Direct interaction between SNAP-23 and L-type Ca^{2+} channel

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Abstract

During secretion, membrane-bound secretory vesicles dock and fuse at the base of porosomes in the cell plasma membrane. Among other proteins, the porosome is composed of SNAREs and Ca^{2+}-channels. Ca^{2+}-channels and SNAREs have been implicated in cell secretion. Several immunoprecipitation and binding studies suggest the physical interaction of the t-SNARE proteins, Syntaxin-1 and SNAP-25 with various Ca^{2+}-channels. In this study, using yeast two-hybrid and immunoanalysis, we demonstrate for the first time, direct interaction of SNAP-23 and a L-type Ca^{2+}-channel at the plasma membrane in pancreas.

Keywords: SNAP-23 • ; L-type Ca^{2+} channel • yeast two-hybrid system

Introduction

Atomic force microscopy (AFM) in combination with electron microscopy (EM) and biochemical approaches, has helped in the discovery of the fusion pore or porosome at the cell plasma membrane, where secretory vesicles dock and fuse to release their contents [1–7]. The structure of the porosome in both exocrine [1, 2, 5, 6], neuroendocrine cells [3, 4], and in neurons [7], at nanometer-subnanometer resolution, and its dynamics in real time, have been determined. Immunochemical studies further demonstrate that t-SNAREs, NSF, actin, vimentin, α-fodrin and the calcium channels α1c and β 3, are part of the fusion pore complex

[5, 6]. The localization and possible arrangement of SNAREs at the fusion pore, was demonstrated from combined AFM, immunoAFM and electrophysiological measurements. Our studies reveal that the fusion pore or porosome is a cup-shaped lipoprotein structure, the base of which has t-SNAREs and allows for docking and fusion of secretory vesicles, and the release of secretory products.

Calcium influx through voltage-gated Ca^{2+}-channels (VGCC's) has been implicated in triggering fusion of secretory vesicle membranes at the cell plasma membrane [8]. Soluble N-ethylmalemide-sensitive factor attachment protein receptor (SNARE) complex, consisting of t-SNAREs or target SNARE proteins (syntaxin and SNAP-25/23) at the plasma membrane and v-SNARE or vesicle associated membrane protein (synaptobrevin or VAMP) at the vesicular membrane, plays an indispensable role in mediating

