sonication for 2 min, and extrusion using an extruder with membranes of different pore size, to obtain different size vesicles. Large unilamellar vesicles were formed following sonication for 2 min. Liposomes ranging in size from 0.2–2 μm in diameter were obtained, as assessed by zeta sizer and AFM. Proteoliposomes were prepared by gently mixing the immunoisolated neuronal porosome complex obtained from solubilized synaptosome membrane, followed by three freeze/thaw cycles to enhance reconstitution at the vesicles membrane. As previously demonstrated in published studies (Cho *et al.*, 2004), these immunoisolated neuronal porosomes are functional when reconstituted into artificial lipid (PC:PS or PE:PC) membrane. These functional studies have previously been performed using an EPC9 electrophysiological setup, having cis and trans compartments, separated by a bilayer. The immunoisolated neuronal porosome complex is then reconstituted to the bilayer (Cho *et al.*, 2004). The porosome preparation is reconstituted into the lipid bilayer, which is formed by brushing PC:PS or PE:PC liposomes onto a 200-mm hole in the bilayer chamber until a stable bilayer with a capacitance between 100 and 250 pF is obtained. Addition of isolated synaptic vesicles into the cis compartment of the bilayer chamber containing calcium in the buffer solution, results in synaptic vesicle fusion, monitored as an increase in capacitance of the porosome-reconstituted bilayer (Cho *et al.*, 2004).

Photon correlation spectroscopy (PCS)

Photon correlation spectroscopy and right angle scattering, well-known techniques for the measurement of size of submicrometre particles and macromolecules, were performed using published procedure (Jeremic *et al.*, 2005, 2006; Cho *et al.*, 2007). PCS measurements were performed using a Zetasizer Nano ZS, (Malvern Instruments, Worcestershire, U.K.). The size distribution of isolated porosomes was determined using built-in software provided by Malvern Instruments. Prior to determination of porosome hydrodynamic radius, calibration of instrument was performed using latex spheres of known size. In PCS, subtle fluctuations in the sample scattering intensity were correlated across microsecond time scales. The correlation function is calculated, from which the diffusion coefficient is determined by the instrument. Using the Stokes–Einstein equation, hydrodynamics radius can be calculated from the diffusion coefficient. The intensity size distribution, which is obtained as a plot of the relative intensity of light scattered by particles in various size classes, is calculated from correlation function using the built-in software. The particle scattering intensity is proportional to the molecular weight squared. Volume distribution, which assigns more realistic weights to both small and large particles, is calculated from the intensity distribution using Mie theory. The transforms of the PCS intensity distribution to volume distributions was obtained using software by Malvern Instruments.

Transmission electron microscopy of rat brain tissue

Rat brain was perfused with normal saline solution followed by phosphate buffer (pH 7.4) containing 2.5% glutaraldehyde. After perfusion, rat brain was removed and diced into 1-mm^3 pieces. Brain slices were post-fixed in phosphate buffer containing 1.5% osmium tetroxide, dehydrated in graded ethanol and acetone and embedded in araldite. Brain tissue blocks were trimmed and sectioned. Sections 40–50-nm thick were obtained and mildly stained using lead citrate, followed by their examination using a JEOL JEM-100C transmission electron microscope (JEOL USA, Inc., Peabody, MA, USA).

Electron microscopy of the porosome complex

Aliquots (2.5 μL) of immunoisolated porosomes from rat brain synaptosomes were adhered to carbon-coated 400-mesh copper grids (Cu-400CN, Pacific Grid-Tech, San Francisco, CA, USA) previously rendered hydrophilic by glow discharge for 10 s. The grids were washed with drops of deionized water and then exposed to drops of 2% (w/v) uranyl nitrate as previously reported (Pettersen *et al.*, 2004). Images at 80 000× magnification were recorded at low defocus on 4 K × 4 K Gatan UltraScan charge-coupled device (CCD) under low electron dose conditions using a Tacnai 20 electron microscope (Philips Electron Optics/FEI, Eindhoven, The Netherlands) operating at 200 kV. Each pixel of the micrographs corresponds to 1.4 Å at the level of the specimen. Particles in micrographs were selected and windowed using EMAN software (Ludtke *et al.,* 1999). Each window size is 160 pixels, corresponding to 225 Å in specimen.

