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Calcium dependent modification of distal C-terminal sequences of glycine transporter GlyT1

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ABSTRACT

Glycine transporter GlyT1 plays important role in maintaining accurate glycine concentration in local brain microenvironment. Transporting efficiency of GlyT1 is strongly affected by the state of its distal C-terminus, which regulates transporter trafficking and cellular surface density. Using selected range of antibody epitopes against C-terminal region of GlyT1 we investigated its changes during calcium overload, the ubiquitous phenomena of several brain pathologies. We show that immunoreactivity against the last 12 amino acids of GlyT1C-terminal region exhibits robust calcium dependent decline, while the immunoreactivity of closely located region shows relatively small changes. Process is fully blocked by calcium chelation and inhibited by cysteine proteases inhibitors as well as inhibitors of protein kinase C. Distal GlyT1C-terminal end contains PDZ binding motif responsible for GlyT1 interaction with trafficking and clustering proteins. Its removal/modification could be part of the mechanism changing glycine homeostasis during physiological/pathological conditions characterized by elevated calcium.

1. Introduction

Amino acid glycine acts as a brain neurotransmitter, influencing both inhibitory and excitatory brain activity (Betz, 1992; Johnson and Ascher, 1987). Two glycine transporters GlyT1 and GlyT2, members of the family of sodium dependent neurotransmitter transporters play major role in the regulation of brain neurotransmitter glycine pools (Nelson, 1998). They control local glycine concentration in various brain regions, where their localization has been described using immunohistochemical and pharmacological methods (Borowsky et al., 1993; Jursky and Nelson, 1995, 1996; Zafra et al., 1995; Cubelos et al., 2005a; Luccini et al., 2008). Further complexity and compartmentalization is reflected by the existence of species specific GlyT1a, b, c as well as GlyT2a, b, c transporter subtypes (Kim et al., 1994; Ponce et al., 1998; Jursky and Baliova, 2002; Ebihara et al., 2004).

Similarly as in the case of other transporter family members, acute regulation of glycine concentration during brain activity is achieved by coupling the glycine transporters cycle to sodium/chloride gradient across the membrane and cycling of the transporters molecules between membrane and intracellular compartments (Nelson, 1998; Masson et al., 1999; Robinson, 2002). Cell surface localization is influenced by the transporter N-terminal interactions with Syntaxin 1A and its C-terminal interactions with trafficking and clustering proteins (Geerlings et al., 2001; Cubelos et al., 2005a,b).

To study the glycine transporter function *in vivo*, genes for GlyT1 and GlyT2 has been deleted in mouse (Gomeza et al., 2003a,b). Despite that both knock-outs die soon after they are born, several important facts about the function of both transporters have been learned from these models. While mice deficient in GlyT1 resemble symptoms of non-ketonic hyperglycinemia, GlyT2 absence leads to hyperplexia (Gomeza et al., 2003a,b; Rees et al., 2006; Eulenburg et al., 2006; Harvey et al., 2008). Experiments further suggest that GlyT1 transporter is responsible for termination of inhibitory glycinergic transmission on strychnine sensitive glycine receptor and GlyT2 rather serves for refilling the vesicles in glycinergic presynaptic terminals.

NMDA receptor is colocalized with GlyT1 (Cubelos et al., 2005a) and contains glycine/D-serine co-agonist site. It was generally assumed that glycine acts as major co-agonist at this site *in vivo*. Recent studies however indicate that glial and neuronal-derived D-serine rather than glycine acts as endogenous ligand of NMDA receptor (Mothet et al., 2000; Panatier et al., 2006; Wolosker, 2007). Glycine concentration in cerebrospinal fluid is low micromolar and it should saturate glycine site on NMDA receptor.

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GlyT1 inhibitors however potentiate NMDA responses, which indicate that transporters lower the glycine concentration under saturating level. Recent discovery of specific glycine transporters inhibitors allowed modification of the local glycine concentration *in vivo*. Such strategy seems to be very promising for treatment of schizophrenia and other psychoses caused by hypothetical hypofunction of NMDA (Sur and Kinney, 2007; Boulay et al., 2010; Javitt, 2009). Heterozygous GlyT1 knock-out mice are viable and have doubled extracellular glycine concentration. It was however recently reported, that they exhibit abnormal redistribution of hippocampal synaptic NMDA receptors into extra synaptic sites (Imamura et al., 2008). Thus despite mice seems to be phenotypically normal and even some increase of memory retention is observed, elevated brain glycine concentration may introduce unexpected pathological side-effects.