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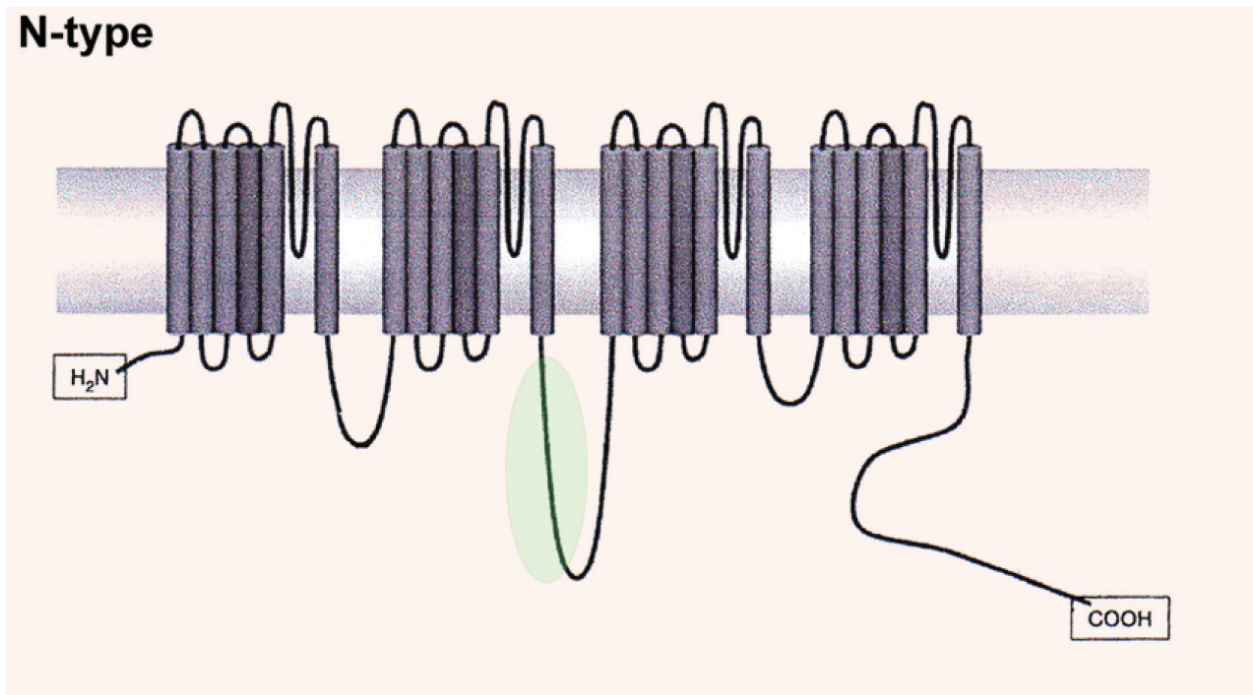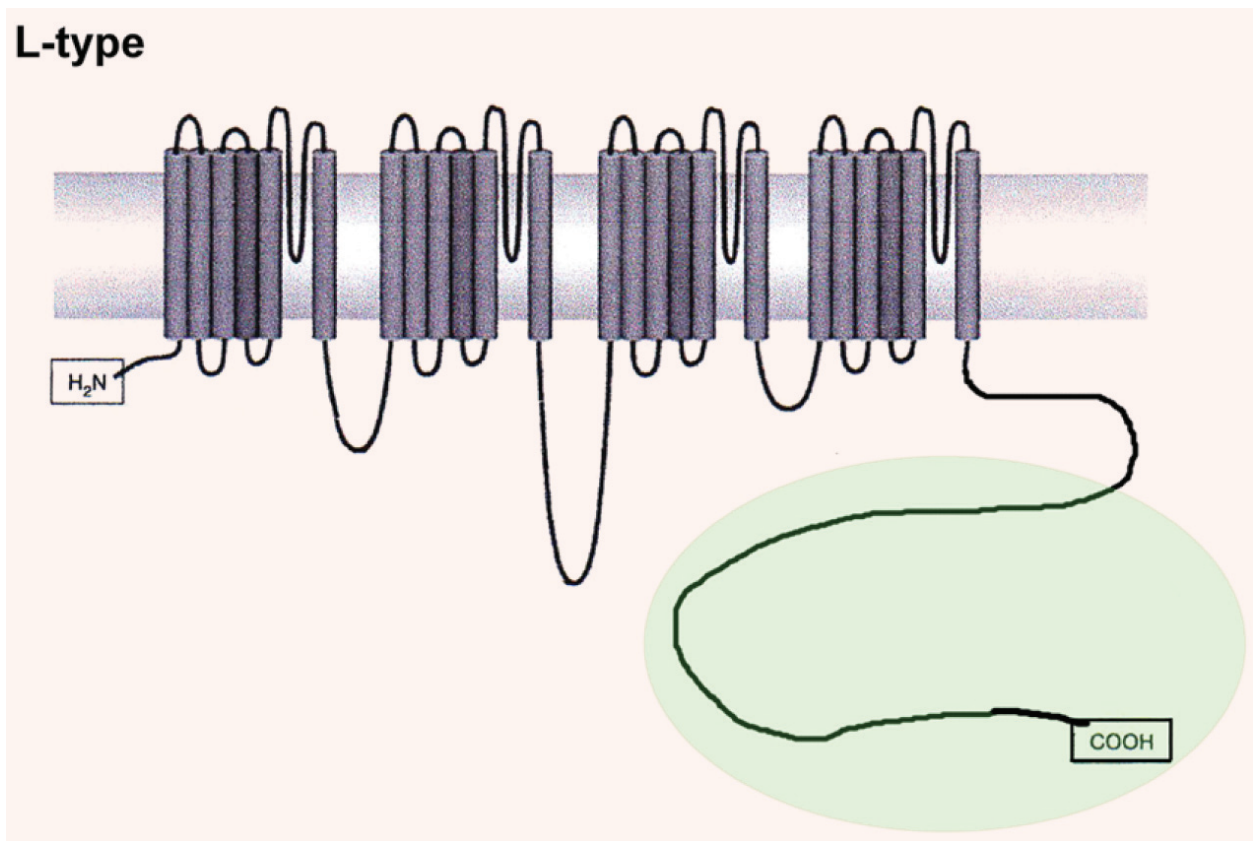Web: http://www.med.wayne.edu/physiology/facultyprofile/jena