Contour mapping

To display the protein structure at greater detail, contour maps of proteins of the neuronal porosome complex was created by SPIDER software (Frank *et al.*, 1996). All particles are first normalized using a mean density of 0 and standard deviation of 10, then, contoured by the lowest ring at 0.5. Outside the protein complex, the background is flattened and smoothed. The protein boundary is defined by the darkest shadow around the particles, which results from the contrast transfer function of electrons.

Porosome topography from electron density maps

Heavy metal staining of the porosome enables an estimation of the relative size and arrangement of proteins in electron micrographs of the complex. Thus, density of negative stained proteins in electron micrographs, reflect the dimension, concentration and arrangement of protein at the various locations. Higher density in the micrograph corresponds to greater amount of protein at that location. Using CHIMERA software developed at UCSF (Pettersen *et al.*, 2004; Goddard

et al., 2005), protein density distributions were determined and revealed in three dimensions. Here, the colours from red, yellow, green to blue, correspond to the protein image density from lowest to the highest. The highest peak in each image is presented at 27 Å.

Atomic force microscopy

Isolated synaptosomes, and immunoisolated neuronal porosomes reconstituted in lipid membrane in PBS, pH 7.5, were imaged using the AFM (BioScope III, Digital Instruments, Santa Barbara, CA). AFM imaging was performed in fluid (PBS, pH 7.5) using the 'tapping' mode. All images presented in this study were obtained in the tapping mode in fluid, using silicon nitride tips with a spring constant of 0.06 N/m, and an imaging force of <200 pN. Images were obtained at line frequencies of 1.98 Hz, with 512 lines per image, and constant image gains. Tip velocity 11.4 mm/s; tip spring constant 0.06 N/m; sample/line 512; integral gain 2.0; proportional gain 1.0; amplitude set point 0.12–0.28 V; drive frequency 7.76–8.12 KHz and drive amplitude 150–400 mV, were used. Topographical dimensions of both native and lipid-reconstituted porosomes were analysed using the NanoScope IIIa version 4.43r8 software, supplied by Digital Instruments.

Results and discussion

Neuronal communication depends on the fusion of 40–50 nm in diameter membrane-bound synaptic vesicles containing neurotransmitters at the presynaptic membrane. In earlier studies (Cho *et al.*, 2004, 2007; Figs 1 and 2), 12–17 nm in diameter cup-shaped neuronal porosomes at the presynaptic membrane have been demonstrated. The porosomes are permanent structures at the presynaptic membrane, where synaptic vesicles transiently dock and fuse to release neurotransmitters. The morphology, isolation, composition and functional reconstitution of porosomes present at the nerve terminal, have also been described (Cho *et al.*, 2004, 2007). In the current study, AFM, EM and electron density measurements followed by contour mapping, and 3D topography, provide for the first time, the arrangement of proteins at nanometres resolution within the neuronal porosome complex. Results from this study demonstrate that proteins at the central plug of the porosome, interact with proteins at the periphery of the complex, conforming to its 8-fold symmetry. Furthermore, at the centre of the porosome complex representing the porosome base, where synaptic vesicles dock and transiently fuse, proteins, possibly comprised t-SNAREs, were found assembled in a ring conformation.