The facts mentioned above indicate that glycine function in brain is tightly regulated or pathologically altered mostly by glycine transporters. Modification of cytosolic regions of transporters, which interconnect them with intracellular regulatory pathways, could be one of the ways to provoke brain glycine concentration changes. We recently described N-terminal truncation of GlyT1 with calpain protease (Baliova and Jursky, 2005). Because the major epitopes of previously used antibodies were located outside the short C-terminal fragment of GlyT1 removed by calpain, we were unable to detect the modification of GlyT1 on distal C-terminal region reported here. We hypothesize that such truncation/modification during the pathological calcium overload could either additionally contribute to abnormal GlyT1 function or it represents certain compensation regulatory feedback.

2. Materials and methods

2.1. Materials

Peroxidase linked anti-rabbit antibodies were from Millipore (Temecula, CA, USA). ECL reagents, Calpain inhibitor I, caspase inhibitor Z-VAD-FMK, Chelerythrine were from Sigma (St. Loius, MO, USA). Oligonucleotides were synthesized by VBC Genomics Bioscience Research (Vienna, Austria). All other chemicals used were of the purest grade available.

2.2. Construction of GST-GlyT1C, GST-GlyT1CΔ3, GST-GlyT1CΔ12, GST-GlyT1C(12), meth-6Xhis-GlyT1C-GST fusion proteins and screening of calpain cleavage sites

For fusion proteins GST-GlyT1C, GST-GlyT1CΔ3, GST-GlyT1CΔ12, regions containing amino acids F561-I638, F561-D635 and F561-G626 of mouse glycine transporter GlyT1b were amplified by PCR reaction using forward EcoRI primer 5′-ttgtacgcagaattccagctctgccgc-3′ identical for all three fragments and reverse SalI primers 5′-caacaagtcgactcatatccgggagtcctg-3′ for GST-GlyT1C, 5′-ctcatgtcgactagtcctggaagcggctgg-3′ for GST-GlyT1CΔ3 and BamHI primer 5′-ctggaggatcctcagcccacgatggggatc-3′ for GST-GlyT1CΔ12. Fragments were inserted into pGEX-5X-1 (GE Healthcare, Freiburg, Germany). Plasmids were transformed into BL21 (DE3) (Novagen, Merck, Darmstadt, Germany) and fusion proteins were purified after 2 h of induction of cultures with 0.3 mM IPTG according to manufacturer instructions.

For the fusion protein Meth-GlyT1C-GST, the GlyT1C-terminal region was amplified using forward NdeI primer 5′-ccattgtaccatatgttccagctctgccgc-3′ and reverse BamHI primer 5′-gcaacaacggatcccctatccgggagtcc-3′. Following digestion with restriction enzymes NdeI and BamHI the DNA was in inserted into pET21a (Novagen, Merck, Darmstadt, Germany) together with downstream in frame DNA sequences coding for glutathione-S transferase (Baliova and Jursky, 2005). Finally forward 5′-catatgcatcaccatcaccatcacc-3 and reverse 5′-atatggtgatggtgatggtgatgc-3′ Meth-His-Tag primers were annealed together and ligated into NdeI site resulting in introduction of methionine initiated Meth-6Xhis-Tag upstream of Meth-GlyT1C coding sequence. Plasmid was transformed into *Escherichia coli* BL21 and overexpresion was achieved after 2 h of induction at 37 °C using 0.3 mM IPTG. Protein was distributed into soluble fraction as well as inclusion bodies. Soluble protein was isolated using Ni^{2+}/NTA-agarose under native condition using homogenization and wash buffer (50 mM sodium phosphate pH 8.0, 0.3 M NaCl, 14 mM 2-ME, 20 mM Imidazole). Elution was achieved by supplementing the same solution with 10 mM EDTA. Eluate was diluted 5× with 25 mM sodium phosphate pH 7.2, 0.150 M NaCl, 1% Triton X-100 and recovered on GST-sepharose. All fusion protein used in this work were eluted with 10 mM glutathione and calpain cleavage as well as cleavage sites determination were performed as previously described (Franekova et al., 2008).