N-type

L-type

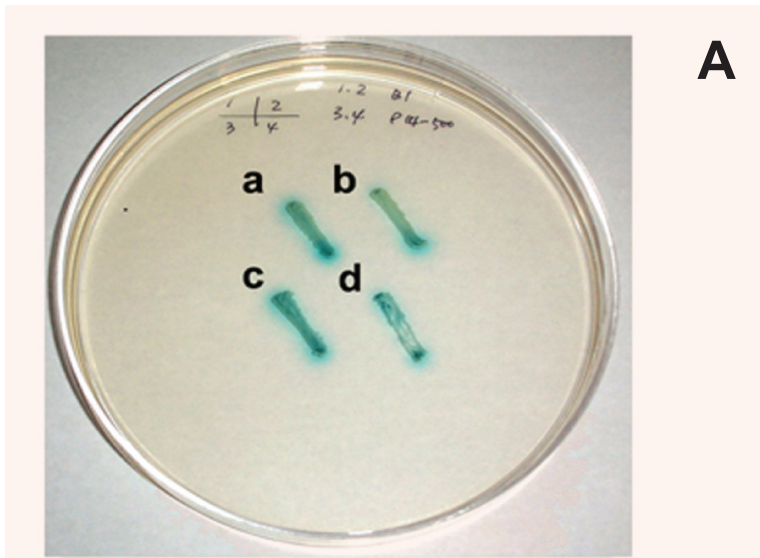**Fig. 1** Transmembrane domain of N- and L-type Ca^{2+}-channel, illustrating major protein interaction sites (green background).