Electron micrographs of presynaptic membrane at the nerve terminal, demonstrate presence of approximately 12-nm cup-shaped porosomes (Cho *et al.,* 2004, 2007; Fig. 1), where 30–50-nm synaptic vesicles dock and fuse to enable the release
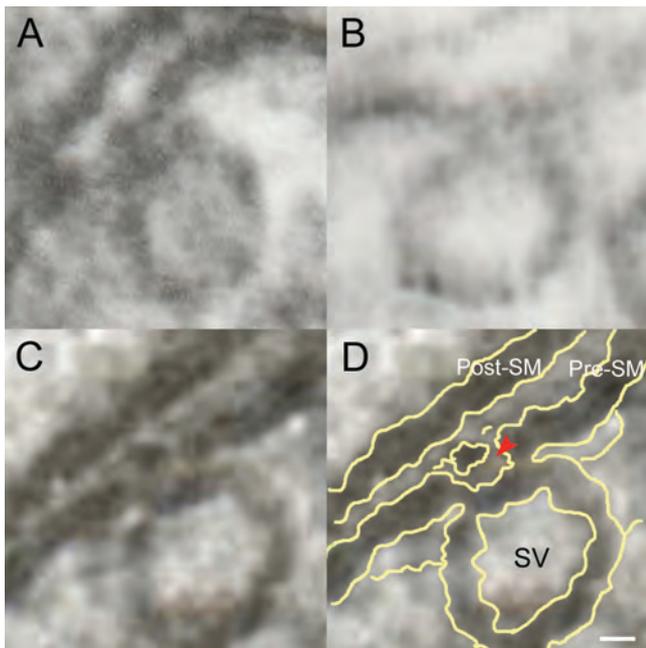
Fig. 1. Electron micrographs of the neuronal porosome complex (A–D). Electron micrographs of cup-shaped neuronal porosomes measuring ∼12 nm, are present at the presynaptic membrane (Pre-SM). Thirty-five to 50-nm synaptic vesicles are seen docked at the base of the neuronal porosome. Note the central plug (red arrowhead) of the porosome complex (D). Bar = 5 nm.

Fig. 2. Neuronal porosome complex, examined using atomic force microscopy and photon correlation spectroscopy. Atomic force micrograph of neuronal porosome at the presynaptic membrane (A), and an isolated neuronal porosome complex, reconstituted in lipid membrane (B). Note the central plug and the eight ridges in the atomic force micrographs of the fusion pore, both at the presynaptic membrane and in the isolated lipid-reconstituted complex. Bridges connecting the ridges with the central plug are also seen in the atomic force micrographs. A schematic diagram of the cup-shaped porosome complex, with a synaptic vesicle docked at its base is shown (C). Photon correlation spectroscopy of the isolated porosome protein complex, demonstrates the structure to be 12–17 nm in size (D).

of neurotransmitters. Studies (Cho *et al.,* 2004, 2007; Fig. 2) using AFM on isolated synaptosomes, confirm the presence of 12–17 nm in diameter cup-shaped neuronal porosomes at the presynaptic membrane. These studies show that porosomes are permanent structures at the presynaptic membrane of the nerve terminal, where synaptic vesicles transiently dock and fuse to release neurotransmitters. Although, the general morphology, isolation, composition and functional reconstitution of neuronal porosomes, have previously been described (Cho *et al.,* 2004, 2007), high-resolution images using AFM of isolated synaptosome preparations in buffered solution, and of reconstituted porosomes in lipid membrane are shown in Fig. 2, to orient the reader. Preparation of lipid membrane and their reconstitution with immunoisolated neuronal porosomes were performed using previously published procedures (Jeremic *et al.,* 2003; Cho *et al.,* 2004, 2007). Isolated synaptosomes, and immunoisolated neuronal porosomes reconstituted in the lipid membrane in PBS, pH 7.5, were imaged using AFM in the tapping mode in fluid, using silicon nitride tips with a spring constant of 0.06 N/m, and an imaging force of <200 pN. Close examination of porosomes in the atomic force micrographs reveal an array of eight globular units arranged at the lip of the porosome opening to the outside. The globular elements appear to be tethered to a central plug-like structure in the atomic force micrographs (Fig. 2A and B).
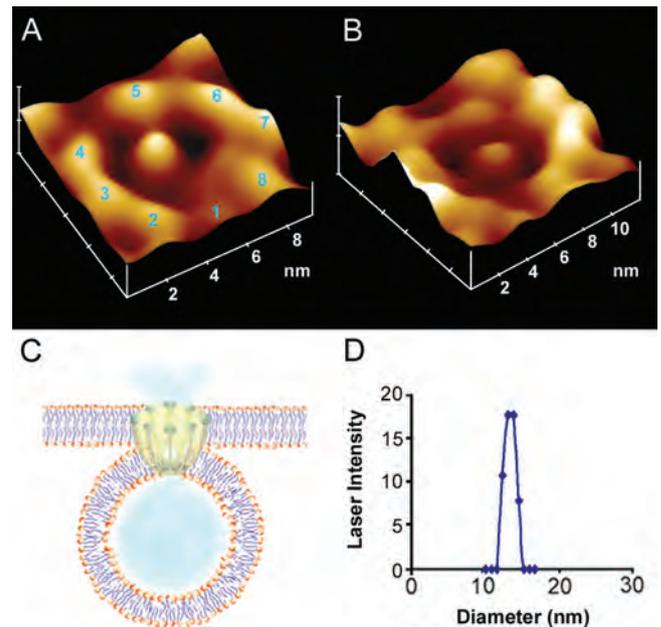
High-resolution negative staining EM (Fig. 3) of the immunoisolated native porosome complexes from rat brain tissue, followed by contour mapping (Fig. 4) and 3D topology measurements (Fig. 5), provide for the first time, the nano arrangement of proteins within the structure. Using SNAP-25-specific antibody, immunoisolation of neuronal porosomes from Triton/Lubrol-solubilized synaptosome membrane is demonstrated, as it readily reconstitutes into artificial PC:PS membrane and exhibits in AFM micrographs, all of the characteristic features exhibited in the native structure (Fig. 2B). Information from Figs 1 and 2A and B, has helped to generate a model of the porosome complex, as a cup-shaped structure having a centrally located plug (Fig. 2C). PCS, further confirms the immunoisolated porosome complex to measure on average, 14 nm (Fig. 2D). In PCS measurements, the size distribution of isolated porosome complexes is obtained from plots of the relative intensity of light scattered by particles of known sizes and a calculation of their correlation function (Jeremic *et al.,* 2005, 2006).

