Transmembrane Topology of Vesicular Glutamate Transporter 2

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Vesicular glutamate transporter (VGLUT) plays an essential role in l-glutamate signaling in neurons and some peripheral tissues through vesicular storage of l-glutamate in secretory vesicles. To investigate the topology of VGLUT in membranes, we prepared site-directed antibodies against the amino-terminal (anti-N), 1st putative loop (anti-L), and carboxyl terminal (anti-C) regions. None of the antibodies reacted with VGLUT2 expressed in COS cells because they could not gain access to the antigen. However, both the anti-N and anti-C antibodies recognized VGLUT2 when the cells were permeabilized with digitonin, while the anti-L antibodies did not. Immunological reactivity to anti-L-antibodies appeared when the cells were permeabilized with Triton X-100. These results suggest that both the amino-terminal and carboxyl-terminal regions of VGLUT2 in membranes face the cytoplasm while the 1st loop faces the lumen.

Key words vesicular glutamate transporter; topology; l-glutamate; secretory vesicle

L-Glutamate, an excitatory amino acid, acts as an intercellular transmitter in the central nervous system and some peripheral tissues.1,2) For l-glutamate signal transmission, l-glutamate is stored in secretory vesicles and then secreted from the cells through exocytosis. Vesicular glutamate transporter (VGLUT) is responsible for the vesicular storage of l-glutamate through an active transport mechanism, and plays a central role in glutamatergic signal transmission.1,2) In 2000, the VGLUT1 moiety was identified.3,4) Successively, two VGLUT1 isoforms, VGLUT2 and 3, were identified.1,2) Molecular biological studies on VGLUT1 gene disruption further demonstrated the essential role of VGLUT in l-glutamate signal transmission.5,6) Although none of functional difference between VGLUT1 and 2 was identified, complementary expression of two isoforms in glutamatergic neurons in brain suggests distinct roles in signal transmission.5,7) Immunohistochemistry of VGLUTs also showed that VGLUT2 is localized in the synapses with high release probability than VGLUT1.8) On the other hand, VGLUT3 is expressed in GABAergic neurons, cholinergic neurons and serotonergic neurons.9) Localization of VGLUT3 other than glutamatergic neurons suggest a novel mode of glutamate signaling. However, very little is known about the molecular nature of the VGLUT moiety as well as the molecular mechanism of l-glutamate transport. As the first step to remedy this, in the present study, we investigated the membrane topology of VGLUT2 expressed in COS7 cells by means of immunological techniques.

MATERIALS AND METHODS

Cell Culture COS7 cells were cultured in DMEM containing 10% fetal calf serum, penicillin, and streptomycin in a humidified incubator at 37°C with 5% CO2. An expression containing 10% fetal calf serum, penicillin, and streptomycin in

Preparation of Antibodies DNA fragments encoding the N-terminus, loop, and C-terminus regions of rat VGLUT2, which correspond to M1-Y71, G86-G134, and G500-S582, respectively,10) were amplified by PCR, and then cloned into the EcoRI site of expression vector pGEX4T-2 for VGLUT2 (Amersham Pharmacia Biotech). The expression plasmids were transformed to E. coli BL21, and the resultant transformants were cultured and harvested after induction with 1 mM isopropyl 1-thio-β-d-galactoside for 3 h. After disruption of the cells, the GST fusion peptides were purified on a glutathione-Sepharose 4B column (Amersham Pharmacia Biotech), and then injected into a rabbit with complete adjuvant 2 times with a 2 week interval. Monoclonal antibodies against tubulin and PDI were obtained from Sigma and Fuji Yakuhin Kogyo, respectively.

Analytical Procedures For immunoblotting, COS7 cells (5×10⁶ cells) were suspended in 2 ml of 20 mM MOPS-Tris (pH 7.0) containing 0.3 mM sucrose, 5 mM EDTA, 10 μg/ml pepstatin A and 10 μg/ml leupeptin, and then homogenized with a Dounce homogenizer. The homogenate was centrifuged at 900×g for 10 min, and the resultant supernatant was centrifuged at 104000×g for 1 h. The pellet (membrane fraction) was suspended in the same buffer, and denatured with the SDS sample buffer containing 1% SDS and 10%β-mercaptoethanol. Polyacrylamide-gel electrophoresis and Western blotting were performed as described,10) and immunoreactivity was visualized by ECL amplification according to the manufacturer’s manual (Amersham). Immunohistochemical analysis was also performed as described10) with a slight modification: COS7 cells on poly l-lysine-coated glass coverslips were fixed in 4% paraformaldehyde for 4% paraformaldehyde for 20 min, washed with PBS, and then incubated in the same buffer. In some experiments, either digitonin at 0.05% or Triton X-100 at 0.1% was included to permeabilize the cellular and organelar membranes for 30 min. Then, samples were further incubated with 2% goat serum in PBS containing 0.5% BSA, and finally reacted with antibodies ×500 diluted in PBS containing 0.5% BSA for 1 h at room temperature. Samples were washed three times with PBS and then reacted with second antibodies for 1 h at room temperature. The second antibodies used were Alexa Fluor 568-labeled anti-mouse IgG at 1 μg/ml or Alexa Fluor 488-labeled anti-rabbit IgG at 2 μg/ml. These second antibodies were obtained from Molecular Probes. Finally, the immunoreactivity was examined.
under an Olympus FV300 confocal laser microscope. DNA sequencing was performed by the chain-termination method.\textsuperscript{11)} Protein concentrations were determined using a BioRad Protein Assay Kit with BSA as a standard.