Fig. 2 Yeast two-hybrid screening of SNAP-23 interacting proteins. (A) Direct interaction between SNAP-23 and candidate proteins were tested on a LacZ assay plate. Out of a total of sixty-eight clones, two clones (clone number 21, 55) were identified as non false-positive genes (a, b). Others were false-positive nuclear genes. One of them (clone number 1) is show (c). Positive control clone was used with p53 and SV40 large T-antigen, which were interact in a yeast two-hybrid assay according to kits recommendation (d). (B) After sequencing, the two positive clones were found to be identical. Amino acids sequences of the two clones had high homology with the C-terminal end-region of L-type Ca^{2+}-channel (Genbank accession No. AF394940).
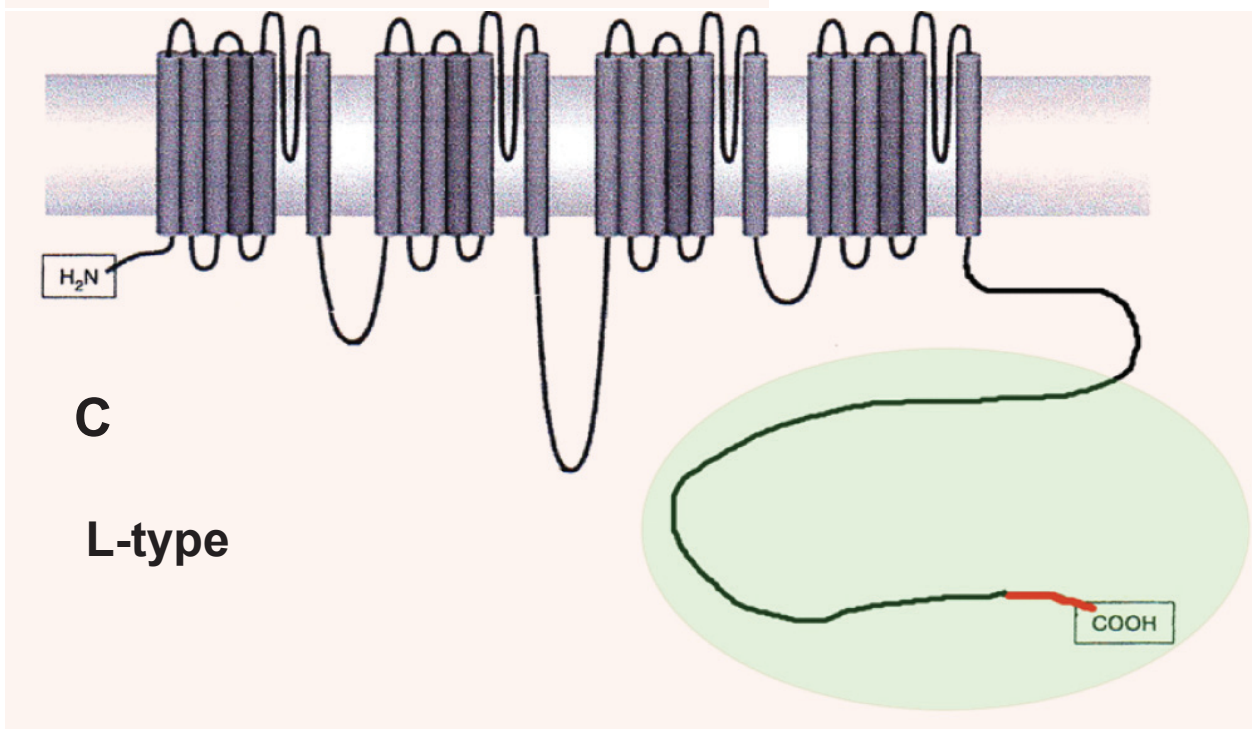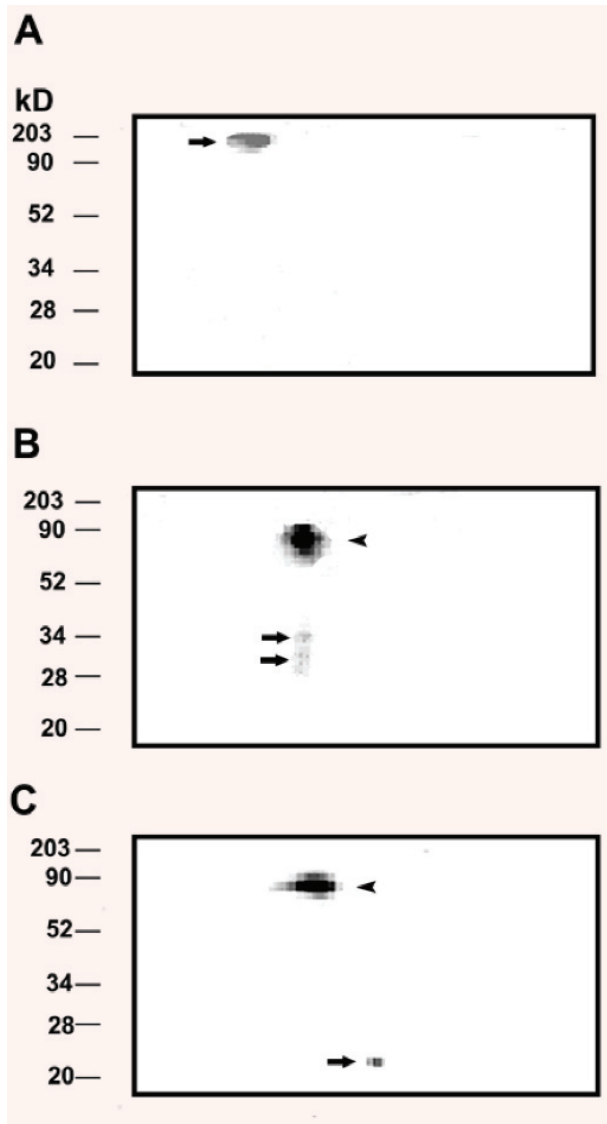
Fig. 3 SNAP-23 immunocomplex from pancreatic plasma membrane contain L-type Ca^{2+}-channel and syntaxin-2. SNAP-23 immunocomplex from solubilized pancreatic plasma membrane, when resolved by 2D gel electrophoresis, electroblotted to nitrocellulose membrane and immunoprobed, demonstrate (depicted by arrows) the presence of L-type Ca^{2+}-channel (A), syntaxin-2 (B) and SNAP-23 (C). A distinct spot with apparent molecular weight of ~70 (depicted by arrowheads) reveals the presence of SNARE complex (B-C).