Negative staining EM was performed using low electron dose, in a Tacnai 20 electron microscope (Philips Electron Optics/FEI) operating at 200 kV (Ludtke *et al.,* 1999). Results
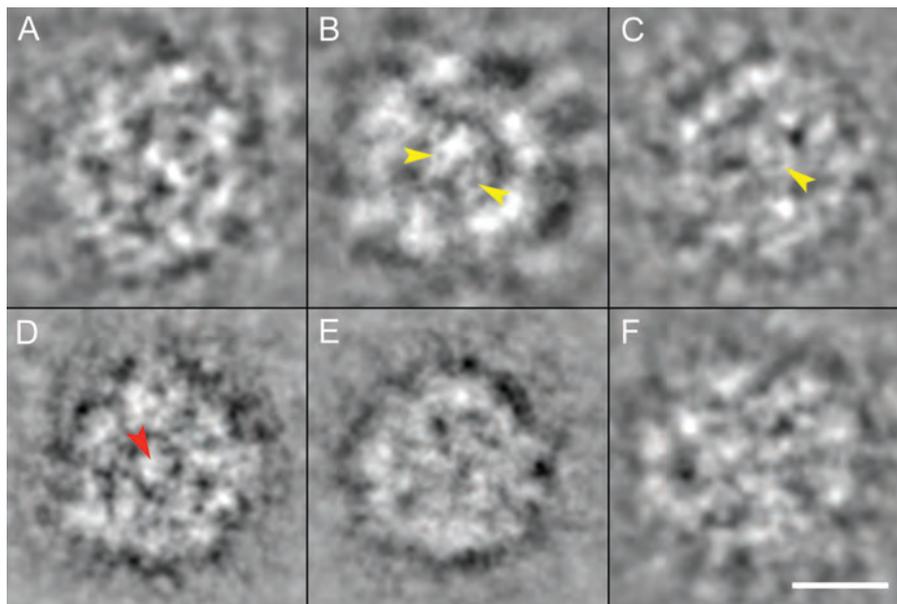
Fig. 3. Negatively stained electron micrographs of isolated neuronal porosome protein complexes (A–F). Note the 12–17-nm complexes exhibiting a circular profile and having a central plug (red arrowhead). Approximately 8 to 10 interconnected protein densities are observed at the rim of the structure, which are connect to a central element via spoke-like structures. At the centre of the structure, which correspond to the porosome base, there are proteins arranged in rings (yellow arrowhead). Bar = 5 nm.