RESULTS AND DISCUSSION

A hydropathy plot of VGLUT2 suggests the presence of twelve transmembranous regions, as shown in Fig. 1A. To reveal the membrane topology of VGLUT2, we prepared site-specific antibodies against the amino- and carboxyl-terminal, and 1st loop regions of VGLUT2 by giving repeated boosters of the synthetic peptides; hereafter referred to as anti-N, anti-C and anti-L antibodies, respectively (Fig. 1A). Immunoblotting with VGLUT2-expressing COS7 cells indicated that these antibodies specifically recognized VGLUT2 as a ~67 kDa protein (Fig. 1B).

The immunological reactivity of the antibodies with VGLUT2 in vesicles should depend on the location, inside or outside, of the peptide segments. To measure the immunological reactivity of the antibodies with VGLUT2, we carefully treated VGLUT2-expressing COS7 cells with or without a detergent. In the absence of a detergent, anti-N, anti-L and anti-C antibodies all did not react with any proteins (Fig. 2). Digitonin is used to permeabilize the plasma membrane, and is exposed on the cytoplasmic face of organelles. As expected, digitonin treatment caused a positive immunoreaction to tubulin, a cytoplasmic face marker,\textsuperscript{12)} but not to PDI, an intravesicular side marker\textsuperscript{13)} (Fig. 2, center panel). Under the conditions used, immunoreactivity with anti-C and anti-N antibodies appeared in digitonin-treated cells, while anti-L antibodies did not react with any proteins (Fig. 2, center panel). Triton X-100 treatment, which is frequently used to permeabilize both plasma membranes and organelles, caused immunological reaction to both tubulin and PDI (Fig. 2, bottom panel). Under the condition used, immunoreactivity with anti-L antibodies appeared, and then all the antibodies exhibited immunoreactivity (Fig. 2, bottom panel). These results indicated that both the N- and C-terminal regions of VGLUT2 face the cytoplasm and the 1st loop region faces the intravesicular luminal space.

The orientation of the N- and C-terminals, and the 1st loop region of VGLUT2 supports the membrane topology shown in Fig. 1A. We have previously shown that VGLUT2 expressed in COS7 cells is distributed in endomembrane sys-

\begin{figure}[h]
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\includegraphics[width=\textwidth]{image1.png}
\caption{Putative Secondary Structure of VGLUT2, and Design and Preparation of Site-Specific Antibodies against VGLUT2}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{image2.png}
\caption{Immunoreactivity of Exogenously Expressed VGLUT2 with VGLUT2 Antibodies in COS 7 Cells}
\end{figure}
tems such as late endosomes, and the trans-Golgi. The VG-LUTs in COS7 cells are functional and transport L-glutamate at the expense of ATP hydrolysis by vacuolar H^+-ATPase. Thus, herein presented membrane topology of VGLUT2 may reflect its physiological orientation. Since the N- and C-terminals comprise about 70 and 84 amino acid residues, respectively, this membrane topology allows modification and/or binding by cytoplasmic proteins, which may affect transport and membrane trafficking of VGLUT2. In fact, the N- and C-terminals both contain the motifs of potential sites of phosphorylation by protein kinase C at positions 3—5, 540—542, and 574—576. The C-terminal contains the motif of sites of phosphorylation by casein kinase II at positions 511—514, 547—550, 568—571, and 574—577. Thus, it is possible that both protein kinase C and casein kinase II modify the VGLUT2 moiety through phosphorylation. This topological model also predicts that the 6th large loop region faces the cytoplasm and might be involved in the modification and/or binding by cytoplasmic proteins.

In conclusion, we obtained the first direct evidence of the membrane topology of VGLUTs. Since VGLUTs belong to the SLC17 type I phosphate transporter family, and there is no available information of topology of SLC17, our result will be helpful for further studies on the structure and function of this superfamily.

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