Ca^{2+}-channels at release sites in a number of cells using combined immunofluorescent imaging and electrophysiological recordings [9–12]. The association of syntaxin 1A, SNAP-25 and synaptotagmin with N-, P/Q and L-type VGCC's (Fig. 1) is suggested from the results of co-immunoprecipitation experiments [13–16]. *In vitro* binding studies suggest that presynaptic N- and P/Q-type Ca^{2+}-channels may interact directly with two presynaptic membrane proteins, syntaxin-1 and synaptotagmin through a specific synprint site at the II-III cytosolic domain, in a Ca^{2+}-indepent [14] and a Ca^{2+}-dependent manner [17, 18]. The II-III domain of the 1c L-type Ca^{2+}-channel subunit that is predominantly expressed in endocrine and neuroendocrine cells, also binds syntaxin-1 and SNAP-25, but with lower affinity compared to the N- and P/Q-channels [16]. Furthermore, studies performed in Xenopus oocytes demonstrate a strong functional relationship between synaptic proteins and Ca^{2+}-channels, since channels exhibit profoundly different kinetic properties in the presence and absence of SNAREs [16, 19].

Close proximity of Ca^{2+}-channels to the releasing sites and the interaction of the Ca^{2+}-channel with synaptic proteins revealed by *in vitro* binding assays, along with functional studies further provide strong but indirect evidence of the physical interaction between these proteins. However, there is no direct evidence that Ca^{2+}-channels physically interact with SNAREs in the native physiological state. Co-immunoprecipitation of SNAREs and Ca^{2+}-channels does not demonstrate a direct interaction between the two proteins, since the proteins could be indirectly connected *via* an intermediate protein or proteins. Similarly, immuno-fluorescent and immuno-electron microscopic studies lack in resolution at the single protein level, and therefore can only reveal the proximity of Ca^{2+}-channels with synaptic proteins [10, 12, 20]. Therefore, to assess the direct interaction of protein(s) with the t-SNARE SNAP-23 in the exocrine pancreas, a yeast-two hybridization system [21–27] was employed. In addition, our results were further confirmed by using immunoprecipitation and immunoblot assays. In the present study reveals a direct physical interaction between an L-type voltage-dependent Ca^{2+}-channel and the SNARE protein SNAP-23.

this fusion event. A number of recent functional and structural studies suggest physical and functional interaction of VGCC's with SNAREs [9–19]. Studies demonstrate the co-localization of

Materials and methods

Yeast two-hybrid screen.

A male Sprague Dawley rat weighting 80-100 g was euthanized by CO_2 inhalation. The pancreas was dissected and diced into 0.5 mm^3 with a razor blade. To isolate total pancreatic RNA, the diced pancreas was agitated for 20 min. on ice in a siliconized glass tube containing TRIZOL reagent (Invitrogen, Carlsbad, CA) as described by the manufacture's instruction. A full-length cDNA of SNAP23 (Genbank accession No. AF052596) was generated by RT-PCR from mRNAs of rat pancreatic tissue with the following forward and reverse primers: 5'-GAAGATCTGAATTCAT-GGCCGAGGACGCAGA-CATG-3' and 5'-GGGGTACCGTCGACTTAAC-CACTTCCCAGCATCTT-3'. The resulting PCR product was digested, cloned into the EcoR I and Sal I sites of pGBKT7 vector (BD Biosciences Clontech, Palo Alto, CA), downstream of the GAL4 DNA-binding motif, and sequenced for confirmation prior to use as bait in the yeast two-hybrid screen. The resulting construct was transformed into the yeast strain Y187 (Clontech). To screen for potential binding partners of SNAP23, a library containing rat pancreatic cDNA was cloned in downstream of the GAL4 activating motif of the pGADT7-RecT vector (Clontech) and transformed into the AH187 strain. Yeast two-hybrid screens were performed according to the instructions of the manufacturer (Clontech, MATCHMAKER Library Construction & Screening Kit). To retest protein-protein interaction in yeast, cotransformation into the AH187 strain was performed with bait plasmids and library plasmids that were isolated from positive colonies.

SNAP-23 immunocomplex isolation and immunoblot analysis.