from this study demonstrate that proteins at the central plug of the porosome complex interact with proteins at the periphery of the structure. Similar to AFM micrographs, approximately 8 to 10 interconnected protein densities are observed at the lip of the porosome complex in the electron micrographs (Fig. 3). The 8 to 10 interconnected protein densities are also connected to the central plug, via spoke-like structures (Fig. 3). Electron density and contour maps and resultant 3D topology profiles of the porosome complex, provide further details of the circular arrangement of proteins, and their connection to the central plug via distinct spokes (Figs 4 and 5). The contour map of proteins within the neuronal porosome
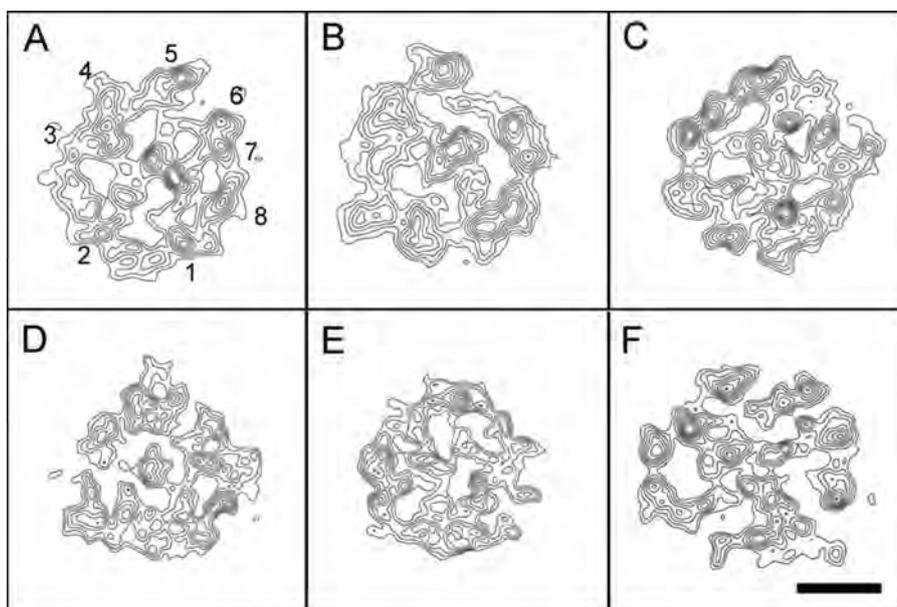

Fig. 4. Electron density maps of negatively stained electron micrographs of isolated neuronal porosome protein complexes (A–F). Note as in Fig. 3, the 12–17-nm complexes exhibiting a circular profile and having a central plug, with 8 to 10 interconnected protein densities at the rim of the complex, and connect to a central element. Bar = 5 nm.
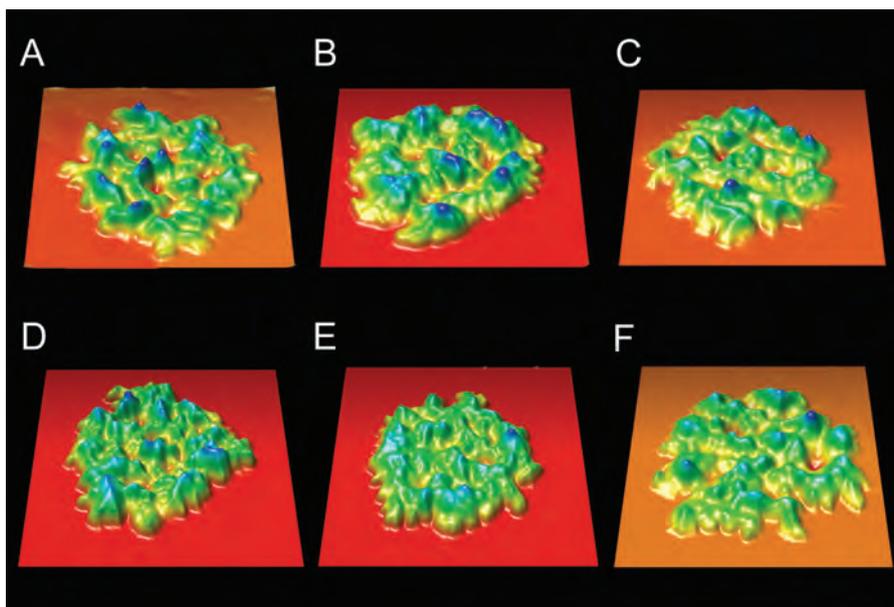
Fig. 5. 3D topography of porosomes obtained from their corresponding electron density maps (A–F). Note the 12–17-nm neuronal porosome complexes, clearly exhibiting a circular profile having a central plug, with distinct radiating spokes from the central element connecting each of the approximately 8 to 10 interconnected protein densities at the periphery of the structures. The colours from yellow, through green to blue, correspond to the protein image density from lowest to the highest. The highest peak in each image represents 27 Å.