2.3. Selective affinity purification of epitopes

Polyclonal rabbit antibodies were raised against whole mouse GlyT1C-terminal region (amino acids 554–638) using pMAL fusion protein as antigen and purified on affinity column as previously described (Jursky and Nelson, 1996). For separation of distal GlyT1C epitopes, DNA primers coding for last 12 amino acids of mouse GlyT1 flanked by EcoRI and HindIII restriction sites 5′-aattcagtaacggctc-cagccgcttccaggactcccggatatgaa-3′ and 5′-agctttcatatccgggagtcctggaagcggctg-gagccgttactg-3′ were synthesized, annealed and ligated into modified (BamHI+1 frame shifted) EcoRI/HindIII digested pET34(+) (Novagen, Merck, Darmstadt, Germany) downstream in frame with cellulose binding protein Tag. Plasmid was transformed into BL21, induced with IPTG and fusion protein was isolated on microcrystalline cellulose. Epitopes against last 12 amino acids of GlyT1C (anti-GlyT1C626–638) were isolated by affinity purification from polyclonal serum using affinity column with immobilized fusion protein using method described previously (Baliova et al., 2004). Fresh affinity column was also used for removal of these epitopes from whole anti-glyT1C antibodies. This resulted in antibodies directed against amino acids 554–625 of GlyT1C (anti-GlyT1C554–626). The specificity of all antibodies was verified by cross absorption with corresponding fusion proteins.

2.4. Site directed oligonucleotide mutagenesis

To replace potentially phosphorylated serine in position S636 with asparagine, GST-GlyT1C construct was mutated using primers P2-F 5′-cgcttccaggacgatcggaatatgagtc-3′, P2-R 5′-gactcatatccgatcgtcctggaagcg-3′. For mutagenesis we used Quick-change mutagenesis kit (Agilent, Stratagene Products, La Jolla, CA, USA) according to manufacturer's suggestions.

2.5. Isolation of synaptosomes and internal calpain activation

Crude mouse hindbrain synaptosomes were isolated as described previously (Baliova et al., 2004) with the following modifications: homogenate was prepared in ice cold 0.35 M sucrose/5 mM Hepes–NaOH, pH 7.4, 10 mM EDTA, 2 mM EGTA. Following 5 min centrifugation at 1000 × *g* to remove nuclei, synaptosomes were recovered from the supernatant by 10 min centrifugation at 12 000 × *g* and washed once with sucrose solution. To remove excess of metal chelators, synaptosomes were washed two times with sucrose solution without EDTA and EGTA. Aliquots of synaptosomes were resuspended in 10 fold volume of 25 mM Hepes/NaOH pH 7.4, with and without the presence of 0.2 mM $CaCl_2$ and incubated for 15 min at 37 °C. Two additional calcium-containing samples were incubated in presence of 50 μM calpain inhibitor I, or general caspase inhibitor 25 μM Z-VAD-FMK. In certain samples 5 mM $MgCl_2$ was added as the necessary component of phosphorylation. Phosphorylation was blocked addition of 17 μM chelerythrine. Additionally, synaptosomes were incubated with $ZnCl_2$, $MnCl_2$ and $CoCl_2$ (final 2 mM each) with and without presence of 2 mM $CaCl_2$. Samples were dissolved in SDS sample buffer, resolved in 7.5% PAGE and transferred to imobilon. Blot was then probed with antibodies anti-GlyT1C554–625 and anti-GlyT1C626–638.

2.6. Western blot data analysis

The relative intensity of immunostaining was quantified with UN-SCAN-IT, Silk Scientific Inc. Utah, USA and plotted with GraphPad Prism 4.00 for Windows, GraphPad Software, San Diego, CA, USA.

3. Results

Despite the presence of several calpain cleavage sites in the recombinant GlyT1C fusion protein sequence, our previous screening of spinal cord synaptosomes did not show marked changes of GlyT1C-terminal immunoreactivity following calcium increase. Polyclonal antigenic determinants are often distributed heterogeneously and successful detection of proteolytic truncation *in vivo* could depend on position of major antibody epitopes (Baliova et al., 2009).

To verify this possibility we decided to determine the exact position of calpain cleavage sites in recombinant GlyT1C fusion protein and to separate possible groups of antibody epitopes directed against peptide fragments released by calpain cleavage. Because protein sequencing by Edman degradation proceeds through the protein N-terminal end, it was possible to use only one set of the proteolytic fragments to determine the calpain cleavage sites. Some of the released fragments were too small to be separated by PAGE. To overcome this limitation, we took advantage of the fact that calpain specificity is mostly determined