To isolate the SNAP-23-associated protein complex from rat pancreatic plasma membrane (PM) fraction, SNAP-23 specific antibody conjugated to protein A-sepharose was used, as previously described [6]. Isolated pancreatic plasma membrane preparations were solubilized in Triton/Lubrol solubilization buffer (0.5 % Lubrol; 1 mM benzamidine; 5 mM ATP; 5 mM EDTA; 0.5 % Triton X-100, in PBS) supplemented with protease inhibitor mix (Sigma). SNAP-23 antibody conjugated to

the protein A-sepharose (Sigma, St. Louis, MO) was incubated with the solubilized membrane for 1 h at R.T. followed by 4 washes 10 vol. each, using a washing buffer (500 mM NaCl, 10 mM TRIS, 2 mM EDTA, pH= 7.5). The SNAP23-immunoisolated protein(s) were resolved by 2D gel electrophoresis. In the first dimension, 16-benzyldimethyl-n-hexadecylammonium chloride (16-BAC) electrophoresis was performed followed by 12.5% SDS-PAGE [28]. The 2D-resolved proteins were electrotransferred to a nitrocellulose membrane, and probed (immunoblotted) using specific antibodies to syntaxin-2 (Alomone Labs, Jerusalem, Israel), SNAP-23 (ABR) and Ca^{2+}- α1c (Santa Cruz, CA). The nitrocellulose was incubated for 1 h at room temperature in blocking buffer (5% non-fat milk in PBS containing 0.1% Triton X-100 and 0.02% NaN3), and immunoblotted for 1 h at R.T. using specific antibodies. Primary antibodies were used at a dilution of 1:3,000 for syntaxin-2 and SNAP-23 and 1:500 for Ca^{2+}-α1c in blocking buffer, respectively. The immunoblotted nitrocellulose sheets were washed in PBS containing 0.1% Triton X-100 and 0.02% NaN3 and incubated for 1 h at R.T. in horseradish peroxidase-conjugated secondary antibody at a dilution of 1:3,000 in blocking buffer. The immunoblots were then washed in PBS buffer, processed for enhanced chemiluminescence and developed using a Kodak 440 image station.

Results and discussion

We explored protein-protein interactions using the yeast two-hybrid system [21, 22] to identify candidate proteins that interact with SNAP-23 in rat pancreas. To perform the two-hybrid screening, the yeast strain AH187 transformed with library construction as target was mated with Y187 transformed with full length SNAP-23 as bait. From a total 1 x 10^8 transformants, sixty-eight positive colonies were identified. DNA sequencing and database searches determined two [10] clones with sequences corresponding to the carboxyl terminal end-region of the L-type Ca^{2+}-channel (Fig 2. A–C). After the plasmids rescued from yeast clones, cotransformation were performed with as bait plasmids to confirm the clones are not false positive (data not shown).

To further assess our yeast two-hybrid results, solubilized rat pancreatic plasma membrane prepa-

ration were immunoprecipitated using SNAP-23 antibody. The resolved proteins where probed with L-type Ca^{2+}-channel antibodies $\alpha 1c$; confirmed its presence as a specific ~200 kd spot (Fig. 3A). The presence of the other t-SNARE protein in exocrine pancreas [29] was also investigated in the SNAP-23 immunoisolates (Fig. 3B–C). Immunopositive spots with apparent molecular masses of 34 kD and 23 kD were detected, when probed with syntaxin-2 and SNAP-23 specific antibodies, respectively. Hence, tight association of 1C subunit with t-SNAREs, syntaxin-2 (Fig. 3B) and SNAP-23 (Fig. 3C), is demonstrated. A distinct spot with apparent molecular weight of ~70kD was also detected, presumably representing the SNARE complex (Fig. 3B and C). The SNAP-23 immunoprecipitate was specific since preimmune rabbit IgG failed to isolate these proteins. Similarly, probing of the resolved SNAP-23 immunoprecipitated complexes with antibodies raised against cardiac isoform of the Ca^{2+}-channel 1C subunit, also failed to yield a signal (data not shown). These high-stringent immunoisolations, followed by immunoanalysis, confirms the physical interaction between Lc-type Ca^{2+}-channels and t-SNAREs in the rat pancreatic plasma membrane. Hence, results from the yeast two-hybrid analysis combined with immunoblot studies demonstrates a direct interaction between L-type Ca^{2+}-channels and SNAP-23 protein in rat pancreatic plasma membrane. Since our earlier study demonstrated the presence of SNAP-23 at the base of porosomes [5], and L-type Ca^{2+}-channels are also part of the porosome complex [5, 6], the present study further demonstrates that L-type Ca^{2+}-channels localize at the porosome base.

Acknowledgements

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