complex was obtained using a published procedure (Frank *et al.*, 1996; Pettersen *et al.*, 2004; Goddard *et al.*, 2005). Furthermore, the centre of the porosome complex representing the porosome base where synaptic vesicles dock and fuse, show ring-like arrangement of proteins, possibly composed of t-SNAREs. Results from these studies provide for the first time the arrangement of proteins at the nanometre scale within the neuronal porosome complex. The next level of understanding of this supramolecular structure requires isolation of the intact complex in bulk, for cryoelectron diffraction studies, which are currently under way.

Acknowledgements

References

Cho, S.-J., Quinn, A.S., Stromer, M.H., Dash, S., Cho, J., Taatjes, D.J. & Jena, B.P. (2002a) Structure and dynamics of the fusion pore in live cells. *Cell Biol. Int.* **26**, 35–42.

Cho, S.-J., Jeftinija, K., Glavaski, A., Jeftinija, S., Jena, B.P. & Anderson, L.L. (2002b) Structure and dynamics of the fusion pores in live GH-secreting cells revealed using atomic force microscopy. *Endocrinology* **143**, 1144–1148.

Cho, W.J., Jeremic, A., Rognlien, K.T., Zhvania, M.G., Lazrishvili, I., Tamar, B. & Jena, B.P. (2004) Structure, isolation, composition and reconstitution of the neuronal fusion pore. *Cell Biol. Int.* **28**, 699–708.

Cho, W.J., Jeremic, A., Jin, H., Ren, G. & Jena, B.P. (2007) Neuronal fusion pore assembly requires membrane cholesterol. *Cell Biol. Int.* **31**, 1301–1308.

Frank, J., Radermacher, M., Penczek, P., Zhu, J., Li, Y., Lasjadj, M. & Leith, A. (1996) SPIDER and WEB: processing and visualization of images in 3D electron microscopy and related fields. *J. Struct. Biol.* **116**, 190–199.

Goddard, T.D., Huang, C.C. & Ferrin, T.E. (2005) Software extensions to UCSF Chimera for interactive visualization of large molecular assemblies. *Structure* **13**, 473–482.

Jena, B.P. (2005) Molecular machinery and mechanism of cell secretion. *Exp. Biol. Med.* **230**, 307–319.

Jena, B.P. (2007) Secretion machinery at the cell plasma membrane. *Curr. Opin. Struct. Biol.* **17**, 437–443.

Jena, B.P., Cho, S.J., Jeremic, A., Stromer, M.H. & Abu-Hamdah, R. (2003) Structure and composition of the fusion pore. *Biophys. J.* **84**, 1–7.

Jeremic, A., Kelly, M., Cho, S.J., Stromer, M.H. & Jena, B.P. (2003) Reconstituted fusion pore. *Biophys. J.* **85**, 2035–2043.

Jeremic, A., Cho, W.J. & Jena, B.P. (2005) Involvement of water channels in synaptic vesicle swelling. *Exp. Biol. Med.* **230**, 674–680.

Jeremic, A., Cho, W.J. & Jena, B.P. (2006) Cholesterol is critical to the integrity of neuronal porosome/fusion pore. *Ultramicroscopy.* **106**, 674–677.

Ludtke, S.J., Baldwin, P.R. & Chiu, W. (1999) EMAN: semiautomated software for high-resolution single-particle reconstructions. *J. Struct. Biol.* **128**, 82–97.

Pettersen, E.F., Goddard, T.D., Huang, C.C., Couch, G.S., Greenblatt, D.M., Meng, E.C. & Ferrin, T.E. (2004) UCSF Chimera – a visualization system for exploratory research and analysis. *J. Comput. Chem.* **25**, 1605–1612.

Schneider, S.W., Sritharan, K.C., Geibel, J.P., Oberleithner, H. & Jena, B.P. (1997) Surface dynamics in living acinar cells imaged by atomic force microscopy: identification of plasma membrane structures involved in exocytosis. *Proc. Natl. Acad. Sci. U S A* **94**, 316–321.